$^{-}$ mice (Fig. 6D). Furthermore, the proportion and number of Mφ/cDCs was increased, even in mice lacking $CD4^+$ T cells (Fig. 6D and E). These data suggest that colon inflammation induces the recruitment of Mφ/cDCs and these newly recruited Mφ/cDCs stimulate Th1 cells or promote differentiation of Th1 cells.

We also assessed whether these Mφ/cDCs that emerged under colitic conditions contributed to acute liver inflammation, which had been initially induced by Fas-activating antibody (Jo2) [18,19]. As shown in Fig. 7A, DSS-treated mice with significant body weight loss underwent Jo2 treatment. The livers of DSS-induced colitic mice in which Jo2 was administered showed significant blood accumulation (Fig. 7B). Consistently, the levels of aspartate aminotransferase and alanine aminotransferase were significantly increased in DSS mice after Jo2 treatment compared with non-DSS mice (Fig. 7C).

Leukocyte Infiltration was not Detected in Acute Colitis Models Under GF Conditions

We investigated whether MAMPs or other bacterial degradation products induce hepatic immune dysregulation and analyzed the livers of mice treated with DSS under GF conditions. Bacteria do not reside in the intestines of GF mice, meaning there is no inflow of bacterial components into the liver. Mice treated with DSS under GF conditions showed severe colitis; however, there was no evidence of leukocyte infiltration (Fig. 8A and B). Consistent with histological data, we observed no significant changes in the number of liver mononuclear cells for controls and DSS-treated mice (Fig. 8C). Flow cytometry data showed that there were no significant changes in the ratios of pDCs to Mφ/cDCs (Fig. 8D); or in the absolute numbers of these cells in the livers of DSS-treated mice (Fig. 8E). DSS-treated GF mice also exhibited severe colitis compared with SPF mice (Fig. 8F), but Mφ/cDCs were not increased (Fig. 8G). These findings indicate that bacterial products play a crucial role in inducing infiltration of Mφ/cDCs into the liver.

Discussion

In this study we demonstrated: (1) hepatic pDCs are decreased and Mφ/cDCs are increased in mice with chronic intestinal inflammation; (2) newly emerged Mφ/cDCs during the development of colitis possess pro-inflammatory characteristics that drive differentiation of naïve T cells toward Th1 cells; (3) Mφ/cDCs that emerge during colitis possibly result in the exacerbation of hepatitis symptoms; and (4) the reciprocal changes we observed in the compartments of the liver's innate immune system during intestinal inflammation were mainly caused by MAMPs, other bacterial degradation products, or bacteria themselves subsequent to the disruption of the intestinal wall. Changes in APC compartments were seen not only in RAG-$2^{-/-}$ RB^{high} mice and DSS-administered mice but also in RAG-$2^{-/-}$ mice retransferred with gut-tropic colitogenic LP $CD4^+$ T cells in SPF conditions; but were not seen in mice of the DSS colitis model in the GF condition that lack commensal bacteria in the gut.

Previous studies have suggested a relationship between intestinal and liver inflammation [2,3,11,26]. These previous reports support our hypothesis that intestinal inflammation skews the balance of immune cells in the liver. However, no expansive research has been conducted to clarify the inflammatory relationship between the liver and the intestine. In this study, we are the first to demonstrate distinctive changes in compartments (pDCs vs. Mφ/cDCs) of hepatic immune cells due to chronic intestinal inflammation. Increased hepatic Mφ/cDCs appeared irrespective of whether the colitis model was acute or chronic. Immunological changes were not only observed in the liver during colitis, but also in ConA-induced liver injury (Fig. 3A) [14], suggesting that these changes are universal phenomena during liver stress.

We hoped to elucidate how these crucial changes of hepatic APCs occur during intestinal inflammation. The liver is between the portal and systemic circulatory systems. The liver receives continuous blood supplements from the intestine via the portal vein and is presumably exposed to MAMPs or other degradation products from viable or non-viable commensal bacteria [8]. We used a GF system to demonstrate the importance of bacterial components in causing immunological dysregulation in the liver. Most models of experimental colitis fail to develop under GF conditions [27,28]; however, we used a DSS model, which is known to result in the development of severe colitis [29,30]. DSS-treated mice under SPF conditions exhibited Mφ/cDC infiltration into the liver (Fig. 5 and 8). This was not observed for DSS-treated mice under GF conditions despite the existence of severe colitis. This means that the accumulation of Mφ/cDCs is not just a consequence of nonspecific inflammation related with colitis. Mice under GF conditions lack the protective effects against colitis from microbiota [31], but also lack the stimulant transferred from the intestine to the liver. These data suggest that stimulation from degradation products from intestinal commensal bacteria play an important role in recruitment of Mφ/cDCs.

Various commensal bacteria that usually reside in the intestine (such as *C. coccoides*, *C. leptum* and *Enterococcus)* were found in ConA-treated and untreated livers [20]. Such a finding lends support to the hypothesis that intestinal bacterial products or bacteria themselves are transferred from the intestine to the liver. The rate (and amount) of uptake of bacteria-derived products, such as microbial DNA and LPS, is thought to increase during colitis because of the fragility of the colon wall [11,32]. Further study is required to estimate the amount and type of bacterial products that stimulate the liver during colitis.

Despite our finding that recruitment of Mφ/cDCs is stimulated by bacterial degradation products or bacteria themselves, several scenarios may be considered as additional mechanisms underlying the accumulation of Mφ/cDCs. First, activated Mφ/cDCs themselves migrate from the intestine to the liver. Alternatively, circulating monocytes accumulate in the liver stimulated by pro-inflammatory cytokines transferred via the portal vein, such as TNF-α produced by LP $CD4^+$ T cells or APCs in the intestine [30]. However, pro-inflammatory cytokines are produced in the colon of GF mice, so this possibility may only have a partial effect. Whether increased Mφ/cDCs originate from monocytes or resident macrophages in the liver should be explored in future studies. Third, activated T cells migrate from the intestine to the liver and stimulate the liver to recruit and activate circulating or resident Mφ/cDCs. RAG-$2^{-/-}$ LP $CD4^+$ mice (Fig. 3) and DSS-treated RAG-$2^{-/-}$ (Fig. 6) mice show increased Mφ/cDCs without infiltration of T, B, and NKT cells in the liver, which suggests that Mφ/cDCs are recruited to the liver independently of T, B, and NKT cells. However, there still remains involvement of cytokines, DSS itself and other types of the cells such as NK cells and liver sinusoidal endothelial. Macrophages accumulated during both acute and chronic colitis models in the liver produced inflammatory cytokines and promoted differentiation of Th1 cells or activation of NK cells. Thus, it is likely that systemic IFN-γ production leads subsequent upregulation of the sensitivity of FAS-mediated signal in the liver.

The current study suggests that hepatic APC compartments alter in parallel with the progression of colitis, and increased Mφ/

cDCs have pro-inflammatory characteristics. Two major hepatic diseases presenting as extraintestinal manifestations in IBD patients are PSC and autoimmune hepatitis. The prevalence of these liver diseases is reported, both in Crohn's disease and ulcerative colitis, to correlate with the severity and expansion of the intestinal disease [3,26,33]. The active disease is associated with ongoing extraintestinal manifestations in patients with Crohn's disease [26]. The prevalence of PSC was 5.5% in patients with substantial colitis and 0.5% in patients with distal colitis [2]. Therefore, Mφ/cDCs infiltrating into the liver in the colitis models may be involved in the pathogenesis of IBD-related liver diseases. However, liver enzymes demonstrated no significant changes during colitis unlike autoimmune hepatitis or PSC. Thus, we hypothesized that accumulated Mφ/cDCs may increase the susceptibility of hepatitis. We combined the Fas-mediated model of hepatitis with the DSS colitis models to show the clinical importance of our study. Fas-mediated hepatitis models are widely used as a model of hepatitis [18,19]. TNF-α released by activated hepatic macrophages is one of the very important factors that damage hepatocytes, which are highly sensitive to cell-extrinsic stimulation in Fas-mediated hepatitis [19]. We also suggest the importance of the infiltration of macrophages in fulminant hepatitis models [10]. Mφ/cDCs recruited to the liver during colitis in a T cell-independent manner produce pro-inflammatory cytokines and promote Th1 reaction. $CD11b^{+}CD11c^{low/+}$ Mφ/cDCs are already detectable in healthy WT mice (Fig. 2B), but previous studies suggest that these resident macrophages including Kupffer cells have a immunoregulatory character, such as producing IL-10 [6,34]. Taken together with the previous studies and our findings, Mφ/cDCs infiltrating into the liver during colitis may contribute to making the hepatitis worse. Some additional stimulation would be needed for breaking down liver tolerance and causing hepatitis or cholangitis which mimics liver diseases associated with IBD.

An additional finding of our study is that pDCs are significantly decreased in the late phase of colitis in the chronic models. Hepatic pDCs plays an important role in maintaining liver homeostasis [9,10,14], so not only increased Mφ/cDCs but decreased pDCs may have effects on the infiltration of mononuclear cells in the liver during chronic colitis. This is known as sustained negative regulation of LPS signaling in response to a second LPS stimulus. pDCs express certain toll-like receptors (TLR) [14], with the number of spleen pDCs decreasing following TLR treatment [35]. These previous reports suggest that pDCs are one of the initial sensors of MAMPs or other degradation products, and possibly decrease when the endotoxin tolerance is broken down. Further studies will be needed to address this issue.

In conclusion, the findings from our study suggest that dysregulation in the balance of liver APCs, especially in the recruitment of pro-inflammatory Mφ/cDCs, during colitis occurs in a T-cell-independent manner for chronic and acute murine colitis models. This report may provide the basis for a novel strategy to treat intractable immune diseases in the liver. We have also highlighted the relevance of the relationship between liver and intestine immunology during intestinal inflammation.

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Author Contributions

Conceived and designed the experiments: YM AY TK. Performed the experiments: YM SM NN AH. Analyzed the data: YM T. Sujino T. Sato KM T. Hisamatsu HE T. Hibi NK. Wrote the paper: YM AY TK.

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Endoscopic imaging of parasites in the human digestive tract

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ABSTRACT

There are various diagnostic approaches for parasitic infections, including microscopic identification of parasites in the stool or biopsy samples from the intestinal mucosa, antigen testing of feces or serum, polymerase chain reaction (PCR) testing, and serology. Endoscopy is sometimes used for direct confirmation of parasite infection and as a therapeutic option for removal. In recent years, innovations in endoscopy have advanced remarkably with regards to endoscopic devices as well as diagnostic and therapeutic endoscopical methods. Several new endoscopic devices are now used for diagnostic and therapeutic approaches to parasitic infections. In the present article, we have focused on in vivo imaging of parasitic infections. In vivo images of parasites were obtained using various endoscopic methods such as high-definition endoscopy, super-magnifying endoscopy, and video capsule endoscopy.

Contents

1. Introduction

The incidence and prevalence of parasitic infection remain high worldwide [1–4]. In developing countries, controlling parasitic infection is crucial for public health. There are various diagnostic approaches for parasitic infections, including microscopic identification of parasites in the stool or biopsy samples from the intestinal mucosa, antigen testing of feces or serum, polymerase chain reaction (PCR) testing, and serology. Endoscopy is sometimes used for direct confirmation of parasite infection and as a therapeutic option for removal.

In recent years, innovations in endoscopy have advanced remarkably with regards to endoscopic devices as well as diagnostic and therapeutic endoscopical methods. Several new endoscopic devices are now used for diagnostic and therapeutic approaches to parasitic infections.

In the present article, we have focused on in vivo imaging of parasitic infections. In vivo images of parasites were obtained by various endoscopic tools, ranging from conventional to newly developed devices. We have also discussed and described endoscopic innovations.

2. *Anisakis* visualized and removed by endoscopy

Anisakiasis is a common parasitic disease that is caused by *Anisakis* larvae. Anisakiasis patients have a typical history of consumption of raw fish and present with epigastric pain, nausea, and vomiting. Diagnosis of anisakiasis is usually made by identifying *Anisakis* larvae. Endoscopy is mainly used for diagnosing gastric anisakiasis [5–11], while computed tomography (CT) is mainly used for intestinal anisakiasis [12,13]. Another option is serological testing [14,15]. Endoscopy can be used to directly diagnose anisakiasis as well as to subsequently remove the larvae by using biopsy forceps (Fig. 1). Many case reports have illustrated gastric anisakiasis [5–11], a few reports have highlighted esophageal anisakiasis [16,17], and colonic cases are relatively rare [13,18–23]. Only one case report has described enteric anisakiasis detected using video capsule endoscopy (VCE). Celestino et al. [6] reported a case of anisakiasis observed using a magnifying endoscope. Nakagawa et al. [24] compared magnified endoscopic images between hookworm and

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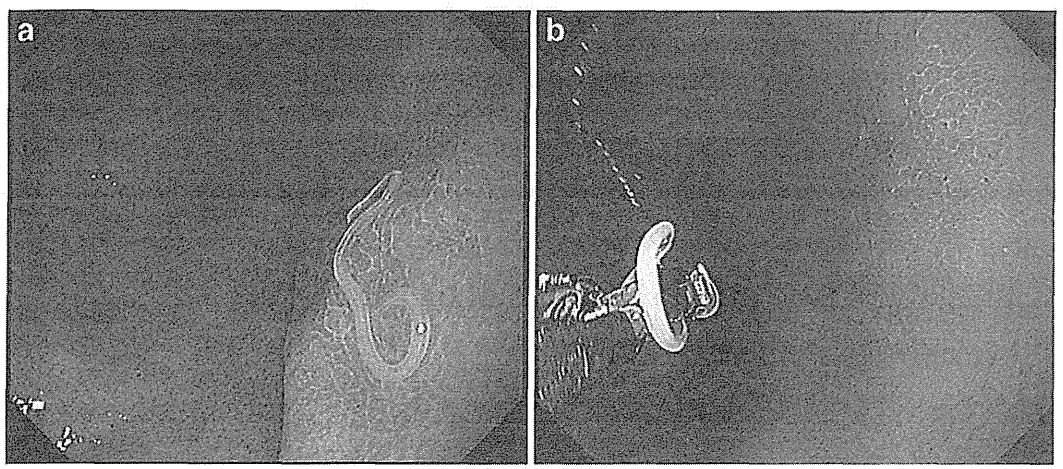

Fig. 1. Endoscopic view of an *Anisakis* larva. (1a) The *Anisakis* larva was seen sticking to the gastric wall. (1b) The *Anisakis* larva could be removed with forceps.

Anisakis. A magnifying endoscope (GIF-H260Z, Olympus Medical Systems, Tokyo) can obtain high-definition images with 85× magnification and is mainly used to distinguish between malignant and benign mucosa [25,26].

High-definition endoscopic images of our case of Anisakiasis are shown in Fig. 1. An *Anisakis* larva sticking to the edematous gastric wall is shown in Fig. 1a. The *Anisakis* larva could be removed by biopsy forceps.

3. *Entamoeba histolytica* visualized using super-magnifying endoscopy

Amoebic colitis is distributed worldwide, and is known to be a sexually transmitted disease [27]. Some cases of amoebic colitis that exhibit chronic symptoms are misdiagnosed as ulcerative colitis and treated with corticosteroids [28]. Importantly, the usage of corticosteroids is detrimental in such cases. Therefore, it is essential that the diagnosis of amoebic colitis is made promptly and accurately in order to prevent fulminant worsening of the disease. Accurate diagnosis of amoebic colitis relies on the microscopic identification of amoebic trophozoites in the stool or colonic mucosa of patients. Moreover, there are a variety of laboratory tests that use antigen testing of feces or serum, PCR, and serology. However, these are neither sensitive nor specific, even in combination with a patient's history, endoscopic findings, and other laboratory tests. Thus, it is necessary to develop better diagnostic tools for amoebic colitis.

Recently, super-magnifying endoscopes have been developed, which allow us to obtain images at the cellular level. Currently, there are two devices available that have the ability to allow in vivo microscopic inspection: confocal laser endomicroscopy (CLE) (Pentax, Tokyo) [29] and an endocytoscopy system (ECS) with a high magnification light microscopy device (Olympus Medical Systems, Tokyo) [30–32]. CLE based on tissue fluorescence uses local and/or intravenous contrast agents to generate images. The ECS is based on the principle of contact light microscopy [33–35]. ECS observation also requires pre-treatment with methylene blue or toluidine blue staining [36]. Most clinical studies reported to date have used CLE integrated into the distal tip of a conventional upper endoscope (iCLE: EG-3870CIK, Pentax, Tokyo) or colonoscope (EC-3870CILK, Pentax) [37]. A smaller number of studies have used probe-based CLE (pCLE) (Mauna Kea Technologies, Paris, France) inserted through the accessory channel of a traditional endoscope [37]. Similarly, the ECS is classified as a probe-based ECS (pECS) or an integrated-scope type ECS (iECS) (Fig. 2) [38–40].

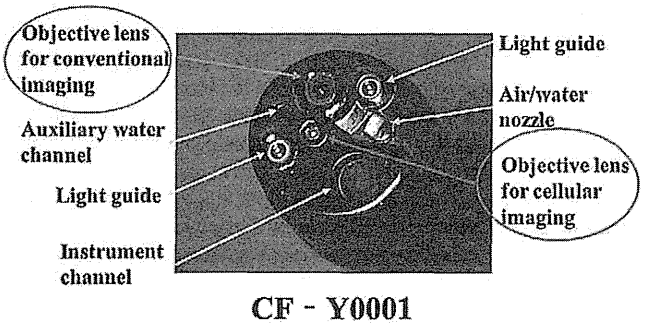

Fig. 2. Integrated-scope type endocytoscope (CF-Y0001).

In the field of ophthalmology, confocal laser microscopy (Heidelberg Retina Tomograph 2, Rostock Cornea Module, Heidelberg Engineering GmbH, Dossenheim, Germany) has been used to diagnose *Acanthamoeba* keratitis [41–45]. On the other hand, the ECS has been used to obtain real-time in vivo histology for cancer [30–32,46–48]. Previously, we reported the utility of the ECS for predicting the histopathological activity of ulcerative colitis and its usefulness as a real-time diagnostic tool for amoebic colitis [49].

4. ECS procedures for detecting *E. histolytica* trophozoites

We use an iECS (ECS, CF-Y0001, Olympus Medical Systems, Tokyo) to detect amoebic trophozoites; this system is shown in Fig. 2. This scope can be switched easily from conventional view to a super-magnifying view (×450) by using a button located at the top of the endoscope. A conventional colonoscopic image of amoebic colitis is shown in Fig. 3a. Irregular shallow ulcers with marginal redness, edema, and mucus exudates are seen in the rectum. Subsequently, we changed the conventional view to a super-magnifying view (Fig. 3b). The observation area of the epithelial surface is 400 μm × 400 μm, and the bar represents 100 μm (Fig. 3b). Without methylene blue staining, *E. histolytica* trophozoites were hardly detectable. In order to better visualize *E. histolytica* trophozoites, the lesions were stained with 1.0% methylene blue for 2 min, followed by a few washes with dimethicone solution. As shown in Fig. 3b, following staining, we were clearly able to visualize the body of amoebic trophozoites in the mucus surrounding the lesions. Numerous bluish amoebic trophozoites with a characteristic round shape (white arrows) were easily found in one field of view. We noticed that the size of

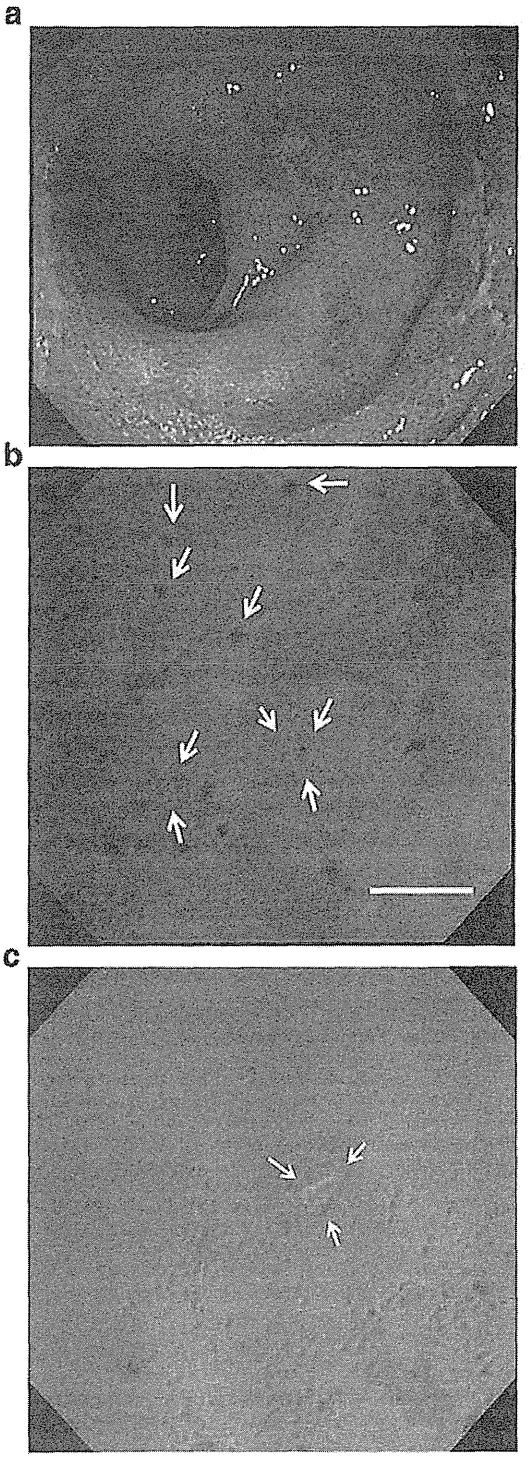

Fig. 3. a. Conventional colonoscopic image of amoebic colitis. b. Endocytoscopy system image of the rectal lesion. c. Endocytoscopy system image of live *E. histolytica* trophozoites.

E. histolytica trophozoites detected by the ECS were appreciably smaller relative to the trophozoites detected by traditional hematoxylin and eosin staining. We found that methylene blue staining could make the cytoplasm collapse, resulting in the observation of nuclei that were smaller in size. Biopsy samples were obtained from the lesion, and histological findings corresponded with those of the ECS. Interestingly, in one case only, non-stained *E. histolytica* trophozoites with amoeboid movement were clearly visualized using the ECS (Fig. 3c). An amorphous amoeba was also seen on the surface of the aphthous lesion. The small spots

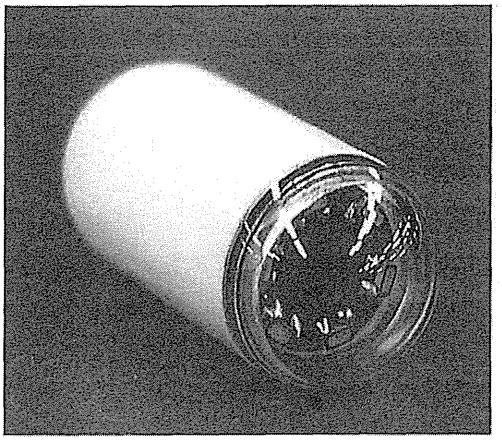

Fig. 4. Video capsule endoscope (EC-1, Olympus Medical Systems).

are red blood cells, and amoebic trophozoite phagocytosis of floating red blood cells could be observed.

5. Tapeworm visualized by VCE

Tapeworms are classified as fish tapeworms (*Diphyllobothrium latum*), pork tapeworms (*Taenia solium*), and beef tapeworms (*T. saginata*). Fish tapeworms are prevalent in Europe and East Asia, in countries where raw or undercooked freshwater fish is consumed. In Japan, the main pathogenic tapeworm is the fish tapeworm *D. nihonkaiense*, which is considered as a separate species from *D. latum*. On the other hand, in Europe, *D. latum* is the most common fish tapeworm [50]. Several reports [51–54] have shown in vivo imaging of tapeworms detected by conventional colonoscopy. In addition, we have successfully detected beef tapeworm (*T. saginata*) by using VCE and radiography [55]. VCE was first reported by Iddan [56] in the year 2000. It allows visualization of the small intestinal mucosa and facilitates detection of small intestinal abnormalities. Several studies have shown the high efficiency of VCE for detecting certain disorders such as obscure gastrointestinal bleeding [57], suspected Crohn's disease [58,59], small bowel tumors [60], and small intestinal mucosal injury associated with the use of non-steroidal anti-inflammatory drugs [61]. The size of the video capsule is 11 mm × 26 mm (Fig. 4). The camera within the capsule can obtain 2 pictures per second. Patients only have to swallow the capsule, and thus it is considered as a non-invasive tool for small intestinal investigations. Recently, esophageal and colon capsules were also developed [62–65], and in the gastric field, a magnetically maneuverable capsule was reported [66]. Several case reports [67–75] have shown VCE pictures of tapeworms, and this technique has the potential to diagnose tapeworm infection effectively. Representative images of fish tapeworms detected by VCE (EC-Z0001, Olympus Medical Systems, Tokyo) are shown in Fig. 5. strobila (Fig. 5a), scolex (Fig. 5b), and uterine loops (Fig. 5c) of fish tapeworms are clearly visualized by VCE.

6. Future perspectives

Diagnostic approaches for parasitic infection have changed dramatically, including PCR and enzyme-linked immunosorbent assay (ELISA). Development of endoscopic devices has also been progressing rapidly. Super-magnifying endoscopes can provide real-time in vivo cellular level imaging, and allow direct detection of amoebic trophozoites. VCE can provide small intestinal images and show pictures of the largest type of parasite, the tapeworm. The ECS is a prototype endoscope at this time, and is not available on the market. The cost of VCE is expensive, and other diagnostic methods for parasite infection would be

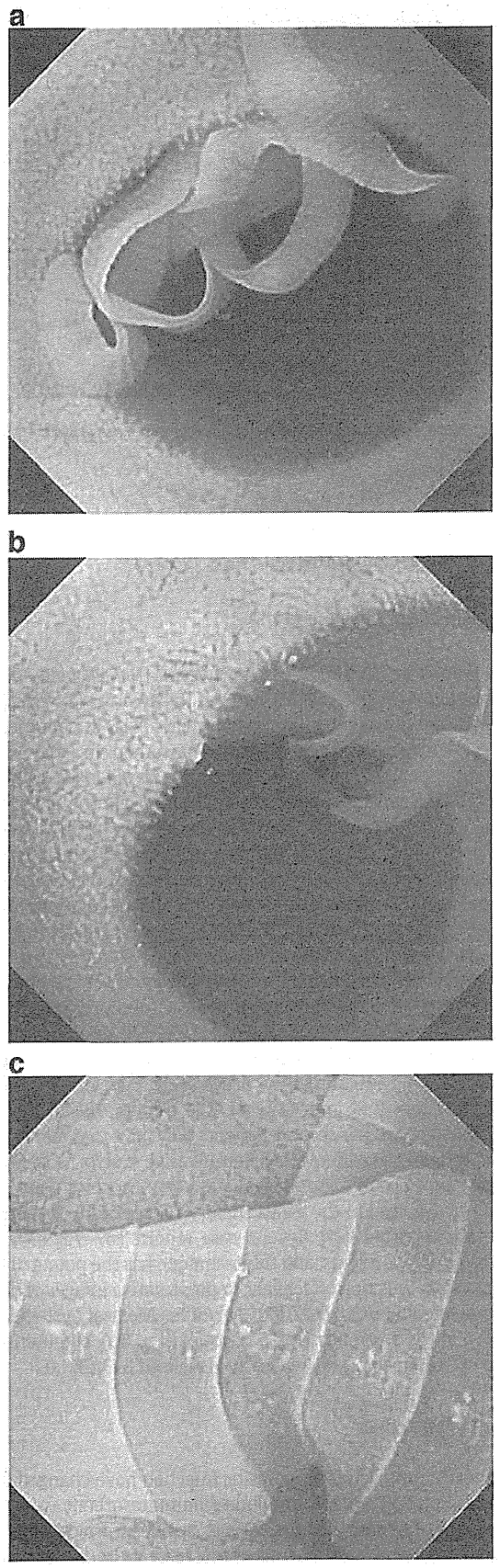

Fig. 5. a. Fish tapeworm observed by video capsule endoscopy (EC-Z0001, Olympus Medical Systems). b. Scolex of fish tapeworm observed by video capsule endoscopy (EC-Z0001, Olympus Medical Systems). c. Uterine loops of fish tapeworm visualized by video capsule endoscopy (EC-Z0001, Olympus Medical Systems).

useful. Such devices will be mass-produced in the future, allowing them to become more generally used in the clinical field.

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RAPID COMMUNICATION

The 2nd edition of consensus statements for the diagnosis and management of intestinal Behçet's disease: indication of anti-TNFα monoclonal antibodies

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Abstract
Background Clinical evidence regarding intestinal Behçet's disease (BD) management is lacking and intestinal lesions are a poor prognostic factor. In 2007, the Japan consensus statement for diagnosis and management of intestinal BD was developed. Recently, the efficacy of anti-tumor necrosis factor (TNF)α monoclonal antibodies (mAbs), and infliximab (IFX) was reported and adalimumab (ADA) was approved for intestinal BD in Japan. This study renewed consensus-based practice guidelines for diagnosis and treatment of intestinal BD focusing on the indication of anti-TNFα mAbs.
Methods An expert panel of Japanese gastroenterology and rheumatology specialists was involved. Clinical statements for ratings were extracted from the literature, a professional group survey, and by an expert panel discussion, which rated clinical statements on a nine-point scale. After the first round of ratings, a panelist meeting discussed areas of disagreement and clarified areas of uncertainty. The list of clinical statements was revised after the panelist meeting and a second round of ratings was conducted.
Results Fifteen relevant articles were selected. Based on the first edition consensus statement, improved clinical statements regarding indications for anti-TNFα mAbs use were developed. After a two-round modified Delphi approach, the second edition of consensus statements was finalized.
Conclusions In addition to standard therapies in the first edition, anti-TNFα mAbs (ADA and IFX) should be considered as a standard therapy for intestinal BD. Colchicines, thalidomide, other pharmacological therapy,

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endoscopic therapy, and leukocytapheresis were deemed experimental therapies.

Keywords Intestinal Behçet's disease · Anti-TNFα mAb · Consensus statements

Abbreviations

ADA	Adalimumab
BD	Behçet's disease
CRP	C-reactive protein
IFX	Infliximab
mAb	Monoclonal antibody
TNF	Tumor necrosis factor

Introduction

Behçet's disease (BD) is a chronic relapsing disease with multiple organ system involvement characterized clinically by oral and genital aphthae, cutaneous lesions, and ophthalmological, neurological, or gastrointestinal manifestations [1, 2]. Approximately 3–16 % of patients with BD have gastrointestinal tract involvement. Gastrointestinal disease typically affects the ileocecal area, although involvement of the esophagus and small intestine has been reported [3]. The most common gastrointestinal symptoms are abdominal pain, diarrhea, and bleeding. Deep ulcers are responsible for the most common intestinal complications, such as severe bleeding and perforation [4]. Various drugs, such as 5-aminosalicylic acid (5-ASA), systemic corticosteroids, and immunosuppressive agents have been used anecdotally to treat intestinal BD. However, the clinical evidence regarding the management of intestinal BD is very limited. In 2007, the Japanese Inflammatory Bowel Disease Research Group, supported by the Japanese Ministry of Health, Labour and Welfare, proposed consensus statements for the management of intestinal BD for the first time [5]. In this consensus, infliximab (IFX) was described as an optional therapy for intestinal BD. In recent years, accumulating evidence on the efficacy of anti-TNFα agents for the management of Crohn's disease and Behçet's uveitis have encouraged the use of anti-TNFα agents for management of intestinal BD. Although clinical studies with high-quality evidence have not been available, several cases of intestinal BD successfully treated by anti-TNFα agents have been reported [6–14]. These case reports mainly showed clinical efficacy in the short term, although some reports showed mid- and long-term efficacy and improved endoscopic findings [15, 16]. Furthermore, on May 16 2013, adalimumab (ADA) was approved as a therapeutic option for intestinal BD in Japan. Currently, the Research Committee for small bowel inflammation of unknown etiology operated by the Health Labour Sciences Research Grant, titled "Research on Measures for Intractable Diseases", was concerned that the approval of anti-TNFα mAb could dramatically change the therapeutic strategy for intestinal BD. Furthermore, the first edition does not contain information regarding anti-TNFα mAbs and is, therefore, outdated. Therefore, consensus statements for the management of intestinal BD should be adjusted to the current clinical settings, especially regarding the indication of anti-TNFα agents (Table 1).

Methods

An overview of the study

The development of the second edition of consensus statements for the diagnosis and management of intestinal BD consisted of three phases. In brief, in the first phase, literature that reported the efficacy of anti-TNFα monoclonal antibodies (mAbs) in intestinal BD were collected by survey using PubMed with the following key words: "intestine", "Behçet's disease", "anti-TNF", "infliximab" and "adalimumab". In addition, results of a questionnaire-based investigation on the actual treatment situation of intestinal BD by infliximab performed by the Japanese Study Group for a project on Research on Measures for BD operated by the Japanese Ministry of Health, Labour and Welfare in 2012 were referred to. During the second phase, expert panelists discussed areas of disagreement and areas of uncertainty regarding improvements of statements from the first edition and revised some of the clinical statements. During the third phase, the revised clinical statements were rated. Ratings of appropriate methods were developed using a modified Delphi approach, where members of the expert panel rated each part of the statements using a nine-point scale from 9 to 1 (9, strongly agree; 1, strongly disagree). Consensus was defined as a median score of ≥ 7, if the difference between the highest score and lowest score

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Table 1 Consensus statements for the diagnosis and management of intestinal Behçet's disease (second edition), by Research Committee for small bowel inflammation of unknown etiology, and Behçet's Disease Research Committee, Ministry of Health, Labour, and Welfare, Japan

Concept of the second edition of consensus statements

According to increased use of anti-TNFα mAb in inflammatory bowel disease, many cases of intestinal Behçet's disease in which anti-TNFα mAb (infliximab, IFX) showed efficacy also have been reported in Japan. The same tendency was observed in foreign countries that have a high prevalence of Behçet's disease, such as Korea. In 2013, adalimumab, humanized anti-TNFα mAb was approved for intestinal Behçet's disease in Japan. In the second edition, statements have focused on where we should place anti-TNFα mAb for the treatment of intestinal Behçet's disease based on relevant literature and expert panel discussion.[a]

Diagnosis

1. Diagnosis of intestinal Behçet's disease can be made if

   A. There is a typical oval-shaped large ulcer in the terminal ileum, OR

   B. There are ulcerations or inflammation in the small or large intestine, and clinical findings meet the diagnostic criteria of Behçet's disease.[b]

2. Acute appendicitis, infectious enteritis, tuberculosis, Crohn's disease, nonspecific colitis, drug-associated colitis and other diseases that mimic intestinal Behçet's disease should be excluded by clinical findings, radiology, and endoscopy before diagnosis of intestinal Behçet's disease is made.

Assessment of severity

Disease severity should be comprehensively assessed by systemic symptoms (e.g., fever, extra-intestinal manifestations), physical examinations of abdomen (e.g., pain, inflammatory mass, rebound tenderness), depth of ulcers and intestinal complications (e.g., bleeding, stricture, fistula), inflammatory mediators (e.g., CRP, WBC, ESR), and anemia.

Treatment objectives

In the treatment of intestinal Behçet's disease, as well as the improvement of abdominal and extra-intestinal symptoms, the achievement of negative levels of CRP could be desirable. In the long-term prognosis, the prevention of progression to disability and poly-surgery is important.

A. Standard treatment

1. In patients with severe symptoms (i.e., abdominal pain, diarrhea, gastrointestinal bleeding) and complications with deep ulcers confirmed by radiology or endoscopy, corticosteroids should be considered for induction therapy. The initial dose of corticosteroids is 0.5–1 mg/kg per day of prednisolone for l–2 weeks. When clinical improvement is observed, prednisolone should be tapered by 5 mg every week and finally stopped. ADA (approved on May 16, 2013 in Japan) could be considered for induction therapy [160 mg at 0 w, 80 mg at 2 w, 40 mg at 4 w, sub-cutaneously (s.c.)]. In responders, scheduled maintenance therapy should be considered (40 mg s.c. every other week). IFX (not approved yet) could also be considered for induction therapy (5 mg/kg at week 0, 2, and 6). In responders, scheduled maintenance therapy every 8 weeks should be considered. In patients with mild to moderate activity, mesalasine (5-ASA) could be effective for induction therapy. In patients treated with corticosteroids, anti-TNFα mAbs and immunomodulators, infectious disease and neoplasm should be surveyed. After initiation of these therapies, the risk of infectious disease and neoplasm should be monitored continuously.
2. In patients who are induced to clinical remission, 5-ASA and colchicine could be used for maintenance therapy. The optimal dose of 5-ASA for adult patients is 2.25–3 g/day. When sulfasalazine (SASP) is used, the optimal dose is 3–4 g/day.
3. Immunosuppressive agents such as azathioprine (AZA)[c] are indicated when patients are corticosteroid-dependent, corticosteroid-resistant, or anti-TNFα mAb-resistant. The initial dose of AZA is 25–50 mg/day. In patients treated with AZA, adverse effects (e.g., neutropenia and liver dysfunction) should be monitored.
4. Total parenteral nutrition (TPN) is indicated for patients with severe systemic symptoms such as fever and for patients with intestinal complications such as stenosis, fistula, bleeding, and impending perforation. TPN is also indicated for patients who cannot orally intake drugs due to severe oral or upper gastro intestinal lesions. It is usually used for a limited period of time considering the risk of catheter infection and thrombosis. After the patient's condition is improved by TPN, enteral nutrition (EN) could be considered.
5. EN using an elementary diet could be effective for induction therapy. It is indicated in particular for patients with refractory disease, severe activity, and disability such as stricture lesions. When EN is introduced, adherence and quality of life of the patients should be considered.
6. Surgery is indicated for patients in whom improvement is not expected by medications. Patients with severe stricture lesions, perforations, large abscesses, and massive gastrointestinal bleedings have an absolute indication. Patients refractory to medications, and with a low quality of life due to intestinal complications such as fistula, have a relative indication of surgery. Minimum length of resection surgery should be considered.
7. Risk of post-operative recurrence is high in patients with volcano shape deep ulcers and fistulas. Post-operative recurrence often occurs at anastomosis. Although a treatment strategy has not been established that can reduce the risk of post-operative recurrence, considering the high risk of post-operative recurrence and poly surgeries, medication by 5-ASA, immunomodulators, metronidazole, anti-TNFα mAb and EN could be considered for post-operative management.
8. In patients with intestinal Behçet's disease complicated with eye lesions, consultation with ophthalmologists is necessary for their management

B. Optional treatment

- Since there are some case reports showing that spraying of absolute ethanol via endoscope has efficacy for ulcers of intestinal Behçet's, it could be considered in refractory patients.

Table 1 continued

- Expecting the efficacy as an anti-rheumatoid arthritis drug, change from 5-ASA to SASP could be considered in patients with arthritis (especially peripheral arthritis).

The authors state that, (1) most of the consensus statements are based on expert opinions, (2) the consensus statements have not been endorsed by any organizations, (3) the consensus statements need to be prospectively reevaluated, (4) the consensus statements do not cover histopathological diagnosis, and (5) the consensus statements do not have any binding force.

[a] The majority of literature regarding anti-TNFα therapy in intestinal Behçet's disease that is referred to for establishment of the second edition described the efficacy of infliximab. On May 16 2013, ADA was approved for intestinal Behçet's disease. The clinical trial of infliximab in intestinal Behçet's disease is currently in progress in Japan.

[b] Diagnosis of Behçet's disease is according to the Japanese criteria proposed in 2003.

[c] Immunomodulators besides AZA, including 6-mercaptopurine, cyclosporine, tacrolimus and methotrexate could be considered, but consultations with specialists who have sufficient experience are required. When considering the use of these drugs, adverse effects should be monitored.

was <4. For the present study, an expert panel composed of gastroenterologists ($n = 6$), gastrointestinal surgeons ($n = 2$), and rheumatologists ($n = 2$) was established. In addition to the expert panel, a moderator (Hisamatsu, T.) and a professional adviser (Ueno, F.) were involved in the study. The moderator organized discussion by the expert panel and moderated the modified Delphi approach. The moderator searched and reviewed the literature and collected clinical statements. The professional adviser surveyed the process of the modified Delphi approach. The second edition of consensus statements proposed by the expert panel was discussed and then recognized by the Research Committee for small bowel inflammation of unknown etiology operated by a Health Labour Sciences Research Grant, Research on Measures for Intractable Diseases, Japan.

Results

Search for literature on intestinal BD and anti-TNFα mAbs

In the first phase, 15 relevant literature items were collected. This literature included 10 case reports, 3 retrospective analyses of more than one patient in a single institute, 1 letter to the editor, and 1 review article ("Appendix"). To date, no randomized controlled trials of anti-TNFα mAbs for the treatment of intestinal BD have been reported.

Development of the second edition of consensus statement

In the second phase, the expert panel discussed the place of anti-TNFα mAb for the treatment of intestinal BD. Based on the literature found, the clinical experience of experts and results of a questionnaire-based investigation, the expert panel agreed that anti-TNFα mAb treatment should be regarded as a standard therapy for intestinal BD, which was an optional treatment in the first edition. With the recognition of anti-TNFα mAb treatment as a standard therapy, the expert panel also discussed the therapeutic goal of intestinal BD. In the second edition, it was proposed that the achievement of negative levels of C-reactive protein (CRP) levels, in addition to the improvement of clinical symptoms, could be desirable as an objective therapeutic goal. The expert panel also proposed that improvement of long-term prognosis such as reducing the risk of surgery should be set as a final goal in the treatment of intestinal BD. Corticosteroid and anti-TNFα mAb were placed as standard therapies, while the expert panel deemed colchicines, thalidomide, endoscopic therapy, and leukocytapheresis to be experimental therapies.

In the first round of the modified Delphi approach, there were no statements with a median score <7. Although median scores were ≥7, three parts of statements did not obtain consensus because the difference between the highest and lowest score was 4. After discussion by the expert panel, the second round was performed, and then consensus was obtained for all statements. Thus, after a two-round modified Delphi approach, the second edition of consensus statements was finalized.

The authors' stated that limitations of the second edition included (1) most of the consensus statements are based on expert opinions, (2) the consensus statements have not been endorsed by any organizations, (3) the consensus statements need to be prospectively reevaluated, (4) the consensus statements do not cover histopathological diagnosis, and, (5) the consensus statements do not have any binding force.

Discussion

BD involves multiple organs, including the eye, nervous system, skin, genitalia, and gastrointestinal tract. About

3–16 % of patients with BD have gastrointestinal tract involvement [3], while most clinical studies of BD published to date concern the management of mucocutaneous lesions and ophthalmological lesions. However, intestinal BD often causes severe gastrointestinal complications, such as massive bleeding and perforation; therefore, intestinal lesions should be considered a poor prognostic factor. Even in high-prevalence areas such as Japan, Korea, the Middle East, and the Mediterranean region, intestinal BD has been treated empirically because data from the literature regarding management of this condition are scant. The consensus of expert opinion in a high-prevalence area should, therefore, be extremely helpful in daily practice. With this background, the first edition of a consensus for the management of intestinal BD was proposed for the first time in 2007 [5]. However, even after its proposal, conventional therapies have been insufficient for the management of intestinal BD. In the current clinical setting, anti-TNFα mAbs have been used to treat patients with intestinal BD. Reports demonstrating the efficacy of anti-TNFα mAbs for the management of intestinal BD are increasing. Furthermore, ADA was approved for intestinal BD in 2013 after an open-label clinical trial in Japan. With this in mind, it was considered that the first edition of the consensus statement should be updated.

The first edition was established in 2007 by the Japanese Inflammatory Bowel Disease Research Group. In 2011, the Research Committee for small bowel inflammation of unknown etiology was established independently from the Japanese Inflammatory Bowel Disease Research Group. To avoid changes in expert panel members affecting the results, some members of the first edition joined the expert panel of the second edition, which also had discussions with the Behçet's Disease Research Committee as well as the first edition expert panel. Finally, the second edition was evaluated and approved by the Research Committee for small bowel inflammation of unknown etiology composed of experts for gastrointestinal disorders including members of the first edition.

The modified Delphi approach used in the second edition also provided panelists with the opportunity to discuss their judgments between the rating rounds as well as in the first edition. Unfortunately, there is not much evidence for the management of intestinal BD. Therefore, the discussion by the expert panel must make practical consensus statements rather than be a simple rating method. In the process for improving the second edition of the consensus statement, several subjects were discussed. First, the expert panel discussed the validity of the efficacy of anti-TNFα mAb therapy in intestinal BD. To date, no clinical trial for anti-TNFα mAb therapy in intestinal BD with high-quality evidence such as a double-blind, randomized, placebo-controlled trial has been reported. Therefore, the expert panel relied on their clinical experience and clinical case reports. All members agreed that anti-TNFα mAb therapy is effective for intestinal BD. Second, the expert panel discussed where anti-TNFα mAb therapy should be placed in the treatment of intestinal BD. Although anti-TNFα mAb therapy was considered an option therapy in the first edition in 2007 [5], the expert panel recommended anti-TNFα mAb as a standard therapy in the second edition. Third, according to the recommendation of anti-TNFα mAb as a standard therapy, the expert panel discussed whether the goals for medication of intestinal BD should be addressed. The expert panel was concerned about the overuse of anti-TNFα mAb without any objective parameters. Unfortunately, practical clinical activity indexes for intestinal BD (e.g., Crohn's disease activity index for Crohn's disease) have not been established. Endoscopic mucosal healing was also discussed, but it was not agreed on because of the lack of evidence in the literature and an impractical setting. Although evidence that CRP is a practical biomarker to assess disease activity of intestinal BD is insufficient, several reports suggested that CRP could reflect disease activity and disease prognosis [17]. In addition, in Crohn's disease, negative CRP levels are considered a therapeutic goal as well as endoscopic mucosal healing by biologics therapy. In this context, the expert panel proposed "treatment objectives" that were not in the first edition and recommended the monitoring of CRP.

The problems that now confront us are the safety monitoring of anti-TNFα mAb use and the determination of whether anti-TNFα mAb treatment can improve the long-term prognosis of intestinal BD by prospective observation.

Conclusions

The second edition of consensus statements for the diagnosis and management of intestinal BD was established. In the second edition, anti-TNFα mAb treatment was recognized and recommended as a standard therapy for the treatment of intestinal BD.

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Conflict of interest Tadakazu Hisamatsu received a research grant from Ajinomoto Pharmaceuticals CO., LTD. and received lecture fees from Abbvie.

Appendix: literature list of intestinal Behçet's disease and anti-TNFα mAbs treatment

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TGF-β1 in Tumor Microenvironments Induces Immunosuppression in the Tumors and Sentinel Lymph Nodes and Promotes Tumor Progression

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Summary: In cancer patients, sentinel lymph nodes (SLNs) are crucial in the induction of antitumor T cells. However, in many cases, SLNs and tumors appear to be in immunosuppressive condition through mechanisms yet to be elucidated. In this study, the role of tumor-derived TGF-β1 in the generation of immunosuppressive microenvironments of tumors and SLNs was investigated. Murine colorectal carcinoma CT26 transduced with TGF-β1 cDNA (CT26-TGF-β1) showed enhanced tumor growth compared with mock-transduced CT26 (CT26-Mock) when implanted in syngeneic Balb/c mice, even though CT26-TGF-β1 shows slower growth in vitro. This enhanced growth was not observed when implanted in immunodeficient mice, suggesting that TGF-β1 enhanced tumor growth by suppressing antitumor T-cell responses. Analysis of immune cells in CT26-TGF-β1-implanted mice revealed impairment of dendritic cells (DCs), decrease of $CD8^+$ T cells, and increase of MDSCs and Tregs in the tumors. Similarly, the SLNs of these mice showed an increase of MDSCs, Tregs, and PD-$L1^+$ DCs, and decrease of T-cell stimulatory activity of DCs accompanied by decreased CD80 expression and TNF-α production. In addition, induction of tumor antigen-specific T cells from SLNs of the CT26-TGF-β1–implanted mice was significantly reduced. These results demonstrate that overproduction of TGF-β1 is critical for the generation of immunosuppressive microenvironments in both tumors and SLNs, which may result in suppression of spontaneous antitumor $CD8^+$ T-cell responses. Therefore, TGF-β1 is an attractive target for restoration of immunosuppressive condition in cancer patients.

Key Words: TGF-β, cancer immune evasion, immunosuppression, sentinel lymph node

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Immunologic conditions, particularly infiltration of $CD8^+$ T cells in tumors, have recently been reported to be different among patients and correlate with prognosis and treatment response of patients.[1–5] However, the mechanisms of the differential immune status among cancer patients have not been well investigated. Sentinel lymph nodes (SLNs) are the lymph nodes where lymphatic flow from a tumor first reaches, and are important lymphoid organs where dendritic cells (DCs) loaded with tumor antigens activate naive T cells to become antitumor effector $CD8^+$ T cells. Therefore, immunologic evaluation of both tumors and SLNs is important to understand immunopathology of tumor microenvironments.

Immunohistochemical studies of various immune cells, such as DCs, myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), and $CD8^+$ T cells, demonstrate that SLNs are generally immunologically suppressed in patients with melanoma and breast cancers, although some reports show activation of DCs (eg, increased expression of CD83 on DCs).[6–11] In patients with colorectal carcinoma, the immunosuppressive status of SLNs has also been reported to correlate with disease progression.[12] As lymphatic fluid from the tumor contains a variety of biologically active molecules, including immunosuppressive cytokines produced by cancer cells and tumor-infiltrating cells, immunologic condition of SLNs is thought to be highly influenced by the characteristics of primary cancer tissues even before cancer cells metastasize to the lymph nodes.[13] However, the precise mechanisms involved in immunosuppression of SLNs with regard to its relationship with tumor microenvironments have not been well investigated.

In this study using CT26 mouse colon cancer model with particular focus on TGF-β1, which is produced by most cancer cells and some tumor-infiltrating cells such as MDSCs and Tregs, we have performed comprehensive immunologic analysis of the microenvironments of SLNs and tumor tissues. We demonstrate that overexpression of TGF-β1 by tumor cells induces immunosuppressive condition not only in the tumor tissues, but also in the SLNs through impairment of T-cell stimulatory activity of DCs and increase of immunosuppressive MDSCs and Tregs. These immunosuppressive changes lead to the suppression of antitumor T-cell induction, decreased accumulation of $CD8^+$ T cells, and enhanced tumor growth. Here, we illustrate the importance of TGF-β1 pathway in the formation of tumor-associated immunosuppressive microenvironments in the tumors and SLNs as observed in patients with various cancers, and emphasize again that the TGF-β1 pathway is an attractive target for restoration of immunologic condition of cancer patients.

MATERIALS AND METHODS

Animals and Cell Lines

Balb/c and NOD/SCID mice of 6–8 weeks of age were bred at the animal facilities of Keio University following the guidelines for animal experimentation. CT26, a murine

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colorectal carcinoma cell line, was purchased from American Type Culture Collection and maintained in RPMI1640 with 10% FBS and 1% penicillin.

Preparation of Lentiviral Vectors for TGF-β1 Overexpression

HIV lentiviral vectors for TGF-β1 expression were prepared as previously described.[14] cDNA of murine TGF-β1 (NCBI reference sequence ID, BC013738) was purchased from Thermo and inserted into the multiple cloning sites (MCS) of self-inactivating (SIN) vector, pCSII-CMV-MCS-internal ribosomal site (IRES)-puroR-PRE. CT26 cells were infected with the TGF-β1-expressing HIV vector or the MOCK-puroR HIV vector for control and subjected to puromycin selection (20 μg/mL).

Cell Proliferation Analysis

Cells were seeded (2×10^3 cells/100 μL/well) into 96-well plates. On day 2, 20 μL of Cell Titer AQueous One Solution Reagent (Promega, WI) was added and incubated for 2 hours at 37°C. Optical densities were read by a microplate reader scanning at 450 nm.

Tumor-bearing Mouse Models and SLN Identification

Balb/c or NOD/SCID mice were inoculated SC with TGF-β1 or Mock-transduced CT26 cells in the right hip (Balb/c, n = 6; NOD/SCID, n = 5). The tumor size was measured according to the following formula, Volume = $0.5 \times (\text{width})^2 \times (\text{length})$. Ten days after the tumor inoculation, SLNs were identified by the injection of indigo carmine blue dye along the tumor. The right inguinal lymph nodes were collected as the SLN and the left axilla lymph nodes were used as the non-SLN. We checked tumor micrometastases in SLNs by detecting puromycin-resistant gene within the CSII-CDF-MCS-PRE vector by RT-PCR. The primer sequences used to amplify *Streptomyces alboniger* puromycin N-acetyltransferase (pac) gene mRNA were 5′-GTCGGGCTCGACATCGGCAA-3′ (forward) and 5′-TCC ATCTGTTGCTGCGCGGC-3′(reverse). β-actin [5′-GAT TACTGCTCTGGCTCCTA-3′ (forward) and 5′-GACTCA TCGTACTCCTGCTT-3′ (reverse)] was used as an internal control.

Cytokine Measurement

Cytokines were quantified in cell culture supernatant and serum by ELISA using the following commercial kits: human TGF-β1 [Becton, Dickinson and Company (BD), NJ], mouse IFN-γ (BD), mouse TNF (BD), and mouse IL12 (BD). For measuring TGF-β1 in the cell culture supernatant, cells were seeded at 2×10^5 cells/2 mL/well in 6-well plates, incubated for 48 hours. To activate TGF-β1, samples were incubated with 1 N HCl (1:25) for 1 hour at 4°C and neutralized with 1 N NaOH (1:25) before they were used for the assay.

Flow Cytometry

Tumor tissues were digested in RPMI1640 medium containing 1 mg/mL of collagenase, 2.5 U/mL of hyaluronidase, and 0.1 mg/mL of DNase for 90 minutes at 37°C. Cells from tumor tissues or lymph nodes were stained with fluorescein-conjugated monoclonal antibodies (Abs) for 1 hour and analyzed using Gallios (Beckman Coulter Inc., CA). The Abs used in this study are as follows: CD45-APC-Cy7, CD11c-PECy7, CD80-FITC, CD86-FITC, I-Ad-PE, PDL1-PE, PDL2-APC, CD4-PECy5.5, CD8a-FITC, CD3e-PB, CD25-PECy7, CD11b-FITC, Gr1-PECy5.5 (BD), CD80-APC (Bio Legend, CA), and Foxp3-PE (eBioscience, CA). Specificity of the Abs was confirmed by isotype-matched monoclonal Abs. For the intracellular Foxp3 staining, we used FOXP3 Fix/Perm Buffer Set (BioLegend).

Isolation of DCs From SLNs and Assay for TNF-α Production

Ten days after the tumor inoculation, SLNs and non-SLNs from each tumor-bearing mouse were resected. DCs were positively selected using CD11c Microbeads (Miltenyi Biotec, CA) and were stimulated with 1 μg/mL LPS for 18 hours and the production of TNF-α was measured by ELISA.

Mixed Leukocyte Reaction

T cells were positively selected from normal Balb/c mice splenocytes using CD90.2 (Thy1.2) Microbeads (Miltenyi Biotec). DCs were also prepared as previously described and irradiated by 32 Gy. The 2×10^5 T cells were cocultured for 4 days with anti-mouse CD3e mAbs (BD) and 2×10^4 CD11c cells from SLNs or non-SLNs. After 4-day coculture, IFN-γ production from T cells in the culture supernatants was determined by ELISA.

Detection of Tumor-specific T-Cell Responses

Six days after the tumor inoculation, 0.5 KE Picibanil (OK432) (Chugai Pharm, Tokyo, Japan) was injected intratumorally. Eight days after OK432 injection, whole cells from SLNs or non-SLNs were cultured in RPMI 1640 with 10% FBS and restimulated with 1 μg/mL AH-1 peptide (the self-tumor Ag-derived immunodominant T-cell epitope, $gp70_{423-431}$) for 8 days. Then, T cells were collected using Lymphosepar II (Immuno Biological laboratories, Japan) and cocultured in a 96-well plate in the presence of AH-1 peptide (0, 0.04, 0.2, and 1 mg/mL) with syngeneic splenocytes which had been treated with 10 μg/mL mitomycin C for 2 hours at 37°C and been washed (T cell 2×10^5 cells, splenocytes 1×10^6 cells/200 μL/well). After 24 hours, the concentration of IFN-γ was measured by ELISA.

Statistics

The statistical significance between 2 groups was determined by the Student *t* test. *P* values of <0.05 were considered statistically significant.

RESULTS

TGF-β1 Produced in Tumor Microenvironments Enhances In Vivo Tumor Growth Through Immunologic Mechanisms

To investigate the role of tumor-derived TGF-β1 in the tumor tissues and SLNs, a murine colorectal carcinoma cell line CT26 producing high murine TGF-β1 was generated by lentiviral-mediated stable transfection of cDNA-encoding TGF-β1, and implanted in syngeneic Balb/c mice. TGF-β1 produced in vitro by TGF-β1-transduced CT26 (CT26-TGF-β1) was approximately 10 times higher than that produced by control (CT26-MOCK) (Fig. 1A). Even when 1×10^6 CT26-MOCK or CT26-TGF-β1 cells were implanted in Balb/c mice, the serum TGF-β1 levels were not different from that of mice without tumors, suggesting that tumor-derived TGF-β1 may not have systemic effects in this mouse model (Fig. 1B). Although in vitro cell proliferation of CT26-TGF-β1 was slower than CT26-MOCK

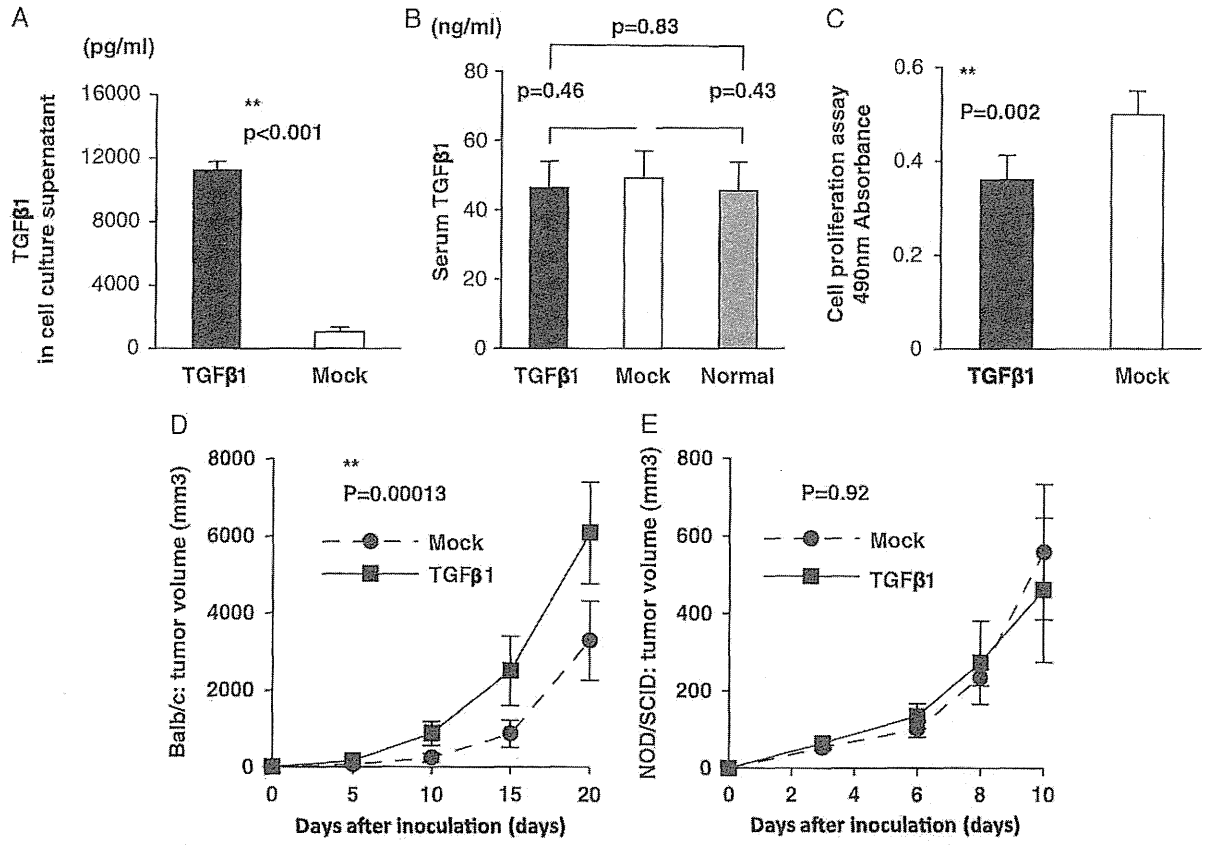

FIGURE 1. In vivo enhanced growth of TGF-β1-overexpressed CT26 only when inoculated in syngeneic mice. A and B, The production of TGF-β1 detected in the culture supernatants (A) and sera from CT26-MOCK-bearing or CT26-TGF-β-bearing mice (B). C–E, In vitro (C) and in vivo growth (D; Balb/c, E; NOD/SCID) of CT26-MOCK and CT26-TGF-β1. Balb/c or NOD/SCID mice were inoculated SC with 1×10^6 (B and D) or 5×10^5 (E) TGF-β1 or Mock-transduced CT26 cells in the right hip (Balb/c, n = 6; NOD/SCID, n = 5). The tumor size was measured according to the following formula, Volume = 0.5 × (width)^2 × (length). The data are representative of 3 (A, B, D, and E) or 2 (C) independent experiments and shown as average ± SD.

(Fig. 1C), the tumors of CT26-TGF-β1 grew significantly larger than CT26-MOCK when implanted in Balb/c mice (Fig. 1D). In contrast, the in vivo growth was not different between CT26-TGF-β1 and CT26-MOCK when implanted in NOD/SCID mice (Fig. 1E), indicating that TGF-β1 produced by tumor cells enhanced in vivo growth of CT26 tumor cells through immunologic mechanisms, such as suppression of antitumor T-cell responses.

Immunosuppression by Tumor-derived TGF-β1 in Primary Tumor Tissues

We then evaluated immunologic effects of TGF-β1 overproduction in primary tumor tissues by analyzing DCs, $CD8^+$ T cells, MDSCs, and Tregs. To avoid the effects of tumor sizes on immune status, we analyzed tumors and SLNs at day 10 after implantation of 5×10^5 tumor cells, when no significant difference of tumor growth was yet observed between CT26-TGF-β1 and CT26-MOCK groups (Fig. 2A). In the CT26-TGF-β1 tumor tissues, the number of $Gr1^+CD11b^+$ MDSCs and the percentage of $CD4^+$ $CD25^+FoxP3^+$ Tregs in $CD4^+$ T cells were significantly increased (Figs. 2B, C). In contrast, the number of DCs was significantly decreased (Fig. 3A). The expression of MHC class II in $CD11c^+$ $CD45^+$ DCs and the percentage of the high MHC class II and CD80-expressing $CD11c^+$ DCs among tumor tissues were significantly different between CT26-TGF-β1 and CT26-MOCK (Figs. 3B, C). Furthermore, significantly less infiltration of $CD8^+$ T cells was observed in the CT26-TGF-β1 tumor tissues (Fig. 3D). These results indicate that tumor-derived TGF-β1 generates immunosuppressive conditions in primary tumor tissues by both decreasing DCs with high CD80 and MHC class II expression and increasing immunosuppressive cells including Tregs and MDSCs, and may subsequently suppress spontaneous $CD8^+$ T-cell response in the tumor microenvironment.

Tumor Microenvironments With TGF-β1 Overproduction Increases MDSCs and Tregs in SLNs

We then evaluated whether tumor-derived TGF-β1 produced in the tumor microenvironments has similar immunosuppressive effects on SLNs. It was confirmed that there was no tumor metastasis in the SLNs as no RT-PCR amplification of puromycin resistance gene expressed in the CT26-TGF-β1 and CT26-MOCK was observed (data not shown). We identified SLNs as described in the Materials and methods section. In the CT26-TGF-β1-bearing mice, the number of $Gr1^+CD11b^+$ MDSCs and the percentage of $CD4^+$ $CD25^+FoxP3^+$ Tregs in $CD4^+$ T cells in SLNs significantly increased compared with those in CT26-MOCK-bearing mice, similar to the observations in

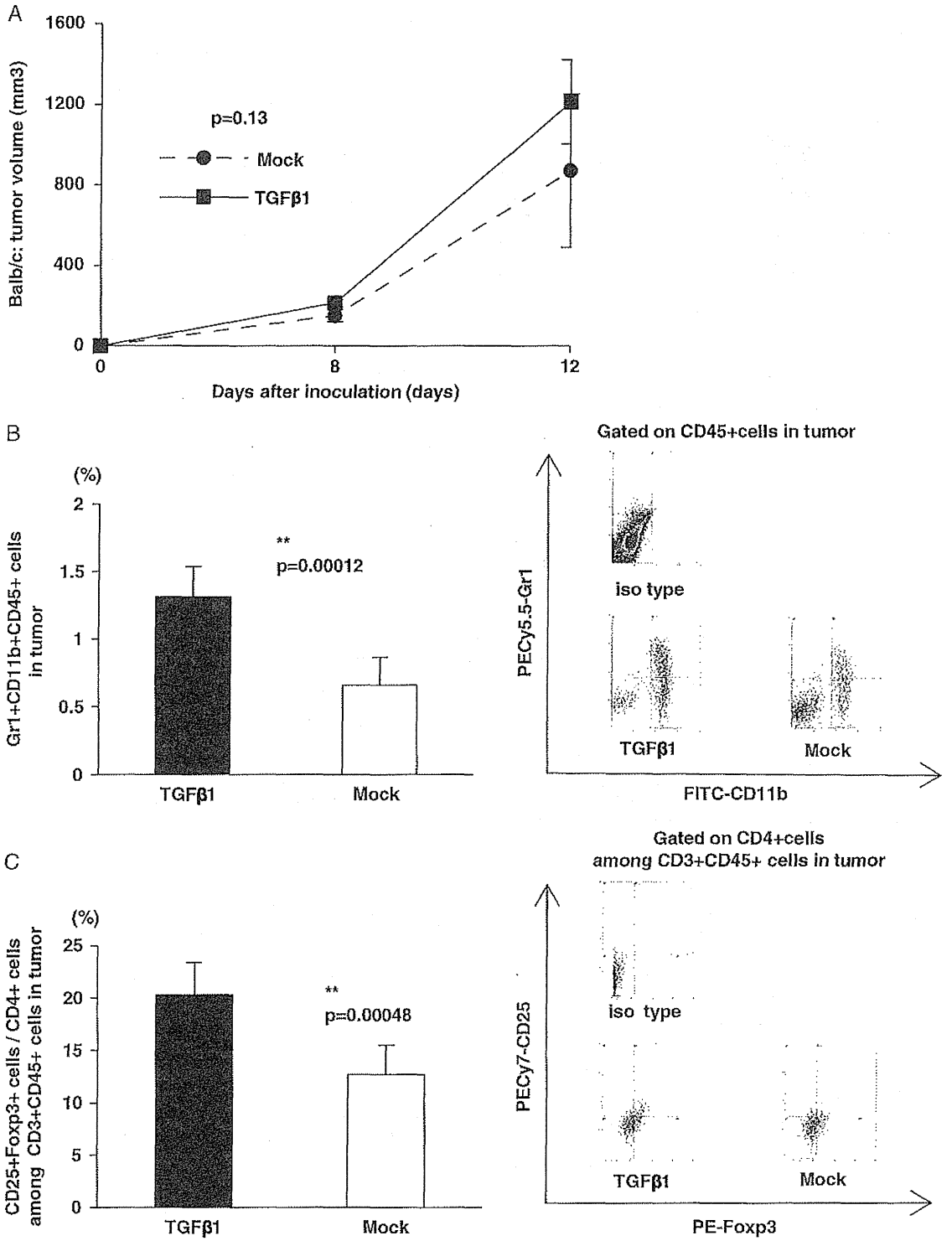

FIGURE 2. Tumor-derived TGF-β1 induces the immunosuppressive microenvironments by increasing MDSCs and Tregs. Six Balb/c mice were inoculated SC with 5×10^5 TGF-β1 or Mock-transduced CT26 cells in the right hip. At day 12, the tumor growth was not significantly different between CT26-MOCK and CT26-TGF-β1 (A). In addition, we selected the timing of FACS analysis as 10 days after inoculation to evade the direct effects of tumor size to SLNs. The infiltrations of MDSCs (B) and Tregs (C) within primary tumor tissues (CT26-MOCK and CT26-TGF-β1) were analyzed by FACS at day 10. The percentage of $CD45^+Gr1^+CD11b^+$ MDSCs in tumor sites increased in CT26-TGF-β1-bearing mice (B). The percentage of Tregs among $CD4^+$ cells in tumor sites was increased in CT26-TGF-β1-bearing mice (C). The data are representative of 2 independent experiments and shown as average ± SD. Corresponding FACS dot plots are shown for each bar graph.

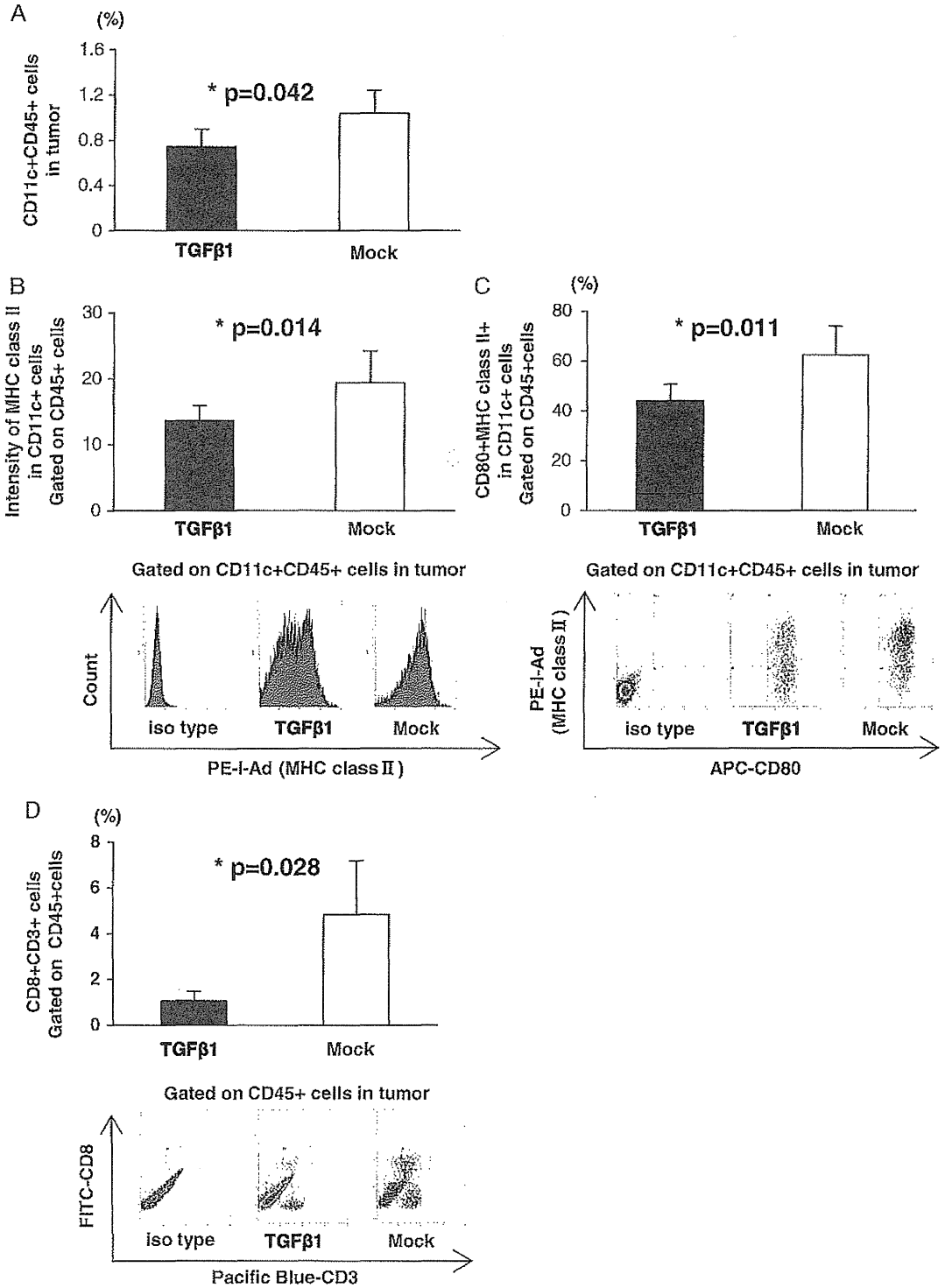

FIGURE 3. Tumor-derived TGF-β1 suppresses DC infiltration and expression level of MHC class II and CD80 among DCs, and decreases $CD8^+$ T cells in primary tumor sites. The phenotypes of tumor-infiltrating DCs were analyzed by FACS at day 10. A, CT26-TGF-β1-bearing mice show the significantly less number of tumor-infiltrating DCs. B, The expression level of MHC class II among $CD45^+$ $CD11c^+$ DCs in tumor site was lower in CT26-TGF-β1-bearing mice. C, The proportion of high MHC class II and $CD80^+$ cells among $CD45^+CD11c^+$ DCs in tumor sites was lower in CT26-TGF-β1-bearing mice. D, The percentage of $CD3^+CD8^+$ T cells among $CD45^+$ cells in tumor sites was significantly decreased in CT26-TGF-β1-bearing mice. The data are representative of 2 independent experiments and shown as average ± SD. Representative FACS dot plots and histogram are shown for each bar graph.