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Distribution of regulatory T cells in inflammatory colorectal polyps of miniature dachshunds

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ABSTRACT

Inflammatory colorectal polyp (ICRP) is an emerging disease in Miniature Dachshunds (MDs). Animals with this disease exhibit multiple polyps with severe neutrophil infiltration that respond to immunosuppressive therapy. Macrophages in polypoid lesions have been described to play an important role in neutrophil infiltration in the lesion by producing IL-8. In contrast, IL-10, an anti-inflammatory cytokine, was also reported to be upregulated in polypoid lesions, but its significance in the pathogenesis of ICRP has not been clarified. Regulatory T cells (Tregs) are the main source of IL-10 production and contribute to the maintenance of intestinal homeostasis. Therefore, the objective of this research was to compare the distribution of Tregs in polypoid lesions of ICRPs and the association between the distribution and expression of pro- or anti-inflammatory cytokines. Tissue biopsy specimens of polypoid lesions were collected from 28 MDs with ICRP. Those of macroscopically non-polypoid colonic mucosa from 24 MDs with ICRPs and 21 control dogs were further included as controls. Real-time quantitative polymerase chain reaction was used to quantify gene expression of *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-17*, *IL-22*, *IFN-γ*, *TNF-α*, *TGF-β*, and *forkhead box protein P3 (Foxp3)* in each tissue sample. The numbers of Foxp3-positive cells (Tregs) and ionized calcium binding adapter molecule 1 (Iba-1)-positive cells (macrophages) were determined by immunohistochemistry. The gene expression of *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, *IFN-γ*, *IL-17*, *IL-10*, *TGF-β*, and *Foxp3* was significantly upregulated in polypoid lesions relative to control levels. The numbers of Foxp3-positive Tregs and Iba-1-positive macrophages were significantly increased in polypoid lesions compared to those in the non-polypoid colonic mucosa of MDs with ICRPs and control dogs. The upregulation of *IL-10* was moderately correlated with the distribution of Tregs in polypoid lesions from MDs with ICRPs. In addition, the relative upregulation of *IL-1β*, *IL-6*, and *IL-8* in polypoid lesions, compared to expression in non-polypoid colonic mucosa of MDs with ICRPs, was significantly greater than that of *IL-10*. These results indicate that increases in Treg numbers and anti-inflammatory cytokines in polypoid lesions comprise reactive changes in response to the inflammation, which warrants further investigation.

1. Introduction

Inflammatory colorectal polyps (ICRPs) often develop in Miniature Dachshunds (MDs) in Japan, and these are characterized by hematochezia, mucoid feces, and tenesmus. ICRPs in MDs emerge as single or multiple outgrowth located in the colorectal region.

Abbreviations: Foxp3, forkhead box protein P3; Iba-1, ionized calcium binding adapter molecule 1; IBD, inflammatory bowel disease; ICRP, inflammatory colorectal polyp; IHC, immunohistochemistry; MD, miniature dachshund; TBST, tris-buffered saline with 0.1% tween 20; Treg, regulatory T cell

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Histopathologically, this is associated with severe inflammatory infiltration, composed of neutrophils and macrophages, in the thickened mucosa (Ohmi et al., 2012; Uchida et al., 2016). ICRPs in MDs exhibit a good response to immunosuppressive therapy including prednisolone, cyclosporine, or leflunomide (Fukushima et al., 2015; Ohmi et al., 2012). Therefore, they are speculated to comprise a novel form of canine inflammatory bowel disease (IBD) (Ohta et al., 2013).

Although the pathogenesis of both human and canine IBD remains unclear, dysregulation of the mucosal immune system, especially regulatory T cells (Tregs), is thought to contribute to the pathogenesis of IBD in both species. Tregs are essential for maintaining mucosal immune tolerance because of their suppressive effect on effector T cells, which occurs via the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (Sakaguchi et al., 2008). Furthermore, expression of the transcription factor forkhead box protein P3 (Foxp3), which is expressed predominantly in Tregs, is critical for the differentiation of these cells (Ziegler, 2006).

In humans, the number of Tregs is increased in the inflamed mucosa of patients with IBD, which is considered a reactive change that occurs to maintain homeostasis (Wang et al., 2011). In addition, the mRNA levels of pro-inflammatory cytokines are up-regulated in the inflamed mucosa of patients with IBD (Kobayashi et al., 2008; Roberts-Thomson et al., 2011). In contrast, the number of Tregs was found to be decreased in the duodenal mucosa of dogs with IBD (Junginger et al., 2012; Maeda et al., 2016). Moreover, the levels of mRNA encoding pro-inflammatory cytokines are not increased in the inflamed intestinal mucosa of dogs with IBD (Jergens et al., 2009; Tamura et al., 2014). Therefore, the pathogenesis of canine IBD is considered different from that of human disease.

Regarding ICRPs in MDs, recent investigations have demonstrated that the mRNA expression of pro-inflammatory cytokines is increased in the colorectal mucosa of MDs with ICRPs (Igarashi et al., 2014; Ohta et al., 2013). Especially, IL-8, a neutrophil chemotactic factor that is produced by macrophages, was found to be markedly increased in the polypoid lesions of ICPRs in MDs (Tamura et al., 2013). In contrast, the gene expression of *IL-10*, which encodes a cytokine that inhibits T cell proliferation and pro-inflammatory cytokine secretion (Roncarolo et al., 2006), was found to be upregulated in the colorectal mucosa of MDs with ICRPs (Ohta et al., 2013). Thus, concerning cytokine levels, the pathogenesis of ICRPs in MDs seems to share several common features with human IBD.

Nevertheless, to date, there have been no reports on changes in the number of Tregs in the colorectal mucosa of MDs with ICRPs. Therefore, this study aimed to evaluate the distribution of Tregs in the polypoid lesions of ICRPs in MDs, as well as the association between the distribution and expression levels of pro- or anti-inflammatory cytokines.

2. Materials and methods

2.1. Study population

Colorectal mucosal specimens were obtained from 28 MDs at the Veterinary Medical Center of the University of Tokyo to inspect the cause of chronic hematochezia, mucoid feces, and tenesmus, and were taken endoscopically from both polypoid and non-polypoid lesions between July 2011 and April 2014. All dogs were diagnosed histopathologically with ICRPs by excluding the cases with neoplastic polyp or ICRPs accompanied by adenoma or adenocarcinoma (Uchida et al., 2016; Saito et al., 2018). Twelve ICRP-affected MDs had received treatment with prednisolone (0.25 − 1.0 mg/kg/day). In addition, cyclosporine or leflunomide had been used in four or two ICRP-affected cases, respectively. No anti-inflammatory agent was used in 14 ICRP-affected MDs. The median age of these dogs was 125.5 months (range, 48 − 168 months) with 11 females (three intact and eight spayed) and 17 males (four intact and 13 castrated). The median body weight was 5.55 kg (range, 3.40–7.40 kg). In addition, colorectal mucosal specimens were obtained endoscopically from 21 Beagles that had no gastrointestinal signs or disease based on blood tests, fecal examination, and ultrasound, as healthy controls. The median age of these dogs was 82 months (range, 34–105 months) with 14 females (eight intact and six spayed) and seven males (one intact and six casted). The use of dogs in this study was approved by the Animal Care Committee of the University of Tokyo (Approval No. P11-530, 2 June 2011).

Colonoscopy was performed on all dogs using a VQ-8143B flexible videoendoscope (Olympus Medical Systems Co., Tokyo, Japan). Both polypoid and non-polypoid lesion specimens were collected from the colorectal mucosa of MDs with ICRPs. Similarly, colonic mucosa was collected from control dogs. Mucosal biopsies were taken using FB-54Q-1 biopsy forceps (Olympus Medical Systems Co.) or an electrosurgical snare (ICC 200, ERBE Co., Tubingen, Germany). Biopsy specimens for histopathology were fixed in 10% formalin and stained with hematoxylin and eosin. One or two mucosal specimens collected from polypoid or non-polypoid lesions were used for RNA extraction, and at least four mucosal specimens or a large polypoid tissue excised by polypectomy were submitted for histopathology. Samples for total RNA extraction were placed immediately in RNAlater (Qiagen Inc., Hilden, CA) and stored at −80°C until use. Of these, tissue samples obtained from 24 ICRP-affected MDs and all 21 control dogs were used in our previous study (Igarashi et al., 2014).

2.2. Quantification of Foxp3 and cytokine mRNA expression by qPCR

Total RNA was extracted from all tissue samples (i.e. 28 polypoid lesions and 24 non-polypoid colonic mucosal specimens from ICRP cases, and 21 colonic mucosal samples from control dogs) with a commercially available kit (RNAspin Mini RNA Isolation Kit, GE Healthcare UK Ltd., Buckinghamshire, England) according to the manufacturer's manual. Genomic DNA was removed from the samples with a TURBO DNA-free Kit (Applied Biosystems, Foster City, CA) and stored at −80°C until use.

Reverse transcription was performed using a PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan) to synthesize complementary DNA from total RNA according to the manufacturer's instructions. Subsequently, quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio Inc.) and a Thermal Cycler Dice Real Time System (Takara Bio Inc.) in a final reaction volume of 25 μl. Information on the primers for *Foxp3* and cytokine-encoding mRNA is depicted in Table 1 (Hosein et al., 2015; Maccoux et al., 2007; Maeda et al., 2009; Peters et al., 2007; Schmitz et al., 2012). Hydroxymethylbilane synthase, ribosomal protein L32, and ribosomal protein S18 were used as reference genes, according to a previous study (Igarashi et al., 2014). The amplification conditions were as follows: 95°C for 10 s, 40 cycles of PCR (95°C for 5 s and 60°C for 30 s), and dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). Nuclease-free water and non-reverse transcription controls were used as negative controls. A sample with a known cycle threshold (Ct) value (as a positive control) was included with all sample runs to control for run-to-run Ct variations. The real-time data were analyzed using Thermal Cycler Dice Real Time System software version 4.01A (Takara Bio Inc.). Ct values were determined with the second derivative maximum cycles method.

Gene expression data for *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* in dogs from a previous study (Igarashi et al., 2014) were also used to compare the rate of increase in the polypoid lesion with other genes and to evaluate the correlation of mRNA expression of each gene.

2.3. Immunohistochemistry for Foxp3 and Iba-1

Immunohistochemistry (IHC) was performed using paraffin-embedded tissues, including 16 polypoid lesions from ICRP-affected MDs and 10 colonic mucosal samples from control dogs. In addition, seven non-polypoid colonic mucosal specimens from the 16 ICRP cases were

Table 1
Primer sequences used in the present study.

Gene		Primer sequences (5′–3′)	Product length (bp)	GenBank accession number
IL-1β	Forward	ACCCGAACTCACCAGTGAAATG	110	NM_001037971
	Reverse	GGTTCAGGTCTTGGCAGCAG		
IL-4	Forward	TCTGCTTACTAGCACTCACCAGCAC	125	NM_001003159
	Reverse	GACAGTCAGCTCCATGCACGA		
IL-6	Forward	TCTGTGCACATGAGTACCAAGATCC	125	NM_001003301
	Reverse	TCCTGCGACTGCAAGATAGCC		
IL-8	Forward	CTTCCAAGCTGGCTGTTGCTC	173	NM_001003200
	Reverse	TGGGCCACTGTCAATCACTCTC		
IL-10	Forward	CAGGTGAAGAGCGCATTTAGT	65	XM_850467
	Reverse	TCAAACTCACTCATGGCTTTGT		
IL-17A	Forward	CACTCCTTCCGGCTAGAGAA	72	NM_001165878.1
	Reverse	CACATGGCGAACAATAGGG		
IL-22	Forward	TCCAGCAGCCCTATATCACC	254	XM_538274.2
	Reverse	TTGGCTTAGCTTGTTGCTGA		
IFN-γ	Forward	CTTGGCAAGTTCTTAAATAGCAGCA	151	NM_001003174
	Reverse	TCCTTAGGTTGGATCTTGGTGAGAG		
TNF-α	Forward	CCCAAGTGACAAGCCAGTAGCTC	146	NM_001003244
	Reverse	ACAACCCATCTGACGGCACTATC		
TGF-β	Forward	GGAGCAGCATGTGGAGCTGTA	125	NM_001003309
	Reverse	GCCTCACGACTCCAGTGACATC		
Foxp3	Forward	GGCTCCTGCTGTATCGTAGC	179	NM_001168461.1
	Reverse	CGCATGTTGTGGAATTTGAA		
HMBS	Forward	TCACCATCGGAGCCATCT	112	XM_546491
	Reverse	GTTCCCACCACGCTCTTCT		
RPL32	Forward	TGGTTACAGGAGCAACAAGAAA	100	XM_848016
	Reverse	CACATCAGCAGCACTTCA		
RPS18	Forward	TGCTCATGTGGTATTGAGGAA	116	XM_532106
	Reverse	TCTTATACTGGCGTGGATTCTG		

Foxp3, forkhead box protein P3; HMBS, hydroxymethylbilane synthase; RPL32, ribosomal protein L32; RPS18, ribosomal protein S18.

further included. IHC for Foxp3 and ionized calcium binding adapter molecule 1 (Iba-1) was conducted using serial sections of paraffin-embedded, 4 μm-thick tissues to detect Tregs and macrophages, respectively. Heat-induced antigen retrieval was performed by autoclaving the sections for 10 min at 121˚C in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating the samples with REAL Peroxidase-Blocking Solution (Dako, Glostrup, Denmark) at room temperature for 10 min (Foxp3) or 30 min (Iba-1). The sections were blocked with 5% (Foxp3) or 8% (Iba-1) skim milk in tris-buffered saline with 0.1% tween 20 (TBST) at room temperature for 60 min and then incubated with a rat anti-mouse Foxp3 mAb (1:400; clone FJK-16 s; eBioscience, San Diego, CA) or a rabbit anti-human Iba-1 pAb (1:250; FUJIFILM Wako Pure Chemical Corp, Osaka, Japan) at 4˚C overnight. The cross-reactivity of these antibodies with canine Foxp3 or Iba-1 was confirmed in previous studies (Mizuno et al., 2009; Tamura et al., 2013). The slides for Foxp3 assessment were washed with TBST, incubated with a biotin-labeled anti-rat IgG antibody (Vector Laboratories, Burlingame, CA) at room temperature for 30 min, further washed with TBST, and then incubated with HRP-labeled streptavidin (Dako) at room temperature for 30 min. The slides for Iba-1 analysis were washed with TBST and then incubated with HRP-labeled polymer conjugated with goat anti-rabbit immunoglobulins (Dako) at room temperature for 45 min. The reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride hydrate (Dako).

Ten appropriate areas were randomly chosen in identical fields for both Foxp3- and Iba-1-stained specimens. The number of Foxp3-positive Tregs and Iba-1-positive macrophages in each field was counted using a × 40 objective, a × 10 eyepiece, and a total area of 62,500 μm^2 using image analysis software (Image J; https://imagej.nih.gov/ij/).

2.4. Statistical analysis

Statistical analyses were performed using JMP 12.0.1 (SAS Institute Inc., Cary, NC, USA). The Kruskal–Wallis test was used to test overall differences in the number of Foxp3-positive Tregs and Iba-1-positive macrophages, as well as the mRNA expression of *Foxp3* and each cytokine among polypoid lesions, non-polypoid colonic mucosa of affected dogs, and colonic mucosa of healthy dogs. The Steel–Dwass test was used as a post-hoc test to determine between-group differences. The Dunn's test was used as a post-hoc test to compare the rate of increase in mRNA expression (polypoid lesions/non-polypoid lesions) of *IL-10* relative to that of *Foxp3* and other cytokines. The relationships among the number of Tregs or macrophages and mRNA expression of each gene were evaluated using the Spearman's rank correlation coefficient. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Macroscopic and histopathologic findings

Eight MDs had diffused multiple small polyps in the colorectal region, while other 20 MDs had both large and small polyps in that region. Histopathologically, severe neutrophil infiltration, moderate to severe infiltration with macrophages, lymphocyte, and/or plasma cells in lamina propria, fibrosis, edema, crypt expansion, and mucous hyperplasia were observed in polypoid lesion in all MDs. In addition, histopathology was also performed in non-polypoid colonic mucosa in 17 of 24 MDs with ICRPs; six of them showed no abnormality while 11 of them had mild lymphocytic–plasmacytic colitis based on World Small Animal Veterinary Association guidelines.

3.2. Quantification of mRNA expression levels of Foxp3 and pro- or anti-inflammatory cytokines

The relative expression levels of all cytokines except for *IL-4* were significantly higher in polypoid lesions than in the non-polypoid colonic mucosa of ICRPs in MDs and control dogs (Fig. 1). However, there was no significant difference in the mRNA expression level of *IL-4* among the three groups (Fig. 1). Whereas the mRNA expression levels of *IL-1β* and *IL-8* were significantly higher in the non-polypoid colonic mucosa of ICRPs of MDs compared to that in control dogs, mRNA levels of *Foxp3* were significantly lower in the non-polypoid colonic mucosa of

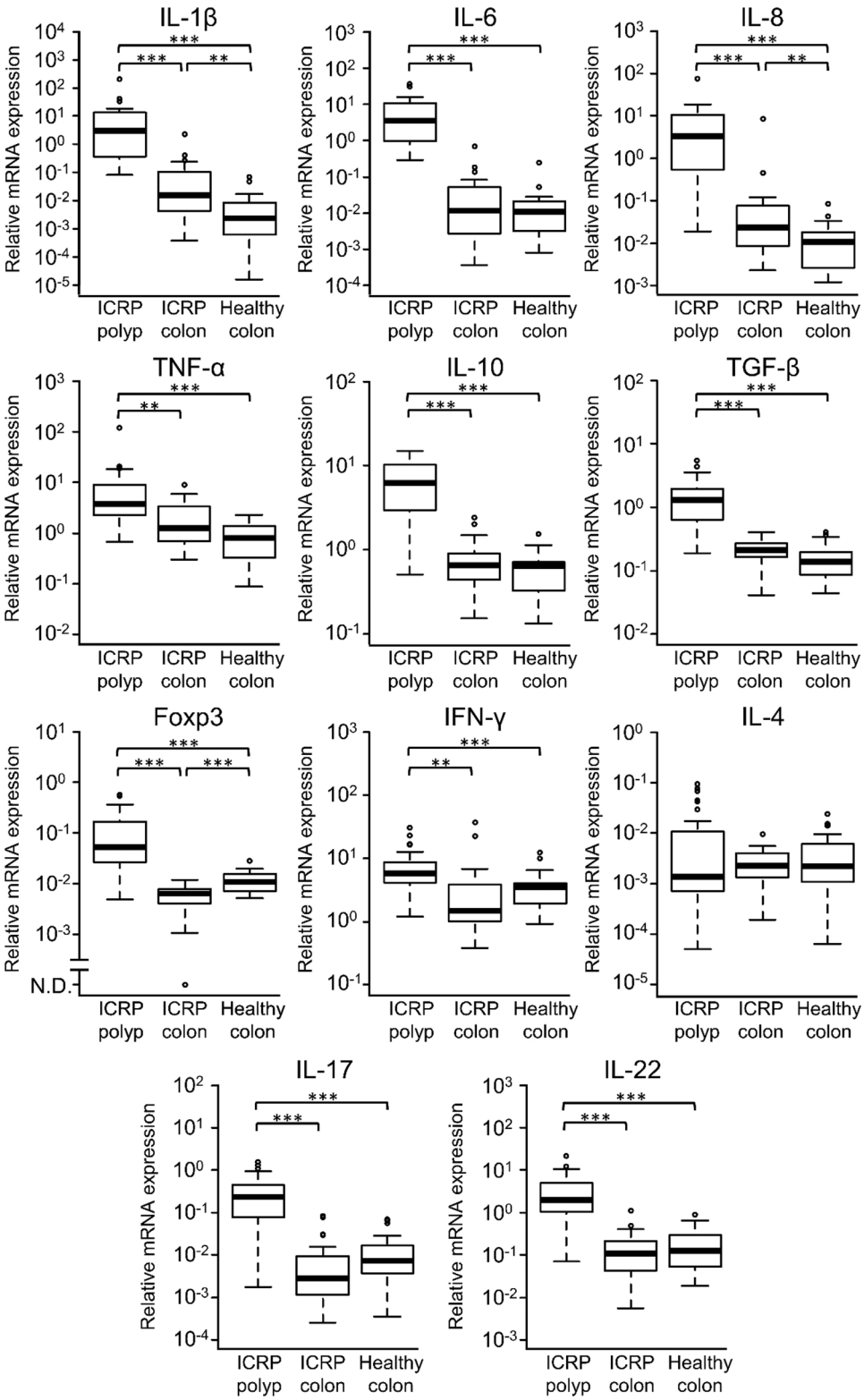

Fig. 1. Relative expression levels of *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, *IL-10*, *TGF-β*, *Foxp3*, *IFN-γ*, *IL-4*, *IL-17*, and *IL-22* mRNA in the polypoid lesion of inflammatory colorectal polyp (ICRP polyp; n = 28), the non-polypoid colonic mucosa (ICRP colon; n = 24), and the colorectal mucosa of control dogs (healthy colon; n = 21). The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; the whiskers indicate the highest and lowest data points within 1.5 times the length of the quartiles; the circles represent outliers. Asterisks indicate statistical differences (*$P <$ 0.05, **$P <$ 0.01, ***$P <$ 0.001); N.D., not detected.

ICRPs in MDs compared to that in in control dogs (Fig. 1). Comparing the rate of increase in mRNA expression (polypoid lesions/non-polypoid lesions) of *IL-10* to that of *Foxp3* and other cytokines in MDs with ICRPs, rates for of *IL-1β*, *IL-6*, and *IL-8* were significantly higher than those of *IL-10* (Fig. 2). In contrast, the rate of *IL-4* was significantly lower than that of *IL-10* (Fig. 2).

3.3. Foxp3-positive Tregs and Iba-1-positive macrophages in the colorectal mucosa

In polypoid lesions, mild infiltration of Foxp3-positive Tregs and moderate infiltration of Iba-1-positive macrophages were observed in the lamina propria (Fig. 3A and B). In contrast, there were minimal to mild infiltration of Foxp3-positive Tregs and Iba-1-positive macrophages in both the non-polypoid colonic mucosa of ICRPs in MDs and

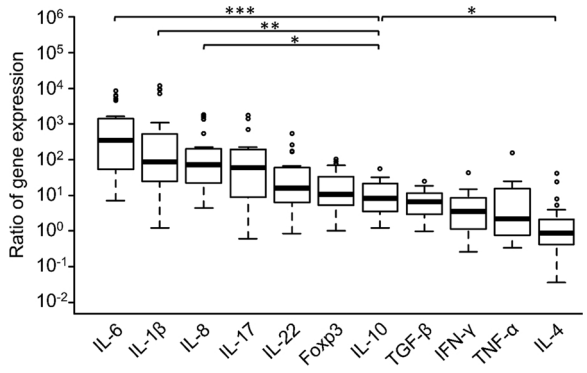

Fig. 2. Ratios of mRNA expression in the polypoid lesions compared to those in the non-polypoid colonic mucosa of inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds (MDs; n = 24). For only *Foxp3*: n = 23 (due to the fact that expression was not detected in one sample). The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; the whiskers indicate the highest and lowest data points within 1.5 times the length of the quartiles; the circles represent outliers. Asterisks indicate statistical differences compared to *IL-10* mRNA expression (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

control dogs (Fig. 3C − F). The numbers of both Foxp3-positive Tregs and Iba-1-positive macrophages were significantly increased in polypoid lesions compared to those in the non-polypoid colonic mucosa of ICRPs in MDs or control dogs (Fig. 4A and B). Whereas the number of Foxp3-positive Tregs was significantly higher in the non-polypoid colonic mucosa of ICRPs in MDs compared to that in control dogs (Fig. 4A), there was no significant difference in the number of Iba-1-positive macrophages between the non-polypoid colonic mucosa of ICRPs in MDs and that of control dogs (Fig. 4B).

3.4. Correlation among the number of Foxp3-positive Tregs and mRNA expression levels of Foxp3 and IL-10

We next examined correlations among the mRNA expression levels of *IL-10* and *Foxp3* with other genes and the number of Foxp3-positive Tregs or Iba-1-positive macrophages (Table 2). Data of correlation among all gene expression and the number of Foxp3- or Iba-1-positive cells were listed in a supplementary Table 1. The most relevant association with *Foxp3* gene expression was found in *IL-17* gene expression ($r = 0.780$, $P < 0.001$); that with *IL-10* gene expression was *TNF-α* gene expression ($r = 0.910$, $P < 0.001$).

4. Discussion

This study demonstrated the distribution of Tregs and macrophages in the polypoid lesions of ICRPs in MDs and the association between the distribution and expression levels of genes encoding pro- or anti-inflammatory cytokines. The results showed that the numbers of Tregs and macrophages were significantly increased concomitant with the upregulation of several pro- and anti-inflammatory cytokines in polypoid lesions. Moreover, the rates at which mRNA expression increased in the polypoid lesion, as compared to levels in the non-polypoid colonic mucosal ICRPs of MDs, were significantly higher for pro-inflammatory cytokines than for *IL-10*.

In the present study, we observed an increase of Foxp3-positive Tregs in the polypoid lesions of ICRPs in MDs. This is an opposite result to that previously reported for canine IBD (Junginger et al., 2012; Maeda et al., 2016), but is consistent with reports of human IBD (Wang et al., 2011). In addition, we also detected the upregulation of *Foxp3*, *IL-10*, and *TGF-β* mRNA in polypoid lesions, which is in line with a previous study (Ohta et al., 2013). Furthermore, previous studies have also shown that the mRNA transcription of anti-inflammatory cytokines (*IL-10* and *TGF-β*) or *Foxp3* is increased in the human intestinal mucosa during IBD (Eastaff-Leung et al., 2010; Melgar et al., 2003). Since higher correlations were observed among Th17 cytokines (i.e., IL-17 and IL-22), proinflammatory cytokines (IL-1*β*, IL-6, IL-8, and TNF-*α*), and Treg status (IL-10, TGF-*β*, and Foxp3) than those with Th1 (IFN-γ) or Th2 (IL-4) cytokines, infiltration of Tregs was speculated to occur concurrently with Th17 and macrophage infiltration, rather than with Th1 or Th2 cells. There were moderate positive correlations for any combination of Foxp3-positive Treg numbers and the expression levels of *Foxp3* or *IL-10* in the polypoid lesions of ICRPs in MDs. Thus, it was indicated that increased infiltration of Foxp3-positive Tregs resulted in an elevated production of anti-inflammatory cytokines including IL-10 and TGF-β in canine ICRPs. Tregs play an important role for regulating mucosal immune tolerance, which suppresses the immune response to non-pathogenic antigens in the intestinal mucosa, by producing IL-10 and TGF-β (Lan et al., 2007). In addition, TGF-β also plays a role in promoting Foxp3 expression and generating Tregs in the intestinal mucosa (Konkel and Chen, 2011). Tregs can be recruited to the inflamed mucosa during human IBD in an attempt to control the inflammation (Pedros et al., 2016). Thus, the increase in the numbers of Tregs and anti-inflammatory cytokines in polypoid lesions of ICRPs in MDs is considered a reactive change in response to the inflammation, as occurs in human IBD.

In contrast, levels of the pro-inflammatory cytokines *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* were found to be higher in the polypoid lesions of ICRPs in MDs, as we reported previously (Igarashi et al., 2014). This was in line with previous studies on the inflammatory lesions of both human IBD and ICRPs in MDs (Szkaradkiewicz et al., 2009; Tamura et al., 2013). These pro-inflammatory cytokines are suggested to exacerbate and maintain chronic intestinal inflammation during human IBD (Neurath, 2014; Roberts-Thomson et al., 2011). In this study, we observed a higher rate of increase in mRNA expression of *IL-1β*, *IL-6*, and *IL-8*, compared to that of *IL-10*. These results suggest that although Tregs are increased in polypoid lesions compared to numbers in the non-polypoid colonic mucosa of ICRPs in MDs and control dogs, they do not appropriately function to suppress the severe inflammation that occurs in the polypoid lesion. Further analysis on the mechanism of this phenomenon is required. In contrast, there was no difference in the climb rate of *TNF-α* expression compared to that of *IL-10*. However, a recent study showed that TNF-α induces IL-8 secretion from monocytes in vitro (O'Dwyer et al., 2016). Thus, the upregulation of TNF-α could also promote inflammation in polypoid lesions, even if the activity of this cytokine appears to be weak in such lesions.

IL-17, which is a pro-inflammatory cytokine within the IL-17 family and is produced by Th17 cells, mediates the upregulation of IL-1β, IL-6, IL-8, and TNF-α, leading to the recruitment of neutrophils in the intestinal mucosa (Neurath, 2014). In contrast, IL-22, which is a cytokine of the IL-10 family and is produced by Th17 cells and human Th22 cells, induces the production of anti-bacterial peptides and facilitates the maintenance of the intestinal epithelial barrier (Nikoopour et al., 2015). In the present study, *IL-17* and *IL-22* mRNA expression levels were also upregulated in the polypoid lesions of ICRPs in MDs. This result is consistent with the observed inflamed mucosa of human IBD (Jiang et al., 2014). Moreover, in an environment rich in pro-inflammatory cytokines, with high IL-1β and IL-6 levels, Tregs lose their suppressive function and convert to IL-17-producing Tregs; this might indicate that Tregs cannot exert suppressive activity despite the high expression of Foxp3 in the intestinal mucosa during human IBD (Eastaff-Leung et al., 2010).

A recent study reported that ICRPs in MDs sometimes develop into colorectal adenoma and adenocarcinoma (Saito et al., 2018). In humans, tumor-infiltrating Tregs inhibit the anti-tumor immune response and promote tumor angiogenesis (Facciabene et al., 2012). Similarly, in dogs, tumor-infiltrating Tregs are detected in various neoplasms, and their abundance contributes to reduced survival times (Maeda et al., 2016; Sakai et al., 2018). Further, glucocorticoids increase the Treg

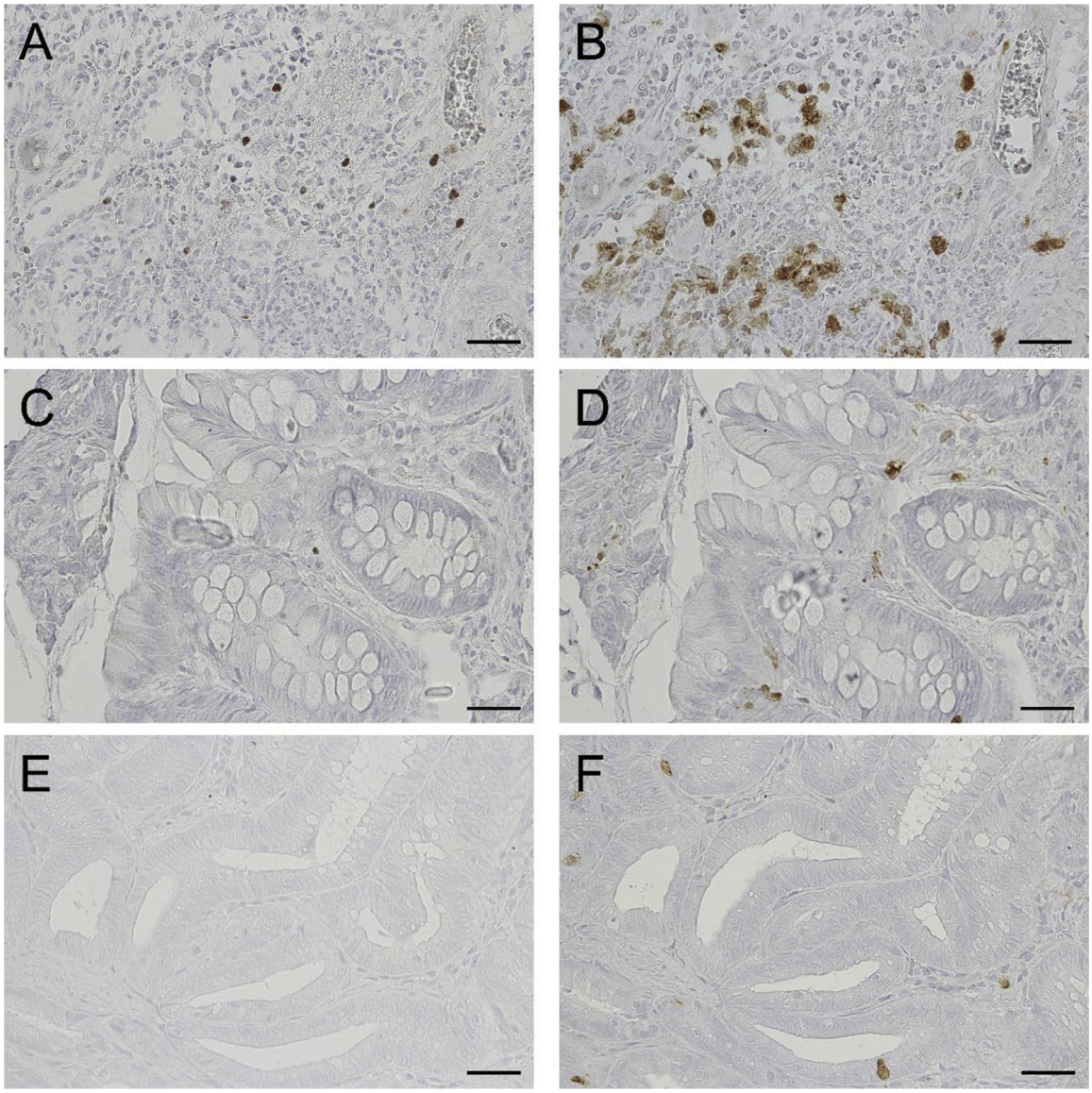

Fig. 3. Immunohistochemistry (IHC) for forkhead box protein P3 (Foxp3)- and ionized calcium binding adapter molecule 1 (Iba-1)-positive cells. Comparisons were made among the colorectal mucosa obtained from the polypoid lesions (A and B), non-polypoid colonic mucosa of miniature dachshunds (MDs) with inflammatory colorectal polyps (ICRPs) (C and D), and colonic mucosa of control dogs (E and F). (A, C, and E) IHC for Foxp3. (B, D, and F) IHC for Iba-1. (A and B) Few to moderate number of Foxp3-positive regulatory T cell (Treg) and Iba-1-positive macrophage infiltration in the polypoid lesions were observed. (C) Minimal Foxp3-positive Tregs were observed. (D) There was mild Iba-1-positive macrophage infiltration in the lamina propria. (E) No Foxp3-positive Tregs were observed. (F) Minimal Iba-1-positive macrophages were observed. Scale bar = 30 μm.

population and shift the immunological balance to a Treg-centered environment (Liberman et al., 2018), whereas NSAIDs reduce the Treg population and function within the tumor (Sharma et al., 2005). Thus, further studies should be performed to determine the mechanisms underlying tumorigenesis by compared ICRPs with colorectal neoplasms, in addition to the association between Treg distribution and prognosis or therapeutic response in MDs with ICRPs.

There were several limitations to this study. First, we used whole endoscopic biopsy specimens to detect the mRNA expression of cytokines. This does not always correlate with protein production, which remains undetermined in this study; however, the amount of IL-8 protein and the number of IL-8-producing macrophages have been reported to be increased in ICRP lesions in MDs (Tamura et al., 2013). Second, we did not examine the distribution of $CD4^+$ T cell subsets, especially Th1, Th2, Th17, and Th22 cells in the lamina propria. These helper T cells are suggested to produce various cytokines in the colorectal mucosa of ICRPs in MDs. Hence, each $CD4^+$ T cell subset in the lamina propria should also be distinguished by IHC or flow cytometry if canine-specific antibodies are available. Third, the cellular source of *IL-10* gene or protein production and its distribution in the lesion should be confirmed by double-labeling immunofluorescence analysis or in situ hybridization in the future. Fourth, all whole endoscopic biopsy specimens were used to measure the numbers of Tregs and macrophages in the lamina propria in the current study. Although biopsy specimens obtained from full-thickness surgical excision are desirable for a more precise evaluation of cell distribution, it was ethically difficult to collect all samples by surgical excision. Fifth, this study did not consider the severity of ICRPs because of the limited number of cases. ICRPs with early stage have been documented to exhibit mild lymphocyte and macrophage infiltration, whereas those with later stages show severe neutrophil infiltration (Uchida et al., 2016). Therefore, the association between Treg infiltration and histopathological stage should be evaluated in a larger population to clarify the role of Tregs in the pathogenesis of ICRPs. Finally, we used normal Beagles which was not an

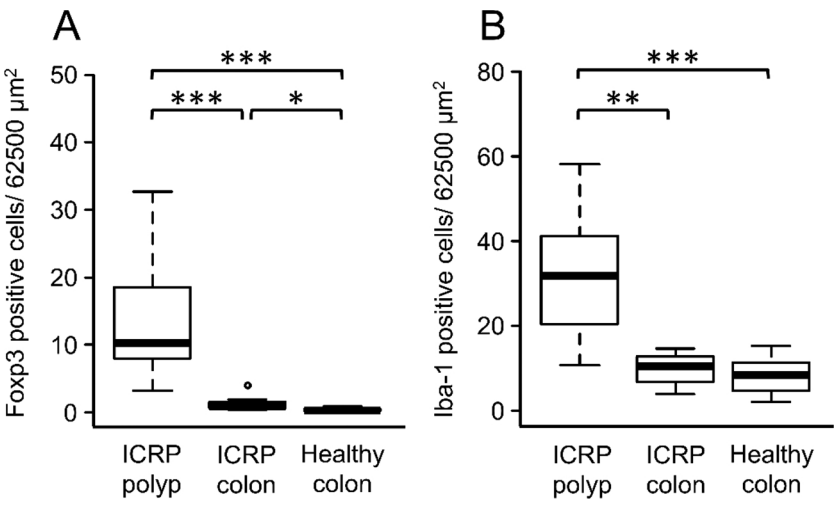

Fig. 4. The numbers of Foxp3-positive cells (A) and Iba-1-positive cells (B) in the polypoid lesion (inflammatory colorectal polyps; ICRP polyps; n = 16), the non-polypoid colonic mucosa (ICRP colon; n = 7), and the colorectal mucosa of control dogs (healthy colon; n = 10). The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; the whiskers indicate the highest and lowest data points within 1.5 times the length of the quartiles; the circles represent outliers. Asterisks indicate statistical differences (*$P <$ 0.05, **$P <$ 0.01, ***$P <$ 0.001).

Table 2
Correlation coefficient between Foxp-3 or IL-10 mRNA expressions and other cytokine genes or the number of Foxp3- or Iba-1-positive cells.

	Foxp3 gene	*IL-10*
Foxp3 gene		0.749
IL-1β	0.729	0.883
IL-4	−0.122	0.191
IL-6	0.763	0.821
IL-8	0.754	0.898
IL-10	0.749	
IL-17	0.780	0.870
IL-22	0.723	0.773
IFN-γ	0.682	0.563
TNF-α	0.694	0.910
TGF-β	0.613	0.829
Foxp3$^+$ cell	0.664	0.549
Iba-1$^+$ cell	0.527	0.561

Each data represents the value of Spearman's r.

ideal control group for the study in terms of age, gender, and breed. Since it was difficult to collect the colonic tissues from client-owned healthy MDs due to the ethical problem. In addition, difference in the treatment status received prior to colonoscopy also affect the results of the study. However, these confounding factors should not interfere with the difference between polypoid lesion and non-polypoid colonic mucosa, which were obtained from same cases. Moreover, the upregulation of gene expression and increase of Foxp3$^+$ or Iba-1$^+$ cell infiltration observed in this study were mostly restricted to the polypoid lesion, thus we believe this limitation may not affect the conclusion.

In conclusion, the number of Foxp3-positive Tregs was increased in the polypoid lesions of ICRPs in MDs, which was moderately correlated with the upregulation of anti-inflammatory cytokines and *Foxp3* expression in these lesions. These results indicate that the increase in the number of Tregs and the upregulation of anti-inflammatory cytokine expression in polypoid lesions is a reactive change in response to the chronic and severe inflammation, which appeared to be insufficient in mitigating the development of inflammatory lesions. Further investigation of the therapeutic response in terms of the differential distribution of Tregs in polypoid lesions is required. Furthermore, the potential tumorigenic mechanisms associated with ICRPs should be elucidated by comparing ICRPs with colorectal adenoma and adenocarcinoma.

Declaration of Competing Interest

The authors of this paper do not have a financial or personal relationship with other individuals or organizations that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109938.

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