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Effect of ultra low fat diet on regulatory T cells of dogs with intestinal limphangiectasia secondary to chronic enteropathy: A pilot study

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Abstract

Ultra low fat diet (ULFD) has shown a good therapeutic effect in dogs with intestinal lymphangiectasia secondary to CE (IL-CE). As we hypothesized that the mechanism of ULFD may involve the resolution of immunological impairment in IL-CE dogs, our study aimed to investigate the effect of ULFD on the circulating regulatory T cell (Treg) of IL-CE dogs. Treg frequency of pre- and post-ULFD blood samples from dogs with IL-CE (n = 3) were measured using flow cytometry. Treg frequency in IL-CE group before ULFD treatment showed lower tendency when compared to healthy control (mean±SD; 2.0±1.6% and 4.3±1.4%, respectively), and showed increased tendency after ULFD (mean±SD; 4.5±3.5%). The results suggested that ULFD might restore circulating Treg frequency of IL-CE dogs.

Key Words: IL-CE, Treg, ULFD

Intestinal lymphangiectasia (IL) in dogs is a dilation of intestinal lymph vessels which subsequently causing leakage of protein-rich lymph into the intestinal lumen. It may occur as a primary disorder or as a result of lacteal obstruction secondary to another disorders such as inflammatory and neoplastic diseases of the intestine. One of the most common inflammatory disease which attributed with IL is chronic enteropathy (CE)[18)]. CE is caused by an unknown

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etiology and characterized by histopathologic finding of lymphoplasmacytic or eosinophilic enteritis[1,2,5,29]. Pathogenesis of CE is suspected to involve an interaction between dietary components, intestinal microflora, and host genetic susceptibility which disrupt the immune system and cause uncontrolled inflammation. Management of CE dogs is commonly started with only dietary modification since marked response has been shown in more than 50% of CE dogs. CE dogs which do not show good response toward single treatment using dietary modification may require concurrent antibiotics, steroid, and/or other immunosuppressive agents[23].

Ultra low fat diet (ULFD) was first introduced as home-cooked diet that composed by turkey breast and potatoes to manage primary and secondary IL in dogs[18]. ULFD was suggested to reduce the workload of intestinal lymph vessels during fat absorption[17]. Despite its good response, the therapeutic mechanism exhibited by ULFD in IL dogs secondary to CE (IL-CE) is still unclear. ULFD may act as an elimination diet with fewer potential allergen because it contains not more than two types of protein and carbohydrate sources. We also hypothesize that the mechanism may involve the resolution of immunological impairment in IL-CE dogs.

Regulatory T cells (Treg) is a subset of T lymphocytes which regulate the immune homeostasis and defined by the expression of CD4, CD25, and the transcription factor Forkhead box p3 (Foxp3)[3,21]. Treg has been reported to be involved in the pathogenesis of inflammatory bowel disease (IBD) and other immune-mediated diseases in human[12,14]. In human IBD, previous studies suggested that redistribution of Treg toward the active inflammation site resulting in a reduction of circulating Treg and an increase of Treg number in the intestinal mucosa[14,24,25,28]. CE in dogs has been known to share some common pathogenesis with human IBD as both diseases are thought to be generated from disruption of immune homeostasis[4,23]. Similar to the situation in human IBD, reduction in peripheral blood Treg number was reported in dogs with CE[27]. Nevertheless, different from human IBD, the reduction of Treg in the intestinal mucosa was reported in dogs with CE[10,13]. In addition, there has been developing interest on the use of Treg as a biomarker as well as the therapeutic target in both diseases. The dynamic of Treg was reported following treatment and suggested to correlate with disease activity in IBD patients as well as CE dogs[20,24]. Considering the above background, the aim of this study was to investigate the effect of ULFD on the circulating Treg of dogs with IL-CE with the expectation to further enrich the currently available monitoring biomarkers and therapeutic agents for CE dogs.

Dogs presenting to Hokkaido University Veterinary Teaching Hospital with IL-CE from March 2016 to April 2017 were included. Diagnosis of IL-CE was based on the following criteria: (1) Gastrointestinal (GI) signs unresponsive to symptomatic treatment (≥3 weeks); (2) exclusion of possible causes of GI signs (i.e. infection, neoplasia, endocrinal disease, exocrine pancreatic insufficiency) by blood test, urinalysis, fecal examination, X-ray, and ultrasonography; (3) histopathological evaluation of intestinal biopsy revealed lymphoplasmacytic or eosinophilic enteritis with IL[29]. A histopathological diagnosis was made using hematoxylin and eosin (HE) stained specimens according to World Small Animal Veterinary Association (WSAVA) criteria by an American College of Veterinary Pathologists board-certified pathologist (Y.K.). In addition, 10 healthy beagles (7 intact males, 3 intact females, age 1-3 years) which belong to Hokkaido University laboratory animal facility were enrolled as control. All procedures in this study were approved by the Hokkaido University Animal Care and Use Committee (No. 16-0094), and the informed consent was obtained from owner of all dogs.

Dogs included in IL-CE group were prescribed for ULFD. To evaluate the effect of ULFD on the frequency of circulating Treg, peripheral blood samples were collected on the first visit before

Table 1. Signalment, Body Weight, Albumin, and CCECAI Score of Dogs with Intestinal Lymphangiectasia Secondary to Chronic Enteropathy (IL-CE) before and after Ultra Low Fat Diet Treatment.

Dog	Signalment			Body Weight (kg)		Albumin (g/dL)		CCECAI Score	
	Breed	Age (Year)	Sex	Pre-ULFD	Post-ULFD	Pre-ULFD	Post-ULFD	Pre-ULFD	Post-ULFD
1	Mix	7	M	11.27	10.7	1.6	2.1	6	5
2	Welsh Corgi	5	F	14.66	11.94	2.3	2.4	7	3
3	Japanese Spitz	6	SF	6.32	6.1	1.6	2.1	2	2

M: male; F: female; SF: spayed female; ULFD: ultra low fat diet; CCECAI: canine chronic enteropathy clinical activity index

ULFD treatment (pre-ULFD) and 1 to 3 weeks after initiation of ULFD treatment (post-ULFD). During this period, dogs did not receive any treatment other than ULFD. In addition, each dog was given a clinical score by the canine chronic enteropathy clinical activity index (CCECAI) scoring system, and the scores were recorded before and after treatment. The scoring criteria included attitude/activity, appetite, vomiting, feces consistency, feces frequency, weight loss, plasma albumin concentration, ascites and peripheral edema, and pruritus. By the CCECAI, the above mentioned 9 prominent gastrointestinal signs were scored from 0 to 3 according to the magnitude of change. Total CCECAI score was classified as clinically insignificant (0-3), mild (4-5), moderate (6-8), and severe (9 or greater)[2].

The formulation of ULFD diet was based on a previous study[17]. ULFD was composed of 1 part of chicken breast without skin plus 2 parts of rice or white potato. Chicken breast is composed mainly of protein with hardly any fat, and provides 125 kcal per 100 g[22]. Rice and white potato are composed mainly of carbohydrates with little protein, and provide 168 kcal and 84 kcal per 100 g respectively[22]. The feeding amount of ULFD was calculated on the basis of the daily energy requirement (DER) which was obtained by multiplying resting energy requirement (RER, $BW^{0.75}$ x 70) with correction factor 1.0 (used for poorly nourished dogs). The total feeding amount was subsequently divided into two meals per day, and the food was prepared by the owner at home.

The frequency of circulating Treg was measured using flow cytometry. All blood samples were processed within 24 hr. Peripheral blood mononuclear cells (PBMCs) were separated by Percoll density gradient centrifugation. The PBMCs (1×10^6-10^9 cells/ml) were stained for surface expression of CD4 (anti-canine CD4, FITC, 10 µg/ml, clone YKIX302.9, Bio-Rad, Hercules, CA, USA) and CD25 (anti-canine CD25, PE, 2.5 µg/ml, clone P4A10, eBioscience, San Diego, CA, USA) at room temperature in the dark for 30 min. After washing with PBS, the cells were fixed, permeabilized and stained for intracellular expression of Foxp3. Foxp3 was stained using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and anti-Foxp3 mAb (rat anti-mouse Foxp3, APC, 10 µg/ml, clone FJK-16, eBioscience, San Diego, CA, USA). Foxp3 staining was performed at room temperature in the dark for 30 min. In all reactions, isotype-matched antibody was utilized as a negative control. The stained PBMC was subsequently analyzed by flow cytometer (BD FACSVerse™ ver1.2 suite 1.0.2, Nippon Becton Dickinson, San Jose, CA, USA) to determine the percentage of CD4+CD25+Foxp3+ cells in the CD4+ lymphocytes. Pre-ULFD Treg frequency of IL-CE group was compared with control group using student's *t* test. Pre-ULFD and post-ULFD Treg frequency were compared by match-paired *t* test. Significances were determined by P value <0.05.

Three dogs were included in IL-CE group (Table 1). Pre-ULFD blood samples were collected in all 3 dogs at first visit before starting ULFD treatment; whereas post-ULFD samples were collected at 10, 19, and 22 days after initiation of ULFD treatment in dog 1 to 3, respectively.

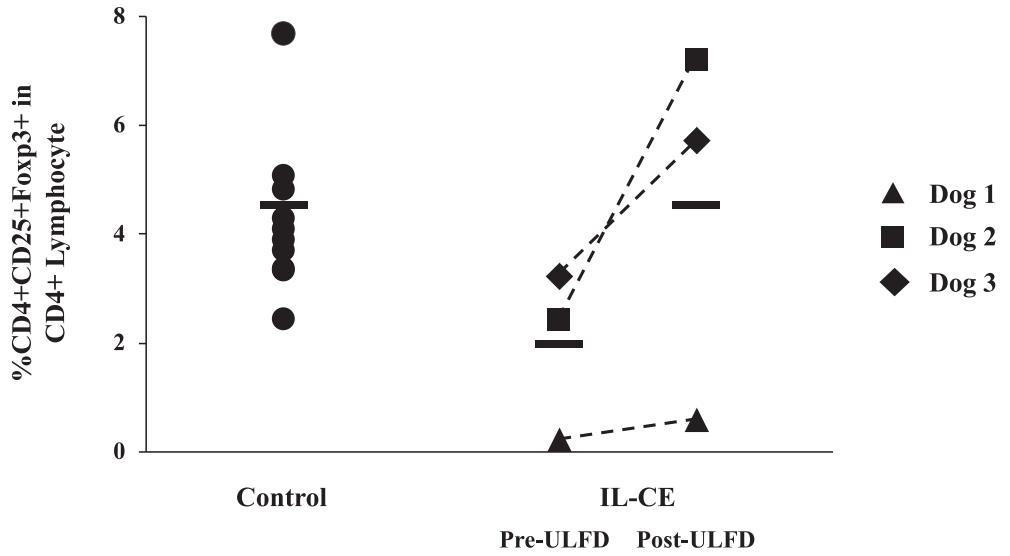

Fig. 1. Scatter plot of circulating Treg frequency in control group, and intestinal lymphangiectasia dogs secondary to chronic enteropathy (IL-CE) before and after ultra low fat diet treatment (pre-ULFD and post-ULFD, respectively). The circulating Treg frequency in IL-CE dogs at pre-ULFD showed lower tendency compared to control group ($P = 0.1031$). Treg frequency exhibited increased tendency in all 3 IL-CE dogs at post-ULFD compared to pre-ULFD ($P = 0.1838$).

The circulating Treg frequency in IL-CE group before ULFD treatment showed lower tendency compared to control group (mean±SD; 2.0±1.6% and 4.3±1.4%, $P = 0.1031$) (Fig. 1). Post-ULFD Treg frequency (mean±SD; 4.5±3.5%) exhibited increased tendency in all 3 IL-CE dogs compared to pre-ULFD ($P = 0.1838$) (Fig. 1).

Histopathological examination of the duodenum showed lymphoplasmacytic enteritis with lymphangiectasia in all dogs. All dogs in IL-CE group showed hypoalbuminemia (reference range: 2.6-4.0 g/dL) at first visit, and the albumin concentration increased but still lower than reference range at the time of post-ULFD Treg frequency evaluation (Table 1). In addition, 2 dogs showed moderate CCECAI score at first visit which subsequently decreased after ULFD treatment. The remaining one dog showed clinically insignificant score that did not change after treatment (Table 1).

Before ULFD treatment, Treg frequency in the peripheral blood of IL-CE dogs tent to be lower than control group. This finding is consistent with a previous report that suggest the role of Treg suppression in the pathogenesis of CE, even though the exact mechanism is still unclear[27]. In human IBD, a decrease in Treg frequency in the circulation was considered to be a result of Treg redistribution in consequence of active inflammation in the intestine[14]. This assumption was strengthened by the finding of an increase on mucosal Treg number of IBD patients[14,24,25,28]. In contrast, a reduction of mucosal Treg was observed in CE dogs[10,13]. Therefore, it is unknown whether mechanism of decreased circulating Treg number in CE dogs is similar to human IBD or not. Another possibility was the impaired proliferation of circulating Treg which is known to mostly consist of natural Treg produced in thymus[7]. To investigate Treg involvement on the pathogenesis of CE, further study to evaluate the circulating and mucosal Treg simultaneously is warranted. In addition, we found that 1 of our IL-CE dogs showed a much lower Treg frequency in comparison to the other 2 dogs. It is possible that this particular dog was at the stage of immune response and/or the disease course in which the Treg proliferation were more severely impaired in comparison to other 2 dogs in the IL-CE group. These factors were mentioned

in a study evaluating Treg frequency in dogs with atopic dermatitis[9]. The other possible factors mentioned in the corresponding study including age difference, disease severity, and concurrent therapies seem not to be applicable in our dogs[9].

Post-ULFD Treg frequency in the peripheral blood of IL-CE dogs showed an increasing tendency compared to pre-ULFD values. This result indicates that ULFD might influence the circulating Treg frequency of IL-CE dogs. Increase of Treg percentage in the peripheral blood of IBD patients after treatment with prednisolone was previously reported[24]. In CE dogs, study on the response of circulating Treg toward a treatment is limited. However, a previous study suggested an increase of mucosal Treg after treatment with a multistrain probiotic product[20].

The exact mechanism of increased Treg frequency in peripheral blood after ULFD treatment remains to be elucidated. ULFD contains only chicken breast, potato and/or rice which can be considered as an elimination diet[11]. This diet was reported to solve GI signs in dogs with suspected food responsive enteropathy[26]. Therefore, it could be one possible mechanism for the improvement of clinical sign in our IL-CE dogs following ULFD treatment. In human IBD, elimination diet was also reported to be one of the alternative treatment[19]. Elimination diet is thought to reduce the potential food antigen that provide a better intestinal microenvironment. As it was reported that poor microenvironment result in Treg suppression in the intestine[15], ULFD treatment might improve Treg function in the intestine and indirectly balance the Treg frequency in intestine and circulation of IL-CE dogs.

ULFD therapeutic mechanism might also involve interleukin 2 (IL-2) which has an important role in the production and maintenance of Treg. IL-2 is a cytokine which has been known to be important in proliferation and differentiation of Treg. The Treg marker CD25 which has high affinity toward IL-2 is functionally essential in the Treg production[15,21]. In a previous report, feeding a diet with low fat content have been reported to retain IL-2 production in mice model of autoimmune disease[16]. Furthermore, studies in mice and humans suggested that the expression of cytokines such as interleukin 10 (IL-10) and transformation growth factor beta (TGF-β) is necessary for Foxp3 expression[16,30]. In mouse model of colitis, the increase of Treg by probiotic was reported to involve IL-10 and TGF-β[8]. Therefore, it is necessary to monitor the expression level of these cytokines in the peripheral blood to clarify the relationship between ULFD, cytokine level, and dynamic of Treg.

In this study, the clinical effect of ULFD was determined by evaluating plasma albumin concentration and CCECAI score. Post-ULFD albumin concentration increased compared to pre-ULFD albumin concentration in all IL-CE dogs, but the concentrations were still less than the reference value (2.6-4.0 g/dl). In addition, the CCECAI score decreased after ULFD treatment compared to before ULFD treatment in 2 of all 3 dogs. These results suggested that all 3 dogs of IL-CE group showed a partial response toward ULFD as a single treatment.

This study had several limitations. First, only small number of IL-CE dogs were included in the current study. Further study with a larger number of cases is necessary to validate the results obtained in this pilot study. Second, control dogs used in this study was not age-matched. Therefore, the age difference could be one of the confounding factors that limit our results, because it was reported that age-related changes and immunosenescence influence the number of lymphocytes in dogs[6,10].

From this pilot study, we can conclude that the increase of Treg frequency in peripheral blood of IL-CE dogs was observed after ULFD treatment. This result may help us understanding the ULFD mechanism. ULFD may not only reduce lacteal pressure, but also induce immune homeostasis by restoring the Treg number.

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