Evaluation of Selected Cytokine Gene Expression in Colonic Mucosa from Dogs with Idiopathic Lymphocytic-plasmacytic Colitis

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ABSTRACT. Lymphocytic-plasmacytic colitis (LPC) is a common form of inflammatory bowel disease (IBD) affecting the canine large intestine. Cytokines are thought to be involved in the pathogenesis of IBD. However, to date, few studies have investigated cytokine mRNA expression in dogs with LPC. In this study, we investigated mRNA transcription levels of T helper cell cytokines, such as IFN-γ, IL-4, IL-17 and IL-10 and pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IL-8, IL-12 and IL-23, in colonic mucosa from LPC dogs by quantitative real-time RT-PCR. No significant differences were detected in cytokine mRNA expressions between dogs with LPC and controls, except for IL-23p19. Dogs with LPC failed to express a predominant cytokine profile in inflamed colonic mucosa as opposed to human IBD.
KEY WORDS: canine, colon, cytokine, lymphocytic-plasmacytic colitis, mRNA

Idiopathic lymphocytic-plasmacytic colitis (LPC) is a common form of inflammatory bowel disease (IBD) affecting the canine large intestine [5]. Although the etiology of LPC is still unknown, it has been suggested that dysfunction of the mucosal immune system, with loss of tolerance to luminal antigens, plays a possibly important role in the pathogenesis of canine LPC [4]. Previous investigations into canine LPC have revealed changes in immune cell populations and inflammatory cytokine mRNA expressions in the large-intestinal mucosa [13, 15].

Data implicating the involvement of mucosal cytokines in the pathogenesis of canine LPC are limited. Dogs with LPC lack consistent patterns of cytokine mRNA expressions. Ridyard *et al*. reported that IL-2 and TNF-α mRNA expressions are increased in colonic mucosa from dogs with LPC when compared to healthy controls and concluded that LPC is associated with the activation of helper T (Th) 1 type CD4+ lymphocyte [13]. On the other hand, Jergens reported that LPC dogs failed to express a predominant pro-inflammatory Th1- or Th2- cytokine bias in inflamed colonic mucosa [3]. These previous studies analyzed cytokine mRNA expressions using semi-quantitative PCR methods, and there is no report about mucosal cytokine gene expressions in colonic biopsies from dogs with LPC using quantitative real-time PCR.

Therefore, the aim of the present study was to investigate the Th cytokine and pro-inflammatory cytokine mRNA expressions profile in the colonic mucosa from dogs with LPC, using quantitative real-time PCR method.

Dogs (n=6) brought to Hokkaido University Veterinary Teaching Hospital from July 2010 to November 2012 for the investigation of large intestinal disease (diarrhea, tenesmus, hematochezia and increased frequency of defecation) persisting for more than 3 weeks and subsequently diagnosed histopathologically with LPC were included in this study. Dogs treated with corticosteroids within 2 weeks prior to presentation were excluded. Breed, sex, age, body weight and disease severity are shown in Table 1. Disease severity of dogs was scored based on the Canine Chronic Enteropathy Clinical Activity Index (CCECAI) [1]. Total CCECAI score classifies the disease as clinically insignificant (score 0–3), mild (score 4–5), moderate (score 6–8), severe (score 9–11) or very severe (score>12).

Control colonic mucosal specimens were obtained endoscopically from 12 healthy dogs (6 beagles and 6 mongrels). These included 8 intact females and 4 intact males. Eight dogs were 1 year old (range, 16 to 23 months; median 22 months), one mongrel was 8 years old, and three mongrels were 9 years old. The median body weight was 12.1 kg (range, 7.9 to 15.8 kg). No clinical signs suggestive 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. Use of dogs in this study was approved by the Laboratory Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University (Approval no. 13–0142).

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Breed	Sex	Age (Years)	BW (kg)	CCECAI score
Border Collie	NF	8.2	13.5	11 (severe)
Bernese Mountain Dog	F	2.5	33.1	8 (moderate)
Pembroke Welsh corgi	NM	11.7	13.4	12 (severe)
Pomeranian	M	6.3	2.0	3 (insignificant)
Shih-Tzu	NF	11.0	8.9	7 (moderate)
mongrel	F	10.5	16.4	6 (moderate)

BW, body weight; CCECAI, canine chronic enteropathy clinical activity index; NF, neutered female; F, female; NM, neutered male; M, male.

Colonoscopy was performed under general anesthesia using a VQ-8143A flexible video endoscope (Olympus Medical Systems Corp., Tokyo, Japan). Multiple mucosal biopsies were taken using FB-55Q-1 biopsy forceps (Olympus Medical Systems Corp.). Colonic specimens for total RNA extraction were immediately submerged in RNAlater Solution (Ambion Inc., Austin, TX, U.S.A.) and stored at −80°C until use. Samples for histopathology were placed in 10% formalin and embedded in paraffin, and hematoxylin and eosin-stained sections were prepared. Total RNA was extracted using a RNeasy Protect Mini Kit (Qiagen Inc., Valencia, CA, U.S.A.), according to the manufacturer's instructions, and genomic DNA was removed from the samples with a commercially available kit (RNase-Free DNase set; Qiagen Inc.). cDNA was synthesized from 0.5 μg total RNA using High Capacity RNA-to-cDNA Master Mix containing the Oligo dT primer and random primer (Applied Biosystems, Foster City, CA, U.S.A.).

Primers for IFN-γ, IL-4, IL-17, IL-10, 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 used. Quantitative reverse transcription PCR (qPCR) was either designed using Primer-BLAST, based on the canine GenBank sequences or based on previous reports [10, 16]. For accurate quantification, *SDHA* was chosen as the reference gene by geNorm [17] from three candidate reference genes (*GAPDH*, *SDHA* and *HMBS*) as described previously [12, 16]. Sequences of primer pairs used for qPCR are shown in Table 2. The specificities of these primers in amplifying each target mRNA by sequential analysis of PCR products were confirmed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and carried out using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Each qPCR 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 were 95°C for 10 min and 40 cycles PCR (95°C for 15 sec and 60°C for 1 min), followed by dissociation (95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec). Reaction efficiency was determined for each primer set, using 10-fold dilutions (10^7 molecules μl^{-1} to 10^1 molecules μl^{-1}) of plasmids ligated with each cytokine

and reference gene, and the correlation coefficient was>0.98. Cycle threshold (C_t) values that indicated the point where the threshold intersected with amplification curves of PCR reaction were determined using software (7300 SDS Software; Applied Biosystems). Melting curve analysis did not show misprinting in any of the reactions.

All samples were examined in duplicate, and each PCR reaction included a non-template control. Absolute quantification of cytokine mRNA was performed by converting the sample C_t values to a 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.

Statistical analyses were performed using a statistical program, JMP 9 (SAS Institute Inc., Cary, NC, U.S.A.). Normality of distribution was assessed by the Shapiro-Wilk W test. The nonparametric Wilcoxon rank sum test was used to compare the results between LPC and controls. A value of $P<0.05$ was considered significant.

No significant differences were detected in cytokine mRNA expressions between dogs with LPC and control dogs, except for IL-23p19 (Fig. 1).

In the present study, we examined the cytokine mRNA expression profile in the colonic mucosa of canine LPC using the qPCR method. There was no evidence of up-regulation of each cytokine mRNA expression, except for IL-23p19 mRNA expression, in the colonic mucosa from dogs with LPC when compared to healthy controls. Recently, we reported significantly increased expression of pro-inflammatory cytokine genes, such as IFN-γ, IL-17, IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40 and IL-23p19, in the colorectal mucosa of dogs with inflammatory colorectal polyps [10, 16]. In this study, we used the same qPCR method. Thus, obvious pro-inflammatory cytokine up-regulation is unlikely in the colonic mucosa of dogs with LPC.

The current study also demonstrated that Th cytokines, such as IFN-γ, IL-4, IL-17 and IL-10 mRNA expressions, in the colonic mucosa from dogs with LPC were not different from those in healthy controls. In addition, our recent study of the expressions of IFN-γ, IL-17 and IL-10 mRNA in the duodenal mucosa of dogs with lymphocytic-plasmacytic enteritis (LPE) indicated that there was no difference in these cytokine mRNA expressions when compared to those in healthy controls [9]. Thus, there was no evidence of the up-regulation of typical Th cytokine mRNA in the duodenal or colonic mucosa of dogs with IBD.

The results of the present study indicated that only IL-23p19 mRNA expression was increased in the colonic mucosa from dogs with LPC. IL-23 is a heterodimeric cytokine and comprises p19 and p40 subunits [6, 14]. IL-23p19 is thought to be produced by macrophages and plays important roles in the promotion of Th17 cell differentiation [2, 14]. However, not only IL-23p19 but also IL-23p40, IL-6 and TGF-β are required for the differentiation and maintenance of Th17 cells in humans [6]. Therefore, IL-17A mRNA expression was not increased in the colonic mucosa of dogs with LPC. Moreover, there was no correlation between IL-23p19 mRNA expression and the CCECAI score in dogs with LPC. The significance of increased IL-23p19 mRNA

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

Primer set		Primer sequence (5′–3′)	Position	GenBank accession number
IFN-γ	Forward	GCGCAAGGCGATAAATGAAC	492–573	NM_001003174
	Reverse	CTGACTCCTTTTCCGCTTCC		
TNF-α	Forward	TCTCGAACCCCAAGTGACAAG	241–393	NM_001003244
	Reverse	CAACCCATCTGACGGCACTA		
IL-4	Forward	TCACCAGCACCTTTGTCCAC	113–256	NM_001003159
	Reverse	CGCTTGTGTTCTTTGGAGCA		
IL-17	Forward	GGAATCTGCACCGCAATGAGGAC	231–378	NM_001165878
	Reverse	CGCAGAACCAGGATCTCTTGCTGG		
IL-10	Forward	CGGGAGGGTGAAGACTTTCT	148–291	NM_001003077
	Reverse	GGCATCACCTCCTCCAAGTA		
IL-1β	Forward	CAAGTCTCCCACCAGCTCTGTA	154–234	NM_001037971
	Reverse	GGGCTTCTTCAGCTTCTCCAA		
IL-6	Forward	TTAAGTACATCCTCGGCAAAATCT	212–297	NM_001003301
	Reverse	CAGTGCCTCTTTGCTGTCTTCA		
IL-8/CXCL8	Forward	CTCTCTGTGAAGCTGCAGTTCTG	59–139	NM_001003200
	Reverse	GGAAAGGTGTGGAGTGTGTTTTT		
IL-12p35	Forward	GTGCCTCAACCACTCCCAA	117–217	NM_001003293
	Reverse	CAATCTCTTCGGAAGTGCAGG		
IL-12p40	Forward	CAGCAGAGAGGGTCAGAGTGG	527–635	NM_001003292
	Reverse	ACGACCTCGATGGGTAGGC		
IL-23p19	Forward	CAAGGGGAGAAAAACAGCAG	303–381	XM_538231
	Reverse	TGCTGTCCGTTCTGTGAGTC		
GAPDH	Forward	ATCACTGCCACCCAGAAGAC	535–667	NM_001003142
	Reverse	TCAGCTCAGGGATGACCTTG		
SDHA	Forward	GCCTTGGATCTCTTGATGGA	685–776	XM_535807
	Reverse	TTCTTGGCTCTTATGCGATG		
HMBS	Forward	TCACCATCGGAGCCATCT	323–434	XM_546491
	Reverse	GTTCCCACCACGCTCTTCT		

CXCL8, CXC chemokine ligand 8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDHA, succinate dehydrogenase complex, subunit A ; HMBS, hydroxymethyl- bilane synthase.

expression in the development and maintenance of colonic mucosal inflammation remains unclear. Thus, further studies are necessary for clarification of the importance of increased IL-23p19 mRNA expression in the pathogenesis of canine LPC.

In this study, two dogs showed clinically severe disease. In these dogs, IL-1β mRNA expression showed an increasing tendency, although there was no significant difference. Therefore, there is a possibility that IL-1β mRNA expression would be significantly increased in dogs with LPC if the case number was larger. Thus, IL-1β mRNA expression may be involved in the disease severity of dogs with LPC.

A recent study demonstrated increased nucleotide oligomerization domain two (NOD2) mRNA expression and NFkappaB activation in the colonic mucosa of dogs with LPC [11]. NOD2 has been shown to recognize bacterial components, and this interaction leads to the activation of NFkappaB. Activation of NFkappaB could lead to not only pro-inflammatory cytokines but also chemokine production in human IBD [8]. Maeda *et al*. reported the up-regulation of some chemokines and chemokine receptors in the duodenal mucosa of dogs with LPE when compared to controls [7]. Thus, chemokines but not pro-inflammatory cytokines may play important roles in the infiltration of inflammatory cells, such as lymphocytes and plasma cells, in dogs with LPC.

The major limitation of the current study was the small number of cases, because of the limited availability of large intestinal IBD cases visiting our facility. Further studies with a larger number of cases are needed to validate our findings in the current study.

In conclusion, no distinct cytokine profile was found in the inflamed intestinal mucosa of dogs with LPC at the level of mRNA expression. There was no clear evidence of the contribution of a cytokine profile in the pathogenesis of canine LPC.

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. [Medline] [CrossRef]
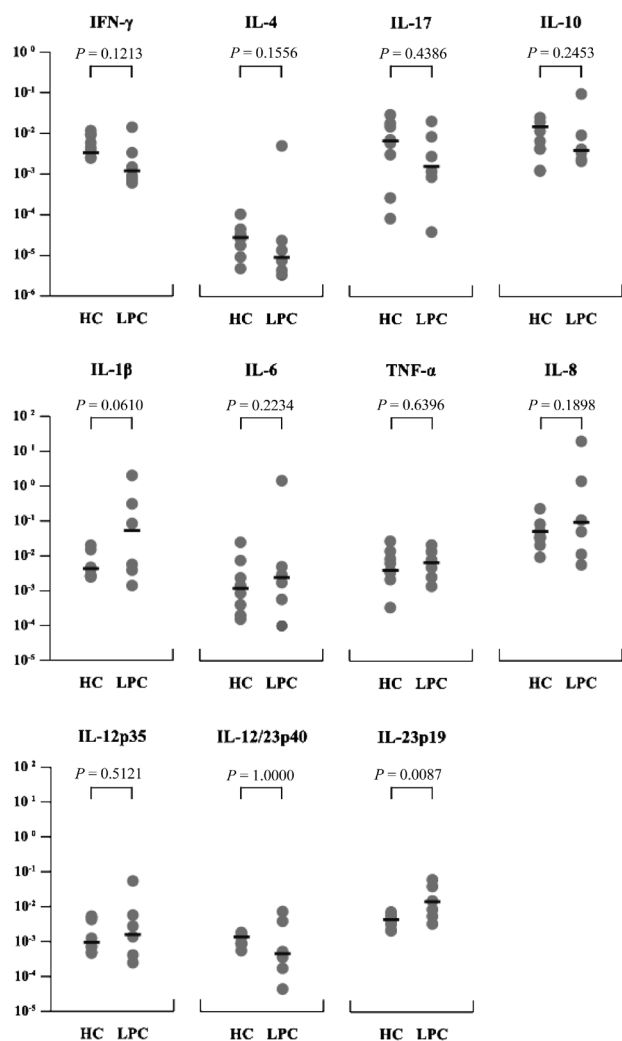
Fig. 1. Relative expression of cytokine (IFN-γ, IL-4, IL-10, IL-17, IL-1β, IL-6, TNF-α, IL-8, IL-12p35, IL-12/23p40 and IL-23p19) mRNA in colonic specimens from healthy controls (HC, n=12) and dogs with LPC (LPC, n=6). Transcription of succinate dehydrogenase complex, subunit A (*SDHA*) mRNA was used as an internal control. The horizontal lines correspond to the median of the relative quantity in each group.

2. 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 CD4T cells. *J. Interferon Cytokine Res.* **31**: 363–371. [Medline] [CrossRef]
3. 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. [Medline]
4. 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. [Medline] [CrossRef]
5. Guildford, G.1996. Idiopathic inflammatory bowel disease. pp.

451–486. *In*: Strombeck's Small Animal Gastroenterology, 3rd ed. (Guildford W. G., Center, S. A., Strombeck, D. R., Williams, D. A. and Meyer, D. J. eds.), Saunders, Philadelphia.
6. Iwakura, Y. and Ishigame, H. 2006. The IL-23/IL-17 axis in inflammation. *J. Clin. Invest.* **116**: 1218–1222. [Medline] [CrossRef]
7. 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. [Medline] [CrossRef]
8. McCormack, G., Moriarty, D., O'Donoghue, D. P., McCormick, P. A., Sheahan, K. and Baird, A. W. 2001. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm. Res.* **50**: 491–495. [Medline] [CrossRef]
9. 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.* Available online at http://dx.doi.org/10.1292/jvms.13-0008.
10. 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. [Medline] [CrossRef]
11. Okanishi, H., Hayashi, K., Sakamoto, Y., Sano, T., Maruyama, H., Kagawa, Y. and Watari, T. 2013. NOD2 mRNA expression and NFkappaB activation in dogs with lymphocytic plasmacytic colitis. *J. Vet. Intern. Med.* **27**: 439–444. [Medline] [CrossRef]
12. 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. [Medline] [CrossRef]
13. 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. [Medline] [CrossRef]
14. 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. [Medline] [CrossRef]
15. Stonehewer, J., Simpson, J. W., Else, R. W. and Macintyre, N. 1998. Evaluation of B and T lymphocytes and plasma cells in colonic mucosa from healthy dogs and from dogs with inflammatory bowel disease. *Res. Vet. Sci.* **65**: 59–63. [Medline] [CrossRef]
16. Tamura, Y., Ohta, H., Torisu, S., Yuki, M., Yokoyama, N., Murakami, M., Lim, S. Y., Osuga, T., Morishita, K., Nakamura, K., Yamasaki, M. and Takiguchi, M. 2013. Markedly increased expression of interleukin-8 in the colorectal mucosa of inflammatory colorectal polyps in miniature dachshunds. *Vet. Immunol. Immunopathol.* **156**: 32–42. [Medline] [CrossRef]
17. 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**: H0034. [Medline] [CrossRef]