JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 50 (1): 21–28, 2017
doi: 10.1267/ahc.16035

Frequency of CD4+CD161+ T Cell and Interleukin-10 Expression in Inflammatory Bowel Diseases

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Received October 21, 2016; accepted December 5, 2016; published online February 22, 2017

Mucosal immune dysregulation associated with T cells plays a critical role in the development of inflammatory bowel diseases (IBD). However, the definite significances of these cells in IBD still remain unclear. Therefore, we investigated the population and expression of $CD4^+CD161^+$ T cells in the colonic lamina propria mononuclear cells (LPMCs) in patients with IBD by analyses using flow cytometry and immunohistochemistry. Interleukin-10 (IL-10) mRNA levels in both LPMCs and $CD4^+$ T cells in lamina propria (LP-$CD4^+$ T cells) were measured using a real-time quantitative reverse transcription-polymerase chain reaction. IL-10 production was investigated with immunohistochemistry. The results revealed that the population of $CD4^+CD161^+$ T cells was significantly decreased in active ulcerative colitis (UC) compared with inactive UC ($P < 0.05$). The $CD4^+CD161^+$ T cell population was inversely correlated with disease activity in patients with UC ($r = -0.6326$, $P = 0.0055$), but there was no significant correlation in those with Crohn's disease. Over-expression of IL-10 mRNA in both LPMCs and LP-$CD4^+$ T cells were detected in active UC. Immunohistochemistry revealed decreased frequency of $CD161^+$ cells and increased IL-10 positive cells in active UC. The frequency of $CD4^+CD161^+$ T cells and IL-10 expression was supposed to be associated with the pathological status of mucosal immunoregulation in IBD.

Key words: $CD4^+CD161^+$ T cell, IL-10, inflammatory bowel diseases, immunohistochemistry

I. Introduction

The development and causes of inflammatory bowel diseases (IBD) are extensively affected by genetic susceptibility, intestinal microbiota, environmental factors, and host immune dysregulation, all of which are interrelated [3, 21]. However, the exact etiology and pathogenesis of IBD remains unclear. The clinical features of the two major forms of IBD, ulcerative colitis (UC) and Crohn's disease, differ to some extent [16], but both exhibit inappropriate and ongoing activation of the mucosal immune system [21]. T lymphocytes play an important role in the production of proinflammatory cytokines and in immunoregulatory function.

CD161 encoded by *KLRB1* (human killer cell lectin-like receptor subfamily B, member 1) is a C-type lectin-like receptor that exhibits both activating and inhibitory function in natural killer (NK) cells [22, 23] and is expressed on invariant natural killer T (NKT) cells and mucosal-associated invariant T (MAIT) cells, which are involved in IBD [7, 9]. Except for this, CD161 is detected in 20% of peripheral T cells [18], particularly in $CD4^+$ T cells in the lamina propria in human intestine [20], where a more frequent population exists in the lamina propria than MAIT

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Table 1. *Characteristics of patients*

	Patients with UC $n = 26$	Patients with CD $n = 28$	Controls $n = 29$
Age (years)	10–77 (49.5)	19–60 (34.5)	32–79 (65.4)
Male/female	18/8	18/10	18/11
Months of disease duration	5–432	18–300	(—)
(mean ± SD)	(105.5 ± 106.6)	(126.9 ± 87.0)	
Localization	23/2/1[†]	12/2/13/1[‡]	
Medications			
Corticosteroids	20	7	
5-ASA	21	19	
Immunosupressive agents	13	7	
Infliximab	0	11	
UCDAI (score: 0–4/5–8/9–12)	6/8/12	not applicable	
CDAI (remission/ active)	not applicable	13/15	

[†] Number of patients with extensive or pancolitis/left-sided colitis/proctitis.
[‡] Definitions of disease location according to the Vienna classification of Crohn's disease as follows: Location: L1 (terminal ileum)/L2 (colon)/L3 (ileocolon)/L4 (upper gastrointestinal).
Abbreviations: UC, ulcerative colitis; UCDAI, ulcerative colitis disease activity index; CD, Crohn's disease; CDAI, Crohn's disease activity index.

cells in the epithelium [9].

CD4^{+}CD161^{+} T cells possess the potential to influence immunoregulatory and proinflammatory functions by producing interleukin (IL)-10 and interferon (IFN)-γ in thymocytes, and IL-17 in Crohn's disease and rheumatoid arthritis [1, 12]. However, the function of these cells is still unresolved especially in UC. In general, IL-10 is defined as an immunoregulatory cytokine that closely downregulates the production of proinflammatory cytokines. Important evidence of the immunoregulatory role of IL-10 was provided by IL-10 knockout mice who developed chronic enterocolitis [14]. On the other hand, over-expression of IL-10 mRNA is revealed paradoxically in the active inflammatory site of UC [2, 17].

By these previous results, we hypothesized that the population of CD4^{+}CD161^{+} T cells and IL-10 production may affect the intestinal inflammation in patients with IBD, although the functional role of IL-10 was still controversial as far as its association with immune responses in IBD was concerned. Therefore, in the present study, we investigated the expression and population of CD4^{+}CD161^{+} T cells in the lamina propria and its association with IL-10 production in patients with IBD.

II. Materials and Methods

Patients

Patients' demographics and history of drug therapy are summarized in Table 1. We enrolled 83 patients with IBD, colorectal carcinoma, and benign conditions who underwent surgery at the Faculty of Medicine, University of Miyazaki Hospital from January 2007 to December 2008. Patients suffering from UC were judged by clinical and endoscopic symptoms according to the Ulcerative Colitis Disease Activity Index (UCDAI) [25] and remission was defined as UCDAI score 0 or 1, with maximum score in severe disease was 12. The disease status of patients with Crohn's disease was estimated using the Crohn's Disease Activity Index (CDAI) [8] and remission was defined as CDAI ≤150, with active disease defined as >150.

The study protocol was reviewed and approved by the Ethics Committee of Miyazaki University (authorization number 2006-271) and conforms to the provisions of the World Medical Association's Declaration of Helsinki in 1995 (as revised in Tokyo 2004). Informed consent was obtained from all patients before their entry into this study.

Preparation of lamina propria mononuclear cells

Lamina propria mononuclear cells (LPMCs) were isolated from macroscopically inflamed and noninflamed mucosa of freshly obtained surgical specimens from patients with IBD. More than one part of the specimen was taken to investigate the active inflammatory site and inactive inflammatory site. All specimens were taken from resected colonic material from patients with UC and Crohn's disease. The grade of inflammation was estimated at this time using hematoxylin and eosin staining. Grossly normal tissue was taken at least 5 cm proximal to or distal from any macroscopically detectable lesions from control patients according to the method of Fuss *et al.* [5]. LPMCs were isolated using a modification of the method of Bull and Bookman [4]. Briefly, mucosal strips were washed in Hanks' balanced salt solution without calcium and magnesium (HBSS-CMF, Life Technologies, Japan Ltd.) containing 1 mmol/L of dithiothreitol for 30 min at room temperature to remove mucus. The epithelium was removed using sequential washes in HBSS-CMF containing 1 mM EDTA, and the mucosal strips were incubated for 8 hr at 37°C in HBSS-CMF containing 0.2 mg/mL purified type 3 collagenase (Worthington Biochemical Corp., Freehold, NJ, USA), 0.2 mg/mL deoxyribonuclease I (Worthington Biochemical), 250 U/mL penicillin, 250 µg/mL streptomycin,

and 2.5 mM HEPES buffer (Life Technologies Japan, Ltd.). T cells were isolated by filtering cell suspensions through a Nylon Fiber (Wako Pure Chemical Industries, Ltd., Osaka, Japan) column made in our laboratory. The supernatant was pelleted and resuspended in HBSS-CMF containing RPMI medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and layered on a Ficoll-Paque PLUS solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and density gradient centrifugation was performed for 5 min at 2,100 × *g*. Trypan blue-exclusion analysis indicated that the final LPMCs preparations contained greater than 95% viable cells. For magnetic sorting of $CD4^+$ T cells from LPMCs, we used 20 µl of CD4 MicroBeads per $\leq 10^7$ LPMCs, MS column and VarioMACS™ Separator (Miltenyi Biotec, Auburn, CA), according to manufacturer's protocol. Although the number of samples between all resected specimens and measurement for the flow-cytometric analysis were not matched, LPMCs were investigated by flow cytometry only when they were obtained in sufficient cell numbers from the specimen to investigate. Otherwise, LPMCs were investigated only by real-time PCR analysis.

Flow cytometric analysis

LPMCs were analyzed using three-color flow cytometry methods according to the protocol published by Ikeda *et al.* [10]. Briefly, freshly isolated cells (1×10^6 cells/mL) were incubated for 30 min on ice with the antibodies, washed with PBS, and then analyzed using a BD FACSCalibur system with CellQuest software (Becton Dickinson, San Jose, CA, USA). Antibodies were used as follows: fluorescein isothiocyanate (FITC)-labeled anti-CD4 (clone RPA-T4) and phycoerythrin (PE)-labeled anti-CD3 (clone UCHT1) were purchased from Bay Bioscience Co., Ltd. Allophycocyanin (APC)-labeled anti-CD161 (clone DX12) was purchased from Becton Dickinson.

RNA isolation and Real-time PCR analysis

LPMCs were diluted in RLT lysis buffer (included in the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany)) containing 1% β-mercaptoethanol and stored at −80°C before RNA isolation. Total RNA was extracted using the RNeasy Plus Mini Kit. Five hundred micrograms of RNA was reverse transcribed using the ReverTra-Ace cDNA synthesis Kit (Toyobo, Osaka, Japan) at 25°C for 10 min and then at 37°C for 120 min with an iCycler (Bio-Rad, Hercules, CA, USA). The cDNA (diluted 1:50) was amplified using probe and primer sets of TaqMan Gene Expression Assays (Life Technologies). TaqMan Universal PCR Master Mix, No AmpErase UNG Kit was used with the primers as follows: IL-10, Hs00174086_m1; *ACTB* (encoding β-actin), Hs99999903_m1. Using an ABI Prism 7000 system (Life Technologies), amplification was performed for 50 cycles, including denaturation at 95°C for 15 s and primer annealing and extension at 60°C for 1 min. A specific RNA-copy standard was prepared from peripheral blood mononuclear cells treated with anti-CD3 monoclonal antibody (clone UCHT 1) and anti-CD28 monoclonal antibody (clone CD28.2) stimulation (eBioscience, San Diego, CA, USA) for 48 hr. Assays were performed in triplicate. Results are expressed as cytokine mRNA copies per *ACTB* unit to estimate the average IL-10 mRNA content.

Immunohistochemistry

Paraffin-embedded tissues were cut into 4 µm-thick sections. The sections were deparaffinized with toluene and rehydrated through graded ethanol series. To retrieve antigens for anti-KLRB1 (CD161) rabbit polyclonal antibody (Atlas Antibodies, Bromma, Sweden; product number HPA039113), specimens were autoclaved at 120°C for 15 min in 10 mM citrate buffer, pH 6.0 [24]. For anti-CD4 rabbit monoclonal antibody (Abcam, Cambridge, MA, USA; product number ab133616), we used Tris-EDTA (pH 9.0) according to manufacturer's protocol. For anti-IL-10 mouse monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany, product number sc-8438), we used microwave at 95°C for 10 min in Tris-EDTA (pH 9.0). After inhibition of endogenous peroxidase activity with 3% H_2O_2 in methanol for 20 min, the sections were preincubated with normal goat serum and 5% BSA in PBS for 1 hr to block non-specific binding of antibodies. Unless otherwise specified, all reactions were conducted at room temperature. The sections were reacted primarily with 0.56 µg/ml of anti-CD4 rabbit monoclonal antibody, 8 µg/ml of anti-IL-10 mouse monoclonal antibody, and 10 µg/ml of anti-CD161 rabbit polyclonal antibody overnight, respectively. Following this, they were reacted with HRP-goat anti-rabbit IgG or HRP-goat anti-mouse IgG for 1 hr. The HRP sites were visualized with 3-amino-9-ethylcarbazole (AEC) or 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin [19]. As a negative control, normal mouse or rabbit IgG was used at the same concentration instead of the primary antibodies in every experiment.

Statistical analysis

Nonparametric statistics were applied. The Mann-Whitney rank sum test was used to detect differences between unpaired groups. Correlation was tested with Spearman's rank correlation coefficient. $P < 0.05$ was considered significant. Analyses were performed using GraphPad Prism 5.0 (MDF Co., Ltd., Japan).

III. Results

Population of $CD4^+CD161^+$ T cells is reduced in patients with active UC and Crohn's disease

We investigated the population of $CD4^+CD161^+$ T cells on CD3-gated T cells both in the active and inactive inflammatory site of UC, Crohn's disease and controls (Fig. 1A). The terms active and inactive indicate active and inactive inflammatory sites in the patients. The population of $CD4^+CD161^+$ T cells was significantly decreased in patients with active UC (11.0 ± 5.07%) and Crohn's disease

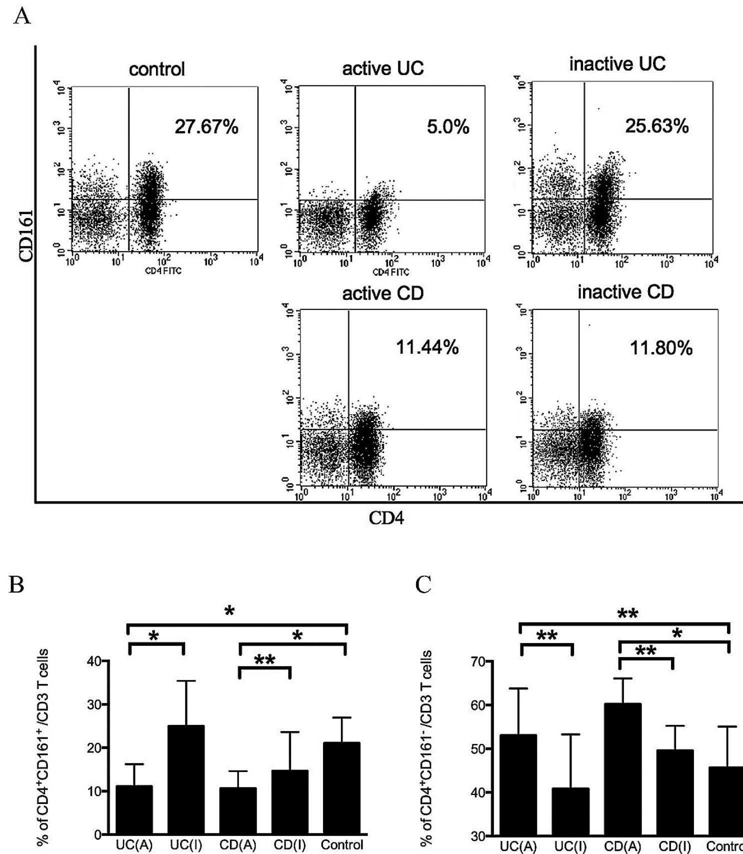

Fig. 1. Representative flow cytometric analysis of $CD4^{+}CD161^{+}$ T cells (**A**) and frequency of $CD4^{+}CD161^{+}$ T cells (**B**) and $CD4^{+}CD161^{-}$ T cells (**C**) in patients with active and inactive UC and Crohn's disease. $CD4^{+}CD161^{+}$ T cells were gated on $CD3^{+}$ T cells. The percentage of $CD4^{+}CD161^{+}$ T cells is shown in each upper-right quadrant (**A**). T cells were derived from active UC (UC (A), $n = 17$), inactive UC (UC (I), $n = 7$), active Crohn's disease (CD (A), $n = 9$), inactive Crohn's disease (CD (I), $n = 6$), and controls ($n = 14$) (**B, C**). The y-axis indicates the population of $CD4^{+}CD161^{+}$ T cell. Columns represent averages, and error bars represent standard deviation. Mann-Whitney rank sum test was applied. $*P < 0.05$, $**P$ = not significant.

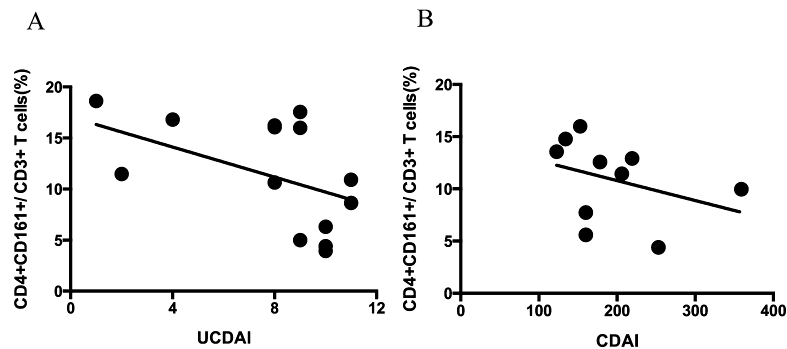

Fig. 2. Correlation between disease activity and population of $CD4^{+}CD161^{+}$ T cells. $CD4^{+}CD161^{+}$ T cells were derived from the patients with UC estimated by UCDAI in **A** ($n = 17$), and from the patients with Crohn's disease estimated by CDAI in **B** ($n = 10$). The x-axis indicates the UCDAI score in **A** and CDAI score in **B**. The y-axis indicates the population of $CD4^{+}CD161^{+}$ T cell. Spearman's rank correlation test was applied.

(11.5 ± 4.73%) patients compared with control patients (21.0 ± 5.88%) (Fig. 1B). A significant difference was found between patients with active and inactive UC (24.9 ± 10.4%) but not for patients with active or inactive Crohn's disease (14.6 ± 8.99%). In contrast, analysis of $CD4^{+}CD161^{-}$ T cells revealed no significant difference between active UC (53.0 ± 10.7%), inactive UC (40.8 ± 12.5%) and control (45.6 ± 9.43%) (Fig. 1C). Moreover, there was no significant difference in the population analysis of peripheral blood mononuclear cells of the two classes of patients (data not shown).

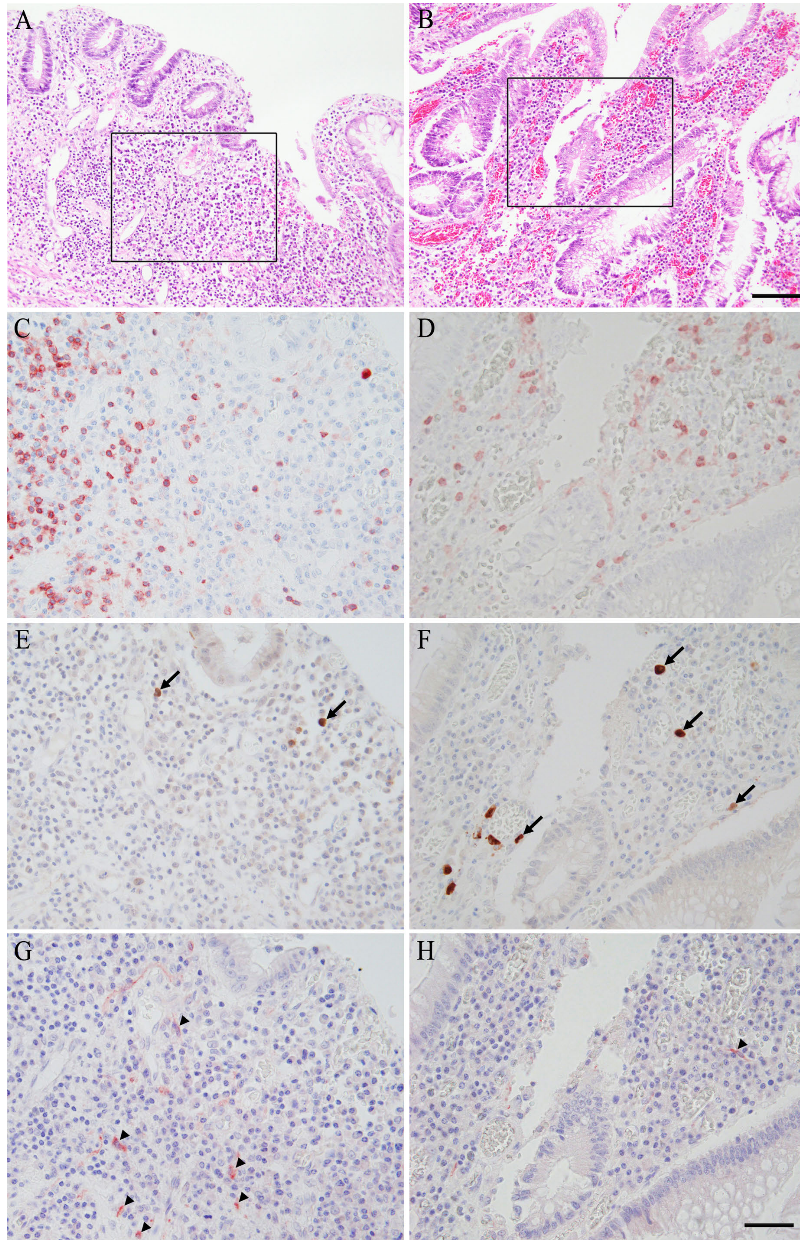

Fig. 3. Expression of CD4, CD161 and IL-10 in colonic mucosa of active and inactive inflammatory site of UC. H&E stain (**A, B**) and immunohistochemical localization of CD4 (**C, D**), CD161 (**E, F**) and IL-10 (**G, H**) in paraffin-embedded serial sections of colonic mucosa from active and inactive inflammatory site of UC. Panels in the left column (**A, C, E, G**) represents active inflammatory site of UC. Panels in the right column (**B, D, F, H**) represents inactive inflammatory site of UC. Boxed areas were enlarged and shown in **C** to **H**. Arrows indicate positive cells for CD4 expressing CD161. Arrowheads indicate IL-10 positive cells. Magnification ×200, Bar = 100 µm in H&E, Magnification ×400. Bar = 50 µm in immunohistochemistry.

Disease activity of UC inversely correlated with $CD4^+CD161^+$ T cell population

We next investigated the correlation between disease activity and population of $CD4^+CD161^+$ T cell. There was an inverse correlation between the population of $CD4^+CD161^+$ T cell and UCDAI ($r = -0.6326$, $P = 0.0055$) (Fig. 2A). In contrast, there was no significant correlation between the population of $CD4^+CD161^+$ T cell and CDAI ($r = -0.5836$, $P = 0.0775$) (Fig. 2B).

Expression of CD4, CD161 and IL-10 in active and inactive UC

Representative figures of H&E stain and immunohistochemistry were made from the specimen of active and inactive UC from a patient with UCDAI score 2 (Fig. 3A–H). CD161 positive cells were fewer in the specimen from the patients with higher UCDAI score compared with the patients with lower UCDAI score (data not shown).

Immunohistochemical staining in the colonic mucosa

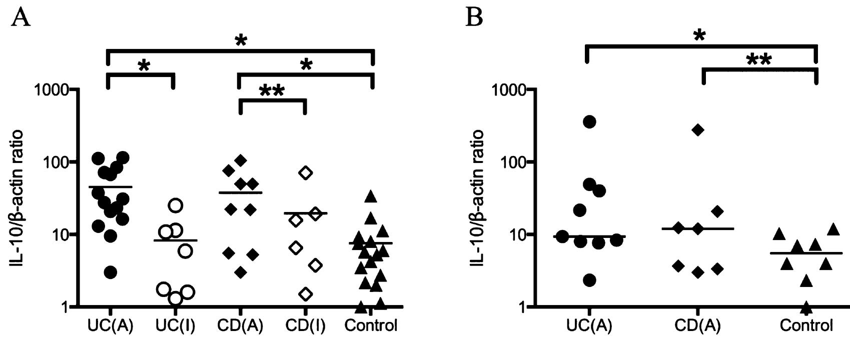

Fig. 4. Real-time PCR analysis of IL-10 mRNA expression by LPMCs. LPMCs were derived from active UC (n = 14), inactive UC (n = 7), active Crohn's disease (n = 9), inactive Crohn's disease (n = 6), and controls (n = 16) (**A**). LP-CD4$^+$ T cells were derived from LPMCs of active UC (UC (A), n = 9), active Crohn's disease (CD (A), n = 7), and controls (n = 8), respectively (**B**). The y-axis indicates the relative value in the ratio of the target gene copy number to that of *ACTB*. Horizontal bars represent median. Mann-Whitney rank sum test was applied. *$P < 0.05$. **P = not significant.

of active UC revealed that the CD4 positive cells were abundant in lymphoid aggregates in lamina propria beneath the ulcerated epithelium (Fig. 3C). Several CD161 positive cells were detected in the area of lymphoid aggregates (Fig. 3E). IL-10 positive cells were scattered in inflammatory infiltrates in lamina propria, parenchyma and ulceration sites (Fig. 3G). In inactive UC, CD4 positive cells were extensively detected beneath the non-ulcerated epithelium (Fig. 3D). Interestingly, higher frequency of CD161 positive cells were detected in inactive UC compared with active UC (Fig. 3F). IL-10 weakly positive cells were slightly detected in colonic mucosa of inactive UC patients by immunohistochemistry (Fig. 3H).

Over-expression of IL-10 mRNA in both LPMCs and LP-CD4$^+$ T cells

We next investigated expression of IL-10 mRNA in the LPMCs. The level of expression of IL-10 mRNA in LPMCs isolated from patients with active UC or active Crohn's disease was significantly higher compared with those of patients with inactive UC, inactive Crohn's disease, or controls (Fig. 4A). Subsequently, the expression level of IL-10 mRNA in lamina propria (LP)-CD4$^+$ T cells was significantly higher in patients with UC compared with controls (Fig. 4B).

IV. Discussion

In the present study, we found that the expression and population of CD4$^+$CD161$^+$ T cells was decreased significantly in inflamed lesions on the colonic mucosa of patients with IBD and that the population of these cells inversely correlated with disease activity in patients with UC. Over-expression of IL-10 mRNA and increased IL-10 production was revealed in active UC.

CD161 is a human cell differentiation molecule firstly defined in mouse as NK 1.1 molecule expressed in invariant NKT cells [15]. Recently it was reported that, while CD161 is expressed by MAIT cells in healthy controls and IBD patients and is mainly detected in the peripheral blood, as well as scattered in the crypts of intestinal epithelium where it plays important roles in gut mucosal immunity by secreting cytokines in IBD patients, it was found no significant relationship with regard to the clinical activity of IBD [9]. In contrast, we investigated the population of CD4$^+$CD161$^+$ T cells abundant in the lamina propria as to whether it can be used as a biomarker for disease activity. Our data of reduced frequency of CD4$^+$CD161$^+$ T cells in active inflammatory sites compared with inactive sites in the patients with UC, and inverse correlation between UCDAI and population of CD4$^+$CD161$^+$ T cells might provide a new biomarker of UC.

In contrast, an unexpected finding was that gross and microscopic findings were different between active and inactive Crohn's disease, whereas the population of CD4$^+$CD161$^+$ T cells and IL-10 production did not differ. This population data in Crohn's disease are consistent with that of Kleinschek *et al.* [12] who reported that Th17 cells expressing CD161. This finding may explain the differences in clinical features between UC and Crohn's disease, indicating that Crohn's disease is a systemic disease with the potential for relapse in the entire gut.

While the reason of depletion of CD4$^+$CD161$^+$ T cells in active IBD remains unclear, several mechanisms might be involved, such as the expression of proapoptotic features activated by chronic inflammation [9] and the loss of surface markers by bacterial infection [27]. In fact, increased frequency of these cells was reported in the synovial fluid of the patients with rheumatoid arthritis [18] and in the tumor and malignant effusion of breast cancer [11], in which the local environment is germ-free. We believe further studies are needed to determine whether the populations of these cells are altered in the intestinal bacterial flora.

IL-10 is defined as an anti-inflammatory cytokine that downregulates the production of proinflammatory cytokines and T helper 1 cytokines in general. The data on IL-10 deficient mice by Kuhn *et al.* showing chronic noninfectious intestinal inflammation exhibited immunoregulatory role of IL-10 [14]. The inhibitory function of secreting

proinflammatory cytokines, such as IL-6, IFN-γ and tumor necrosis factor-α, by IL-10 was reported in IBD *in vitro* [6, 13]. On the contrary, Melgar *et al.* reported the over-expression of IL-10 mRNA in mucosal T cells in active UC [17]. We also demonstrated high expression of IL-10 mRNA of LPMCs and LP-CD4^{+} T cells in UC. A comparative analysis of IL-10 mRNA from the intestinal mucosa with diverticulitis and IBD revealed similar levels of IL-10 production, but IL-1β expression was higher in IBD compared with diverticulitis [2]. Taking these facts into consideration, insufficiency of IL-10 production to regulate proinflammatory cytokine might be involved in the inflammatory site in UC. Otherwise, high-dose of IL-10 may result in the induction of proinflammatory cytokines in Crohn's disease [26]. Therefore, the function of IL-10 might be influenced by local environmental factors and degree of concentration.

We could not detect the CD4^{+}CD161^{+} T cells producing IL-10 in the serial sections analysed by immunohistochemistry, but we detected higher IL-10 mRNA expression in CD4^{+} T cells, indicating that CD4^{+}CD161^{-} T cells might also contribute to the production of IL-10. Cell sorting and intracytoplasmic staining with flow cytometric analysis or multi-chromatic fluorescent imaging in immunohistochemistry may reveal whether these cells have the ability to produce IL-10 in active UC. A limitation of our study is that there were few unmedicated patients, therefore we could not exclude the influence of corticosteroids or other immunosuppressive agents in these patients.

In summary, we found that the proportion of CD4^{+}CD161^{+} T cells was decreased in active IBD. We found an inverse correlation between the percentage of CD4^{+}CD161^{+} T cells and disease activity in UC but not in Crohn's disease. The difference in this cell population between UC and Crohn's disease may explain the distinct immunopathology of these diseases. Reductions in the population of CD4^{+}CD161^{+} T cells and over-expression of IL-10 in patients with IBD may be involved in deficient mucosal immunoregulation. Therapy to improve the proportion of CD4^{+}CD161^{+} T cells and to regulate IL-10 may resolve intestinal inflammation.

V. Acknowledgments

The authors thank former professor Kazuo Chijiiwa, Department of Surgery, Faculty of Medicine, University of Miyazaki. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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