Study on the pro-inflammatory cytokine expression profile in dogs with inflammatory bowel disease

（犬の炎症性腸疾患における炎症性サイトカイン発現解析に関する研究）

Yu Tamura

Laboratory of Veterinary Internal Medicine

Department of Veterinary Clinical Sciences

Graduate School of Veterinary Medicine

Hokkaido University, Japan

March 2014

ABBREVIATIONS

Ab: antibody

CCECAI: canine chronic enteropathy clinical activity index

CD: Crohn’s disease

cDNA: complementary DNA

Ct: cycle threshold

CXCL: CXC chemokine ligand

DAPI: 4', 6'-diamidino-2-phenylindole

DNA: deoxyribonucleic acid

DW: distilled water

ELISA: enzyme-linked immunosorbent assay

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HC: healthy controls

HMBS: hydroxymethyl-bilane synthase

Iba1: ionized calcium binding adapter molecule 1

IBD: inflammatory bowel disease

ICRPs: inflammatory colorectal polyps

IL: interleukin

LPC: lymphocytic-plasmacytic colitis

LPE: lymphocytic-plasmacytic enteritis

mAb: monoclonal antibody

mRNA: messenger RNA

OD: optical density

PBS: phosphate-buffered saline

RNA: ribonucleic acid

RT-PCR: reverse transcription-polymerase chain reaction

SDHA: succinate dehydrogenase complex, subunit A

Th: helper T

TNF: tumor necrosis factor

UC: ulcerative colitis

CONTENTS

GENERAL INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are the most common forms of human IBD. CD involves any part of the gastrointestinal tract, most commonly the terminal ileum or the perianal region, in a non-continuous fashion. The microscopic features of CD include thickened submucosa, transmural inflammation, fissuring ulceration, and non-caseating granulomas [3, 9, 32]. In contrast, UC is characterized by inflammation that is limited to the colon: it begins in the rectum, spreads proximally in a continuous fashion and frequently involves the periappendiceal region. Bloody stools and irregular crypt architecture are solely or more often observed in UC patients. Histologically, UC shows superficial inflammatory changes limited to the mucosa and submucosa with cryptitis and crypt abcesses [3, 32, 74]. The numbers of patients with human IBD are increasing more and more all over the world including Japan [37, 45]. Though the etiology is still unknown, genetic and environmental factors are thought to be involved in the pathogenesis of human IBD [32, 35]. Genome-wide association studies in human IBD patients identified genetic risk loci about barrier function, innate immune defense, and lymphocyte activation [32]. The intestinal microbiota has a critical role in this formation and maintenance of gut immune systems [12, 14, 37, 53]. Previous reports revealed that the rearing environment is a major factor in the determination of the intestinal microbiota profiles in mice [11, 15]. Improved hygiene is believed to result in a limited exposure to micro-organisms. Such exposure is thought to be necessary in the formation of intestinal microbiota and development of immune system in the gut, and decreased its future inflammatory responses [11, 35].

Breakdown of this regulation are thought to lead to the development of human IBD [12, 35].

Intestinal T cells and macrophages are considered to be key players involved in abnormal cytokine production in human IBD [2]. The cytokine responses characterizing human IBD are the key pathophysiologic elements that govern the initiation, evolution, and ultimately, the resolution of these forms of inflammation [67]. TNF-α is one of the most important pro-inflammatory cytokines of human IBD. In fact, anti-TNF therapy has a beneficial effect of IBD treatment when compared to standard therapy such as 5-aminosalicylate and corticosteroids [13, 45, 69]. Moreover, pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-12, and IL-23, are clearly up-regulated in inflamed intestinal mucosa of IBD patients when compared to those in healthy controls [33, 63]. Furthermore, macrophages are thought to be major contributors to the production of these pro-inflammatory cytokines in the gut [19, 50]. Increased levels of IL-1β, IL-6, and TNF-α in the serum or in the intestinal mucosa were related to disease activity [52]. IL-8, a major chemotactic and activating peptide for neutrophils, induces direct migration, the expression of surface adhesion molecules, the release of enzymes, and the production of reactive oxygen metabolites [65]. IL-8 protein level in mucosal biopsies from patients with active CD was positively correlated with the disease activity [27]. Recently, it was revealed that polymorphic variants of IL-8 gene affect the susceptibility of human IBD [72]. IL-12 and IL-23 belong to the IL-12 family of pro-inflammatory heterodimeric cytokines and comprise IL-12p35/IL-12p40 and IL-23p19/IL-12p40 subunits. They promote the differentiation and maintenance of helper T (Th)1 cells and Th17 cells, respectively [40, 63]. Furthermore, protective effect of anti-IL-12p40 mAbs has been well documented in the colitis of IBD model mouse

[10, 48]. The production of IL-23 by the cells of innate immunity is a response to the stimulation or endogenous signals on pattern recognition receptor, indicating a potential role for T cells in the reinforcement of the IL-23 response [52]. Polymorphisms within the *IL-23 receptor* gene locus have been also reported to be involved in IBD patients [8].

Canine IBD is a group of disorders that is characterized by persistent gastrointestinal signs and histological evidence of intestinal inflammation. That is a common cause of chronic vomiting and diarrhea in dogs and must be differentially diagnosed from other possible causes such as infection, food allergy, neoplasia, and exocrine pancreatic insufficiency [66, 73]. Lymphocytic-plasmacytic enteritis (LPE) and Lymphocytic-plasmacytic colitis (LPC) are the most common form of canine IBD [18, 28]. Histopathological features of LPE are diffuse lymphocytic-plasmacytic infiltration to the lamina propria in the duodenal mucosa and that of LPC are lymphocytic-plasmacytic infiltration to the lamina propria in the colonic mucosa [28]. The treatment of canine IBD is focused on the suppression of intestinal inflammation. Glucocorticoids are used most frequently as immunosuppressants [41]. It has been suggested that dysfunction of mucosal immune system with loss of tolerance to luminal antigens possibly plays important roles in the pathogenesis of canine IBD [18, 29]. To date, there are some reports about intestinal barrier, intestinal microbiota, and mucosal pro-inflammatory cytokine expressions in dogs with IBD; however, definitive cause of canine IBD is still unknown [29, 59, 68].

Inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds are recently recognized in Japan as a major cause of large bowel diarrhea in this breed [56]. The most typical clinical signs of ICRPs are hematochezia, tenesmus, and mucoid feces.

ICRPs are characterized by the formation of multiple small polyps (small polyps) in the large bowel mucosa, accompanied by a large space-occupying solitary polyp (large polyp) formation. ICRPs are thought to be a novel form of canine IBD because of the unknown etiology, the existence of idiopathic inflammation, and the effectiveness of immunosuppressive therapy. Histopathological features of ICRPs are crypt expansion, excess mucous secretion, mucous hyperplasia, and inflammatory cell infiltration consisting of mainly macrophages and neutrophils. Dogs with ICRPs usually respond to immunosuppressive drugs such as prednisolone and cyclosporine; however, some of them are resistant to these therapies [56]. Moreover, some of them sometimes form polypoid adenomas in the colorectal mucosa secondary to ICRPs [25]. To date, there is a report describing the expressions of selected Th cytokine such as IL-17A, IFN-γ, IL-4, and IL-10 [58]; however, there is no report describing pro-inflammatory cytokines.

The principal aim of this thesis was to clarify the involvement of pro-inflammatory cytokines in the pathogenesis of canine IBD. Therefore, several pro-inflammatory cytokine expressions in dogs with LPE, LPC, and ICRPs were examined. In Chapter 1, the author investigated pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, in the intestinal mucosa of dogs with LPE and LPC by quantitative real-time RT-PCR. In Chapter 2, same as Chapter 1, the author investigated pro-inflammatory cytokine expressions in the colorectal mucosa of dogs with ICRPs by quantitative real-time RT-PCR.

CHAPTER 1

PRO-INFLAMMATORY CYTOKINE EXPRESSIONS IN THE INTESTINAL MUCOSA OF DOGS WITH LYMPOCYTIC-PLASMACYTIC ENTERITIS AND LYMPOCYTIC-PLASMACYTIC COLITIS

INTRODUCTION

Canine inflammatory bowel disease (IBD) is a group of disorder that is characterized by persistent gastrointestinal signs and histological evidence of intestinal inflammation. Lymohocytic-plasmacytic enteritis (LPE) and lympocytic-plasmacytic colitis (LPC) are the most common forms of canine IBD, and the definitive cause of IBD is unknown. Histopathological features of LPE are diffuse lymphocytic-plasmacytic infiltration to the lamina propria in the duodenal mucosa and that of LPC are lymphocytic-plasmacytic infiltration to the lamina propria in the colonic mucosa. Dysfunction of mucosal immune system with loss of tolerance to luminal antigens possibly plays important roles in the pathogenesis of canine IBD [18, 28].

Several studies reported expression levels of pro-inflammatory cytokine mRNA, such as IL-1β, IL-6, TNF-α, IL-8, and IL-12, in the duodenal mucosa from dogs with LPE; as a result, there was no distinct cytokine profile in the inflamed mucosa of dogs with LPE [17, 29, 38, 60]. In addition, there was no difference in pro-inflammatory cytokine mRNA expressions except for TNF-α in the colonic mucosa from dogs with LPC when compared to those in healthy controls. One report detected the up-regulation of TNF-α mRNA expression, but another report did not detected increased expression of TNF-α mRNA in the colonic mucosa from dogs with LPC when compared to that in healthy controls [28, 60]. Moreover, no report described IL-23 mRNA expression in the intestinal mucosa of dogs with LPE and LPC. Thus, at present, there is no consensus of pro-inflammatory cytokine expressions in the intestinal mucosa from dogs with LPE and LPC.

Since most of the previous studies used semi-quantitative RT-PCR methods, the

aim of Chapter 1 was to determine pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, in the intestinal mucosa of dogs with LPE and LPC by quantitative real-time RT-PCR.

MATERIALS AND METHODS

Dogs

Dogs (n = 24) with chronic gastrointestinal signs were referred to Hokkaido University Veterinary Teaching Hospital from November 2008 to November 2012. Eighteen dogs exhibited clinical signs of small intestinal disease, two dogs exhibited clinical signs of large intestinal disease, and four dogs exhibited clinical signs of both small intestinal and large intestinal disease. Breeds of the dogs included Yorkshire Terrier (n = 4), Border Collie (n = 2), French Bulldog (n = 2), Maltese (n = 2), Basset Hound (n = 1), Beagle (n = 1), Bernese Mountain Dog (n = 1), Boston Terrier (n = 1), Chihuahua (n = 1), Italian Greyhound (n = 1), Jack Russell Terrier (n = 1), Miniature Schnauzer (n = 1), Pembroke Welsh Corgi (n = 1), Pomeranian (n = 1), Shiba Inu (n = 1), Shih-Tzu (n = 1), Toy Poodle (n = 1), and mongrel (n = 1). The criteria for the diagnosis of IBD were as follows: (1) chronic diarrhea with or without vomiting of at least 3-week duration; (2) exclusion of other identifiable causes of chronic gastrointestinal signs by blood examination, urinalyses, parasitic and bacterial analyses of fecal samples, abdominal ultrasonography, and serum trypsin-like immunoreactivity; and (3) Histopathological evidence of intestinal inflammations. Food-responsive diarrhea was ruled out by a complete remission of clinical signs during 2-week on an elimination diet. Antibiotic-responsive diarrhea was ruled out by a lack of complete response to 2-week therapeutic trials of metronidazole (10 mg/kg q12h) or tylosin (20 mg/kg q12h). However, dogs diagnosed with gastric or intestinal tumor by endoscopy and treated with corticosteroids within 2-week were then excluded [38, 73]. All the dogs diagnosed with IBD were scored based on the Canine Chronic Enteropathy Clinical Activity Index

(CCECAI) to assess the severity of the disease [1]. The total CCECAI score was considered clinically insignificant (score 0-3), mild (score 4-5), moderate (score 6-8), severe (score 9-11), or very severe (score >12). Biopsy samples in the duodenal mucosa from dogs with LPE (n = 22) and those in the descending colonic mucosa from dogs with LPC (n = 6) were obtained within the lamina propria by endoscopy. Biopsy specimens for histologic examination were fixed in neutral-buffered 10% formalin, embedded in paraffin, and routinely stained with hematoxylin and eosin. In addition, one mucosal biopsy from each of the inflamed mucosa was immediately placed in RNAlater (Ambion, Austin, TX, USA) and stored at -80℃ until further use.

As healthy control dogs, 6 Beagles and 2 mongrels were used. These included 6 intact females and 2 intact males. Eight dogs were 1 year old (range, 16 to 23 months; median 22 months). In addition, four colonic samples were obtained from 4 healthy mongrels. These included one 8 years old dog and three 9 years old dogs. No clinical signs of gastrointestinal disease were observed in these dogs. Hematologic, serum biochemical, fecal, and abdominal ultrasonographic examinations were performed on all dogs to assess their health status. The use of dogs in this study was approved by the Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University (Approval no. 08-0332).

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, and genomic DNA was removed from the samples with a commercially available kit (RNase-Free DNase set; Qiagen). cDNA was synthesized from 0.5 μg of total RNA using High Capacity RNA-to-cDNA Master

Mix containing the Oligo dT primer and random primer (Applied Biosystems, Foster City, CA, USA).

Generation of positive controls for quantitative PCR

To obtain standard curves for quantitative RT-PCR analysis, parts of the canine sequences for IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40, IL-23p19, and three reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex, subunit A (SDHA), and hydroxymethyl-bilane synthase (HMBS) were cloned into plasmid vectors. Primers were designed using published gene sequences using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the canine GenBank sequences and previous reports [20, 62] (Table 1). PCR was performed with Takara Ex Taq (Takara Bio, Shiga) according to the manufacturer's instructions using the following steps: initial denaturation at 95ºC for 1 min; 35 cycles of denaturation at 95ºC for 30 sec, annealing at 61 to 77ºC for 30 sec, extension at 72ºC for 1 min; final extension at 72ºC for 7 min. Reactions were performed in 25 μl total volume containing 1 μl canine cDNA, 400 pmol cytokine-specific primers. Amplified fragments were visualized on a 1.5% agarose gel. Then, they were ligated into a plasmid vector (TOPO TA Cloning Kit; Invitrogen, Carlsbad, CA, USA). Nucleotide sequences were determined by a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and confirmed with published sequences.

Transcription analysis of cytokine mRNA by real-time RT-PCR

Transcription levels of pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, were examined. Tissue samples were

collected from dogs with LPE (n = 22), LPC (n = 6), and controls (n = 12). Each quantitative real-time RT-PCR reaction was performed in 25 μl, containing 200 nM of each primer and 1 μl cDNA in addition to Power SYBR Green PCR Master Mix (Applied Biosystems) using a 7300 Real-Time PCR System (Applied Biosystems). The amplification conditions consisted of initial denaturation at 95ºC for 10 min and 40 cycles of PCR reaction (95ºC for 15 sec and 60ºC for 1 min), followed by dissociation (95ºC for 15 sec, 60ºC for 30 sec, and 95ºC for 15 sec). Sequences of primer pairs are shown in Table 2. These primers were designed using Primer-BLAST based on the canine GenBank sequences and previous reports [42, 61, 75]. Standard curves for each gene were prepared using 10-fold dilutions (10^0-10^8 molecules μl^{-1}) of plasmids containing the cloned amplicon and reaction efficiency was determined. Standard curves were considered appropriate for efficiency calculation only if a cycle threshold (Ct) value was obtained for at least six serial dilutions, the correlation coefficient was > 0.98 and there was no evidence of misprinting on melting curve analysis. cDNA samples were analyzed in duplicate. Absolute quantification of cytokine mRNA was performed by converting the sample Ct values to the concentration (copies per μl) based on the standard curves. The target amount was then divided by the amount of *SDHA* to obtain a normalized target value and expressed as relative amounts.

Statistical analysis

Statistical analyses were performed using the computer program JMP Pro 10 (SAS Institute, Cary, NC, USA). Normality distribution was assessed by the Shapiro-Wilk W test. The nonparametric Wilcoxon rank sum test was used to compare the results between the two groups. A value of $P < 0.05$ was considered significant.

RESULTS

Clinical and pathological findings

Twenty-four dogs were diagnosed as LPE (n = 22) and/or LPC (n = 6) according to the criteria. The median age of the LPE dogs was 6.7 years (ranging from 1.1 to 10.8 years). The median body weight of the LPE dogs was 6.3 kg (ranging from 1.7 to 33.1 kg). CCECAI scores of dogs with LPE were 4/22 clinically insignificant, 7/22 mild, 6/22 moderate, 4/22 severe, 1/22 very severe, and the median CCECAI score of LPE dogs was 5 (ranging from 2 to 17). The median age of the LPC dogs was 9.4 years (ranging from 2.5 to 11.7 years). The median body weight of the LPC dogs was 13.5 kg (ranging from 2.0 to 33.1 kg). CCECAI scores of dogs with LPC were 1/6 clinically insignificant, 3/6 moderate, 2/6 severe, and the median CCECAI score was 7.5 (ranging from 3 to 12).

Quantification of cytokine expression in the intestinal mucosa

In the preliminary experiment, the transcription levels of *GAPDH*, *SDHA*, and *HMBS* mRNA in the intestinal tissue samples were quantified for validation of accuracy as calibrator genes. Of these three quantified genes, the expression of *SDHA* mRNA was the most stable among the three candidate reference genes using geNorm [70]; therefore, the transcription of *SDHA* mRNA was selected as the reference gene in this study (data not shown).

There was no significant difference in IL-1β, IL-6, and IL-8 mRNA expression in the duodenal mucosa between dogs with LPE and controls (Fig. 1). On the contrary, TNF-α, IL-12p35, IL-12/23p40, and IL-23p19 mRNA expression was significantly

decreased in the duodenal mucosa from dogs with LPE when compared to those in controls (Fig. 1).

There was no significant difference in each cytokine expression except for IL-23p19 mRNA in the colonic mucosa between dogs with LPC and controls (Fig. 2). IL-23p19 mRNA expression was only significantly increased in the colonic mucosa from dogs with LPC when compared to that in controls (Fig. 2).

Table 1. Sequences of oligonucleotide primers used for RT-PCR

Primer set		Primer sequence (5'-3')	Position	GenBank accession number
IL-1β	Forward Reverse	GAGGTTCCAATGTGAAGTGC CCTGTAACTTGCAGTCCACC	83-373	NM_001037971
IL-6	Forward Reverse	GAACTCCCTCTCCACAAGC TTCTTGTCAAGCAGGTCTCC	60-385	NM_001003301
TNF-α	Forward Reverse	ACTCTTCTGCCTGCTGCACTTTGG GTTGACCTTTGTCTGGTAGGAGACGG	138-504	NM_001003244
IL-8/CXCL8	Forward Reverse	ACTTCCAAGCTGGCTGTTGC GGCCACTGTCAATCACTCTC	10-181	NM_001003200
IL-12p35	Forward Reverse	AGCCTCACCGAGCCCAGGA TAAGGCACAGGACCGTCATAAAAG	90-382	NM_001003293
IL-12/23p40	Forward Reverse	GCCCCCGGAGAAATGGTGGTC TTGTGGCACACGACCTTGGCTG	121-908	NM_001003292
IL-23p19	Forward Reverse	ACACGTCTCATTTCCCTTGG TCTCAAATCTGGCTGGCTCT	151-456	XM_538231
GAPDH	Forward Reverse	CCTCCTGCACCACCAACTGC CCACCCGGTTGCTGTAGCCA	443-967	NM_001003142
SDHA	Forward Reverse	GGACAGAGCCTCAAGTTTGG GGCATCCTTCCGTAATGAGA	547-958	XM_535807
HMBS	Forward Reverse	GGCGGAAGGAAACGGCCCAA GCCAGCTGGGCTTCTCGTGG	30-989	XM_546491

Table 2. Sequences of oligonucleotide primers used for quantitative real-time RT-PCR

Primer set		Primer sequence (5'-3')	Position	GenBank accession number
IL-1β	Forward Reverse	CAAGTCTCCCACCAGCTCTGTA GGGCTTCTTCAGCTTCTCCAA	154-234	NM_001037971
IL-6	Forward Reverse	TTAAGTACATCCTCGGCAAAATCT CAGTGCCTCTTTGCTGTCTTCA	212-297	NM_001003301
TNF-α	Forward Reverse	TCTCGAACCCCAAGTGACAAG CAACCCATCTGACGGCACTA	241-393	NM_001003244
IL-8/CXCL8	Forward Reverse	CTCTCTGTGAAGCTGCAGTTCTG GGAAAGGTGTGGAGTGTGTTTTT	59-139	NM_001003200
IL-12p35	Forward Reverse	GTGCCTCAACCACTCCCAA CAATCTCTTCGGAAGTGCAGG	117-217	NM_001003293
IL-12/23p40	Forward Reverse	CAGCAGAGAGGGTCAGAGTGG ACGACCTCGATGGGTAGGC	527-635	NM_001003292
IL-23p19	Forward Reverse	CAAGGGGAGAAAAACAGCAG TGCTGTCCGTTCTGTGAGTC	303-381	XM_538231
GAPDH	Forward Reverse	ATCACTGCCACCCAGAAGAC TCAGCTCAGGGATGACCTTG	535-667	NM_001003142
SDHA	Forward Reverse	GCCTTGGATCTCTTGATGGA TTCTTGGCTCTTATGCGATG	685-776	XM_535807
HMBS	Forward Reverse	TCACCATCGGAGCCATCT GTTCCCACCACGCTCTTCT	323-434	XM_546491

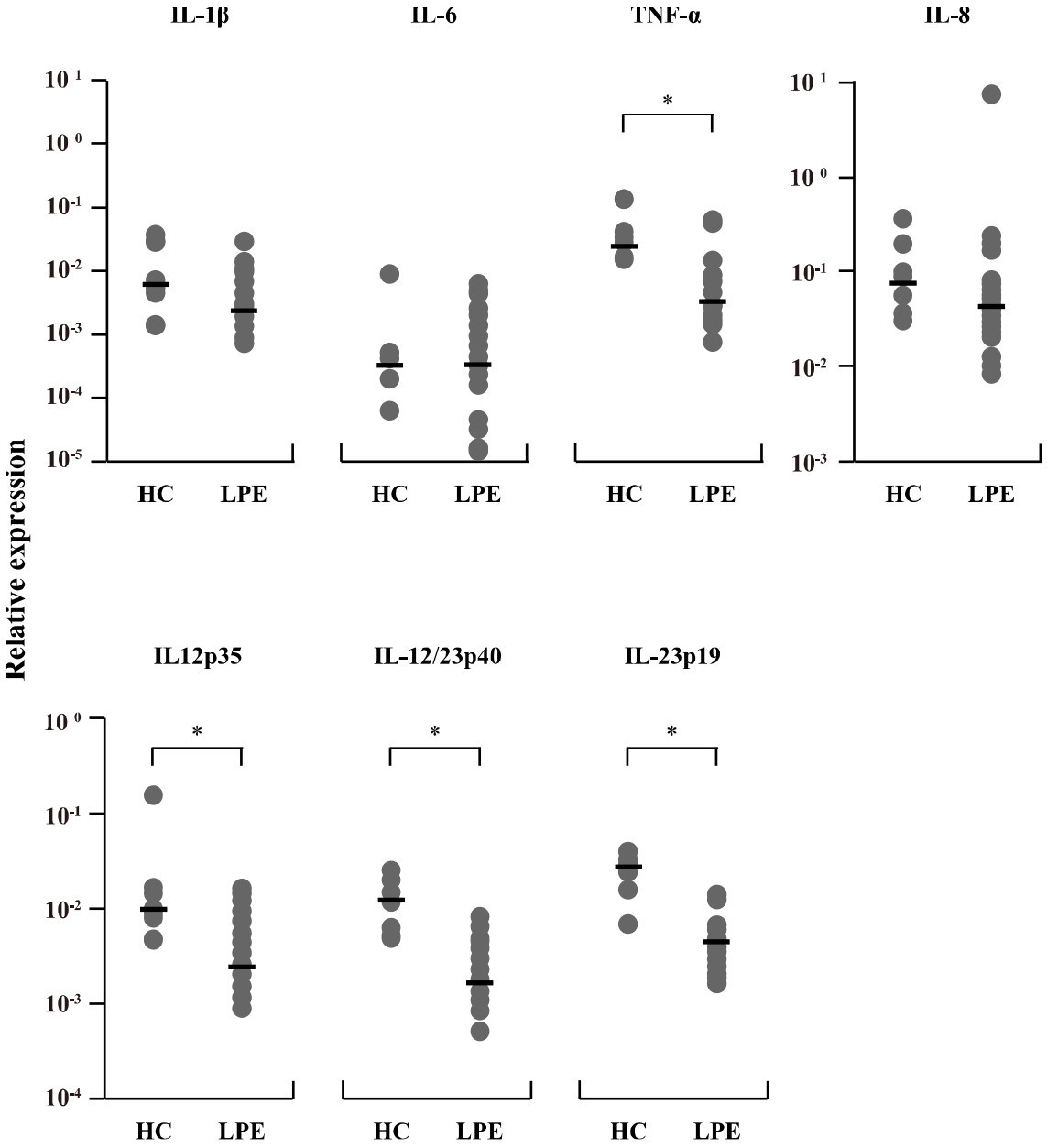

Fig. 1. Relative expressions of cytokine mRNAs (IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40, and IL-23p19) in the duodenal mucosa from healthy controls (HC, n = 8) and LPE dogs (LPE, n = 22). The horizontal lines correspond to the median of the relative quantity in each group. Asterisks indicate statistical differences (*$P < 0.05$).

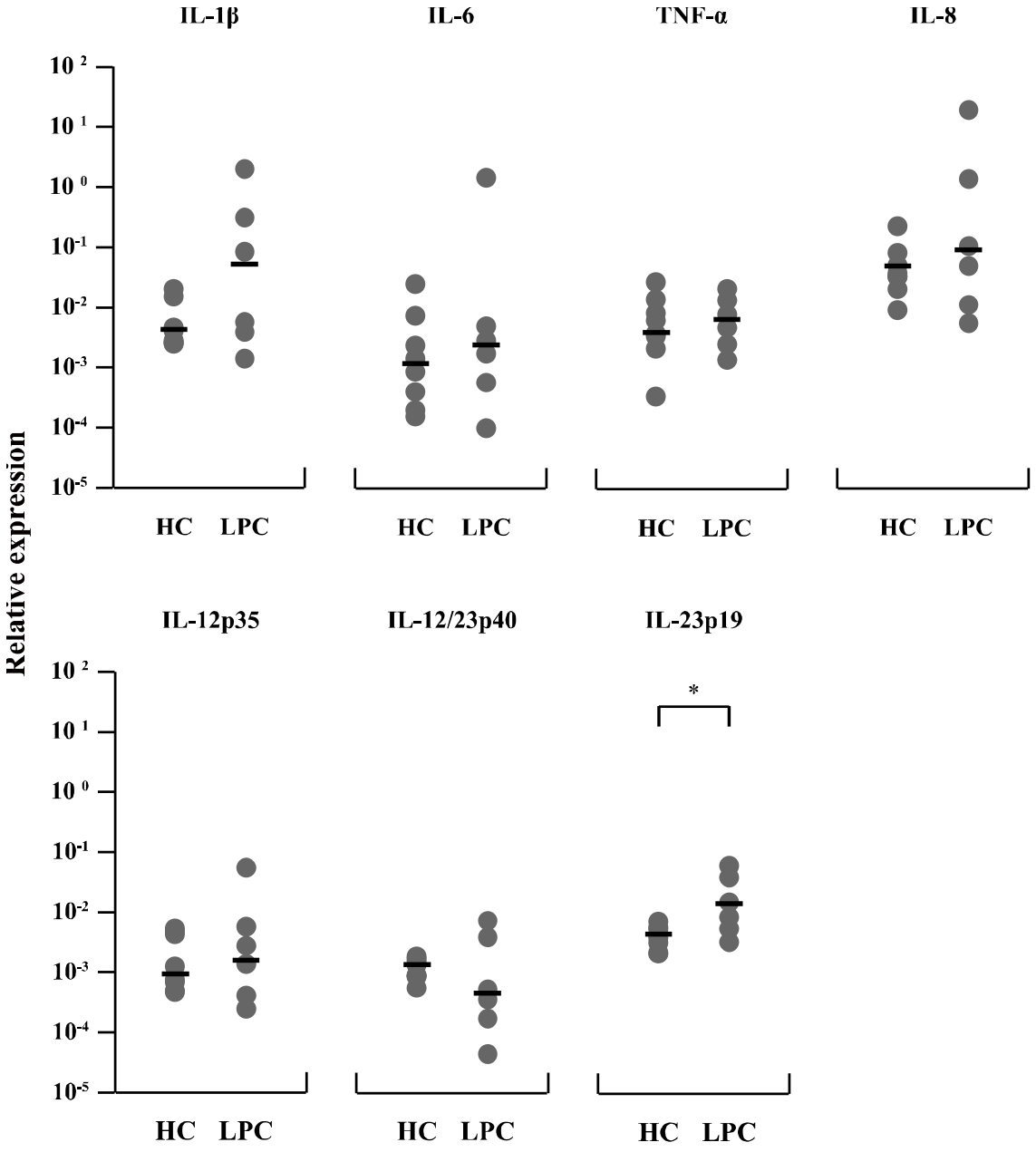

Fig. 2. Relative expressions of cytokine mRNAs (IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40, and IL-23p19) in the colonic mucosa from healthy controls (HC, n = 12) and LPC dogs (LPC, n = 6). The horizontal lines correspond to the median of the relative quantity in each group. Asterisks indicate statistical differences (*$P < 0.05$).

DISCUSSION

In Chapter 1, the author aimed to determine pro-inflammatory cytokine expressions in canine IBD by evaluating the amounts of mRNA in the duodenal and colonic mucosa from dogs with LPE and LPC. In contrast to findings in human IBD, duodenal and colonic samples from dogs with IBD used in this study did not show the up-regulation of IL-1β, IL-6, TNF-α, IL-8, IL-12p35, and IL-12/23p40 mRNA expressions when compared to those in controls. Thus, there was no clear evidence of the up-regulation of pro-inflammatory cytokine mRNA expressions in the intestinal mucosa from dogs with LPE and LPC.

Pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IL-8, IL-12, and IL-23, were clearly up-regulated in inflamed areas in human IBD [33, 63]. Evidence from recent research suggests that CD is driven by Th1 producing TNF-α, IFN-γ and Th17 producing IL-17 [40]. TNF-α expression levels are increased in inflammatory lesions of CD patients, and they play important roles in the induction and persistence of intestinal mucosal inflammation [63]. Indeed, the blockage of TNF-α by anti-human TNF-α monoclonal antibodies dramatically decrease mucosal inflammatory activity and disease severity of patients with CD [39]. IL-17 mRNA expression in the inflamed mucosa and Th17-related cytokine expression of $CD4^+$ T cells in the isolated lamina propria from both UC and CD was found to be increased when compared to that in normal controls [16]. Moreover, IL-17 produced by Th17 appeared to be promoted by IL-23 [34]. IL-23 is thought to be produced by macrophages and plays important roles in the promotion of Th17 cell differentiation [8]. Therefore, IL-23 is the key cytokine in the IL-23/IL-17 pathway of human IBD.

Recent research described about $CD4^+$ T cell cytokine expressions, such as IL-17, IFN-γ, IL-4, and IL-10, in the duodenal mucosa of dogs with LPE. In that study, there was no significant difference in each cytokine expression between dogs with LPE and controls [57]. This observation suggests that there is also no evidence of the involvement of Th1 or Th17 in dogs with LPE. On the contrary, IL-23p19 mRNA expression was only significantly increased in the colonic mucosa from dogs with LPC when compared to that in controls. However, IL-17A mRNA expression was not increased in the colonic mucosa of LPC dogs (unpublished data). Therefore, the significance of increased IL-23p19 mRNA expression in the development and maintenance of colonic mucosal inflammation remains unclear. Thus, further studies are necessary to clarify the biological significance of increased IL-23p19 mRNA expression in the pathogenesis of canine LPC.

In this study, the reason for decreased pro-inflammatory cytokine mRNA expressions was uncertain. Previous report described that IL-10 mRNA expression level in the duodenal mucosa of IBD dogs was not different from healthy control dogs [57]; therefore, increased IL-10 expression might be not the cause of decreased pro-inflammatory cytokine mRNA. Previous report demonstrated that several chemokines and chemokine receptors were significantly increased in the duodenal mucosa from dogs with LPE [38]. Thus, chemokines and chemokine receptors are thought to play important roles in migration of inflammatory cells such as lymphocytes and plasma cells.

There were two limitations of the current study. Firstly, cytokine mRNA expression levels did not always reflect protein expression levels. Thus, cytokine protein levels in lamina propria should be analyzed either by ELISA, flow cytometry, or

immunohistochemistry when canine-specific antibodies are available. Secondly, case numbers were small, in particular LPC dogs because of limited availability of large intestinal cases in my facility. Further studies with a large number of cases are needed to validate this investigation in LPC dogs.

In conclusion, different from human IBD, no distinct cytokine profile was found in canine IBD at the level of mRNA expression in the inflamed intestinal mucosa. No clear evidence was obtained for the contribution of cytokine profile to the pathogenesis of canine IBD. The results of Chapter 1 raise the possibility that the mechanism responsible for the pathogenesis of canine IBD is different from that of human IBD.

SUMMARY

Pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, in the intestinal mucosa of dogs with LPE and LPC were investigated by quantitative real-time RT-PCR. As a result, TNF-α, IL-12p35, IL-12/23p40, and IL-23p19 mRNA expressions were significantly decreased in the duodenal mucosa from dogs with LPE when compared to those in controls. IL-23p19 mRNA expression was only significantly increased in the colonic mucosa from dogs with LPC when compared to that in controls. Thus, different from human IBD, no clear evidence was obtained for the contribution of cytokines to the pathogenesis of canine LPE and LPC.

CHAPTER 2

PRO-INFLAMMATORY CYTOKINE EXPRESSIONS IN MINIATURE DACHSHUNDS WITH INFLAMMATORY COLORECTAL POLYPS

INTRODUCTION

Inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds were recently recognized as a major cause of large bowel diarrhea in this dog breed in Japan [56]. ICRPs are characterized by the formation of multiple small polyps (small polyps) around the large bowel mucosa, almost invariably restricted to the descending colon and rectum, sometimes accompanied by space-occupying solitary large polyp formation (large polyp). ICRPs are histopathologically characterized by proliferative changes of the mucosal epithelium and severe inflammatory cell infiltration, mainly consisting of macrophages and neutrophils (Fig. 3). To date, the etiology of ICRPs is unknown; however, based on the existence of idiopathic inflammation and the effectiveness of immunosuppressive therapy, ICRPs are thought to be a novel form of IBD.

There is no report describing pro-inflammatory cytokine expression in the colorectal mucosa of dogs with ICRPs. Based on the histopathological features of ICRPs, the author speculated that the up-regulation of pro-inflammatory cytokines plays a key role in the pathogenesis of ICRPs. The aim of Chapter 2 was to explore the expression of key pro-inflammatory cytokines in ICRPs and to detect the major source of that cytokine. Therefore, pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*, was quantified in the colorectal mucosa from dogs with ICRPs by real-time PCR. The author also measured IL-8 protein level by ELISA and investigated its localization by immunofluorescence microscopy.

MATERIALS AND METHODS

Dogs

Eleven Miniature Dachshunds brought to Hokkaido University Veterinary Teaching Hospital (n = 6), Miyazaki University Veterinary Teaching Hospital (n = 2), and Yuki Animal Hospital (n = 3) because of signs of chronic colitis, including excess fecal mucus, hematochezia, tenesmus, and increased frequency of defecation were diagnosed with ICRPs based on histological findings of a previous report [56]. The median age of these dogs was 8.9 years (ranging from 7 to 14.6 years) with 4 females (2 intact and 2 neutered) and 7 males (4 intact and 3 neutered). The median body weight was 6.2 kg (ranging from 2.9 to 6.8 kg). These cases presented between April 2010 and February 2013. Routine hematology, serum biochemistry, urinalysis, and fecal examination were unremarkable in all dogs, but serum CRP concentration was elevated in 4 cases. All dogs underwent a colonoscopy under general anesthesia. In this study, large polyp was defined as a solitary space-occupied polyp or greater than 5 mm in size, and small polyp was defined as multiple polyps and less than 5 mm in size.

Colonoscopy demonstrated a large polyp in the colorectal area in eleven cases and small polyps in the descending colon and rectal area in six cases. Six cases had both large and small polyps, while five cases had only a large polyp. Biopsy samples from the large polyps (n = 11) and small polyps (n = 6) were obtained by endoscopy or mucosal pull-through surgical excision. Biopsy specimens for histological examination were fixed in neutral-buffered 10% formalin, embedded in paraffin wax, and routinely stained with hematoxylin and eosin. In addition, one mucosal biopsy from each of the large and small polyps was placed immediately in RNAlater (Ambion) and stored at

-80℃ until further use.

Healthy control dogs were same as Chapter 1. In addition, control samples were also taken from two Miniature Dachshunds (7-year-old and 15-year-old castrated males). Biopsy specimens were taken from non-involved areas of the colon with colorectal adenocarcinoma. The median body weight of these control dogs was 12.0 kg (ranging from 5.4 to 15.8 kg).

Transcription analysis of cytokine mRNA by real-time RT-PCR

RNA extraction, cDNA synthesis, and gene expression analysis of cytokine mRNA were performed as described in Chapter 1. Tissue samples were collected from large polyps (n = 11), small polyps (n = 6), and controls (n = 14).

Quantification of IL-8 protein levels by ELISA

Colorectal specimens were collected from large polyps (n = 6), small polyps (n = 5), and controls (n = 14). The samples for ELISA were rinsed twice in phosphate-buffered saline (pH 7.4) (PBS), placed in empty micro tubes and stored at -80ºC until use. Samples were homogenized for 30 sec in 500 µl homogenization buffer (PBS containing 0.05% Tween 20) with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) using a mechanical tissue homogenizer (Ultra-Turrax T25; Janke & Kunkel, Staufen im Breisgau, Germany). Tissue homogenates were centrifuged briefly and the supernatants were frozen at -20ºC. IL-8 level was measured using commercial ELISA kits specific for canine CXCL8/IL-8 (R & D Systems, Minneapolis, MN, USA). Absorbance was read at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). All samples were examined in duplicate and the

mean OD value was calculated. Total protein concentrations were determined by the Bradford method [7] (Protein assay kit; Bio-Rad Laboratories) and cytokine levels were normalized. Serum samples from 4 ICRP dogs and 12 healthy control dogs were taken and stored at -20ºC until analysis. IL-8 level was measured as described above.

Immunofluorescence microscopy

Colorectal mucosa specimens (large polyps; n = 6, small polyps; n = 5, and controls; n = 12) were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Four-micrometer-thick sections were deparaffinized and boiled for 30 min in 0.01 M citrate buffer (pH 6.0) to achieve antigen retrieval. Sections were washed with PBS, followed by incubation with 10% normal rabbit serum for 30 min. For single labeling, sections were incubated with anti-canine IL-8 goat polyclonal Ab (4 μg/ml; R & D Systems) for 2 hours at room temperature. After incubation, sections were washed with PBS, followed by the incubation with rabbit anti-goat IgG Ab conjugated with Hilyte Fluor 488 (5 μg/ml; AnaSpec, Fremont, CA, USA) for 1 hour at room temperature. After washing with PBS, sections were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI; AnaSpec) for 5 min at room temperature. Slides were washed with PBS, followed by distilled water, and then embedded in fluorescent mounting medium (Dako Cytomation, Glostrup, Denmark). For dual labeling, sections were incubated with anti-IL-8 Ab and anti-human CD3 mouse mAb (Clone F7.2.38, Dako) or anti-Iba1 rabbit polyclonal Ab (Wako, Osaka). Cross reactivity of the anti-CD3 Ab with canine tissues was confirmed by previous report [46]. After blocking with 10% normal rabbit serum for 30 min, sections were incubated with anti-IL-8 Ab and anti-CD3 Ab for 2 hours at room temperature. After incubation, sections were

incubated with rabbit anti-goat IgG Ab conjugated with Hilyte Fluor 488. After washing with PBS, sections were blocked with 10% normal goat serum and incubated with goat anti-mouse IgG Ab conjugated with Hilyte Fluor 555 (5 μg/ml; AnaSpec) for 1 hour at room temperature. Sections were counterstained with DAPI and embedded in fluorescent mounting medium. In the preliminary experiment, the author used anti-Myeloid/Histiocyte Ab (3.75 μg/ml: Clone MAC 387, Dako), anti-human CD163 Ab (10 μg/ml: Clone No. AM-3K; Trans Genic, Hyogo), and anti-Iba1 Ab (2 μg/ml) for macrophage staining. Cross reactivity of AM-3K Ab with canine tissues was confirmed by previous report [76]. Among these antibodies, anti-Iba1 Ab and anti-Myeloid/Histiocyte (MAC387) Ab clearly detected macrophages in canine lymph node sections (data not shown). However, MAC387 Ab reacted with not only macrophages but also neutrophils in canine tissues (data not shown); therefore anti-Iba1 Ab was selected as macrophage specific marker in this study. The decision of macrophages and neutrophils was morphologically determined. For dual labeling with IL-8 and Iba1, sections were blocked with 5% skim milk and incubated with anti-IL-8 Ab and anti-Iba1 Ab (2 μg/ml) for 2 hours at room temperature. Sections were washed with PBS, followed by the incubation with donkey anti-rabbit IgG Ab conjugated with Hilyte Fluor 555 (5 μg/ml; Invitrogen) for 1 hour at room temperature. After incubation, sections were washed with PBS, followed by blocking with 10% normal rabbit serum for 30 min at room temperature. After incubation, sections were incubated with rabbit anti-goat IgG Ab conjugated with Hilyte Fluor 488 (5 μg/ml) for 1 hour at room temperature. Sections were counterstained with DAPI and embedded in fluorescent mounting medium. Sections incubated with normal goat, rabbit or mouse IgG were included as a negative control sample. To examine whether neutrophils secrete IL-8, the

author additionally dual stained with anti-IL-8 Ab and MAC387 Ab using similar method as double staining of anti-IL-8 Ab and anti-CD3 Ab. Sections were examined and analyzed using a LSM 700 Laser Scanning Microscope (Zeiss, Oberkochen, Germany) and LSM Software ZEN 2011 (Zeiss). IL-8- or Iba1-positive cells were enumerated by a single investigator (Y.T.) with a fluorescence microscope (HS BZ-9000; Keyence, Osaka) and HS BZ-II analysis application. Five areas were chosen at random for each standardized area, and positively stained cells were counted.

Statistical analysis

Statistical analyses were performed using a computer program JMP Pro 10 (SAS Institute). Normality distribution was assessed by the Shapiro-Wilk W test. The Kruskal-Wallis test was used to test for overall differences in cytokine mRNA expression and colorectal mucosal IL-8 protein level among large polyps, small polyps, and controls. The Steel-Dwass test was used to test for between-group differences. Wilcoxon rank sum test was used to compare the serum IL-8 protein level between ICRPs and controls. One-way analysis of variance was used to test for overall differences in IL-8- or Iba1-positive cell counts among large polyps, small polyps, and controls. The Tukey-Kramer HSD test was used to analyze between-group differences. The relationships between IL-8 positive cells and both tissue IL-8 protein level and serum IL-8 protein level in dogs with ICRPs and healthy controls were evaluated using the Spearman rank correlation coefficient. A value of $P < 0.05$ was considered significant.

RESULTS

Transcription levels of cytokines

The relative expression levels of all cytokines examined in large polyps were significantly higher than those in normal colorectal mucosa (Fig. 4). In addition, relative expression levels of IL-1β, IL-6, IL-8, and IL-12p35 mRNA in small polyps were significantly higher than those in normal colorectal mucosa (Fig. 4). Compared to small polyps, IL-1β, IL-8, and IL-23p19 mRNA were significantly increased in large polyps (Fig. 4). Among these cytokines, IL-8 mRNA expression was markedly increased in large polyps (25,000-fold) and small polyps (300-fold) compared to that in the normal colorectal mucosa.

IL-8 protein levels

Because IL-8 mRNA expression was markedly increased in colorectal mucosa of dogs with ICRPs, the author next examined the IL-8 protein level in the colorectal mucosa. IL-8 protein was significantly elevated in large polyps compared to that in the normal colorectal mucosa (Fig. 5A). IL-8 protein in small polyps also showed a significant increase when compared to controls (Fig. 5A). The author also examined serum IL-8 protein level in a small number of clinical cases. As a result, serum IL-8 protein level in ICRP dogs was significantly increased when compared to control dogs (Fig. 5B).

IL-8-expressing cells in the colorectal mucosa

To detect IL-8-expressing cells in the colorectal mucosa, samples were stained

with anti-IL-8 Ab. As shown in Fig. 6, there were few IL-8-positive cells in normal colonic mucosa (Fig. 6A). In contrast, IL-8 positive cells were increased in small and large polyps of ICRP dogs (Fig. 6B and C). In particular, IL-8-positive cells were markedly increased in large polyps of ICRP dogs (Fig. 6C and Fig. 10A). IL-8-positive cells were located within the lamina propria and not in intestinal epithelial cells (Fig. 6A-C, Fig. 7A and D, Fig. 8A, and Fig. 9A). To confirm the cellular origin of IL-8 in small and large polyps of ICRP dogs, samples were double stained with fluorescence-labeled anti-IL-8 Ab (Fig. 7A and D, Fig. 8A, and Fig. 9A) and anti-Iba1 Ab (specific for macrophages) (Fig .7B and E), anti-CD3 Ab (Fig. 8B), or MAC387 Ab (Fig. 9B). All of the IL-8-positive cells were positive for Iba1 (Fig. 7C and F). IL-8/CD3 double-positive cells were not observed in colorectal mucosa from control dogs or ICRP dogs (Fig. 8A-C). In addition, IL-8/MAC387 double-positive cells were sparsely observed (Fig. 9A-C). Both Iba1-positive macrophages and IL-8/Iba1 double-positive macrophages were increased in large polyps compared to those in small polyps and controls (Fig. 10A and B). The percentage of IL-8-positive macrophages in controls, small polyps, and large polyps was 15%, 44%, and 50%, respectively (Fig. 10C).

Linear correlation between IL-8 protein levels and IL-8 positive cells

A significant positive correlation between tissue IL-8 protein levels and IL-8 positive cells was detected (r_s = 0.68, P < 0.01) (Fig. 11A). Moreover, a significant positive correlation between serum IL-8 protein levels and IL-8 positive cells was also detected (r_s = 0.78, P < 0.01) (Fig. 11B).

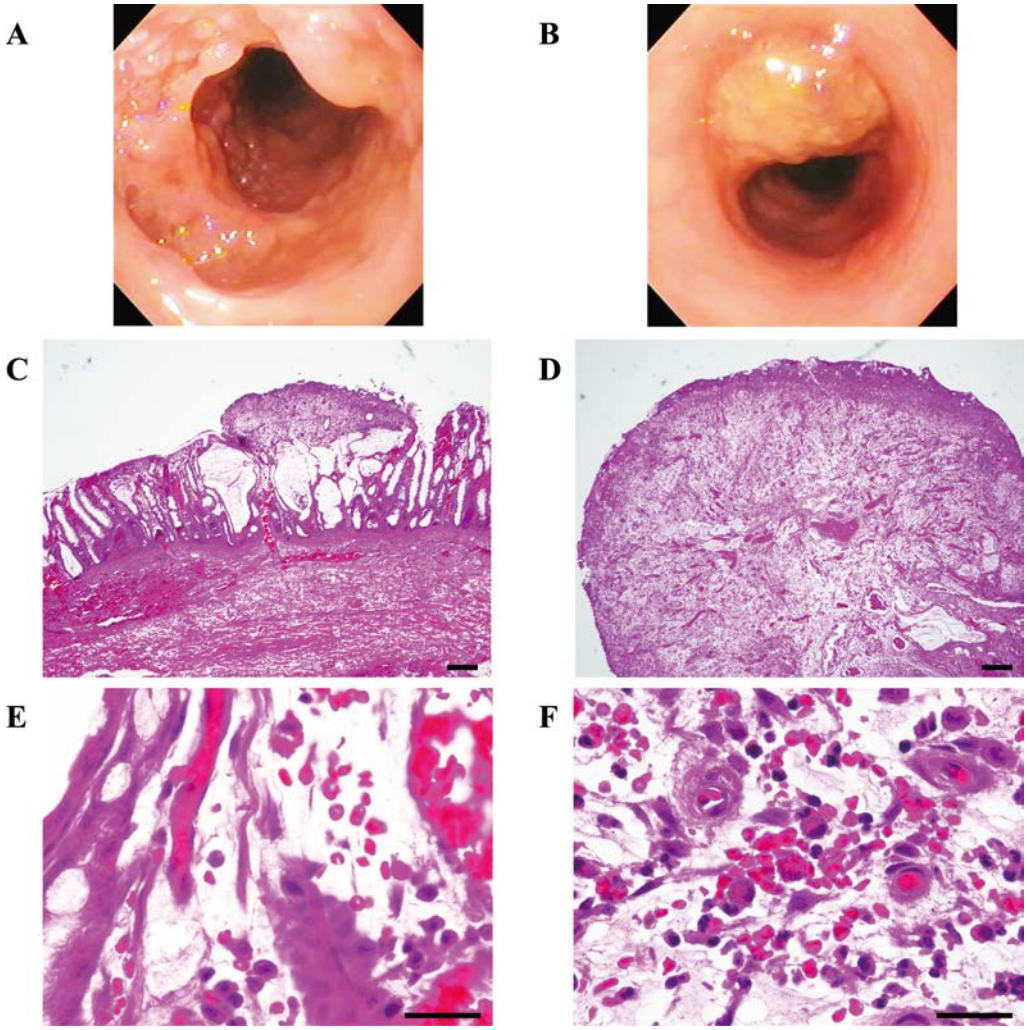

Fig. 3. Endoscopic (A and B) and microscopic (C-F) findings of small polyps (A, C, and E) and a large polyp (B, D, and F) in a miniature dachshund with ICRPs (hematoxylin and eosin). (A) Multiple small polyps in the colorectal area. (B) A solitary space-occupying large polyp in the rectal area. (C) Crypt expansion, excess mucous secretion, and mucous hyperplasia are observed (Scale bar = 200 μm). (D) Severe edema and inflammatory cell infiltration in lamina propria are observed (Scale bar = 200 μm). (E) Infiltration of small number of lymphocytes, macrophages, and neutrophils. (Scale bar = 20 μm). (F) Large number of macrophages and neutrophils are observed (Scale bar = 20 μm).

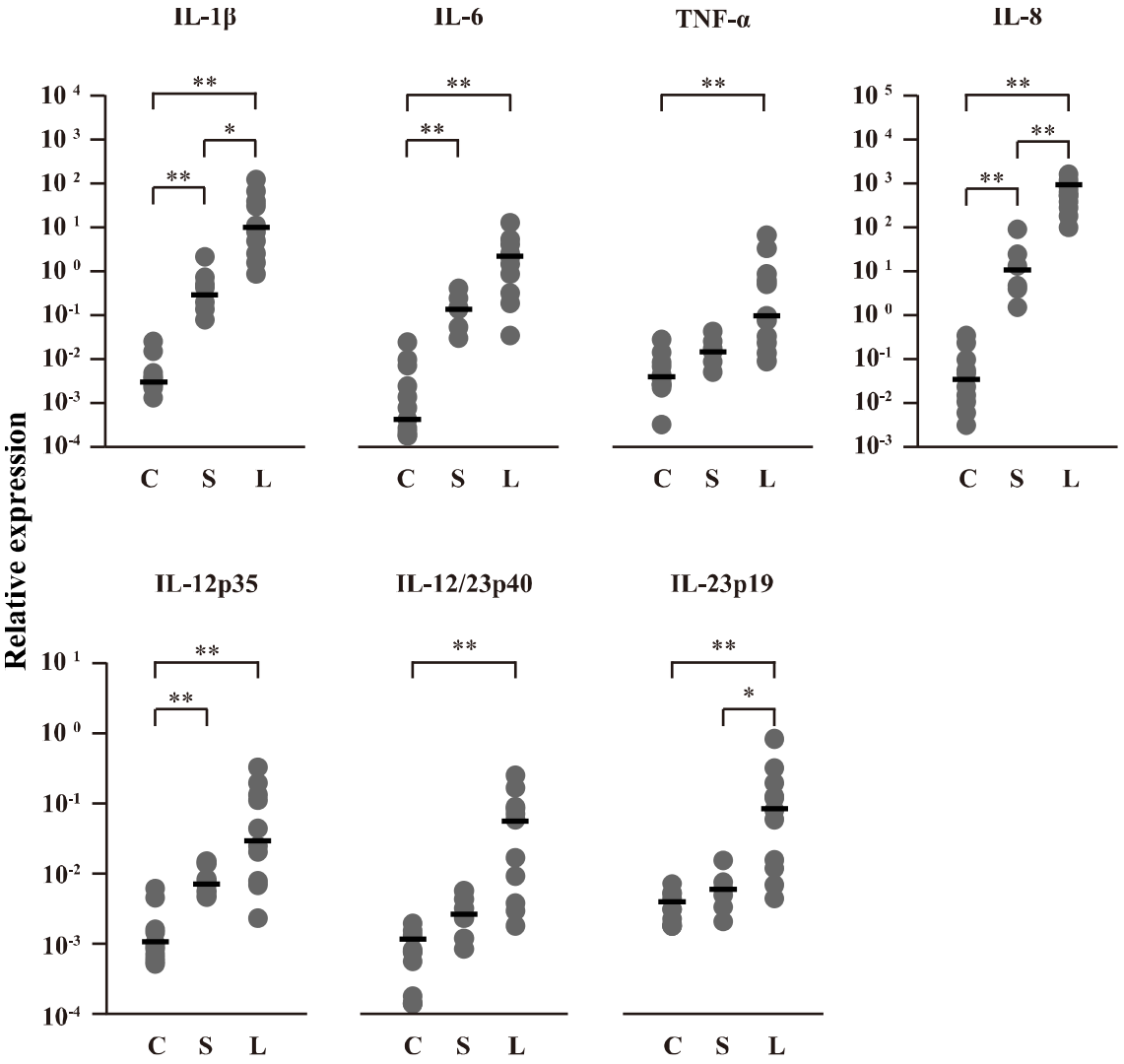

Fig. 4. Relative expression of pro-inflammatory cytokine mRNAs (IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40, and IL-23p19) in the colorectal mucosa from controls (C, n = 14), small polyps (S, n = 6), and large polyps (L, n = 11). The horizontal lines correspond to the median of the relative quantity in each group. Asterisks indicate statistical differences (*$P < 0.05$, **$P < 0.01$).

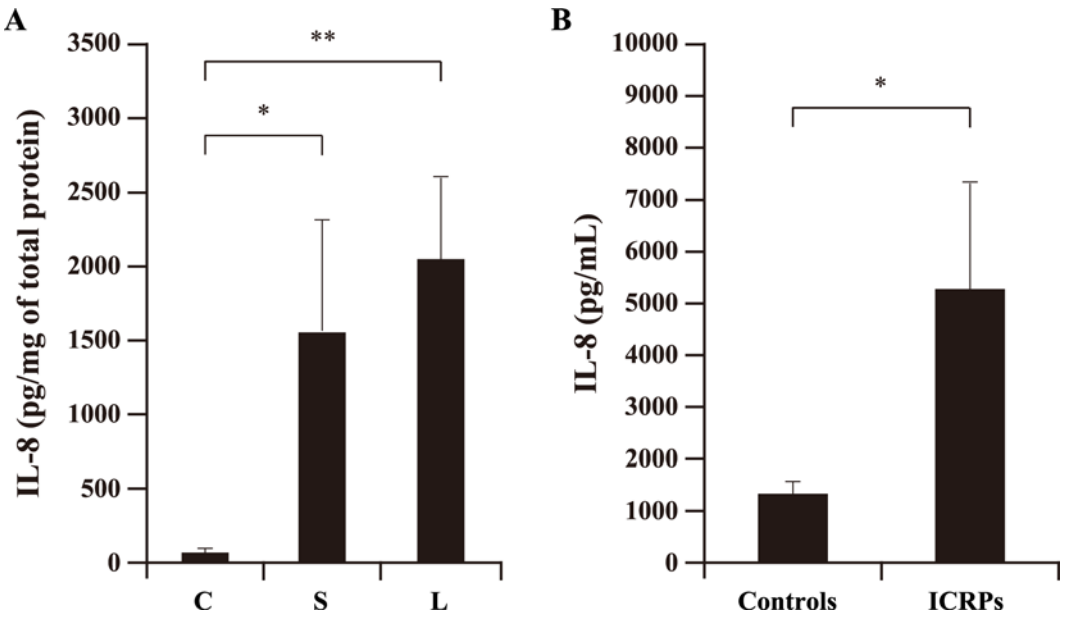

Fig. 5. Protein levels of IL-8 in the colorectal mucosa and serum. (A) IL-8 protein in the colorectal mucosa from controls (C, n = 14), small polyps (S, n = 5), and large polyps (L, n = 6). The protein level was normalized to the total protein concentration in each dog and is expressed as pg/mg total protein. (B) IL-8 protein in the serum from controls (n = 12) and ICRPs (n = 4). The protein level is expressed as pg/mL. Data are shown as mean ± SEM. Asterisks indicate statistical differences (*$P < 0.05$, **$P < 0.01$).

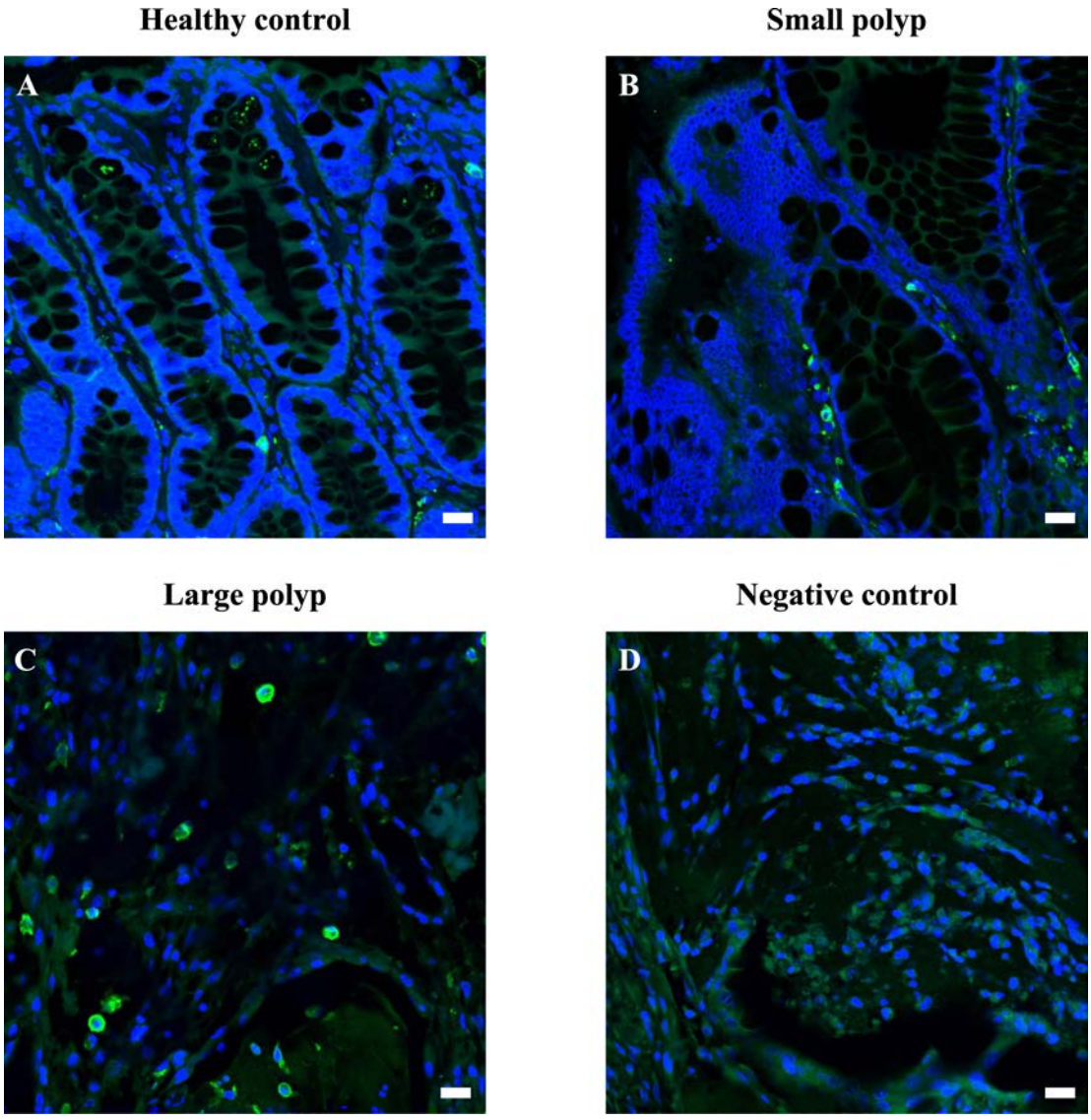

Fig. 6. Photomicrographs of representative immunofluorescence microscope results for single labeling of IL-8 protein in the colorectal mucosa obtained from healthy control (A), small polyp (B), large polyp (C), and negative control using a large polyp specimen stained with normal goat IgG (D). Scale bars = 20 μm.

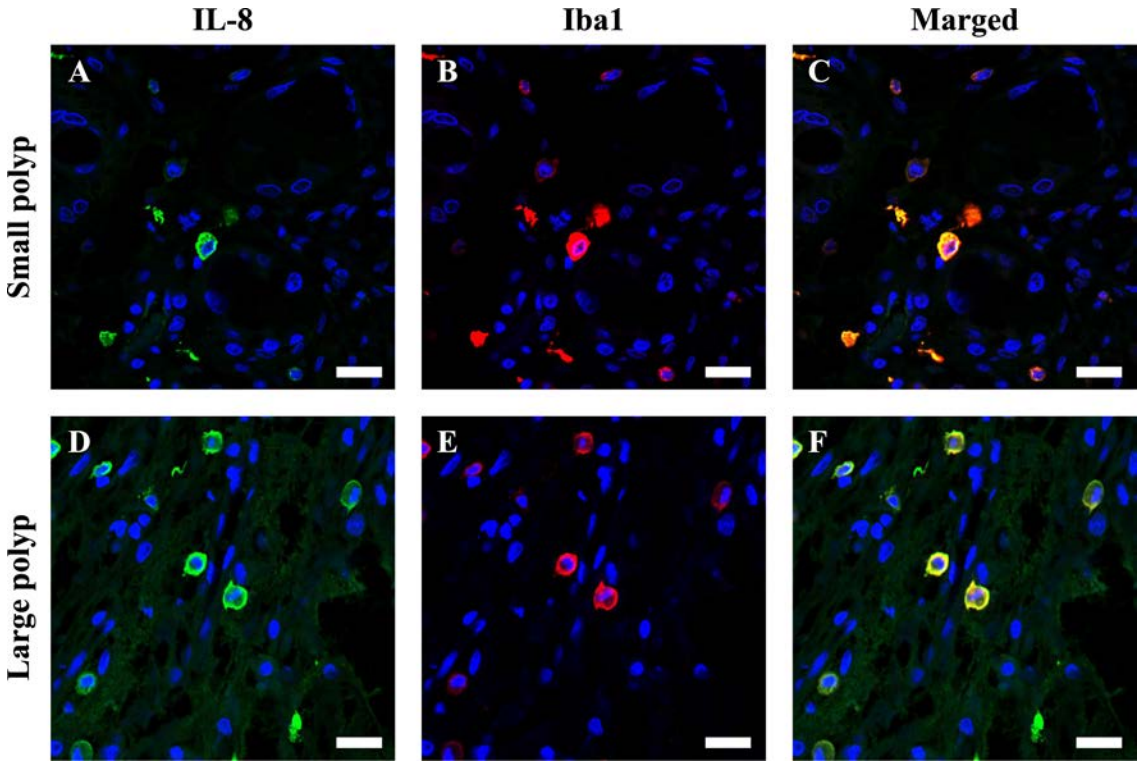

Fig. 7. Photomicrographs of representative immunofluorescence microscope results for localization of IL-8 protein in the colorectal mucosa obtained from small polyps (A-C) and large polyps (D-F). These show dual-immunofluorescent labeling of IL-8 (green: A and D) and Iba1 (red: B and E). Cells showing coexpression of IL-8 and Iba1 are visualized in yellow (C and F). Scale bars = 20 μm.

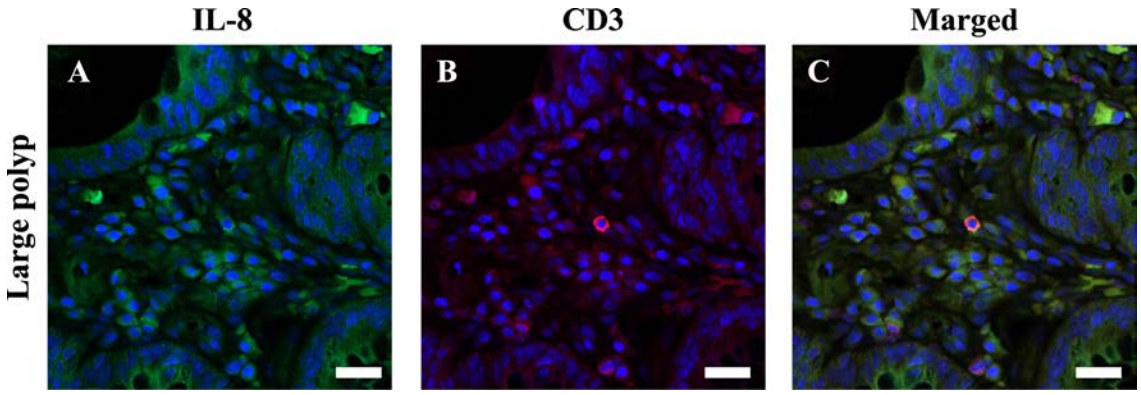

Fig. 8. Photomicrographs of representative immunofluorescence microscope results for localization of IL-8 protein in the colorectal mucosa obtained from large polyps. These show dual-immunofluorescent labeling of IL-8 (green: A) and CD3 (red: B). Cells showing coexpression of IL-8 and CD3 are not observed (C). Scale bars = 20 μm.

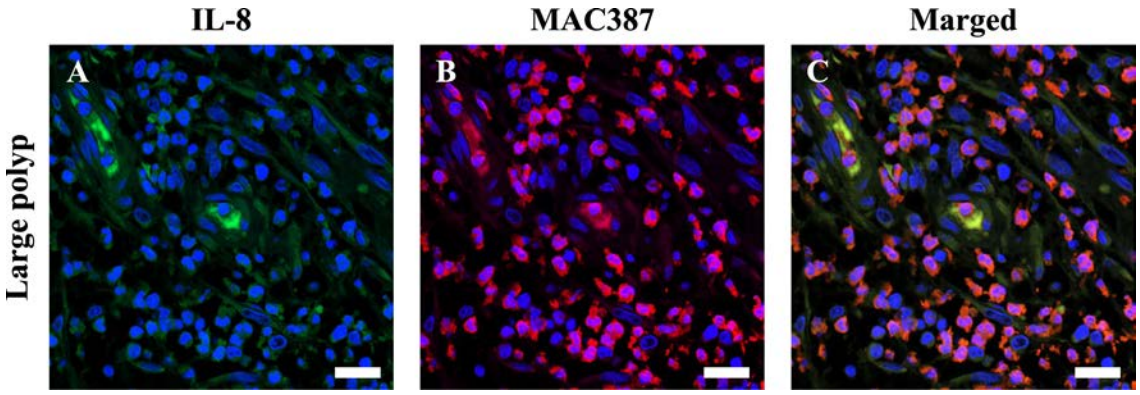

Fig. 9. Photomicrographs of representative immunofluorescence microscope results for localization of IL-8 protein in the colorectal mucosa obtained from large polyps. These show dual-immunofluorescent labeling of IL-8 (green: A) and MAC387 (red: B). Cells showing coexpression of IL-8 and MAC387 are visualized in yellow (C). Scale bars = 20 μm.

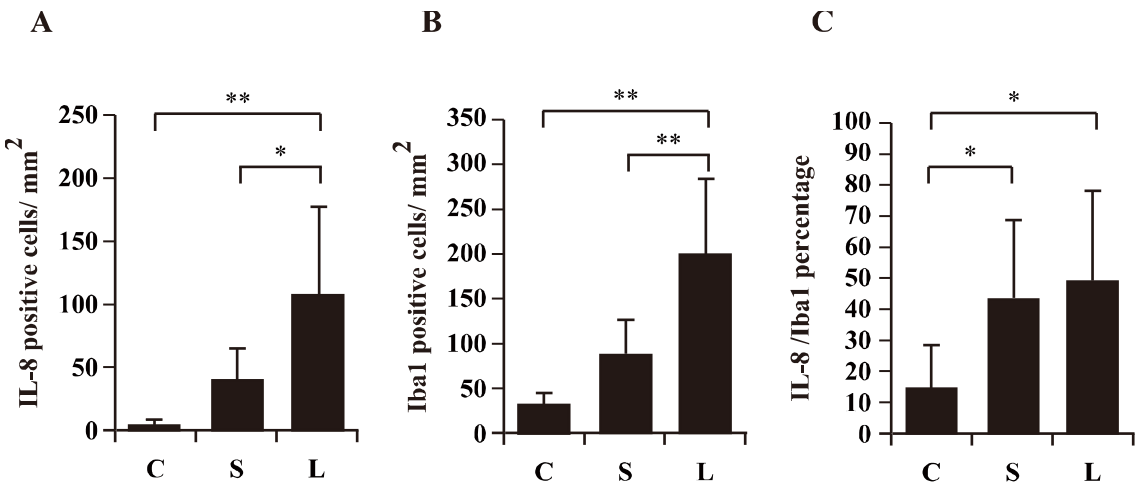

Fig. 10. Cell count results of IL-8- and Iba1-positive cells in the colorectal mucosa. IL-8-positive (A) and Iba1-positive (B) cell number. Results are expressed as the number of positive cells per field (mm^2). The mean and SD are shown. (C) Percentage of IL-8-positive cells to Iba1-positive cells. The mean and SD are shown. C: healthy controls (n = 12), S: small polyps (n = 5), and L: large polyps (n = 6). Asterisks indicate statistical differences (*$P < 0.05$, **$P < 0.01$).

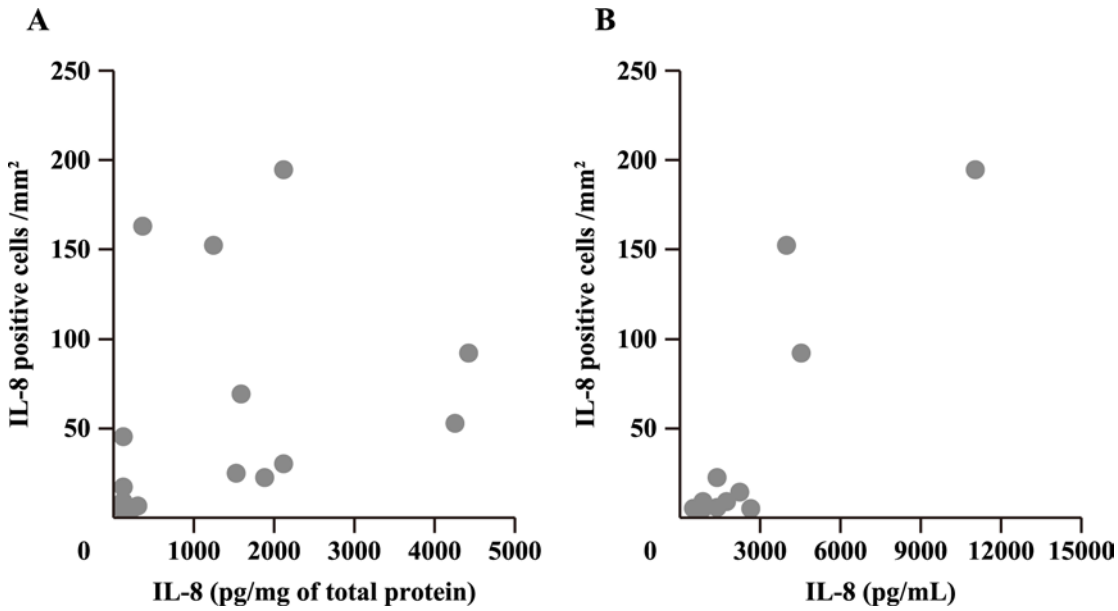

Fig. 11. Linear correlation between IL-8 protein levels (x-axis) and numbers of IL-8 positive cells (y-axis) in healthy dogs and dogs with ICRPs. (A) Tissue IL-8 levels were positively correlated with IL-8 positive cells in the colorectal mucosa (n = 23: Controls, n = 12; Small polyps, n = 5; Large polyps, n = 6) (r_s = 0.6840, P = 0.0012). (B) Serum IL-8 levels in dogs were positively correlated with IL-8 positive cells (n = 16: Controls, n = 12; ICRP dog, n = 4) (r_s = 0.7790, P = 0.0028).

DISCUSSION

In Chapter 2, the author aimed to identify the key pro-inflammatory cytokines in the colorectal lesion of ICRPs in Miniature Dachshunds. A significant increase in the transcription levels of *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19* mRNA was found within large polyps of ICRP dogs when compared to controls. This up-regulation of pro-inflammatory cytokine expressions is consistent with previous reports of human IBD [52, 63]. Among these pro-inflammatory cytokines, *IL-8* gene expression was markedly increased.

IL-8 is known as chemokine CXCL8, which is a potent chemoattractant for neutrophils [65]. This effect has also been confirmed in dogs [44]. Results of the present study showed that IL-8 mRNA transcription and protein levels were significantly increased in large polyps when compared to controls. Increased IL-8 mRNA and protein has been reported in colonic mucosa from patients with CD and UC [27, 47]. Histopathologically, neutrophil infiltration is common in colonic mucosa from both active UC and CD patients [47]. Indeed, IL-8 expression and concentration in the colon correlate with the disease activity of IBD [31, 43]. ICRPs are characterized histopathologically by severe neutrophil infiltration in the colorectal mucosa. Thus, similar to the situation in human IBD, IL-8 might play an important role in the recruitment of neutrophils in the colorectal mucosa of dogs with ICRPs.

In human IBD, serum IL-8 protein level was also increased and associated with disease activity [64]. Although the number of ICRP dogs used in this study was small, serum IL-8 protein level was increased in ICRP dogs when compared to controls (Fig. 5B). In human necrotizing enterocolitis, serum IL-8 levels declined after resection of

local lesion [6]. Likewise, the origin of serum IL-8 in dogs with ICRPs was considered to be derived from colorectal polyps. The usefulness of serum IL-8 in estimating disease activity of ICRP dogs remains unclear; however, there was positive correlation between serum IL-8 protein levels and IL-8 positive cells (Fig. 11B). Therefore, serum IL-8 may be useful as a predictive marker for polyp formations. Further study involving a greater number of cases will clarify its usefulness in ICRP dogs.

In the colonic mucosa of human IBD, a major source of IL-8 is macrophages [19, 24]. In this study, the author analyzed the cellular source of IL-8 in the colorectal mucosa from dogs with ICRPs. As a result, IL-8 protein was detected in Iba1-positive macrophages, but not in intestinal epithelial cells or neutrophils. Moreover, IL-8-positive macrophage subsets were significantly increased in large polyps compared to small polyps and controls. These results suggest that inflammatory macrophages producing pro-inflammatory cytokine such as IL-8 were increased instead of inhibitory intestinal resident macrophages in the colorectal region of dogs with ICRPs.

In the present study, the author used anti-Iba1 Ab for macrophage staining although anti-Iba1 Ab is not a standard marker of macrophage. Iba1 (ionized calcium binding adapter molecule 1) is a protein that is specifically expressed in macrophage/microglia and is involved in RacGTPase-dependent membrane ruffling and phagocytosis [26, 30]. Anti-Iba1 Ab used in this study was raised against the synthetic peptide of C-terminal lesion of Iba1 which are conserved among species including dogs, and specifically reacts with human and rodent macrophage/microglia. In mice, this Ab is regarded as pan-macrophage marker [36]. In the preliminary study described above, the author morphologically confirmed clear and specific macrophage labeling without neutrophil staining by anti-Iba1 Ab using canine lymph node sections and colorectal

specimens of ICRP dogs. Thus, anti-Iba1 Ab was selected as a macrophage specific marker among several antibodies used for macrophage staining.

In human neutrophils and canine polymorphonuclear leukocytes, IL-8 mRNA expressions are increased under the process of phagocytosis or under the influence of lipoteichoic acid stimulation *in vitro* [4, 5]. By using immunofluorescence microscopy, neutrophils were not thought to be a major source of IL-8 protein in the colorectal mucosa of ICRP dogs (Fig. 9A-P). However, it is possible that neutrophils produce minute quantities of IL-8, but they infiltrate the inflamed colorectal mucosa in numbers large enough to contribute to IL-8 protein accumulation.

Both IL-1β and TNF-α are also important pro-inflammatory cytokines released from activated macrophages, and they share many pro-inflammatory activities [63]. IL-1β and TNF-α induce local inflammation and this inflammatory process leads to the disruption of the epithelial barrier [22, 63]. This permits easy access for the luminal microbiota and dietary antigens into resident cells in the lamina propria, thus stimulating pathological immune cell responses. In addition, they induce other pro-inflammatory cytokine production, including IL-8 and IL-6 [21, 63]. In human IBD, IL-1β and TNF-α expression levels are increased in inflammatory lesions of CD and UC patients, and they play important roles in the induction and persistence of intestinal mucosal inflammation [52]. Indeed, the blockage of TNF-α by anti-human TNF-α mAb markedly decreased mucosal inflammatory activity and the disease severity of patients with CD and UC [39]. In dogs with ICRPs, similarly to human IBD, IL-1β and TNF-α were significantly increased in large polyps in the colorectal area. Thus, they might play important roles in the induction and persistence of local inflammation and IL-8 production from macrophages.

IL-6 is mainly produced by activated macrophages and mesenchymal cells, and its production is stimulated with IL-1β and TNF-α [63]. Elevated levels of IL-6 in the serum and in mucosal biopsies taken from patients with IBD have been described, and the serum IL-6 concentration is related to disease activity [54]. IL-6 induces local tissue inflammation and C-reactive protein production in the liver [71]. In addition, according to an analysis of auto immune arthritis in F759 mice, an IL-6 positive-feedback loop is formed between Th17 cells and fibroblasts or vascular endothelial cells at the local inflammatory site, thus up-regulating several cytokines and chemokines [51, 55]; therefore, IL-6 may mediate regional inflammation and could be involved in the production of IL-8 indirectly.

In human IBD, microbial antigen-activated macrophages produce IL-12 and IL-23, thereby leading to the development of Th1 and Th17 immunity, respectively [40, 63]. Evidence from recent research suggests that CD is driven by Th1 cells producing IFN-γ, and Th17 cells producing IL-17 [23]. In the colorectal mucosa from ICRP dogs, IL-23p19 mRNA expression was increased in large polyps compared to small polyps and controls. In contrast, mRNA expression of IL-12p35 was increased in large and small polyps compared to controls, although there was no difference in IL-12p35 mRNA expressions between large and small polyps. These results show that Th17 cytokines might play important roles in the formation of a solitary large polyp. In human cystic fibrosis airway epithelial cells, IL-17A synergistically increased IL-8 production in airway epithelial cells when stimulated by bacterial antigen [49]. Indeed, Ohta et al. [58] found significant up-regulation of IL-17A mRNA in large polyps compared to small polyps and controls; therefore, the IL-23/IL-17 axis might induce IL-8 overproduction, and this leads to severe neutrophil inflammation and large polyp

formation in the colorectal mucosa of ICRP dogs.

In this study, all of ICRP dogs formed a large polyp, and several cases also formed multiple small polyps in the colorectal area. In contrast, a previous report described that there were ICRP dogs which had only small polyps [56]. The current study suggests that pro-inflammatory cytokines play important roles in the pathogenesis of ICRPs; however, the difference in the mechanisms of small polyps and large polyp formation was not clarified. There was more severe inflammation in the large polyp than small polyps; however, this infiltration may not be secondary to tissue trauma in the large polyp because of its sterile nature and responsiveness to immunosuppressive therapy but not to antibiotics. In addition, anatomical factor is thought to be important for the large polyp formation because rectal mucosa is exposed to feces for a long period of time when compared to other intestinal segments. However, further study is needed to clarify the difference between the formation of large and small polyps.

There were some limitations of the current study. Firstly, the function of intestinal macrophages isolated from the colorectal mucosa of ICRP dogs was not analyzed in this study. Thus, in a future study, IL-8 protein level in lamina propria macrophages should be analyzed by flow cytometry. Secondly, IL-8 protein concentration was quantified using total protein extracted from the whole colorectal mucosa. Thus, IL-8 protein in large polyps might be underestimated because of severe hemorrhage in large polyp lesions and blood contamination of large polyp specimens. Thirdly, IL-8 is a secretory protein; thus, it could be difficult to detect small amounts of IL-8 protein by immunostaining. Therefore, there is a possibility that intestinal epithelial cells and neutrophils are also the source of IL-8 in the colorectal mucosa of dogs with ICRPs. Thus, it is necessary to examine IL-8 mRNA expression using *in situ*

hybridization methods. Finally, the control dogs used in this study included only two Miniature Dachshunds because of the limited availability of normal colorectal mucosa from Miniature Dachshunds.

In conclusion, the present study demonstrated that pro-inflammatory cytokine mRNA expression was significantly increased in colorectal mucosa of dogs with ICRPs. In particular, *IL-8* expression was markedly up-regulated, and the number of IL-8-positive macrophages was increased in large polyps. These pro-inflammatory cytokines, especially IL-8, might play important roles in neutrophil recruitment in the colorectal mucosa and polyp formation. Further studies are necessary to clarify the mechanisms of the formation of large and small polyps in dogs with ICRPs.

SUMMARY

To explore key mediators in the pathogenesis of ICRPs, the author investigated pro-inflammatory cytokine expressions, such as *IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19* in the colorectal mucosa of dogs with ICRPs by quantitative real-time RT-PCR. Among these cytokines, *IL-8* expression was markedly up-regulated in large polyps. To examine IL-8 protein expression, the author analyzed IL-8 protein level and its localization in colorectal mucosal specimens of ICRP dogs by ELISA and immunofluorescence microscopy. IL-8 protein was significantly increased in large polyps and serum in dogs with ICRPs when compared to controls. By immunofluorescence microscopy, IL-8 was only localized in macrophages, but not in mucosal epithelial cells and neutrophils. The number of IL-8-positive macrophages was significantly increased in large polyps compared to controls. These results suggest that IL-8 is produced mainly by macrophages and may induce neutrophil infiltration in the colorectal area of ICRP dogs.

GENERAL CONCLUSION

The goal of this study was to clarify whether several pro-inflammatory cytokines are involved in the pathogenesis of dogs with LPE, LPC, and ICRPs. The findings of the present study suggest that the involvement of pro-inflammatory cytokines in dogs with LPE and LPC was different from that of human IBD. In contrast, pro-inflammatory cytokine expressions in dogs with ICRPs were similar to that of human IBD.

In Chapter 1, the author revealed that relative expression of TNF-α, IL-12p35, IL-12/23p40, and IL-23p19 mRNA was significantly decreased in the duodenal mucosa of dogs with LPE when compared to control dogs. Moreover, there was no significant difference in each cytokine mRNA expression except for IL-23p19 mRNA in the colonic mucosa between dogs with LPC and controls. Thus, there is no clear evidence for the up-regulation of pro-inflammatory cytokine mRNA expressions in the intestinal mucosa of dogs with LPE and LPC. These results suggested that the pathogenesis of canine LPE and LPC is different from that of human IBD.

In Chapter 2, the author revealed that the relative expression levels of all cytokines (*IL-1β*, *IL-6*, *TNF-α*, *IL-8*, *IL-12p35*, *IL-12/23p40*, and *IL-23p19*) examined in large polyps were significantly higher than those in normal colorectal mucosa. In addition, relative expression levels of IL-1β, IL-6, IL-8, and IL-12p35 mRNA in small polyps were significantly higher than those in normal colorectal mucosa. Among these cytokines, IL-8 expression was significantly elevated in large polyps and small polyps when compared to that in the normal colorectal mucosa. Serum IL-8 protein level in ICRP dogs was also significantly increased when compared to that in control dogs. By immunofluorescence microscopy, IL-8 was only localized in macrophages, but not in

mucosal epithelial cells and neutrophils. The number of IL-8-positive macrophages was significantly increased in large polyps when compared to controls. These results suggested that IL-8 is produced mainly by macrophages and may induce neutrophil infiltration in the colorectal area of ICRP dogs.

In conclusions, involvement of pro-inflammatory cytokines in dogs with LPE and LPC may be different from human IBD. On the other hand, there is clear evidence for the up-regulation of pro-inflammatory cytokine expressions, in particular IL-8, in dogs with ICRPs. Further study is needed to clarify the etiology of canine IBD.

JAPANESE SUMMARY（要　旨）

犬の炎症性腸疾患（IBD）は慢性の消化器症状を示す原因不明で難治性の疾患群である。小腸性に起こるリンパ球形質細胞性腸炎（LPE）と大腸性に起こるリンパ球形質細胞性結腸炎（LPC）が最も一般的な病型である。LPE と LPC の診断は、除外診断により他の疾患を除外し、病変部の病理組織検査によりリンパ球と形質細胞の浸潤が粘膜に認められたものとされる。一方で、炎症性結直腸ポリープ（ICRPs）は近年国内のミニチュア・ダックスフンドで主に認識されてきている結直腸の炎症性疾患である。内視鏡像では多発性の小ポリープと内腔を占拠するような孤立性の大ポリープとに分けられ、病理組織学的には多数の好中球とマクロファージの浸潤が認められる。病因は不明であるが、免疫抑制療法には反応を示すことから、犬の IBD の新たな病型ではないかと考えられてきている。犬の IBD の病因に関して、管腔内に存在する常在細菌叢などの刺激に対する粘膜免疫の破綻が重要な役割を担っていると考えられている。

人の IBD であるクローン病（CD）と潰瘍性大腸炎（UC）の病態生理には、腸管の免疫抑制の破綻が中心的な役割を担っている。CD と UC の両疾患において、炎症部位における炎症性サイトカイン（IL-1β、IL-6、TNF-α、IL-8、IL-12p35、IL-12/23p40 及び IL-23p19）は健常者に比べて有意に増加しており、病態形成に重要な役割を担っている。一方で、犬においては LPE と LPC では炎症性サイトカインの発現解析を行った報告はあるものの、その数は少なく、一貫した見解は得られていない。また、ICRPs に関しては今までに炎症性サイトカインの発現解析を行った報告はない。そこで本研究の目的は、犬の LPE、LPC 及び ICRPs における炎症性サイトカインの発現を解析することである。

第 1 章では、定量リアルタイム PCR 法を用いて LPE と LPC の腸管粘膜における炎症性サイトカインの遺伝子発現解析を行った。その結果、LPE では TNF-α、IL-12p35、IL-12/23p40 及び IL-23p19 遺伝子発現量が健常犬に比べて有意に減少しており、LPC では IL-23p19 遺伝子発現量のみが健常犬に比べて有意に上昇し

ていた。これらのことより、炎症性サイトカインの発現量は LPE と LPC の病態形成に深く関与していない可能性が示唆された。

第 2 章では、定量リアルタイム PCR 法を用いて ICRPs の小ポリープと大ポリープ病変部における炎症性サイトカインの遺伝子発現解析を行った。その結果、大ポリープでは測定した全ての炎症性サイトカイン遺伝子発現量が健常犬に比べて有意に上昇しており、小ポリープでは IL-1β、IL-6、IL-8 及び IL-12p35 遺伝子発現量が健常犬に比べて有意に上昇していた。中でも、IL-8 遺伝子発現量が顕著に上昇していたため、ELISA 法を用いて IL-8 蛋白質量を定量した。IL-8 蛋白質は ICPRs の血中と病変部の両方において健常犬と比べて有意に上昇していることが明らかになった。そこで、IL-8 産生細胞を特定するために免疫組織化学染色を行ったところ、IL-8 陽性細胞はマクロファージに特異的なマーカーである Iba1 陽性細胞と共染色されることが明らかとなり、その陽性細胞数は健常結腸組織、小ポリープ、大ポリープの順に増加していることが示された。これらのことより、ICRPs の病態形成にはマクロファージから産生される IL-8 が重要である可能性が考えられた。

本研究により、犬の LPE 及び LPC と ICRPs では病変部における炎症性サイトカインの発現動態が異なることが明らかとなった。LPE と LPC では、病変部における炎症性サイトカインの発現上昇はほぼ認められず、人の IBD とは病態が異なる可能性が示唆された。一方、ICRPs では病変部における炎症性サイトカインの発現量は上昇しており、特に顕著に上昇していた IL-8 はマクロファージから産生されている可能性が示唆され、ICRPs の病態形成に深く関わっていることが考えられた。

REFERENCES

1. Allenspach, K., Wieland, B., Gröne, A. and Gaschen, F. 2007. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. *J. Vet. Intern. Med.,* **21:** 700-708.

2. Atreya, R. and Neurath, M. F. 2005. Involvement of IL-6 in the pathogenesis of inflammatory bowel disease and colon cancer. *Clin. Rev. Allergy Immunol.,* **28:** 187-196.

3. Baumgart, D. C. and Sandborn, W. J. 2007. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet,* **369:** 1641-1657.

4. Bazzocchi, C., Mortarino, M., Comazzi, S., Bandi, C., Franceschi, A. and Genchi, C. 2005. Expression and function of Toll-like receptor 2 in canine blood phagocytes. *Vet. Immunol. Immunopathol.,* **104:** 15-19.

5. Bazzoni, F., Cassatella, M. A., Rossi, F., Ceska, M., Dewald, B. and Baggiolini, M. 1991. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J. Exp. Med.,* **173:** 771-774.

6. Benkoe, T., Reck, C., Gleiss, A., Kettner, S., Repa, A., Horcher, E. and Rebhandl, W. 2012. Interleukin 8 correlates with intestinal involvement in surgically treated infants with necrotizing enterocolitis. *J. Pediatr. Surg.,* **47:** 1548-1554.

7. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72:** 248-254.

8. Che Mat, N. F., Zhang, X., Guzzo, C. and Gee, K. 2011. Interleukin-23-induced interleukin-23 receptor subunit expression is mediated by the Janus kinase/signal

transducer and activation of transcription pathway in human CD4 T cells. *J. Interferon Cytokine Res.*, **31:** 363-371.

9. Crohn, B. B., Ginzburg, L. and Oppenheimer, G. D. 1932. Regional ileitis A pathologic and clinical entity. *JAMA.*, **99:** 1323-1329.

10. Davidson, N. J., Hudak, S. A., Lesley, R. E., Menon, S., Leach, M. W. and Rennick, D. M. 1998. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J. Immunol.*, **161:** 3143-3149.

11. Delcò, F. and Sonnenberg, A. 1998. Military history of patients with inflammatory bowel disease: an epidemiological study among U.S. veterans. *Am. J. Gastroenterol.*, **93:** 1457-1462.

12. Diehl, G. E., Longman, R. S., Zhang, J. X., Breart, B., Galan, C., Cuesta, A., Schwab, S. R. and Littman, D. R. 2013. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature*, **494:** 116-120.

13. Dretzke, J., Edlin, R., Round, J., Connock, M., Hulme, C., Czeczot, J., Fry-Smith, A., McCabe, C. and Meads, C. 2011. A systematic review and economic evaluation of the use of tumour necrosis factor-alpha (TNF-α) inhibitors, adalimumab and infliximab, for Crohn's disease. *Health Technol. Assess.*, **15:** 161-163.

14. Friswell, M., Campbell, B. and Rhodes, J. 2010. The role of bacteria in the pathogenesis of inflammatory bowel disease. *Gut Liver,* **4:** 295-306.

15. Friswell, M. K., Gika, H., Stratford, I. J., Theodoridis, G., Telfer, B., Wilson, I. D. and McBain, A. J. 2010. Site and strain-specific variation in gut microbiota profiles and metabolism in experimental mice. *PLoS One,* **5:** e8584.

16. Fujino, S., Andoh, A., Bamba, S., Ogawa, A., Hata, K., Araki, Y., Bamba, T. and Fujiyama, Y. 2003. Increased expression of interleukin 17 in inflammatory bowel

disease. *Gut,* **52:** 65-70.

17. German, A. J., Hall, E. J. and Day, M. J. 2001. Immune cell populations within the duodenal mucosa of dogs with enteropathies. *J. Vet. Intern. Med.,* **15:** 14-25.

18. German, A. J., Hall, E. J. and Day, M. J. 2003. Chronic intestinal inflammation and intestinal disease in dogs. *J. Vet. Intern. Med.,* **17:** 8-20.

19. Grimm, M. C., Elsbury, S. K., Pavli, P. and Doe, W. F. 1996. Interleukin 8: cells of origin in inflammatory bowel disease. *Gut,* **38:** 90-98.

20. Gröne, A., Frisk, A. L. and Baumgärtner, W. 1998. Cytokine mRNA expression in whole blood samples from dogs with natural canine distemper virus infection. *Vet. Immunol. Immunopathol.,* **65:** 11-27.

21. Gu, Y., Hu, X., Liu, C., Qv, X. and Xu, C. 2008. Interleukin (IL)-17 promotes macrophages to produce IL-8, IL-6 and tumour necrosis factor-alpha in aplastic anaemia. *Br. J. Haematol.,* **142:** 109-114.

22. He, F., Peng, J., Deng, X. L., Yang, L. F., Camara, A. D., Omran, A., Wang, G. L., Wu, L. W., Zhang, C. L. and Yin, F. 2012. Mechanisms of tumor necrosis factor-alpha-induced leaks in intestine epithelial barrier. *Cytokine*, **59:** 264-272.

23. Hibi, T. and Ogata, H. 2006. Novel pathophysiological concepts of inflammatory bowel disease. *J. Gastroenterol.,* **41:** 10-16.

24. Hommes, D. W., Radema, S. A., Jansen, J., Smit, F., Fockens, P., Zhao, Y., Ceska, M., Tytgat, G. N. and van Deventer, S. J. 1995. Production and cellular source of interleukin-8 in ulcerative colitis. *Inflamm. Bowel Dis.,* **1:** 108-116.

25. Igarashi, H., Ohno, K., Ohmi, A., Tsukamoto, A., Nakashima, K., Fujino, Y., Uchida, K. and Tsujimoto, H. 2013. Polypoid adenomas secondary to inflammatory colorectal

polyps in 2 miniature dachshunds. *J. Vet. Med. Sci.,* **75:** 535-538.

26. Imai, Y., Ibata, I., Ito, D., Ohsawa, K. and Kohsaka, S. 1996. A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage. *Biochem. Biophys. Res. Commun.,* **224:** 855-862.

27. Izutani, R., Loh, E. Y., Reinecker, H. C., Ohno, Y., Fusunyan, R. D., Lichtenstein, G. R., Rombeau, J. L. and Macdermott, R. P. 1995. Increased expression of interleukin-8 mRNA in ulcerative colitis and Crohn's disease mucosa and epithelial cells. *Inflamm. Bowel Dis.,* **1:** 37-47.

28. Jergens, A. E., Moore, F. M., Haynes, J. S. and Miles, K. G. 1992. Idiopathic inflammatory bowel disease in dogs and cats: 84 cases (1987-1990). *J. Am. Vet. Med. Assoc.,* **201:** 1603-1608.

29. Jergens, A. E., Sonea, I. M., O'Connor, A. M., Kauffman, L. K., Grozdanic, S. D., Ackermann, M. R. and Evans, R. B. 2009. Intestinal cytokine mRNA expression in canine inflammatory bowel disease: a meta-analysis with critical appraisal. *Comp. Med.,* **59:** 153-162.

30. Kanazawa, H., Ohsawa, K., Sasaki, Y., Kohsaka, S. and Imai, Y. 2002. Macrophage/microglia-specific protein Iba1 enhances membrane ruffling and Rac activation via phospholipase C-gamma -dependent pathway. *J. Biol. Chem.,* **277:** 20026-20032.

31. Keshavarzian, A., Fusunyan, R. D., Jacyno, M., Winship, D., MacDermott, R. P. and Sanderson, I. R. 1999. Increased interleukin-8 (IL-8) in rectal dialysate from patients with ulcerative colitis: evidence for a biological role for IL-8 in inflammation of the colon. *Am. J. Gastroenterol.,* **94:** 704-712.

32. Khor, B., Gardet, A. and Xavier, R. J. 2011. Genetics and pathogenesis of inflammatory bowel disease. *Nature,* **474:** 307-317.

33. Klapproth, J. M. and Sasaki, M. 2010. Bacterial induction of proinflammatory cytokines in inflammatory bowel disease. *Inflamm. Bowel Dis.,* **16:** 2173-2179.

34. Kobayashi, T., Okamoto, S., Hisamatsu, T., Kamada, N., Chinen, H., Saito, R., Kitazume, M. T., Nakazawa, A., Sugita, A., Koganei, K., Isobe, K. and Hibi, T. 2008. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut,* **57:** 1682-1689.

35. Koloski, N. A., Bret, L. and Radford-Smith, G. 2008. Hygiene hypothesis in inflammatory bowel disease: a critical review of the literature. *World J. Gastroenterol.,* **14:** 165-173.

36. Köhler, C. 2007. Allograft inflammatory factor-1/Ionized calcium-binding adapter molecule 1 is specifically expressed by most subpopulations of macrophages and spermatids in testis. *Cell Tissue Res.,* **330:** 291-302.

37. Loh, G. and Blaut, M. 2012. Role of commensal gut bacteria in inflammatory bowel diseases. *Gut Microbes,* **3:** 544-555.

38. Maeda, S., Ohno, K., Nakamura, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., Goto-Koshino, Y., Fujino, Y. and Tsujimoto, H. 2011. Quantification of chemokine and chemokine receptor gene expression in duodenal mucosa of dogs with inflammatory bowel disease. *Vet. Immunol. Immunopathol.,* **144:** 290-298.

39. Magro, F. and Portela, F. 2010. Management of inflammatory bowel disease with infliximab and other anti-tumor necrosis factor alpha therapies. *BioDrugs,* **24 Suppl 1:** 3-14.

40. Mahida, Y. R. 2000. The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. *Inflamm. Bowel Dis.,* **6:** 21-33.

41. Malewska, K., Rychlik, A., Nieradka, R. and Kander, M. 2011. Treatment of inflammatory bowel disease (IBD) in dogs and cats. *Pol. J. Vet. Sci.,* **14:** 165-171.

42. Marsella, R., Nicklin, C. and Lopez, J. 2006. Studies on the role of routes of allergen exposure in high IgE-producing beagle dogs sensitized to house dust mites. *Vet. Dermatol.,* **17:** 306-312.

43. Matsuda, R., Koide, T., Tokoro, C., Yamamoto, T., Godai, T., Morohashi, T., Fujita, Y., Takahashi, D., Kawana, I., Suzuki, S. and Umemura, S. 2009. Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid naïve ulcerative colitis during active and quiescent disease. *Inflamm. Bowel Dis.,* **15:** 328-334.

44. Matsumoto, Y., Mohamed, A., Onodera, T., Kato, H., Ohashi, T., Goitsuka, R., Tsujimoto, H., Hasegawa, A., Furusawa, S. and Yoshihara, K. 1994. Molecular cloning and expression of canine interleukin 8 cDNA. *Cytokine,* **6:** 455-461.

45. Matsuoka, K. and Hibi, T. 2013. Treatment guidelines in inflammatory bowel disease: the Japanese perspectives. *Dig. Dis.,* **31:** 363-367.

46. Milner, R. J., Pearson, J., Nesbit, J. W. and Close, P. 1996. Immunophenotypic classification of canine malignant lymphoma on formalin-mixed paraffin wax-embedded tissue by means of CD3 and CD79a cell markers. *Onderstepoort J. Vet. Res.,* **63:** 309-313.

47. Mitsuyama, K., Toyonaga, A., Sasaki, E., Watanabe, K., Tateishi, H., Nishiyama, T., Saiki, T., Ikeda, H., Tsuruta, O. and Tanikawa, K. 1994. IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.,* **96:** 432-436.

48. Mizoguchi, A. 2012. Animal models of inflammatory bowel disease. *Prog. Mol. Biol. Transl. Sci.* **105:** 263-320.

49. Mizunoe, S., Shuto, T., Suzuki, S., Matsumoto, C., Watanabe, K., Ueno-Shuto, K., Suico, M. A., Onuki, K., Gruenert, D. C. and Kai, H. 2012. Synergism between interleukin (IL)-17 and Toll-like receptor 2 and 4 signals to induce IL-8 expression in cystic fibrosis airway epithelial cells. *J. Pharmacol. Sci.,* **118:** 512-520.

50. Mosser, D. M. and Edwards, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.,* **8:** 958-969.

51. Murakami, M., Okuyama, Y., Ogura, H., Asano, S., Arima, Y., Tsuruoka, M., Harada, M., Kanamoto, M., Sawa, Y., Iwakura, Y., Takatsu, K., Kamimura, D. and Hirano, T. 2011. Local microbleeding facilitates IL-6- and IL-17-dependent arthritis in the absence of tissue antigen recognition by activated T cells. *J. Exp. Med.,* **208:** 103-114.

52. Műzes, G., Molnár, B., Tulassay, Z. and Sipos, F. 2012. Changes of the cytokine profile in inflammatory bowel diseases. *World J. Gastroenterol.,* **18:** 5848-5861.

53. Nagalingam, N. A. and Lynch, S. V. 2012. Role of the microbiota in inflammatory bowel diseases. *Inflamm. Bowel Dis.,* **18:** 968-984.

54. Nancey, S., Hamzaoui, N., Moussata, D., Graber, I., Bienvenu, J. and Flourie, B. 2008. Serum interleukin-6, soluble interleukin-6 receptor and Crohn's disease activity. *Dig. Dis. Sci.,* **53:** 242-247.

55. Ogura, H., Murakami, M., Okuyama, Y., Tsuruoka, M., Kitabayashi, C., Kanamoto, M., Nishihara, M., Iwakura, Y. and Hirano, T. 2008. Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity,* **29:** 628-636.

56. Ohmi, A., Tsukamoto, A., Ohno, K., Uchida, K., Nishimura, R., Fukushima, K., Takahashi, M., Nakashima, K., Fujino, Y. and Tsujimoto, H. 2012. A retrospective study of inflammatory colorectal polyps in miniature dachshunds. *J. Vet. Med. Sci.,* **74:** 59-64.

57. Ohta, H., Takada, K., Sunden, Y., Tamura, Y., Osuga, T., Lim, S. Y., Murakami, M., Sasaki, N., Rajapakshage, B. K., Nakamura, K., Yamasaki, M. and Takiguchi, M. 2013. CD4+ T cell cytokine gene and protein expresion in duodenal mucosa of dogs with inflammatory bowel disease. *J. Vet. Med. Sci.*, In press.

58. Ohta, H., Takada, K., Torisu, S., Yuki, M., Tamura, Y., Yokoyama, N., Osuga, T., Lim, S. Y., Murakami, M., Sasaki, N., Nakamura, K., Yamasaki, M. and Takiguchi, M. 2013. Expression of CD4+ T cell cytokine genes in the colorectal mucosa of inflammatory colorectal polyps in miniature dachshunds. *Vet. Immunol. Immunopathol.*, **155:** 259-263.

59. Ohta, H., Yamaguchi, T., Rajapakshage, B. K., Murakami, M., Sasaki, N., Nakamura, K., Hwang, S. J., Yamasaki, M. and Takiguchi, M. 2011. Expression and subcellular localization of apical junction proteins in canine duodenal and colonic mucosa. *Am. J. Vet. Res.*, **72:** 1046-1051.

60. Peters, I. R., Helps, C. R., Calvert, E. L., Hall, E. J. and Day, M. J. 2005. Cytokine mRNA quantification in duodenal mucosa from dogs with chronic enteropathies by real-time reverse transcriptase polymerase chain reaction. *J. Vet. Intern. Med.*, **19:** 644-653.

61. Peters, I. R., Peeters, D., Helps, C. R. and Day, M. J. 2007. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. *Vet. Immunol. Immunopathol.*, **117:** 55-66.

62. Ridyard, A. E., Nuttall, T. J., Else, R. W., Simpson, J. W. and Miller, H. R. 2002. Evaluation of Th1, Th2 and immunosuppressive cytokine mRNA expression within the colonic mucosa of dogs with idiopathic lymphocytic-plasmacytic colitis. *Vet. Immunol. Immunopathol.*, **86:** 205-214.

63. Roberts-Thomson, I. C., Fon, J., Uylaki, W., Cummins, A. G. and Barry, S. 2011.

Cells, cytokines and inflammatory bowel disease: a clinical perspective. *Expert Rev. Gastroenterol. Hepatol.,* **5:** 703-716.

64. Rodríguez-Perálvarez, M. L., García-Sánchez, V., Villar-Pastor, C. M., González, R., Iglesias-Flores, E., Muntane, J. and Gómez-Camacho, F. 2012. Role of serum cytokine profile in ulcerative colitis assessment. *Inflamm. Bowel Dis.,* **18:** 1864-1871.

65. Rot, A. 1991. Chemotactic potency of recombinant human neutrophil attractant/activation protein-1 (interleukin-8) for polymorphonuclear leukocytes of different species. *Cytokine,* **3:** 21-27.

66. Simpson, K. W. and Jergens, A. E. 2011. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. *Vet. Clin. North Am. Small Anim. Pract.,* **41:** 381-398.

67. Strober, W. and Fuss, I. J. 2011. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology,* **140:** 1756-1767.

68. Suchodolski, J. S., Markel, M. E., Garcia-Mazcorro, J. F., Unterer, S., Heilmann, R. M., Dowd, S. E., Kachroo, P., Ivanov, I., Minamoto, Y., Dillman, E. M., Steiner, J. M., Cook, A. K. and Toresson, L. 2012. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One,* **7:** e51907.

69. Thomson, A. B., Gupta, M. and Freeman, H. J. 2012. Use of the tumor necrosis factor-blockers for Crohn's disease. *World J. Gastroenterol.,* **18:** 4823-4854.

70. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.,* **3:** 1-11.

71. Vermeire, S., Van Assche, G. and Rutgeerts, P. 2004. C-reactive protein as a marker for inflammatory bowel disease. *Inflamm. Bowel Dis.,* **10:** 661-665.

72. Walczak, A., Przybylowska, K., Dziki, L., Sygut, A., Chojnacki, C., Chojnacki, J., Dziki, A. and Majsterek, I. 2012. The lL-8 and IL-13 gene polymorphisms in inflammatory bowel disease and colorectal cancer. *DNA Cell Biol.,* **31:** 1431-1438.

73. Washabau, R. J., Day, M. J., Willard, M. D., Hall, E. J., Jergens, A. E., Mansell, J., Minami, T. and Bilzer, T. W. 2010. Endoscopic, biopsy, and histopathologic guidelines for the evaluation of gastrointestinal inflammation in companion animals. *J. Vet. Intern. Med.,* **24:** 10-26.

74. Wilks, S. and Moxon, W. 1875. *Lectures on Pathological Anatomy,* 2nd ed., pp.408, Lindsay and Blakiston, Philadelphia.

75. Wiinberg, B., Spohr, A., Dietz, H. H., Egelund, T., Greiter-Wilke, A., McDonough, S. P., Olsen, J., Priestnall, S., Chang, Y. F. and Simpson, K. W. 2005. Quantitative analysis of inflammatory and immune responses in dogs with gastritis and their relationship to Helicobacter spp. infection. *J. Vet. Intern. Med.,* **19:** 4-14.

76. Yamate, J., Yoshida, H., Tsukamoto, Y., Ide, M., Kuwamura, M., Ohashi, F., Miyamoto, T., Kotani, T., Sakuma, S. and Takeya, M. 2000. Distribution of cells immunopositive for AM-3K, a novel monoclonal antibody recognizing human macrophages, in normal and diseased tissues of dogs, cats, horses, cattle, pigs, and rabbits. *Vet. Pathol.,* **37:** 168-176.

ACKNOWLEDGEMENTS

I would like to extend thanks and my appreciation to those who have encouraged me during years of this study.

First and foremost, I would like to express my gratitude to supervisor, Prof. Mitsuyoshi Takiguchi for providing the opportunity to this study and guidance throughout the duration of this study.

I am also greatly indebted to Prof. Yasuhiro Kon and Prof. Kazuhiko Ohashi for time taken to provide advice, critical comments, and assistance in completing this manuscript, Associate Prof. Masahiro Yamasaki and Assistant Prof. Hiroshi Ohta for their relentless and worthwhile contribution to the planning, execution and success of this work.

I would like to thank Assistant Prof. Kensuke Nakamura and Assistant Prof. Keitaro Morishita for their advice, Prof. Motoshi Tajima, Dr. Yusuke Sakai, Dr. Tomonori Kanazawa, Ms. Sayuri Nakamura, Ms. Aiko Ohnuma, Mr. Tomohiro Nishino, and Mr. Masataka Chihara for technical support and advice.

I am also grateful to graduate school doctoral fellow pupils, in particular Dr. Ryoyo Ikebuchi, Mr. Kensuke Watanabe, and Mr. Shintaro Shichinohe, for providing opportunity to discuss and brush up my research.

I want to also thank all members of the Laboratory of Veterinary Internal Medicine and Veterinary Teaching Hospital for their support, encouragements, and great times.

Finally, I would also like to express my gratitude to my family and relatives for their support and warm encouragement.