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Expression of IL-7Rα^{low}CX3CR1^{+} CD8^{+} T Cells and α4β7 Integrin Tagged T Cells Related to Mucosal Immunity in Children with Inflammatory Bowel Disease

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ABSTRACT

Purpose: The study aimed to investigate the recruiting of T lymphocytes including IL-7Rα^{low} CX3CR1^{+} effector memory (EM) CD8^{+} T cells and α4β7 integrin tagged T cells to inflamed intestinal mucosa.
Methods: Whole blood and mucosal tissues of intestine were collected from 40 children with or without inflammatory bowel disease (IBD). T cell surface staining and immunohistochemistry were done with several antibodies in peripheral blood mononuclear cells (PBMCs) and intestinal mucosa, respectively. Serum levels of cytokines were measured by ELISA.
Results: The frequency of IL-7Rα^{low}CX3CR1^{+} EM CD8^{+} T cells in the PBMC was significantly higher in the ulcerative colitis group than in the control group (57.9±17.80% vs. 33.9±15.70%, p=0.021). The frequency of integrin α4β7^{+} CD4^{+} T cells in the PBMC was significantly lower in the ulcerative colitis group than in the control group (53.2±27.6% vs. 63.9±13.2%, p=0.022). Serum concentration of TNF-α was higher in the Crohn's disease group than in the control group (26.13±5.01 pg/mL vs. 19.65±6.07 pg/mL, p=0.008). Of the three groups, the ulcerative colitis group had the highest frequency of integrin α4β7^{+} T cells based on immunohistochemistry analyses for intestinal tissues, followed by the Crohn's disease group and the control group (4.63±1.29 cells vs. 2.0±0.57 cells vs. 0.84±0.52 cells, p<0.001).
Conclusion: Trafficking immune cells with effector memory CD8^{+} T cells clarified by IL-7Rα^{low}CX3CR1^{+} and integrin α4β7^{+} CD4^{+} T cells might be highly associated with the pathogenesis of ulcerative colitis.

Keywords: CD8; Alpha 4 beta 7; Integrins; Inflammatory bowel diseases; Child

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Conflict of Interest

The authors have no financial conflicts of interest.

INTRODUCTION

Inflammatory bowel disease (IBD), a life-long chronic inflammation in the gastrointestinal tract, is caused by immune dysfunction or hyper-immune response of mucosal immunity to dysbiosis of intestinal microbiome after exposure to unknown environmental etiologies under individual genetic susceptibility [1,2]. Recently, immune cell trafficking and retention have been identified as essential events in the pathogenesis of IBD and the theoretical base for its therapeutic advances [3-6].

Enhanced recruitment of CX3C-chemokine receptor 1^{+} (CX3CR1^{+}) T cells by mucosal endothelial cell-derived fractalkine (CX3C-chemokine ligand 1, CX3CL1) may exacerbate mucosal immune response [7]. However, there is no report specifying the fraction of CX3CR1^{+} CD8^{+} T cells in peripheral blood that contributes to immune cell recruitment to an inflamed intestinal tissue.

Naïve T cells can differentiate into CD4^{+} or CD8^{+} T cells when they are activated by antigen presenting cells originating from an inflamed tissue of the intestine or a secondary lymph node [1,8]. Differentiated CD4^{+} or CD8^{+} T cells tagged by α4β7 integrin can return to systemic blood flow through lymphatic duct. From systemic blood flow, excessive homing lymphocytes tagged with α4β7 integrin to inflamed tissue of the intestine might also affect hyper-immune response and dysfunction of mucosal immunity [8]. However, few reports have shown real infiltration of homing integrin α4β7 positive T cells in the intestinal tissue [9]. Furthermore, studies identifying the difference of circulating and mucosal integrin α4β7 positive T cells between Crohn' disease and ulcerative colitis have not been reported yet. This study hypothesized that such difference might be related to a prominent discrepancy in clinical response to anti-α4β7 integrin (vedolizumab) therapy between Crohn's disease and ulcerative colitis based on previous large-scale randomized controlled studies on adult patients and a retrospective study on pediatric patients with IBD [4,5,10].

The aim of the present study was to investigate recruiting of T lymphocytes including interleukin-7 receptor alpha (IL-7Rα)^{low} CX3CR1^{+} effector memory CD8^{+} T cells and integrin α4β7 tagged T cells as homing lymphocytes to inflamed intestinal mucosa from systemic arterial blood flow. Differences of circulating and mucosal integrin α4β7 positive T cells between Crohn's disease and ulcerative colitis were also investigated.

MATERIALS AND METHODS

Patients

Clinical information and laboratory results of 59 children who were tested by colonoscopy to rule out IBD in Jeju National University Hospital from March 1, 2018 to April 30, 2022 were collected. Of these 59 children, 19 were excluded based on our exclusion criteria (very early onset IBD, children on remission of IBD, infectious colitis, parasite infection, and other underlying diseases). Finally, 40 children were enrolled for this study. Control, Crohn's disease, and ulcerative colitis groups had 16 children, 18 children, and 6 children, respectively. All IBD children except for one with flare of ulcerative colitis were patients initially diagnosed with IBD. All control patients were diagnosed with functional abdominal pain disorders.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were extracted from whole blood cells. T cell surface staining was done with anti-APC-Cy7-CD3, anti-Pacific Blue-CD8, anti-PE-Cy5-CD45RA, anti-PE-Cy7-CCR7 (all from BD Biosciences), anti-FITC-IL-7Rα (R&D Systems), anti-PE-CXC3R1 (BioLegend), anti-Alex Fluor-CD4 (BD Biosciences), and anti-integrin α4β7 antibodies (Novus Biologicals). Finally, PBMCs after surface staining with fluorescent antibodies were read with an LSRFortessa® flow cytometer (BD Biosciences) and analyzed with FlowJo software® (Tree Star).

Immunohistochemistry

Paraffin-embedded ileal and colonic biopsy specimens were de-waxed and rehydrated in ethanol. Tissues were immersed in 0.3% hydrogen peroxide for 40 minutes followed by incubation with goat serum to block nonspecific binding. Tissues were then incubated with primary anti-integrin α4β7 antibody (Novus Biologicals) overnight at 4°C. After washing, tissues were incubated with biotinylated anti-rabbit antibody for 45 minutes. Tissues were washed with phosphate-buffered saline. An avidin-biotin peroxidase complex binding reaction was then performed using an horseradish peroxidase (HRP)-labeled Vectastain Elite ABC kit (Vector). HRP binding sites were detected with 3, 30-diaminbenzidine (DAB, Vector). Counterstaining was performed with hematoxylin. Two to three sections per one sample were analyzed under ×400 magnification with an oil immersion lens (×1,000 magnification) microscope to count positive cells. Images were taken from at least three portions of each section from each sample (two to three sections/sample) with an Olympus DP-72 (Olympus) microscope.

Cytokines analysis

In the afternoon at one day before the test, all sera of 40 children were thawed at room temperature after pulling out frozen samples stored in a deep freezer (−80.0°C). These thawed sera were centrifuged at 15,000 rpm for 5 minutes. Then 50 or 100 μL supernatant of each serum was transferred to a 96-well microplate for ELISA and stored in a 4.0°C refrigerator. Enzyme linked immunosorbent assay (ELISA) was performed to determine serum levels of cytokines in patients utilizing tumor necrosis factor alpha (TNF-α) (Invitrogen), interferon gamma (IFN-γ) (Thermo Fisher Scientific), and CX3CL1 (R&D) ELISA kits according to the manufacturer's instructions.

Statistical analysis and ethical issues

Using SPSS version 25.0 (SPSS Inc.), three groups were compared with Mann–Whitney U-test and Kruskal–Wallis test. p-value <0.05 was defined as statistically significant. This study was approved by the Institutional Review Board of Jeju National University Hospital (approval numbers: JNUH 2017-06-030, JNUH 2019-05-013). Informed written consents were collected from all patients and their parents before participation into this study.

RESULTS

Demographic and clinical characteristics

There were 34 male and 6 female children. The mean age (mean±standard deviation) was 14.44±2.02 years in the control group (n=16), 14.60±1.72 years in the Crohn's disease group (n=18), and 15.70±2.65 years in the ulcerative colitis group (n=6) (p=0.455). White blood cell (6,187±1,343/mm^3 vs. 9,063±2,912/mm^3 vs. 7,340±1,018/mm^3, p=0.002), erythrocyte sedimentation rate (9.0±9.8 mm/hr vs. 62.1±30.5 mm/hr vs. 18.8±9.7 mm/hr, p<0.001), and

carbohydrate reactive protein (261±623 mg/kg vs. 2,793±1,940 mg/kg vs. 3,180±2,366 mg/kg, p<0.001) in the blood were significantly different among the three groups (**Tables 1** and **2**).

Table 1. Demographic and clinical characteristics of 40 children including control and patients with IBD

Variable	Control (n=16)	Crohn disease (n=18)	Ulcerative colitis (n=6)	p-value
Sex				
Male (n=34)	12	17	5	
Female (n=6)	4	1	1	
Age (yr)	14.44±2.02	14.60±1.72	15.70±2.65	0.455
Previous medication	None	None	None, mesalazine in 1 patient	
PCDAI		32.63±12.58		
PUCAI			53.00±17.17	
Laboratory results				
WBC (/mm^3)	6,187±1,343	9,063±2,912	7,340±1,018	0.002
Hemoglobin (g/dL)	13.7±1.2	12.8±1.3	12.8±2.5	0.126
Hematocrit (%)	41.3±3.4	40.5±3.5	39.6±4.9	0.596
ESR (mm/hr)	9.0±9.8	62.1±30.5	18.8±9.7	<0.001
Albumin (g/dL)	4.6±0.2	4.0±0.5	4.1±0.4	0.001
CRP (mg/dL)	0.05±0.07	3.68±3.73	0.49±0.59	0.001
Calprotectin (mg/kg)	261±623	2,793±1,940	3,180±2,366	<0.001
ANCA positive	0	1	0	
ASCA positive	0	7	1	
ANA positive	0	0	0	

Counting of IL7-Rα^{low}CX3CR1^{+} EM CD8^{+} T cells and cytokine analysis were done using peripheral blood mononuclear cells and sera of their blood samples, respectively; EM; all IBD patients except for one with flare of ulcerative colitis were newly diagnosed with IBD.
PCDAI: pediatric Crohn disease activity index, PUCAI: pediatric ulcerative colitis index, WBC: white blood cell, ESR: erythrocyte sedimentation rate, CRP: carbohydrate reactive protein, ANCA: anti-neutrophil cytoplasmic antibody, ASCA: anti-Saccharomyces cytoplasmic antibody, ANA: anti-nuclear antibody, IL7-Rα: interleukin 7 receptor alpha, CX3CR1: CX3C-chemokine receptor 1, EM: effector memory, IBD: inflammatory bowel disease.
p-value <0.05 indicated statistical significance (Kruskal–Wallis test).

Table 2. Demographic and clinical characteristics of 29 children including controls and patients with IBD

Variable	Control (n=7)	Crohn disease (n=16)	Ulcerative colitis (n=6)	p-value
Sex				
Male (n=22)	4	14	4	
Female (n=7)	3	2	2	
Age	13.58±1.75	14.01±2.95	14.30±5.19	0.539
Previous medication	None	None	None, mesalazine in 1 patient	
PCDAI	-	33.1±14.3	-	
PUCAI	-	-	64.0±16.4	
Laboratory results				
WBC (/mm^3)	5,571±1,833	9,381±3,433	8,583±1,287	0.013
Hemoglobin (g/dL)	12.0±4.0	12.6±1.4	11.7±2.3	0.584
Hematocrit (%)	37.3±11.2	40.5±3.6	37.8±5.9	0.555
ESR (mm/hr)	8.2±6.7	57.0±30.4	37.6±34.5	0.003
Albumin (g/dL)	4.7±0.2	4.0±0.5	4.1±0.5	0.021
CRP (mg/dL)	0.028±0.0	2.964±3.2	0.742±0.6	0.001
Calprotectin (mg/kg)	274±335	2,830±2,019	4,172±1,844	0.001
Endoscopic biopsies	T-ileum, Colon	T-ileum, Colon	Colon	

Cells in the mucosa of terminal ileum or colon stained with anti-α4β7 integrin antibody were counted. All IBD patients except for one with flare of ulcerative colitis were newly diagnosed with IBD.
IBD: inflammatory bowel disease, PCDAI: pediatric Crohn disease activity index, PUCAI: pediatric ulcerative colitis index, WBC: white blood cell, ESR: erythrocyte sedimentation rate, CRP: carbohydrate reactive protein, -: not available.
p-value <0.05 indicates statistical significance (Kruskal–Wallis test).

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Flow cytometry and ELISA

The frequency of IL-7Rα^{low}CX3CR1^{+} effector memory (EM) CD8^{+} T cells in the PBMC was significantly higher in the ulcerative colitis group than in the control group (57.9±17.80% vs. 33.9±15.70%, p=0.021) (**Table 3**, **Fig. 1**). The frequency of IL-7Rα^{low}CX3CR1^{+} EM CD8^{+} T cells tended to be higher in the Crohn's disease group than in the control group, although the difference was not statistically significant (45.9±22.4% vs. 33.9±15.70%, p=0.085). The frequency of integrin α4β7^{+} CD4^{+} T cells in the PBMC was significantly lower in the ulcerative colitis group than in control group (53.2±27.6% vs. 63.9±13.2%, p=0.022; **Table 4**). The frequency of integrin α4β7^{+} CD4^{+} T cells tended to be lower in the Crohn's disease group than in the control group, although the difference was not statistically significant (58.8±10.5% vs. 63.9±13.2%, p=0.413).

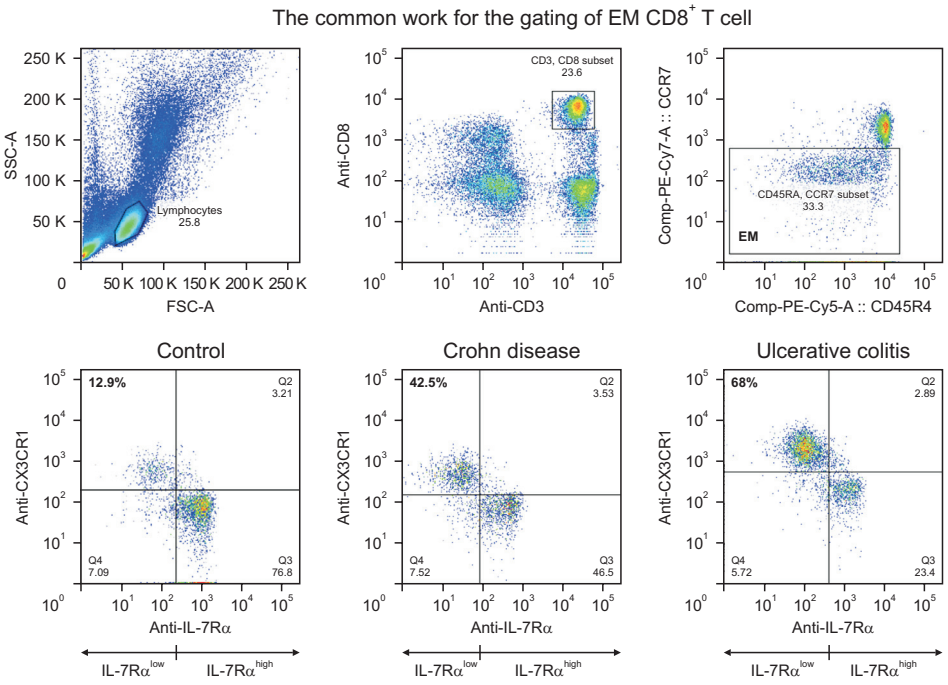

Fig. 1. Examples of cell surface staining for IL-7Rα^{low}CX3CR1^{+} CD8^{+} T cells. The ulcerative colitis group had higher frequency of IL-7Rα^{low}CX3CR1^{+} CD8^{+} T cells in the PBMC than the control group (57.9±17.80% vs. 33.9±15.70%, p=0.021; **Table 3**).
EM: effector memory, UC: ulcerative colitis, IL7-Rα: interleukin 7 receptor alpha, CX3CR1: CX3C-chemokine receptor 1, PBMC: peripheral blood mononuclear cell.

Table 3. Frequency of IL-7Rα^{low}CX3CR1^{+} EM CD8^{+} T cells in PBMC and concentrations of cytokines analyzed by ELISA in serum samples of children with or without IBD

Variable	Control (n=16)	Crohn disease (n=18)	Ulcerative colitis (n=6)	p-valueControl vs. Crohn, UC
IL-7Rα^{low}CX3CR1^{+} (%)	33.90±15.70	45.90±22.40	57.90±17.80	0.085, 0.021
TNF-α (pg/mL)	19.65±6.07	26.13±5.01	30.03±8.50	0.008, 0.101
IFN-γ (pg/mL)	0.47±0.25	0.49±0.18	0.39±0.88	0.856, 0.282
CX3CL1 (pg/mL)	0.47±0.24	0.49±0.16	0.41±0.07	0.897, 0.282

IL7-Rα: interleukin 7 receptor alpha, CX3CR1: CX3C-chemokine receptor 1, EM: effector memory, PBMC: peripheral blood mononuclear cell, IBD: inflammatory bowel disease, UC: ulcerative colitis, TNF-α: tumor necrosis factor alpha, IFN-γ: interferon gamma, CX3CL1: CX3C-chemokine ligand 1.
p-value <0.05 indicates statistical significance (Kruskal–Wallis test).

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Table 4. Frequency of α4β7^{+} T cells in PBMCs of children with or without IBD

Variable	Control (n=7)	Crohn disease (n=16)	Ulcerative colitis (n=6)	*p*-valueControl vs. Crohn, UC
α4β7^{+}CD3^{+}	39.1±5.4	38.1±12.5	31.9±15.8	0.085, 0.021
α4β7^{+}CD4^{+}	63.9±13.2	58.8±10.5	53.2±27.6	0.413, 0.022
α4β7^{+}CD8^{+}	86.9±5.9	83.3±7.7	75.7±9.4	0.492, 0.234

PBMCs: peripheral blood mononuclear cells, IBD: inflammatory bowel disease, UC: ulcerative colitis.
p-value <0.05 indicates statistical significance (Kruskal–Wallis test).

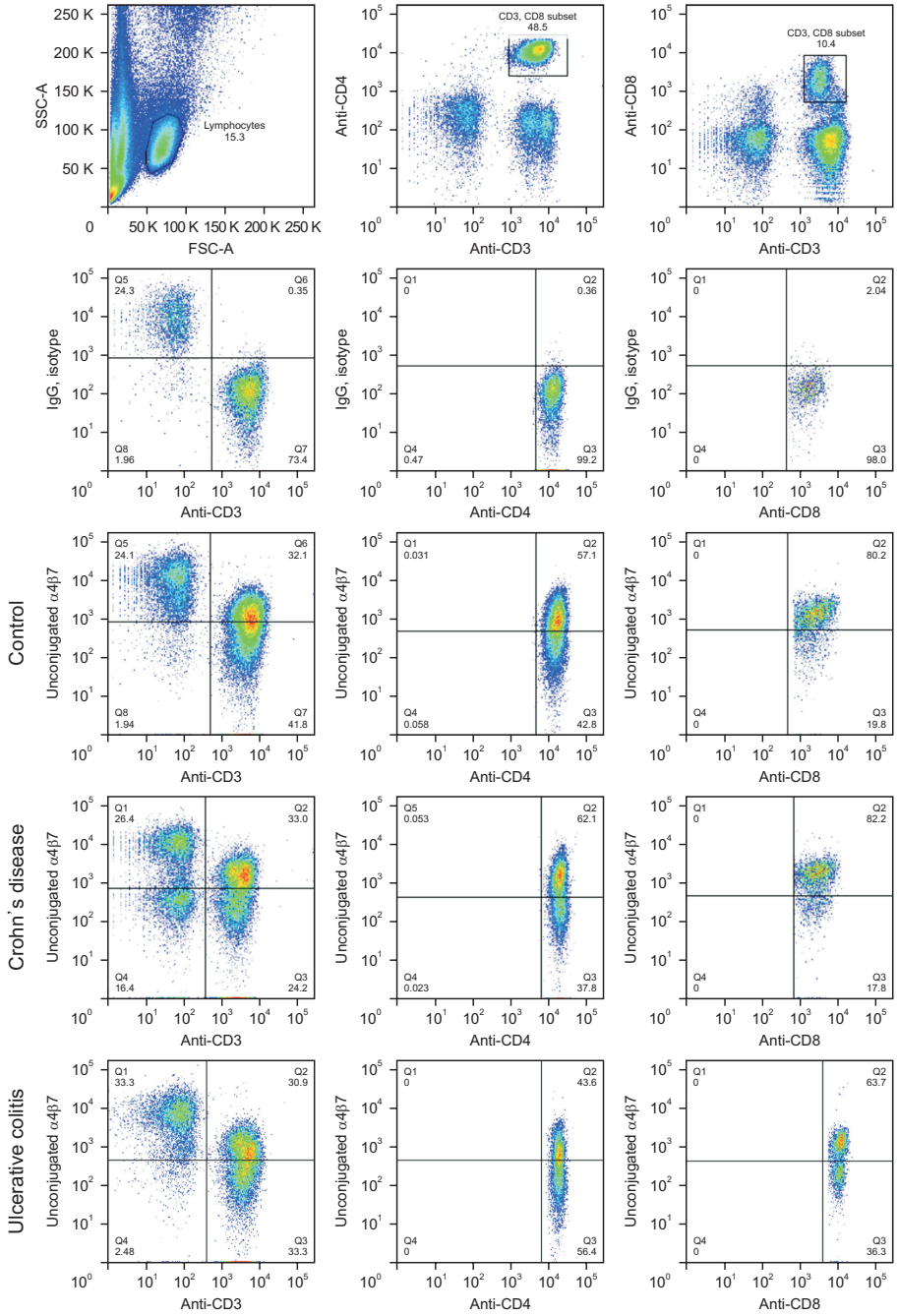

Fig. 2. Examples of cell surface staining for integrin α4β7^{+} T cells. The frequency of integrin α4β7^{+} CD4^{+} T cells in the PBMC was significantly lower in the ulcerative colitis group than in the control group (53.2±27.6% vs. 63.9±13.2%, *p*=0.022; **Table 4**).
UC: ulcerative colitis, PBMC: peripheral blood mononuclear cell.

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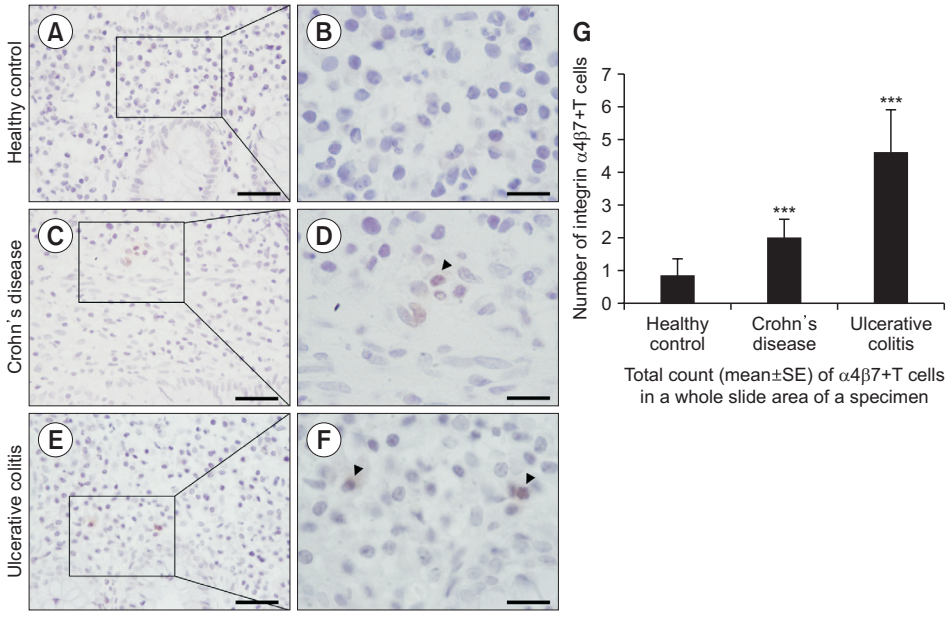

Fig. 3. The frequency of integrin α4β7^{+} T cells in the immunohistochemistry of ileal or colonic mucosa (A, C, E: ×100; B, D, F: ×200). Square bars=100 μm (A-F). Of the three groups, the frequency of integrin α4β7^{+} T cells in one section of the ileal or colonic tissue was higher in ulcerative colitis (E, F) than in Crohn's disease (C, D), higher in ulcerative colitis than in the control (A, B), higher in Crohn's disease than in the control group (4.63±1.29 cells vs. 2.0±0.57 cells vs. 0.84±0.52 cells, p<0.001) (G). Integrin α4β7^{+} T cells (arrowhead) was counted all together in a whole slide area of a specimen (mean±SE).
UC: ulcerative colitis, SE: standard error.
***p<0.001 (Kruskal–Wallis test).

Serum concentration of TNF-α was higher in the Crohn's disease than in the control group (26.13±5.01 pg/mL vs. 19.65±6.07 pg/mL, p=0.008) (**Table 3**, **Fig. 2**). Concentrations of IFN-γ and CX3CL1 were not significantly different among the three groups (**Table 3**, **Fig. 2**).

Immunohistochemistry

Of the three groups, the ulcerative colitis group had the highest frequency of α4β7^{+} T cells in one section of the ileal or colonic tissue, followed by Crohn's disease group and the control (4.63±1.29 cells vs. 2.0±0.57 cells vs. 0.84±0.52 cells, p<0.001) (**Fig. 3**).

DISCUSSION

CX3CR1 is a receptor on the surface of T lymphocytes for fractalkine (CX3CL1) to attach to the endothelial wall which can recruit T lymphocytes from blood flow to inflamed tissues [11]. The CX3CR1 is usually highly expressed in IL-7Rα^{low} CD8 T cells [12-14]. In our study, the expression of IL-7Rα^{low} CX3CR1^{+} CD8^{+} T cells was increased in PBMCs of patients with ulcerative colitis than in the control group (**Table 3**, **Fig. 1**). Our results suggest that IL-7Rα^{low} CX3CR1^{+} CD8^{+} T cell, as one of the trafficking cells to acutely or chronically inflamed tissue of intestine, might be related to the pathogenesis of ulcerative colitis. However, the concentration of CX3CL1 (ligand for IL-7Rα^{low} CX3CR1^{+} CD8^{+} T cells) in the ulcerative colitis group was not significantly different from that in the control group. Unfortunately, the receptor frequency for CX3CL1 and ligand concentration for CX3CR1 in ulcerative colitis and control groups showed discordant trends. Sans et al. [7] have reported that CX3CR1^{+} T cells are enhanced by mucosal endothelial cell-mediated derived fractalkine (CX3CL1) in IBD.

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However, they did not evaluate which subset of T cells were more related to the binding to the fractalkine of mucosal endothelial cell of IBD. Although few research studies have reported the contribution of CX3CL1 (fractalkine) to the recruiting of T lymphocytes to inflamed tissue of the intestine, a phase 1 study on the safety and efficacy of E6011, an anti-fractalkine antibody, in patients with Crohn's disease has been recently conducted [15].

In our study, concentrations of cytokines analyzed by ELISA showed significant increases in the sera of patients with Crohn's disease than in the control group (**Table 3**). Concentrations of IFN-γ and CX3CL1 were not significantly different among the three groups (**Table 3**). TNF-α is a central pro-inflammatory cytokine in IBD pathogenesis. An anti-TNF-α agent that is effective agent as a biological agent for the treatment of IBD is already popular [16,17].

Integrin α4β7 is a transmembrane receptor on the surface of recruiting lymphocytes from blood circulation to different tissues [18]. Integrin α4β7 tagged on gut homing T lymphocyte can bind to a ligand, an adhesive molecule such as mucosal vascular adressin cell adhesion molecule 1 (MAdCAM1) of endothelial wall, which can move T cells from blood flow to inflamed tissue of the intestine [3,8,18,19]. In the present study, frequencies of α4β7^{+} CD4^{+} T cells in PBMC were significantly lower in the ulcerative colitis group than in the control group (**Table 4**, **Fig. 2**). Of course, this finding might be caused by more homing of cells from PBMC to inflamed intestinal tissues. However, the frequency of integrin α4β7^{+} T cells based on immunohistochemistry for the ileal or colonic mucosa was the highest in the ulcerative colitis group, followed by that in the Crohn's disease group. It was the lowest in the control group (**Fig. 3**). These results might suggest the reason why treatment effects with anti-integrin α4β7 (vedolizumab) were lower in Crohn's disease than in ulcerative colitis shown in previous large-scale randomized controlled studies for adults with IBD [4,5]. Meenan et al. [9] have initially reported the expression of α4β7, a gut homing integrin, of circulating and mucosal T cells in IBD. They revealed that the control group had the highest frequency of α4β7^{+} memory T cells in PBMCs, followed by Crohn's disease and ulcerative colitis. They also showed that the control group had the highest frequency of mucosal CD3^{+} α4β7^{+} T cells in colonic mucosa, followed by ulcerative colitis and Crohn's disease. As the second study on the expression of integrin α4β7 in IBD, Souza et al. [20] reported that colonic lamina propria of patients with Crohn' disease and ulcerative colitis showed increased density of CD3^{+} and α4β7 cells than the control group. Contrary to our study results, they could not find a significant difference in mucosal α4β7^{+} T cells between Crohn's disease and ulcerative colitis. Authors of the present study postulate that ulcerative colitis and Crohn's disease have different pathogenesis related to immune cell trafficking to inflamed intestinal tissues. Ulcerative colitis might be highly associated with immune cell trafficking in the mechanism of disease progression.

Limitations of this study are as follows. First, the number of patients was small for each group. Second, staining the colonic mucosa with anti-integrin α4β7 antibody was very difficult. In addition, the count of α4β7^{+} T cells was done in the whole area of the specimen, not in the several high-power field. Only one company has a stock of anti-integrin α4β7 antibody in the market. The authors could not be absolutely sure about the high quality of the antibody. The third was a potential bias in the interpretation due to variations in biopsy sites. Endoscopic biopsies for control were done for the terminal ileum, ascending colon, and sigmoid colon. Because of different distributions of pathologic mucosa between the two IBD groups, most biopsies for Crohn's disease were done for the terminal ileum and/or cecum and ascending colon while most biopsies for ulcerative colitis were done for the sigmoid colon. Finally, authors of the present study did not identify relationships between IL-

7Rα^{low}CX3CR1^{+} effector memory CD8^{+} T cells and Integrin α4β7^{+} T cells as recruiting immune cells to inflamed colonic tissues in ulcerative colitis. It would be interesting to further explore the link between them. Investigating mechanisms underlying gut trafficking of immune cells and involvement of interacting cytokines or chemokines would be valuable. Additional mechanistic experiments, either in vivo or in vitro, would enhance our understanding of the underlying mechanisms. These experiment could explore the link between these cell populations as well as their implications in the pathophysiology of IBD.

In conclusion, trafficking immune cells with effector memory CD8^{+} T cells clarified by IL-7Rα^{low}CX3CR1^{+} and integrin α4β7^{+} CD4^{+} T cells might be highly associated with the pathogenesis of ulcerative colitis. The link between the two cell groups could be researched in the future to clarify the pathogenesis of IBD.

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