Studies on mucosal innate immunity and microflora in miniature dachshunds with inflammatory colorectal polyps

（炎症性結直腸ポリープに罹患したミニチュア・ダックスフンドにおける自然免疫および腸内細菌叢に関する研究）

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Chapter 0 (Introductory Chapter)

1. Introduction

Colorectal polyps are relatively common in dogs, and most are neoplastic (Seiler, 1979; Valerius et al., 1997). However, a recent report showed that Miniature Dachshunds (MDs) are commonly affected by inflammatory colorectal polyps (ICRPs) (Ohmi et al., 2012). ICRPs in MDs were a recently recognized disease in Japan, and these animals commonly develop clinical signs such as hematochezia, tenesmus, and mucoid feces. ICRPs in MDs typically form multiple small polyps restricted to the descending colon and rectum, but they sometimes develop into a space-occupying large polyp. ICRPs commonly show excessive mucous secretion, mucous hyperplasia, and severe inflammatory infiltration (predominantly with neutrophils and macrophages), which is more severe in large polyps than in small polyps (Tamura et al., 2013). MDs with ICRPs respond relatively well to immunosuppressive therapy that includes prednisolone and/or cyclosporine (Ohmi et al., 2012). Therefore, ICRPs are proposed to be a novel form of canine inflammatory bowel disease (IBD) (Ohta et al., 2013).

To date, several studies have investigated the pathogenesis of ICRPs in MDs. Ohta et al. (2013) reported upregulated expression of CD4+ T cell cytokine mRNAs, including IL-17, IFN-γ, and IL-10 at the polypoid lesion. Tamura et al. (2013) also reported upregulation of proinflammatory cytokine genes, including IL-1β, IL-6, IL-8/CXCL8, IL-12p35, IL-12/23p40, IL-23p19, and TNF-α at polypoid lesion; further, this study showed that the macrophages in the colorectal mucosa were the major cellular source of IL-8/CXCL8. Since IL-8/CXCL8 has been shown to induce neutrophil infiltration, the upregulation of IL-8/CXCL8 by macrophages in the polypoid lesion is considered to play an important role in the development of mucosal inflammation commonly observed in ICRPs in MDs. However, no previous study investigated the inflammation trigger.

In human and canine IBD, the etiology of intestinal inflammation has been described as multifactorial, and aberrant interactions between the mucosal immune system and luminal microflora are postulated to lead to the chronic intestinal inflammation (Cerquetella et al., 2010; German et al., 2003; Xavier and Podolsky, 2007). Since the large intestine has the highest density and diversity of bacteria (Hooda et al., 2012), the aberrant host-microbe interaction is speculated to play an important role in the pathogenesis of ICRPs in MDs.

The restriction of ICRPs to the mucosal surface of the colorectal region in MDs is similar to that of ulcerative colitis, a major form of human IBD (Ohmi et al., 2012; Ordás et al., 2012). Although the mechanism of lesion restriction has not been clarified, it is assumed that the colorectal microflora contribute to the pathogenesis. When performing routine medical care, I noted that another characteristic of ICRPs in MDs is that the polypoid lesions frequently occur at the ventral floor of the colorectum; thus, I conducted a preliminary retrospective investigation of the frequency of ICRPs at the ventral floor and the dorsal roof of the colorectum.

I reviewed the medical records of MDs presented to the Veterinary Medical Center of the University of Tokyo between April 2007 and March 2013. Fifty-one dogs were diagnosed during the period and 40 of them had documentation about the lesion angle. Consequently, I found that ICRPs were likely to develop at the ventral floor (85.0% of the MDs had polypoid lesions at the ventral floor and 47.5% at the dorsal roof). However, the lesion angle were determined via rectal examination (n = 23), endoscopy (n = 14), contrast radiography (n = 1), or macroscopic findings at surgery (n = 2); therefore, the data seemed to be too subjective to draw any conclusion. Therefore, I conducted a prospective study (Chapter 0) with a hypothesis that the ICRPs were more likely to

develop on the ventral floor than the dorsal roof of the colorectum.

2. Materials and methods

MDs referred to the Veterinary Medical Center of the University of Tokyo between April 2013 and April 2014 because of clinical signs involving chronic hematochezia and/or tenesmus diagnosed as ICRPs by colonoscopy and histopathology were prospectively included. Dogs with neoplastic polyps were excluded. At colonoscopy, the dorsal angle was placed at the upper end of the image (Figure 1a). Two images were taken from each dog at different sites around the most severe lesion. Subsequently, images of each case at the region of the most severe lesions were stored with the scope angle retained (Figure 1b). Images were trimmed circularly around the centre of the lumen, divided to 12 equal parts (angles i–xii; Figure 1c), and randomly rotated (Figure 1d). These rotated images were subsequently presented to four experienced veterinarians. They were specialized in internal medicine and in diagnostic imaging (radiology) at the Veterinary Medical Center of the University of Tokyo and were blinded to the rotated angle. The ICRP severity at each divided part of the rotated images were scored subjectively (score 0, no polyp exists; 1, small polyps; 2, medium-sized polyps; and 3, large polyps). The mean prevalence and scores were then compared between the ventral floor (angle iv–ix) and dorsal roof (angle x–xii and i–iii) or right- (angle i–vi) and left-sided lateral wall (angle vii–xii) using the Mann–Whitney *U* test (JMP Pro version 10.0.2, SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as $P < 0.05$.

3. Results

In total 14 MDs were diagnosed endoscopically with colorectal polyps, however three of them were excluded based on the histopathological diagnosis of colorectal adenoma. Consequently, 11 MDs were included: seven male dogs (six neutered and one intact) and four neutered female dogs, median age 118 months (range, 48–151 months), and median body weight 5.15 kg (range, 4.30–6.80 kg). All dogs had diffuse small polyps and eight dogs had one to two large polyps as well. All polypoid lesions were localized to the colorectal region approximately 3–10 cm from the anus. As shown in Figures 2 and 3, both the prevalence and scores of ICRP reached the highest at angles iv–viii and v, respectively. The mean prevalence of polyps on the ventral floor was significantly higher than that of the dorsal angles ($P < 0.0001$; Table 1). Furthermore, the mean severity score of polyps on the ventral floor was significantly higher than that of the dorsal roof ($P = 0.0001$; Table 1). Conversely, no significant difference was observed in the mean prevalence or score between right- and left-sided lateral walls ($P = 0.3918$ and 0.2106, respectively; Table 1). Furthermore, similar results were obtained when the scores evaluated by each observer from each image were compared between the ventral floor and the dorsal roof (Figure 4), although there was inter-observer variation in the overall score between two observers (Figure 5).

4. Discussion and general introduction for Chapter 1 and 2

This Chapter revealed that ICRPs in MDs tend to develop larger at the ventral than at the dorsal region. Since this study only investigated macroscopic changes to the mucosal surface, the influence of anatomical factors including distance from regional lymph nodes or mesocolon, distribution of nerve, or blood supply were not determined. Therefore, further anatomical and histological investigations into the associations between these factors and the location of ICRPs might provide a novel insight into the development of mucosal inflammation within the colorectum.

Another possibility responsible for the tendency observed here is the contribution of the aberrant interaction of mucosal immunity with fecal antigens, which has been described as a cause of chronic intestinal inflammation in human and canine IBD (Cerquetella et al., 2010; German et al., 2003; Xavier and Podolsky, 2007). This possibility is based on a hypothesis that the duration of contact between the feces and epithelium would be longer in the ventral floor than in the dorsal roof due to the intestinal gas (unlike in humans, the canine colorectum runs horizontally in the standing position). The fecal component (e.g., food components and metabolites), luminal microbiota, failure of the mucosal barrier function, and mechanical abrasion by the feces may contribute to the aberrant interaction.

There were several limitations to this study. First, only a small number of cases were included; therefore, further follow-up study in a larger-scale is needed. Another limitation was that only a subjective evaluation of disease severity was performed. Endoscopic ultrasound would provide a more objective measurement of polypoid lesion size and localization (Hayashi et al., 2012). However, I believe that this limitation's influence on the result would be small because the score difference resulted from differences between

divided angles of a single image, not from differences between individual cases or observers. Furthermore, there was no inter-observer variations in the difference between the angles (i.e., all four veterinarians scored the polyp size larger at the ventral floor than that at dorsal roof with each image).

In conclusion, the result of this Chapter revealed that ICRPs in MDs develop more severely at the ventral floor of the colorectal mucosa than at the dorsal roof. This result suggests that several factors including the anatomical or histological ventrodorsal differences, contact between epithelium and feces, and fecal components and/or mechanical abrasion might be important in the pathogenesis of this disease.

Given the findings that ICRPs in MDs commonly develop at the colorectum and are likely to occur at the ventral floor of this region, I speculated that the aberrant interaction between the mucosal immunity and fecal microbiota plays an important role in the pathogenesis of ICRPs in MDs.

Innate immune mechanisms recognize microorganisms and are implicated in many inflammatory conditions (Drexler and Foxwell, 2010). Pattern recognition receptors (PRRs), which are the key regulators of the innate immune system in the gastrointestinal mucosa, induce various cellular responses, such as proinflammatory and immunomodulatory responses (Cario, 2010; Fukata and Arditi, 2013). PRRs are expressed in various cell types, including immune and epithelial cells; these receptors activate the intracellular signaling cascades, including nuclear factor-kappa B (NF-κB), in response to the recognition of pathogen-associated molecular patterns (PAMPs) and they induce genes involved in antimicrobial host defense, such as proinflammatory cytokines, type I IFNs, and antimicrobial peptides (Akira et al., 2004, 2006; Zhong et al., 2013). To date, a number of studies have reported that dysregulated expression or

dysfunction has a genetic basis in various inflammatory disorders including IBD in humans (Cario, 2010; Corridoni et al., 2014). Similarly, associations between dysregulation or gene polymorphisms of canine PRRs and chronic enteropathy, including IBD, have been reported (Allenspach et al., 2010; Burgener et al., 2008; Kathrani et al., 2010, 2011, 2014; McMahon et al., 2010; Okanishi et al., 2013a, b). Therefore, I investigated the association between ICRPs in MDs and two well-characterized PRR families, toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors, in Chapter 1. Firstly, in Chapter 1-1, I investigated the mRNA expression levels of PRRs in the polypoid lesions of ICRP-affected MDs. Secondly, I evaluated the reactivity of PRRs in ICRP-affected MDs by using peripheral blood-derived monocytes in Chapter 1-2. Thirdly, I explored the gene polymorphisms responsible for the development of ICRPs in MDs in Chapter 1-3.

Subsequently, I investigated the alteration of the fecal microflora associated with ICRPs in MDs in Chapter 2. The gut microbiota have important roles in the nutritional, immunological, and physiologic processes of the host (Hooda et al., 2012; Hooper et al., 2001; Mackie et al., 1999). The commensal microbiota protect the host from pathogens by forming an integral part of the mucosal barrier (Hooda et al., 2012). The mechanism of this protection includes competition for nutrients and mucosal adhesion sites, which physiologically restricts the environment available to invading pathogens (Kanauchi et al., 2005). In addition, gut microbiota have enzymes that digest complex carbohydrates from the diet and ferment endogenous products, including sloughed epithelial cells and mucus; this process results in the production of short chain fatty acids (SCFAs), such as acetic, propionic, and butyric acids (Sunvold et al., 1995a, b). SCFAs, particularly butyric acid, have been shown to inhibit colonic inflammation, carcinogenesis, and oxidative

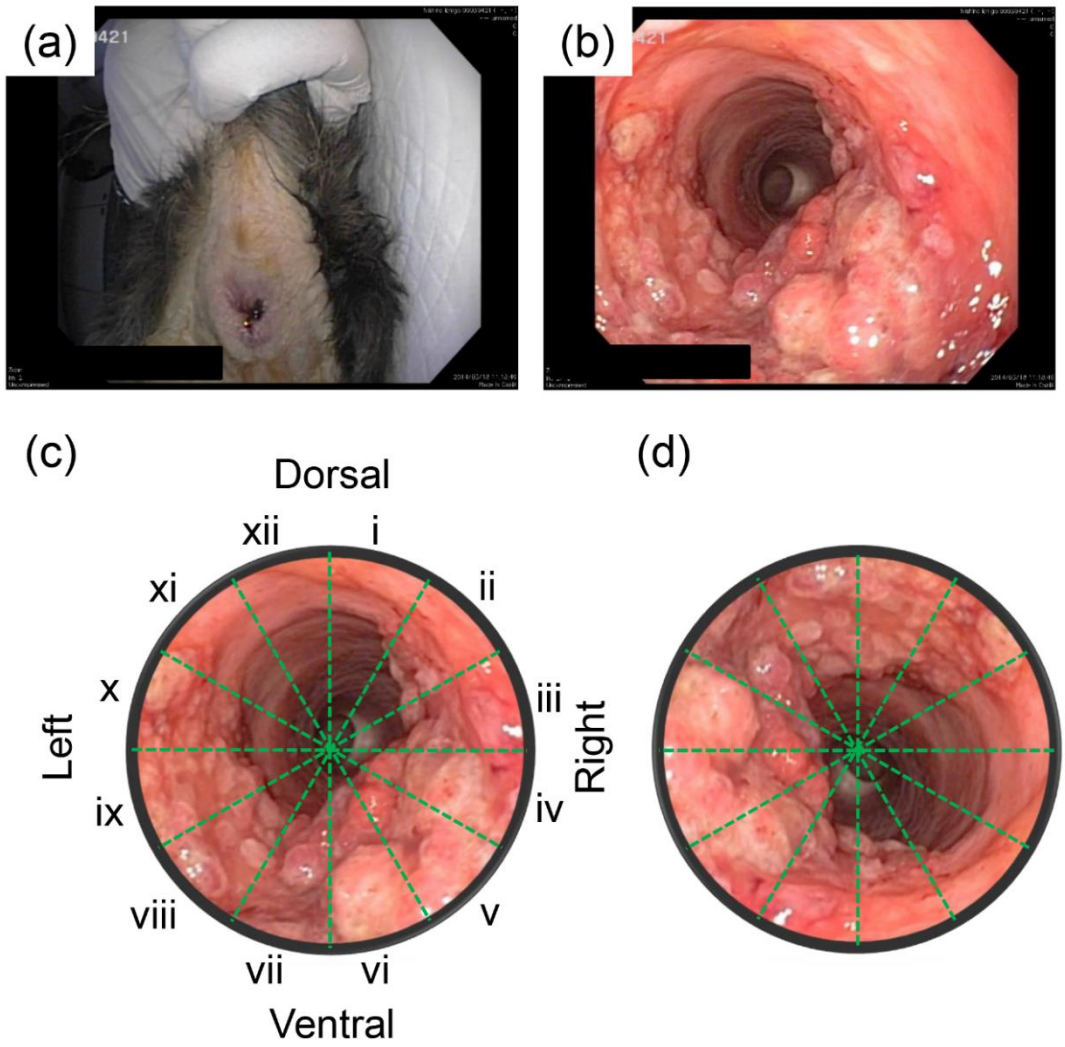
stress, improve colonic defense barrier function, promote satiety, and function as a main energy substrate for colonocytes (Cook and Sellin, 1998; Hamer et al., 2008). Luminal dysbiosis has been reported in human and canine IBD patients, but a cause-effect relationship was not fully clarified (Deng and Swanson, 2014; Honneffer et al., 2014; Wang et al., 2014). Furthermore, decreased fecal SCFA concentrations and the efficacy of SCFA enemas were reported in human IBD (Cummings, 1997; Hamer et al., 2008; Huda-Faujan et al., 2010; Takaishi et al., 2008). Therefore, I characterized the composition of the fecal microbiota in ICRP-affected MDs by using high-throughput 16S rRNA gene sequencing with the Illumina MiSeq platform in Chapter 2-1. Subsequently, I analyzed the fecal SCFA concentrations in ICRP-affected MDs by using high performance liquid chromatography (HPLC) in Chapter 2-2.

Table 1
Mean prevalence and severity scores of each angle at four directional angles.

	Dorsal roof*	Ventral floor*	*P*-value	Left-side*	Right-side*	*P*-value
Mean prevalence (%)	62.5 (20.8–81.3)	91.7 (77.1–100)	<0.0001	79.2 (35.4–95.8)	68.8 (47.9–97.9)	0.3918
Mean severity score	0.83 (0.27–1.44)	1.81 (1.25–2.42)	0.0001	0.96 (0.65–2.31)	1.52 (0.69–1.94)	0.2106

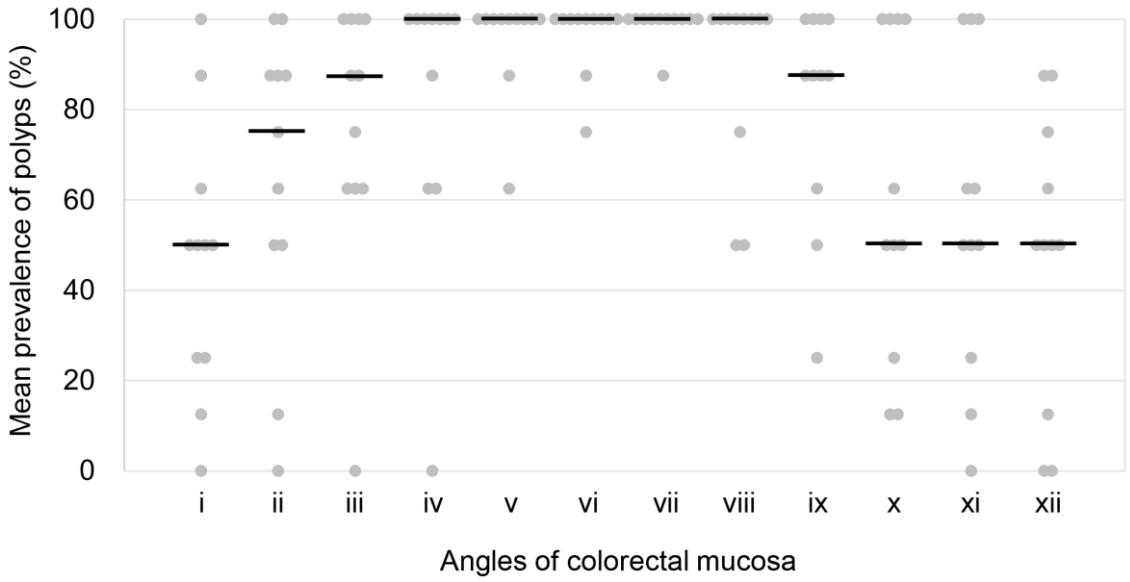
Data are represented as median (range).
*Dorsal roof represent angles i–iii and x–xii, ventral floor iv–ix, left-sided lateral wall vii–xii, and right-sided lateral wall i–vi, respectively.

Figure 1



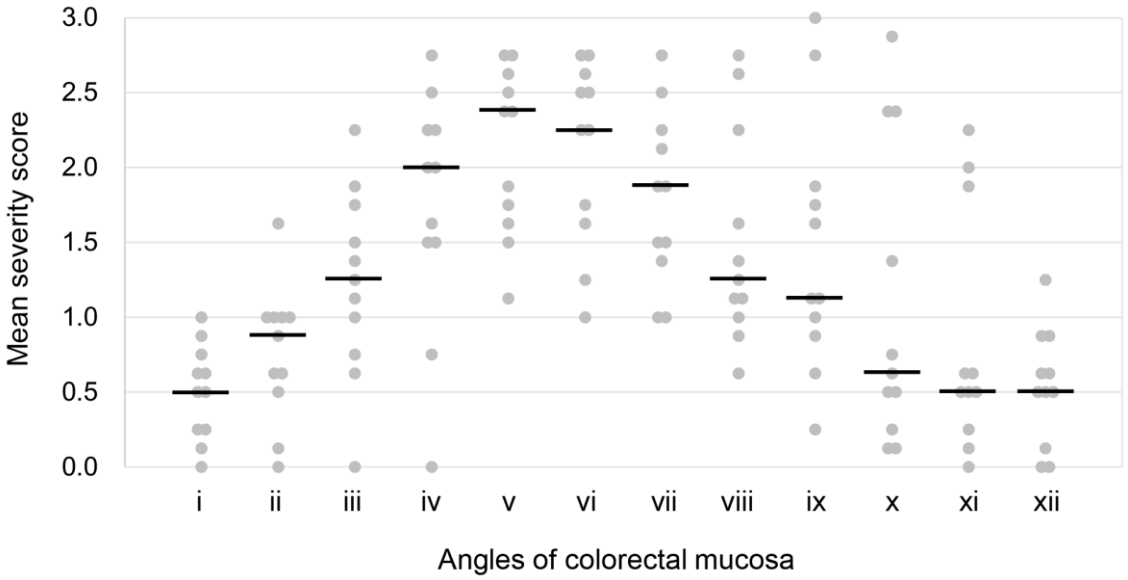
Data from the colonoscopic images obtained in the study of Chapter 0. (a) Before inserting the endoscope into the dog's anus, the dorsal roof was placed at the upper end of the image. (b) Subsequently, the endoscope was inserted into the anus while scope angle was retained (i.e., with the upper side of the image corresponding to the dorsal aspect). Images of each case around the most severe lesion were stored. (c) Each image was circularly trimmed and divided into 12 equal angles. The angles were allocated as angles i–xii. (d) Subsequently, the trimmed images were randomly rotated and presented to four veterinarians blinded to the rotated angle.

Figure 2



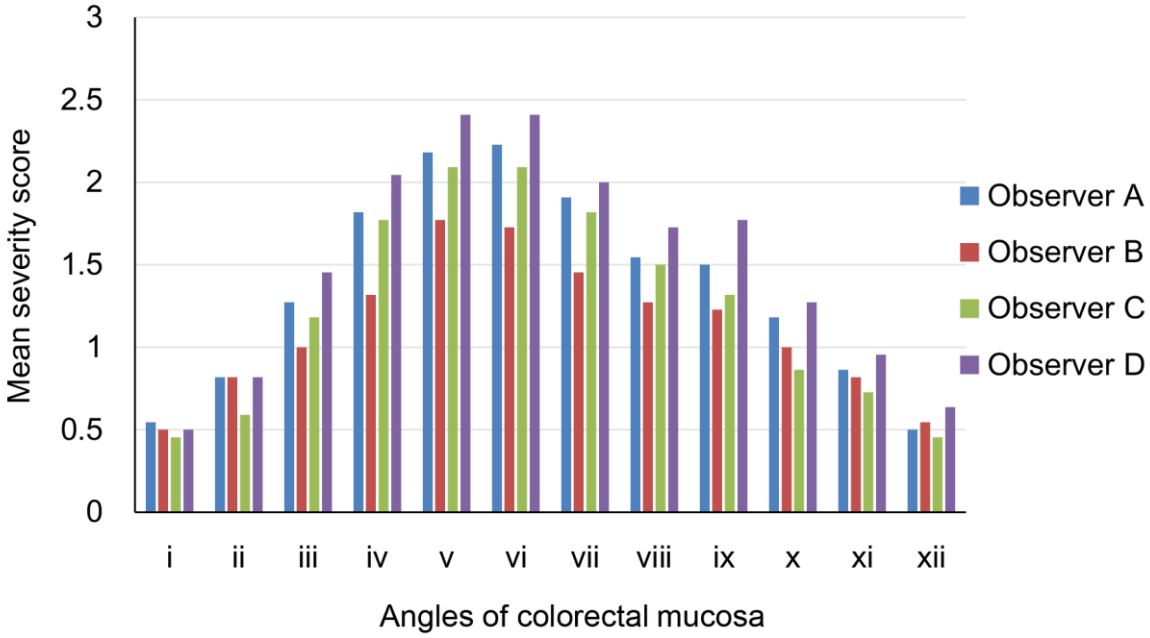
Mean prevalence of inflammatory colorectal polyps (ICRPs) in 11 dogs included in the present study at different angles of colorectal mucosa. The plots indicate the averaged data of each dog derived from two images and four observers. Each angle specified in the X-axis corresponds to that allocated in Figure 1c. The horizontal lines represent the median value of the angle.

Figure 3



Mean severity scores of ICRPs in 11 dogs at different angles of colorectal mucosa. The plots indicate the averaged data of each dog derived from two images and four observers. Each angle specified in the X-axis corresponds to that allocated in Figure 1c. The horizontal lines represent the median value of the angle.

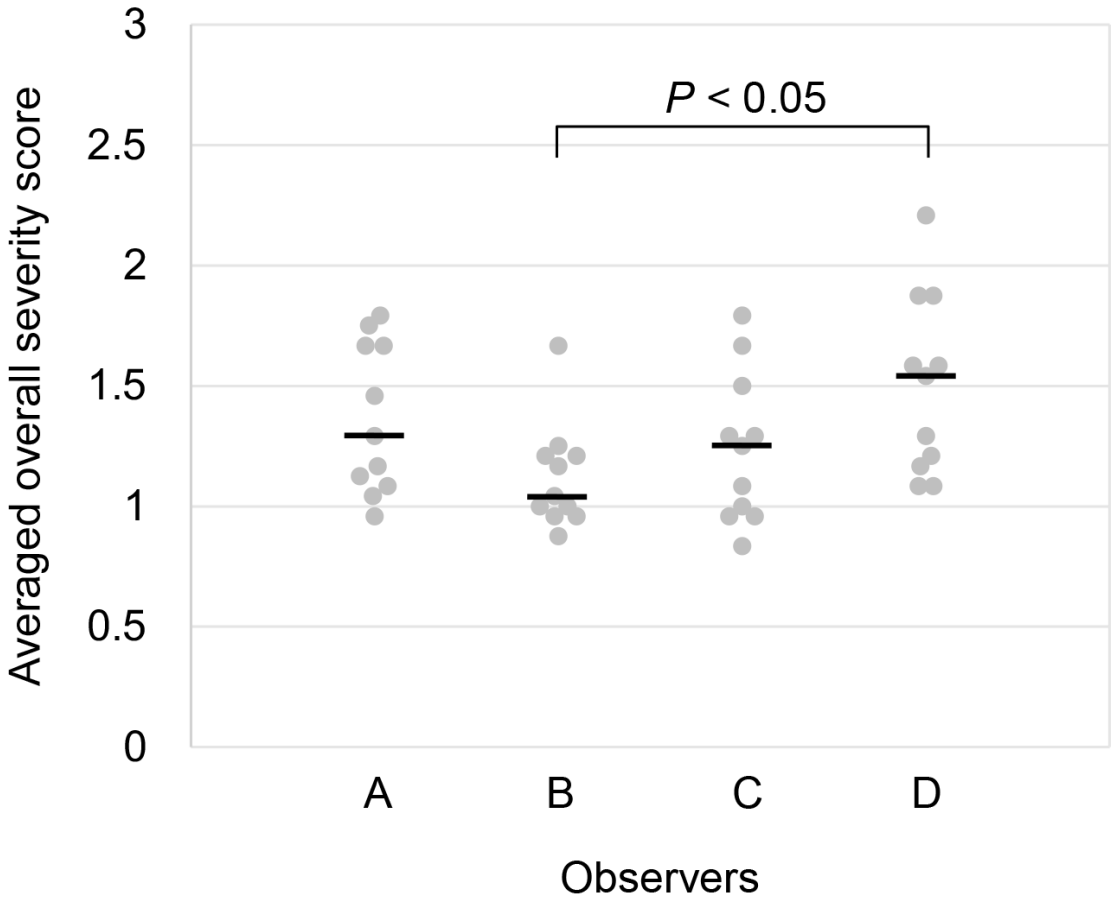
Figure 4



Mean severity scores of ICRPs in 11 dogs evaluated by each observer (A, B, C, and D).

The data represent the averaged value derived from 11 dogs and two images of each dog.

Figure 5



Mean overall scores of each case based on the evaluation by different four veterinarians. The plots indicate the averaged data of each dog derived from two images and all 12 divided angles. Inter-observer variation was identified (i.e., observer D tended to evaluate the score more severely than observer B). Data were statistically analysed using the Kruskal–Wallis test with Dunn's post hoc test. The horizontal lines represent the median value in each observer.

Chapter 1

Analyses on the pattern recognition receptors in Miniature Dachshunds with inflammatory colorectal polyps

Chapter 1-1

Expression profiling of pattern recognition receptors in Miniature Dachshunds with inflammatory colorectal polyps

Abstract

Inflammatory colorectal polyp (ICRP) in Miniature Dachshund (MD) is thought to be a novel form of inflammatory bowel disease (IBD), but its etiology has not been investigated. Dysregulated innate immune conditions have been described to be implicated in the pathogenesis of both human and canine IBD. Therefore, the aim of the current study was to evaluate the messenger RNA (mRNA) expression profiles of pattern recognition receptors (PRRs) and cytokines in ICRPs. Polyp tissues were collected by colonoscopic biopsies from 24 MDs with ICRPs. Non-polypoid colonic mucosa was collected from all MDs with ICRPs and 21 clinically healthy beagles (as the controls). The expression levels of the mRNAs encoding toll-like receptors (TLRs) 1–10; nucleotide-binding oligomerization domain (NOD)-like receptors NOD1 and NOD2; and cytokines IL-1β, IL-6, IL-8/CXCL8, IL-10, TGF-β, and TNF-α were evaluated by quantitative real-time RT-PCR. Three of the 10 well-known candidate reference genes were selected as housekeeper genes based on analyses from the GeNorm, NormFinder, and BestKeeper programs. Levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10, NOD2, and all cytokines were significantly upregulated in the polyps relative to those in the controls. There was significant decrease in the expression levels of TLR3 and NOD1 in the polyp tissues compared to the non-polypoid colonic mucosa obtained from MDs with ICRPs. All upregulated PRR mRNAs were positively correlated with all proinflammatory cytokine mRNAs. This study demonstrated the dysregulation of PRRs and cytokines in ICRPs of MDs, which may play an important role in the pathogenesis of this disease.

1. Introduction

One of the etiologies of human and canine inflammatory bowel disease (IBD) is hypothesized to result from the inappropriate activation of mucosal immunity by various environmental factors such as intestinal microbiota (Wallace et al., 2014; Xavier and Podolsky, 2007). Innate immune mechanisms recognize microorganisms and are thought to be implicated in many inflammatory conditions (Drexler and Foxwell, 2010). Pattern recognition receptors (PRRs) are the key regulators of the innate immune system in gastrointestinal mucosa that induce proinflammatory and immunomodulatory responses in various cell types, including immune and epithelial cells (Akira and Takeda, 2004; Cario, 2010; Fukata et al., 2013). Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors are the most characterized classes of PRRs, and many studies have revealed that the dysregulation of TLRs and/or NOD-like receptors can lead to inflammation (Becker and O'Neill, 2007; Franchi et al., 2008; Shibolet and Podolsky, 2007).

In human IBD, many studies have reported the upregulation of TLR2 and TLR4 messenger RNAs (mRNA) and proteins (Cario and Podolsky, 2000; Frolova et al., 2008; Szebeni et al., 2008). In a recent study, TLR5, TLR8, and TLR9 mRNA levels were also upregulated in ulcerative colitis patients and correlated with inflammatory activity (as determined by endoscopy, histology, and transcription levels of proinflammatory cytokines including IL-6 and TNF-α) (Sánchez-Muñoz et al., 2011). Another study also identified the hyperexpression of NOD2 in intestinal epithelial cells, macrophages, and mast cells in Crohn’s disease patients (Berrebi et al., 2003; Okumura et al., 2009).

Several reports have shown similar results in veterinary medicine. TLR2, TLR4 and TLR9 mRNA levels were also upregulated in the duodenal mucosa, and TLR2 was

weakly correlated with histological activity in dogs with IBD (Burgener et al., 2008; McMahon et al., 2010). In German shepherds with chronic enteropathy, TLR4 mRNA expression was also upregulated, while TLR5 (expressed mainly in CD11+ dendric cells inducing anti-inflammatory cytokines) expression was downregulated (Allenspach et al., 2010). More recently, NOD2 mRNA expression and nuclear factor-kappa B (NF-κB) activity were upregulated in dogs with lymphocytic–plasmacytic colitis (Okanishi et al., 2013a).

Based on the facts that inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds (MDs) show idiopathic inflammation and clinical response to the immunosuppressive therapy, they are thought to represent a novel form of canine IBD (Ohta et al., 2013). As described in Chapter 0, ICRPs in MDs typically occur at the ventral floor of the colorectal mucosa. This finding suggest a possibility that the aberrant response of the host mucosal immunity to fecal antigens play an important role in the pathogenesis of ICRPs in MDs. A recent report showed an increase in proinflammatory cytokines in the colorectal mucosa of MDs with ICRPs (Tamura et al., 2013). However, to date, there are no reports on the status of PRRs in MDs with ICRPs. Therefore, I hypothesized that the dysregulation of PRRs exists in polypoid lesions in MDs with ICRPs, which could be correlated with the expression of proinflammatory cytokines. I conducted quantitative real-time PCR (qPCR) analyses to quantify the expression levels of PRRs and selected cytokines in polypoid lesions and non-polypoid colonic mucosa in MDs with ICRPs and in healthy dogs. It is essential to select the appropriate multiple reference gene for accurate quantification (Peters et al., 2007). Although Peters et al. (2007) have reported stable genes in the colon, duodenum, and duodenal endoscopic biopsies, previous studies have not investigated the appropriate combination of stable

reference genes in colonic mucosa. Thus, I also assessed combinations of the most stably expressed reference genes.

2. Materials and methods

2.1. Animals

Tissue samples were obtained from MDs referred to the Veterinary Medical Center of the University of Tokyo for investigation of chronic hematochezia and/or tenesmus and detected colorectal polyps endoscopically between July 2011 and October 2013. Dogs diagnosed histopathologically with ICRPs were included in this study, while dogs with colorectal adenoma or adenocarcinoma were excluded.

As healthy controls, 21 beagles were used in this study. These dogs had no clinical signs of gastrointestinal disease and showed no abnormalities, as determined by blood test, fecal examination, and ultrasound. The use of dogs in this study was approved by the Animal Care Committee of the University of Tokyo (Approval No. P11-530).

2.2. Sample collection

Colonoscopy was performed in all dogs under sedation (butorphanol) or general anesthesia (premedication of butorphanol and midazolam, propofol and isoflurane) using a VQ-8143B flexible videoendoscope (Olympus Medical Systems Co., Tokyo, Japan). Mucosal specimens of polypoid lesions were collected from MDs with ICRPs. As controls, colonic mucosa without macroscopic polypoid lesions was collected at the descending colon of MDs with ICRPs and from healthy beagles. Multiple mucosal biopsies were taken by using the FB-54Q-1 biopsy forceps (Olympus Medical Systems Co.) or an electrosurgical snare (ICC 200, ERBE Co., Tubingen, Germany). One to two mucosal specimens collected from polypoid lesion or non-polypoid colonic mucosa were used for RNA extraction, and at least four mucosal specimens or a large polypoid tissue resected by polypectomy were submitted for histopathology. Samples for total RNA

extraction were immediately submerged in RNAlater (Qiagen Inc., Valencia, CA, USA) and stored at –80°C until use. Samples for histopathology were placed in 10% formalin, and hematoxylin and eosin-stained sections were prepared.

2.3. Evaluation of candidate reference genes

In total, 30 tissue samples were derived from ten polypoid samples, ten non-polypoid colonic mucosa samples from MDs with ICRPs, and ten colonic mucosa samples from healthy dogs. Total RNA was extracted with a commercially available kit (RNAspin Mini RNA Isolation Kit, GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's manual. Genomic DNA was removed from the samples with a TURBO DNA-free Kit (Applied Biosystems, Foster City, CA, USA) and stored at –80°C until use. RNA was quantified using the DU 730 Life Science UV/Vis spectrophotometer (Beckman Coulter Inc., Brea, CA). The PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan), containing both oligo (dT) and random hexamer primers, was used to synthesize complementary DNA (cDNA) from 100 ng of total RNA according to the manufacturer's instructions. After reverse transcription, qPCR was performed using the SYBR Premix Ex Taq II (Takara Bio Inc.) and Thermal Cycler Dice Real Time System (Takara Bio Inc.). The qPCR assays were performed in triplicate using a 25-μl reaction volume/well. Ten well-known candidate reference genes were tested as follows: β-2 microglobulin (B2M), CG14980-PB, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hydroxymethylbilane synthase (HMBS), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), ribosomal protein L32 (RPL32), ribosomal protein S18 (RPS18), succinate dehydrogenase complex subunit A (SDHA), and TATA box binding protein (TBP) (Maccoux et al., 2007a; Peters

et al., 2007). Information on the primers used in my analyses is depicted in Table 2. Non-RT and no-template controls were also used as negative controls. The amplification conditions were as follows: 95°C for 10 s, 40 cycles of PCR (95°C for 15 s and 60°C for 30 s), and finally dissociation (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s). The real-time data was analyzed by the Thermal Cycler Dice Real Time System software version 4.01A (Takara Bio Inc.). The amplification efficiency calculations derived from standard curves based on a 10-fold dilution series of representative cDNA samples were between 90 and 105%. Ct values were determined as second derivative maximum cycles and assessed for stability of expression across samples using three different statistical algorithms, including GeNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004). The consensus from these programs was used to demonstrate the most stable reference genes.

2.4. Quantification of PRR and cytokine mRNA expression by qPCR

Similarly, total RNA was extracted from all biopsy specimens (RNAspin Mini RNA Isolation Kit) and a cDNA sample was synthesized (PrimeScript RT Reagent Kit). Subsequently, qPCR was performed (SYBR Premix Ex Taq II) in a final reaction volume of 25 µl with the same amplification conditions. Information on the primers for PRRs and cytokines is depicted in Table 3. The primer sequences were obtained from previous studies (Maccoux et al., 2007b; Maeda et al., 2009; Mercier et al., 2012). HMBS, RPL32, and RPS18 were used as reference genes, which were determined to be the most stable genes by the GeNorm, NormFinder, and BestKeeper programs. As required for the ΔΔCt method, all primer sets exhibited >95% efficiency, which was determined by using a 10-fold dilution series from representative cDNA samples. The nuclease-free water was used

as a negative control. A sample with a known Ct value (as a positive control) was included with all sample runs to control for run-to-run Ct variation. All samples were examined in duplicate, and the mean value of ΔCt was calculated. The relative expression of the target gene was reported as an n-fold difference relative to the expression of the reference gene by subtracting the reference Ct values from the target (ΔCt) Ct values.

2.5. Statistical analysis

Statistical analyses were performed using commercially available software (StatMate III; ATMS Co., Ltd., Tokyo, Japan). Data were statistically analyzed using the Kruskal–Wallis test with Dunn's post hoc test to compare PRR and cytokine mRNA expression levels among polypoid lesions, non-polypoid colonic mucosa of diseased dogs, and non-polypoid colonic mucosa of healthy dogs. The relationships of mRNA expression levels between all PRRs and proinflammatory cytokines were evaluated using the Spearman's rank correlation coefficient. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Animals

Twenty-four MDs were diagnosed with ICRPs; seven MDs had diffused multiple small polyps in the colorectal region, while other 17 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 14 of 24 MDs with ICRPs; five of them showed no abnormality while nine of them had mild lymphocytic–plasmacytic colitis based on World Small Animal Veterinary Association guidelines. The median age of dogs with ICRPs was 125.5 months (range, 68–168 months) with ten females (three intact and seven neutered) and 14 males (two intact and 12 neutered). Control samples were obtained from 21 healthy beagles: 14 females (seven intact and seven neutered) and seven males (one intact and six neutered), median age 53 months (range, 48–120 months). All control dogs showed no abnormality in histopathology.

3.2. Reference gene selection

To determine the most appropriate set of reference genes, I evaluated ten candidate reference genes. The mRNAs were ranked in the order of expression stability by the GeNorm program; RPS18, RPL32, and HMBS were the three most stably expressed genes (Figure 6). Similarly, these three genes showed the lowest stability values and standard errors with the NormFinder program (Table 4) and the highest correlation coefficients with the BestKeeper program (Table 5). Therefore, I selected RPS18, RPL32,

and HMBS as reference genes for further analyses.

3.3. Quantification of mRNA expression levels of PRRs and proinflammatory cytokines

Expression of the mRNAs of all PRRs and cytokines was observed in all tissue samples. Of the PRRs investigated in this study, the relative expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10 and NOD2 in polypoid lesions were significantly higher than those in the non-polypoid colonic mucosa of MDs with ICPRs and control dogs (Figure 7). The mRNA expression levels of TLR3 and NOD1 were significantly lower in polypoid lesions than those in the non-polypoid colonic mucosa of MDs with ICRPs (Figure 7). The relative expression levels of all investigated cytokines in polypoid lesions were also significantly higher than those in the non-polypoid colonic mucosa of MDs with ICRPs and control dogs (Figure 8). Furthermore, the mRNA expression levels of TLR3, NOD1, and TNF-α in the non-polypoid colonic mucosas of MDs with ICRPs were also significantly higher than those of the control dogs (Figures 7 and 8).

3.4. Correlation of mRNA expression levels between PRRs and proinflammatory cytokines

As shown in Table 6, all proinflammatory cytokines showed a positive correlation with the mRNA expression levels of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10, and NOD2, which were upregulated in polypoid lesions. The most relevant association was found between TLR4 and IL-8/CXCL8 mRNA levels ($r = 0.8367$, $P < 0.001$).

4. Discussion

In this Chapter, I demonstrated the dysregulation of PRRs and cytokines in the polypoid lesions of MDs with ICRPs. Several PRR and all cytokine genes showed significant upregulation in polypoid lesions. These changes were partially consistent with human and canine IBD.

Proinflammatory cytokines play a key role in the modulation of the mucosal immune system, and disturbances in proinflammatory and immunomodulatory cytokines have been reported in human IBD (Rogler and Andus, 1998; Szkaradkiewicz et al., 2009). On the contrary, in canine IBD, the status and role of proinflammatory cytokines in the etiology of the disease are controversial, and a recent meta-analysis showed the lack of increase in proinflammatory cytokines associated with canine IBD (Jergens et al., 2009). Unlike in canine IBD, a significant upregulation of proinflammatory cytokines has been reported in ICRPs in MDs, which was consistent with the present study (Tamura et al., 2013). Interestingly, recent studies also revealed the difference between canine IBD and ICRPs in MDs in terms of CD4+ T-cell cytokines, including IL-17A, IL-10, and IFNγ; these cytokines did not increase in the duodenal mucosa of dogs with IBD but increased in large polyps in MDs with ICRPs (Ohta et al., 2013; Schmitz et al., 2012). These results indicate that the pathogenesis of inflammation in ICRPs is more similar to that in human IBD rather than that in canine IBD.

Several PRR genes were upregulated in mucosal specimens obtained from the polyps of MDs with ICRPs than those in the non-polypoid colonic mucosa of MDs with ICRPs and healthy dogs. TLR2 and TLR4 were the most characterized TLRs in human and canine IBD. TLR4 recognizes lipopolysaccharide (LPS) and induces various responses, including proinflammatory cytokines. Large amounts of luminal LPS are usually well-

tolerated within a healthy intestine, and downregulation of TLR4 expression is responsible for LPS tolerance in the mouse model (Nomura et al., 2000). Thus, increased TLR4 expression in IBD patients is considered an important factor for the development of inflammation (Cario and Podolsky, 2000). On the other hand, TLR2 recognizes bacterial peptidoglycan and induces proinflammatory and immunomodulatory cytokines and Th2 immune response (Cantó et al., 2006; Dillon et al., 2004). TLR2 expression is also reported to be positively correlated with the induction of proinflammatory cytokines in human ulcerative colitis or the clinical disease severity score of canine IBD (McMahon et al., 2010; Sánchez-Muñoz et al., 2011). Similar to human IBD, TLR2 and TLR4 expression levels were significantly upregulated in polypoid lesions obtained from MDs with ICRPs and may aggravate inflammation through excess recognition of luminal or fecal antigens.

TLR7, TLR8, and TLR9, the relatively well-characterized TLRs in human IBD, were also upregulated in ICRPs and correlated with proinflammatory cytokines, which is consistent with human IBD (Sánchez-Muñoz et al., 2011). TLR9 recognizes bacterial unmethylated CpG DNA motifs and is thought to play a significant role in the induction of IL-8/CXCL8 production in intestinal epithelial cells (Ghadimi et al., 2010). TLR7 and its homolog, TLR8, recognize single-stranded RNA and their expression levels were upregulated in response to innate immunity cytokines (Zarember and Godowski, 2002). Although the functional consequence of TLR7 and TLR8 upregulation in the gut is unclear, Steenholdt et al. (2009) has reported that IL-8/CXCL8 induction in primary colonic epithelial cells is stimulated by TLR8 ligand. IL-8/CXCL8 has been shown to induce neutrophil infiltration, and interestingly, the correlation of mRNA expression levels between TLR8 and IL-8/CXCL8 was the second-highest in this study (Table 6);

therefore, hyperexpression of these TLRs, especially TLR8, may play an important role in the accumulation of neutrophils in colorectal mucosa in MDs with ICRPs.

I also identified the upregulation of NOD2 level in polypoid lesions of ICRPs. NOD2 recognizes peptides derived from bacterial peptidoglycan and induces proinflammatory cytokine production through NF-κB activation (Ogura et al., 2001b). However, it has been suggested that its primary role is to modulate TLR signaling through the induction of IFN regulatory factor 4 and mediate tolerance to bacterial products in the intestines (Hedl et al., 2007; Watanabe et al., 2008, 2004). Therefore, the presence of NOD2 upregulation in ICRPs suggests the failure of this tolerance mechanism; alternatively, it is simply consequence of inflammation.

In the present study, levels of PRRs, including TLR1 and TLR6, were also upregulated in ICRPs. Unlike in MDs with ICRPs, the expression levels of TLR1 and TLR6 did not change in human IBD (Sánchez-Muñoz et al., 2011). TLR1 and TLR6 have been reported to alter the ligand specificity of TLR2, which also leads to the induction of proinflammatory signals (Abreu, 2010; Hajjar et al., 2001). Therefore, dysregulation of TLR1 and TLR6 in ICRPs could contribute to an abnormal innate immune response for TLR2. In addition, TLR1 and TLR6 polymorphisms have been reported to influence the disease extension of Crohn's disease and ulcerative colitis, respectively (Pierik et al., 2006). Thus, they might lead to the localization of ICRPs in the colorectal region.

Interestingly, TLR10 expression level was upregulated in ICRPs. TLR10 has been reported to be expressed on regulatory T (Treg) cells and is implicated in the regulation of human Treg cells (Bell et al., 2007). Moreover, the expression levels of anti-inflammatory cytokines, including IL-10 and TGF-β, were also upregulated in ICRPs, which was consistent with a previous study (Ohta et al., 2013); hence, I assume that the

induction of Treg cells could occur in ICRPs. On the contrary, mRNA expression of TLR5 did not change in the present study. TLR5 recognizes bacterial flagellin and is mainly expressed in CD11c+ dendritic cells in lamina propria, which induce Treg cells and stimulate production of anti-inflammatory cytokines (Uematsu and Akira, 2009). Thus, further studies are needed to investigate the number, distribution, and function of Treg cells and CD11c+ dendritic cells in ICRPs.

In the present study, TLR3 and NOD1 mRNA expression in polypoid lesions was lower than that in non-polypoid colonic mucosa in diseased dogs. In contrast to these results, TLR3 and NOD1 mRNA levels have been previously shown to be stable or upregulated during intestinal inflammation (Cario and Podolsky, 2000; Hisamatsu et al., 2003). The cause of this discrepancy is unknown. However, because the expression of both PRRs has been described in intestinal epithelial cells (Cario and Podolsky, 2000; Rubino et al., 2012), I speculate that this discrepancy might be due to severe inflammatory infiltration and a relative decrease in epithelial cells in biopsy specimens from polyps. In addition, their mRNA expression levels in non-polypoid colonic mucosa of MDs with ICRPs were also higher than that in healthy beagles. However, there were differences in the breed, age, and sex of experimental subjects, which should also be taken into account. Age-related increase of intestinal epithelial TLR3 expression has been reported in mouse and human (Pott et al., 2012), and mRNA expression level of TLR3 in healthy beagles correlated with aging ($r = 0.6077$, $P = 0.007$) while that of other PRRs including NOD1 ($r = 0.1718$, $P = 0.442$) and all proinflammatory cytokines did not show any correlation in this study (Table 7). The MDs with ICRPs were older than the healthy control dogs in the present study; therefore, this may contribute to the difference in the result of TLR3 between non-polypoid colonic mucosa of MDs with ICRPs and that of healthy beagles.

Furthermore, since the TNF-α mRNA expression level was also slightly higher in the non-polypoid colonic mucosa of MDs with ICRPs than that in healthy dogs, a genetic or epigenetic disorder might exist in MDs with ICRPs. However, I also evaluated the mRNA expression levels of all PRRs and proinflammatory cytokines in non-polypoid colonic mucosa of five MDs with colorectal adenoma/adenocarcinoma, and the results were not significantly different from those of MDs with ICRPs and healthy beagles (data not shown). Thus, further investigations using age- and gender-matched dogs with various breeds are required.

There were several limitations in the current study. First, using whole endoscopic biopsy specimens is not ideal because the composition of the samples should vary. Therefore, this would influence the expression levels of PRRs, to some extent. In humans, upregulation of PRRs was induced by the recognition of their ligands or stimulation with inflammatory cytokines (Zarember and Godowski, 2002). The upregulation of PRRs and proinflammatory cytokines may have been caused by two factors: (1) the upregulation of transcription in cells expressing each PRR and (2) infiltration of inflammatory cells. Therefore, further investigations treating specific cell types, which could be performed using laser microdissection (Funke, 2011), need to be conducted. Second, mRNA expression levels are not necessarily correlated with changes in protein production. Thus, if canine-specific antibodies are available, immunohistochemistry, immunofluorescence, and/or flow cytometry could be performed to determine the expressed cells and their localization in the polypoid lesions of ICRPs. Third, I used only mRNA expression levels of proinflammatory cytokines as an indicator of inflammation severity. The disease severity should also be classified by macroscopic or histopathological evaluation; however, because of the absence of objective criteria in these evaluations in ICRPs, they

could not be used in the analysis of the correlation between PRR expression levels and disease severity. Although a morphometric analysis may provide the number or composition of epithelial or inflammatory cell and might solve this problem, it could not be simply applied because the histological construction of ICRPs is commonly collapsed, especially in large polyps (i.e. a region remains the “intestinal epithelial construction” with inflammatory infiltration while another region shows disrupted construction with severe inflammatory infiltration, fibrosis and necrotized histopathology, and often does not involve epitheliums). Therefore, I consider it is not appropriate to count the numbers of each cells in the limited area of the biopsy specimens. If possible, the composition of each cell type in whole biopsy specimens would be counted using flow cytometry, but it was difficult in this study because fresh tissue samples were not available.

In conclusion, the expression of bacteria-responsive PRRs was dysregulated in the polypoid lesions of ICRPs, which support the potential implication of the innate immune system in the pathogenesis of this disease. Since the non-elevated expression of most PRRs and cytokines was observed in non-polypoid mucosa, it was implied that the upregulation of PRRs is not the cause but the consequence of inflammation and may aggravate the disease, as observed in human IBD (Szebeni et al., 2008). However, Burgener et al. (2008) proposed that PRR upregulation is a genetic predisposition because of the absence of significant changes in PRR expression after therapy, despite clinical improvement in canine IBD. Since the expression of some PRRs and TNF-α was also upregulated in non-polypoid colonic mucosa of MDs with ICRPs, further investigation into the genetic background of the MDs is required.

Table 2

Primer sequences of candidate reference genes used in Chapter 1-1.

Gene		Primer sequences (5'-3')	Product length (bp)	Genbank accession number
B2M	Forward	ACGGAAAGGAGATGAAAGCA	99	XM_535458
	Reverse	CCTGCTCATTGGGAGTGAA		
CG14980	Forward	GCAGGAAGGGATTCTCCAG	75	XM_536878
	Reverse	GGGTCCAGTAAGAAATCTTCCATAA		
GAPDH	Forward	CATTGCCCTCAATGACCACT	105	NM_001003142
	Reverse	TCCTTGGAGGCCATGTAGAC		
HMBS	Forward	TCACCATCGGAGCCATCT	112	XM_546491
	Reverse	GTTCCCACCACGCTCTTCT		
HPRT1	Forward	CACTGGGAAAACAATGCAGA	123	AY_283372
	Reverse	ACAAAGTCAGGTTTATAGCCAACA		
RPL13A	Forward	GCCGGAAGGTTGTAGTCGT	87	AJ_388525
	Reverse	GGAGGAAGGCCAGGTAATTC		
RPL32	Forward	TGGTTACAGGAGCAACAAGAAA	100	XM_848016
	Reverse	CACATCAGCAGCACTTCA		
RPS18	Forward	TGCTCATGTGGTATTGAGGAA	116	XM_532106
	Reverse	TCTTATACTGGCGTGGATTCTG		
SDHA	Forward	GCCTTGGATCTCTTGATGGA	92	XM_535807
	Reverse	TTCTTGGCTCTTATGCGATG		
TBP	Forward	CTATTTCTTGGTGTGCATGAGG	96	XM_849432
	Reverse	CCTCGGCATTCAGTCTTTTC		

B2M, β-2 microglobulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; HPRT1, hypoxanthine phosphoribosyltransferase 1; RPL13A, ribosomal protein L13a; RPL32, ribosomal protein L32; RPS18, ribosomal protein S18; SDHA, succinate dehydrogenase complex subunit A; TBP, TATA box binding protein.

Table 3

Primer sequences of pattern recognition receptors (PRRs) and proinflammatory cytokines used in Chapter 1-1.

Gene		Primer sequences (5'-3')	Product length (bp)	Genbank accession number
TLR1	Forward	GCCATCCTACCGTGAACCT	114	NM_001146143.1
	Reverse	GCACTCAACCCCAGAAACTC		
TLR2	Forward	TCGAGAAGAGCCACAAAACC	90	NM_001005264.2
	Reverse	CGAAAATGGGAGAAGTCCAG		
TLR3	Forward	GCAACACCCAGCTACACAGA	130	JF681167
	Reverse	ATGTGGAAGCCAGACAAAGG		
TLR4	Forward	GTGCTTCATGGTTTCTCTGGT	146	NM_001002950.1
	Reverse	CCAGTCTTCATCCTGGCTTG		
TLR5	Forward	TCGTGTTGACAGACGGTTATTT	143	EU551146.1
	Reverse	TCCGGTTGAGGGAAAAGTC		
TLR6	Forward	TCAAGCATTTAGACCTCTCATTCA	109	EU551147.1
	Reverse	CCGTAACTTTGTAGCACTTAAACCT		
TLR7	Forward	GCCCTTTTTCTGATGGTGATT	100	AB248956.1
	Reverse	CGCCGATACCCCTTTATTTT		
TLR8	Forward	TCAGCTACAATGCACACTACTTCC	138	JF681168
	Reverse	ACGCTTCTCAGGTCTTGCTC		
TLR9	Forward	ACTGGCTGTTCCTCAAGTCC	104	NM_001002998.1
	Reverse	AGTCATGGAGGTGGTGGATG		
TLR10	Forward	TGCCAACAACACATCCTTG	145	JF681169
	Reverse	GCAAGCACCTGAAAACAGAA		
NOD1	Forward	GTCACTCACATCCGCAACAC	84	JF681170
	Reverse	CCACGATCTCCGCATCTT		
NOD2	Forward	GCACATCACCTTCCAGTGTTT	98	JF681171
	Reverse	GGCCCATGACAAATGAAGA		
IL-1β	Forward	ACCCGAACTCACCAGTGAAATG	110	NM_001037971
	Reverse	GGTTCAGGTCTTGGCAGCAG		
IL-6	Forward	TCTGTGCACATGAGTACCAAGATCC	125	NM_001003301
	Reverse	TCCTGCGACTGCAAGATAGCC		
IL-8/ CXCL8	Forward	CTTCCAAGCTGGCTGTTGCTC	173	NM_001003200
	Reverse	TGGGCCACTGTCAATCACTCTC		
IL-10	Forward	CAGGTGAAGAGCGCATTTAGT	65	XM_850467
	Reverse	TCAAACTCACTCATGGCTTTGT		
TGF-β	Forward	GGAGCAGCATGTGGAGCTGTA	125	NM_001003309
	Reverse	GCCTCACGACTCCAGTGACATC		
TNF-α	Forward	CCCAAGTGACAAGCCAGTAGCTC	146	NM_001003244
	Reverse	ACAACCCATCTGACGGCACTATC		

CXCL, chemokine C-X-C motif ligand; IL, interleukin; NOD, nucleotide-binding oligomerization domain; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor.

Table 4

NormFinder reference gene stability.

Gene name	Stability value	Standard error
RPL32	0.082	0.030
HMBS	0.099	0.029
RPS18	0.159	0.031
CG14980	0.165	0.031
RPL13A	0.220	0.036
TBP	0.231	0.037
B2M	0.316	0.046
GAPDH	0.378	0.053
HPRT1	0.436	0.060
SDHA	0.620	0.083

Table 5

BestKeeper reference gene stability.

Gene name	Coefficient of correlation	Standard deviation	Covariance (%)
RPL32	0.994	1.089	5.62
HMBS	0.993	1.120	4.42
RPS18	0.992	1.079	5.70
TBP	0.987	0.861	3.43
CG14980	0.985	1.091	4.31
RPL13A	0.984	1.213	5.86
B2M	0.968	1.218	6.55
GAPDH	0.943	1.075	4.56
HPRT1	0.936	1.258	5.10
SDHA	0.834	1.134	5.00

Table 6

Correlations between the messenger RNA (mRNA) expression levels of PRRs and proinflammatory cytokines.

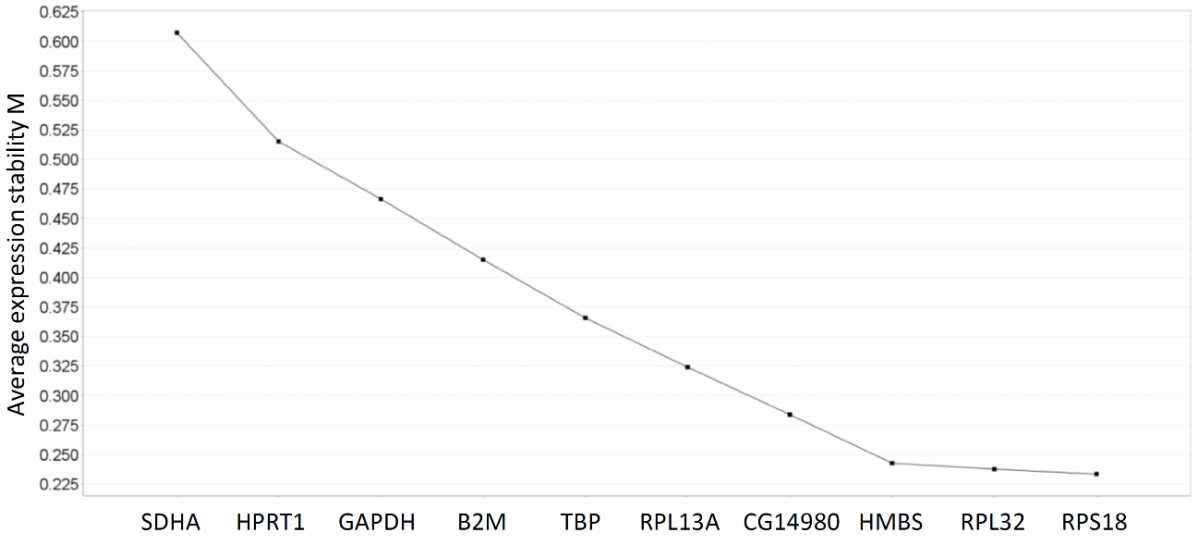
Gene Transcript	IL-1β		IL-6		IL-8/CXCL8		TNF-α	
	r	*P* value	r	*P* value	r	*P* value	r	*P* value
TLR1	0.7326	<0.001	0.6999	<0.001	0.7832	<0.001	0.6879	<0.001
TLR2	0.7062	<0.001	0.7553	<0.001	0.7718	<0.001	0.6665	<0.001
TLR3	–0.1008	0.416	–0.2360	0.057	–0.0914	0.461	–0.0616	0.619
TLR4	0.8051	<0.001	0.7723	<0.001	0.8367	<0.001	0.6970	<0.001
TLR5	0.2473	0.054	0.2835	0.032	0.2732	0.038	0.2161	0.081
TLR6	0.6418	<0.001	0.6259	<0.001	0.7031	<0.001	0.6696	<0.001
TLR7	0.6207	<0.001	0.6763	<0.001	0.6743	<0.001	0.5986	<0.001
TLR8	0.7694	<0.001	0.7938	<0.001	0.8272	<0.001	0.7126	<0.001
TLR9	0.4595	<0.001	0.5145	<0.001	0.5318	<0.001	0.5846	<0.001
TLR10	0.4264	<0.001	0.5224	<0.001	0.5036	<0.001	0.4850	<0.001
NOD1	–0.2789	0.035	–0.4041	0.001	–0.2723	0.038	–0.1151	0.353
NOD2	0.6276	<0.001	0.6860	<0.001	0.6638	<0.001	0.5673	<0.001

Table 7

Correlations between the mRNA expression levels of PRRs or proinflammatory cytokines and aging in healthy beagles.

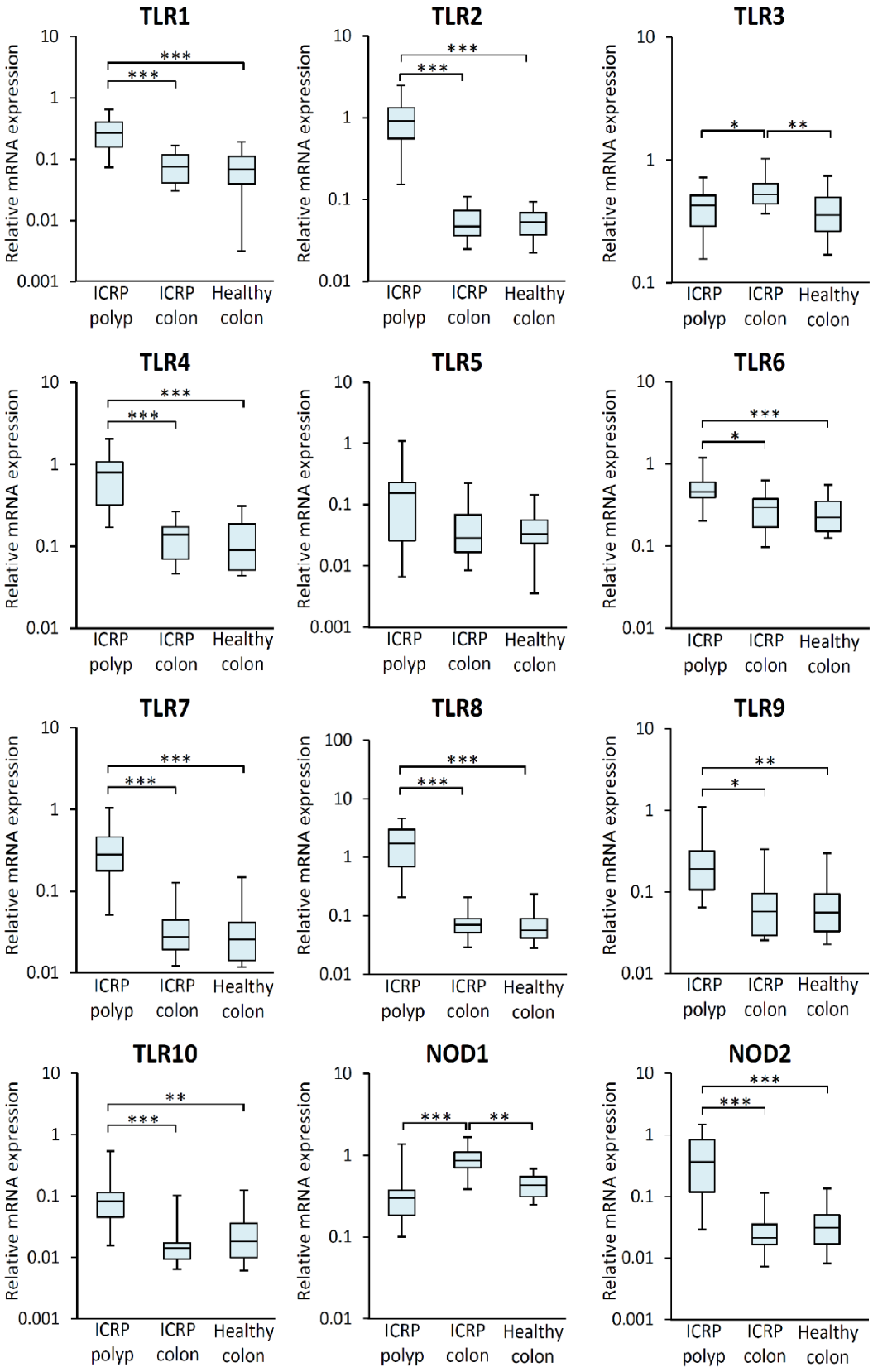
Gene Transcript	Correlation with age	
	r	*P* value
TLR1	–0.1442	0.519
TLR2	–0.0441	0.844
TLR3	0.6077	0.007
TLR4	–0.0678	0.762
TLR5	0.1014	0.650
TLR6	–0.2087	0.351
TLR7	0.1001	0.654
TLR8	0.1534	0.493
TLR9	–0.0263	0.906
TLR10	0.0079	0.972
NOD1	0.1718	0.442
NOD2	0.0092	0.967
IL-1β	0.0856	0.702
IL-6	0.0520	0.816
IL-8/CXCL8	–0.1271	0.570
TNF-α	0.0803	0.719

Figure 6



Average expression stability values and ranking of reference genes. The GeNorm program calculates the gene expression stability (M) of one gene based on the average pair-wise variation between all examined reference genes. The lowest M values characterize genes with the most stable expression levels.

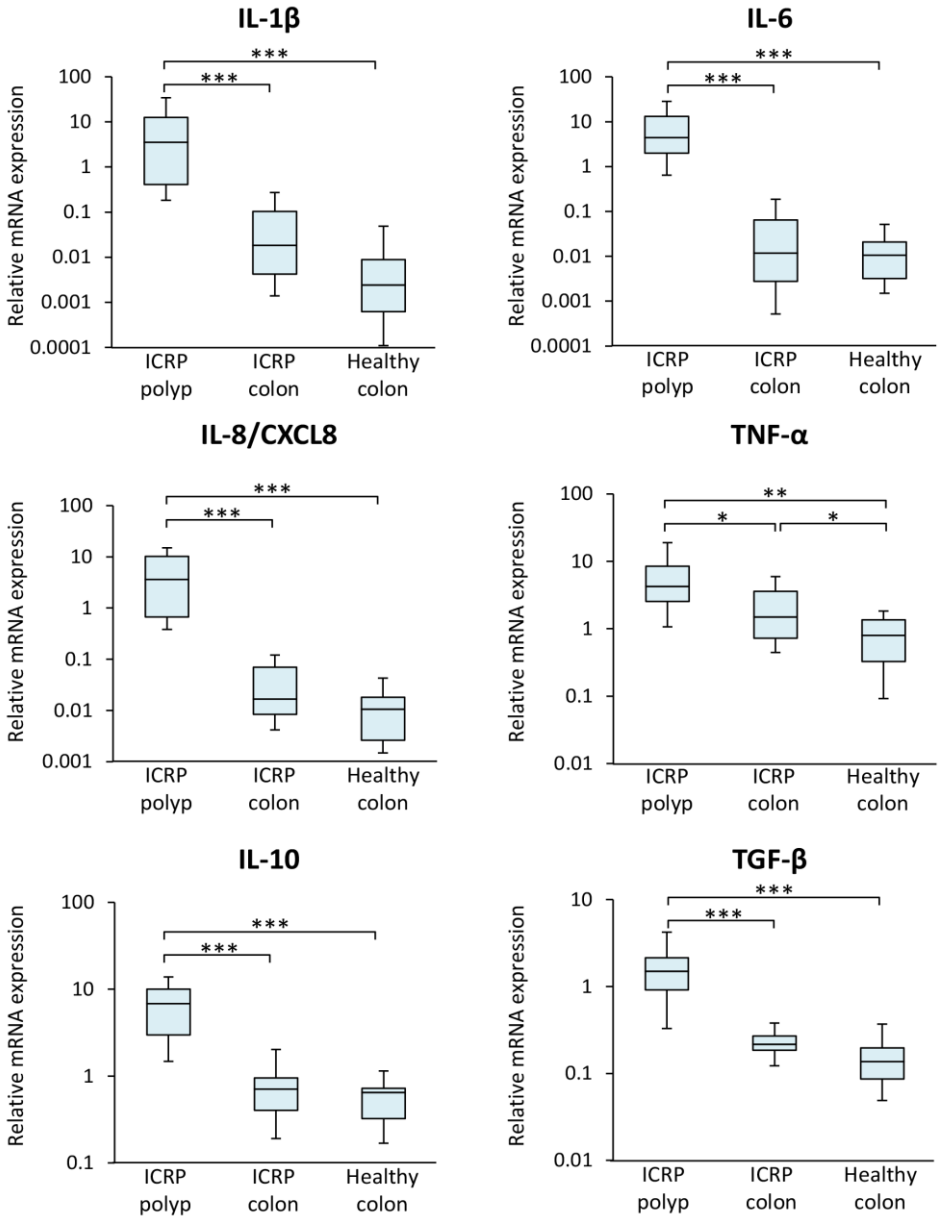
Figure 7


TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10
NOD1
NOD2
Relative mRNA expression
ICRP polyp
ICRP colon
Healthy colon

Legend for Figure 7.

Relative transcription levels of pattern recognition receptor messenger RNA (mRNAs) in polypoid lesions and non-polypoid colonic mucosa of Miniature Dachshunds (MDs) with inflammatory colorectal polyps (ICRPs) (n = 24) and healthy dogs (n = 21). The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; and the whiskers represent the 95th and 5th percentiles. Asterisks indicate statistical differences (*P < 0.05, **P < 0.01, and ***P < 0.001). NOD, nucleotide-binding oligomerization domain; TLR, toll-like receptor.

Figure 8



Relative transcription levels of proinflammatory cytokine mRNAs in polypoid lesions and non-polypoid colonic mucosa of MDs with ICRPs (n = 24) and healthy dogs (n = 21). The top and bottom of the box represent the 75th and 25th percentiles, respectively; the middle line represents the median; and the whiskers represent the 95th and 5th percentiles. Asterisks indicate statistical differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). CXCL, chemokine C-X-C motif ligand; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

Chapter 1-2

Functional analysis of pattern recognition receptors in Miniature Dachshunds with inflammatory colorectal polyps

Abstract

Pattern recognition receptors (PRRs) play a key role in the distinction of pathogens from commensal bacteria and food antigens. Dysfunction resulting from genetic disorders of PRRs have been linked to human and canine IBD. Therefore, I analyzed the reactivity of PRRs in MDs with ICRPs. Twenty-six MDs with ICRPs and 16 control MDs were recruited. Peripheral blood-derived monocytes were obtained from each dog, and then stimulated with PRR ligands for 6 and 24 hr; subsequently, messenger RNA (mRNA) expression levels and protein secretion of IL-1β were quantified using quantitative real-time PCR and ELISA, respectively. The levels of IL-1β mRNA and protein secretion after stimulation with a nucleotide-binding oligomerization domain 2 (NOD2) ligand were significantly greater in monocytes from ICRP-affected MDs than in those from control MDs. In addition, IL-1β protein secretion induced by toll-like receptor (TLR) 1/2, TLR2, and TLR2/6 stimulation was also significantly greater in ICRP-affected MDs. These results suggest that reactivity against NOD2, TLR1/2, TLR2, and TLR2/6 signals is enhanced in ICRP-affected MDs and may play a role in the pathogenesis of ICRPs in MDs. Additional studies of the genetic background of these PRRs should be performed.

1. Introduction

The etiology of human inflammatory bowel disease (IBD) has been described as multifactorial and including the interplay of environment, gut microbiota, mucosal immune system, and genetic background, which also have roles in canine IBD (Cario, 2010; Cerquetella et al., 2010; Xavier and Podolsky, 2007). Pattern recognition receptors (PRRs) distinguish pathogens from commensal bacteria and food antigens by recognizing pathogen-associated molecular patterns (PAMPs), induce cell signaling via activation of nuclear factor-kappa B (NF-κB), and subsequently increase the upregulation of immune response gene expression involving proinflammatory cytokines and co-stimulatory molecules (Abreu, 2010; Cario, 2010; Medzhitov et al., 1997). Therefore, the dysregulated expression or dysfunction of PRRs has been characterized as a cause of various inflammatory disorders, including IBD (Cario, 2010; Corridoni et al., 2014).

I revealed the dysregulated expression of various PRRs in Miniature Dachshunds (MDs) with inflammatory colorectal polyps (ICRPs) in Chapter 1-1. However, the observed dysregulation was mostly restricted to the polypoid lesion, which indicates that the upregulation of PRRs is not the cause but the consequence of inflammation and may aggravate the disease. On the other hand, MDs are commonly affected by ICRPs, suggesting a genetic predisposition (Ohmi et al., 2012), but no report has investigated their genetic background.

To date, many studies of human and canine IBD have reported genetic predispositions, including genetic disorders of PRRs. Although a meta-analysis of genome-wide association studies revealed 163 risk-associated loci for human IBD (Jostins et al., 2012), the genetic backgrounds of PRRs are still of interest because they play crucial roles in the interaction between luminal antigens and host immunity. Genetic variations associated

with human IBD have been reported in a variety of PRR genes including toll-like receptors (TLRs; e.g., TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9) and nucleotide-oligomerization domain (NOD)-like receptors including NOD2 (Cario, 2010; Cummings et al., 2010; Ogura et al., 2001a). Polymorphisms of TLR4, TLR5, and NOD2 are also associated with canine IBD (Kathrani et al., 2010, 2011, 2014).

A defect in PRRs is considered to influence ligand recognition, mucosal immune tolerance, and commensal composition, leading to innate or adaptive immune hypo- or hyperreactivity (Cario, 2010). Many risk-associated genetic variations for human IBD in PRRs have been shown to confer functional disorder (Arbour et al., 2000; Gewirtz et al., 2006; Tanabe et al., 2004); for example, a D299G mutation in human TLR4 gene results in conformational change and hyporesponsiveness to bacterial lipopolysaccharide (LPS), but is considered to induce a signaling disequilibrium of other TLRs leading to intestinal inflammation (Cario, 2010). Furthermore, an IBD risk-associated haplotype of canine TLR5 reportedly includes hyperresponsiveness to bacterial flagellin (Kathrani et al., 2012).

I hypothesized that functional disorder related to genetic background would also exist in MDs with ICRPs. Recently, Tamura et al. (2013) have suggested that macrophages in the colorectal area of ICRP-affected MDs play a key role in neutrophil recruitment via production of proinflammatory cytokines. Therefore, this study aimed to evaluate the reactivity of PRRs in ICRP-affected MDs using peripheral blood-derived monocytes to narrow the candidate PRR genes responsible for development of the condition.

2. Materials and methods

2.1. Animals

ICRP-affected MDs evaluated at the Veterinary Medical Center of the University of Tokyo between April 2012 and November 2013 were recruited for the study. The diagnosis of ICRP was determined based on the colonoscopic and histopathological findings as characterized in a previous study (Ohmi et al., 2012). As controls, MDs owned by veterinarians or veterinary technicians were also recruited. These control MDs were confirmed as having no inflammatory, infectious, or immune-mediated diseases with a health examination. All procedures were conducted according to the animal experimentation guidelines of the University of Tokyo, and informed consent was obtained from the owners of the MDs.

2.2. Cell preparation and culture

Approximately 10 ml of whole blood in ethylenediaminetetraacetic acid (EDTA) was collected from each dog. One milliliter was used for routine blood tests including complete blood count (CBC), and the remainder was used to obtain peripheral blood-derived monocytes modifying the methods as described in previous studies (Bueno et al., 2005; Goto-Koshino et al., 2011). Peripheral blood mononuclear cells (PBMCs) were obtained from the EDTA blood via Ficoll-Paque Plus (GE Healthcare Ltd., Buckinghamshire, UK) density gradient centrifugation. The PBMCs were resuspended in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Biowest, Nuaillé, France) supplemented with penicillin and streptomycin (Sigma–Aldrich). PBMCs were seeded into nine wells of a 12-well plate at a concentration of 1×10^6 cells/well and nine wells of a 48-well plate at 2.5×10^5 cells/well and cultured at 37°C

in 5% CO_2 overnight. To obtain monocytes, I washed the cells twice with Hank's balanced salt solution (Sigma–Aldrich) to remove non-adherent cells, and the adherent cells were then cultured for an additional 6 days and used for experiments.

The viability and purity of peripheral blood-derived monocytes were preliminary assessed using four healthy laboratory beagles. The use of laboratory beagles was approved by the Animal Care Committee of the University of Tokyo (Approval No. P13-774). Adherent cells were harvested by using a cell lifter (Corning, Lowell, MA, USA). Cell viability was assessed by trypan blue staining, and 92–97% of the cells were regarded as viable (100 cells counted per dog, in duplicate). The morphology of these cells was assessed by Wright–Giemsa staining; these cells presented variable cell size, <1.0 of N:C ratios, irregularly round-shaped nuclei with diffuse chromatins, vacuolated cytoplasms, and sometimes multinucleated. Non-specific esterase staining was performed using a commercially available kit (Muto pure chemicals, Tokyo, Japan) according to the manufacturer's manual; 90–96% of the cells were positively stained (100 cells counted per dog, in duplicate).

2.3. Stimulating cells with PAMPs

The monocytes were washed twice with culture medium and cultured in 500 µl of culture medium for the 12-well plate and 200 µl for the 48-well plate with stimulation by the following PAMPs: peptidoglycan-like molecule (iE-DAP; NOD1 ligand, 10 µg/ml), muramyl dipeptide (MDP; NOD2 ligand, 10 µg/ml), synthetic bacterial lipoprotein (Pam3CSK4; TLR1/2 ligand, 500 ng/ml), peptidoglycan from *Escherichia coli K12* (PGN-EK; TLR2 ligand, 20 µg/ml), synthetic diacylated lipoprotein (FSL-1; TLR2/6 ligand, 50 ng/ml), ultrapure LPS from *E. coli K12* (LPS-EK Ultrapure; TLR4 ligand, 10

μg/ml), purified flagellin from *Salmonella typhimurium* (FLA-ST Ultrapure; TLR5 ligand, 100 ng/ml), CpG oligonucleotide (ODN2006; TLR9 ligand, 5 μM) (all from Invivogen, San Diego, CA, USA), and culture medium only (negative control). The stimulation lasted 6 hr for monocytes in the 12-well plate and 24 hr for those in the 48-well plate. Each cell-free supernatant of culture media was collected and stored at –80°C for further analysis. The cells in the 12-well plate were washed twice with Hank's balanced salt solution, and subsequently lysed to extract their total RNA using a commercially available kit (RNAspin Mini RNA Isolation Kit, GE Healthcare Ltd.) according to the manufacturer's instructions and stored at –80°C for further analysis.

2.4. Quantification of PRR and cytokine messenger RNA (mRNA) expression with quantitative PCR

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.). The amplification conditions were as follows: 95°C for 10 s, 40 cycles of PCR (95°C for 15 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 variation. 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 second derivative maximum cycles.

The primers used in my analyses are detailed in Table 8. The primer sequences were obtained from previous studies (Maeda et al., 2009; Mercier et al., 2012; Peters et al., 2007). The most stably expressed reference genes were preliminarily determined using 50 ng total RNA derived from monocytes of seven ICRP-affected MDs and seven control MDs via assessment of ten candidate genes: β-2 microglobulin, CG14980-PB, glyceraldehyde-3-phosphate dehydrogenase, hydroxymethylbilane synthase, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, ribosomal protein L32, ribosomal protein S18, succinate dehydrogenase complex subunit A, and TATA box binding protein (Maccoux et al., 2007a; Peters et al., 2007). Hydroxymethylbilane synthase, succinate dehydrogenase complex subunit A, and TATA box binding protein were selected as the most stable reference genes in the current study using the GeNorm, NormFinder, and BestKeeper programs (data not shown) (Andersen et al., 2004; Pfaffl et al., 2004; Vandesompele et al., 2002).

The amplification efficiency calculated based on standard curves from a 10-fold dilution series of representative complementary DNA samples was >95%, as required for the ΔΔCt method. All samples were examined in duplicate, and the mean ΔCt value was calculated. The relative expression of the target gene was calculated as an n-fold difference relative to the expression of the reference gene by subtracting the reference Ct values from the target (ΔCt) Ct values.

2.5. Quantification of cytokine production by ELISA

The concentrations of IL-1β protein in culture media stimulated with PAMPs for 24 hr were determined using a commercially available ELISA kit (Canine IL-1β VetSet^{TM} ELISA Development Kit, Kingfisher Biotech, St. Paul, MN, USA) according to the

manufacturer's instructions. Each assay was performed in duplicate.

2.6. Statistical analysis

Statistical analyses were performed using a commercially available software package (JMP Pro version 10.0.2, SAS Institute, Cary, NC, USA). The Mann–Whitney *U* test was used to compare results between groups. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Animals

Twenty-six MDs with ICRPs were included in the current study: their median age was 125.5 months (range, 68–153 months), and the group included 16 males (four intact and 12 neutered) and ten females (three intact and seven spayed). Eight of 26 ICRP-affected MDs had clinical histories of other inflammatory disease, most predominantly with chronic gastroenteritis (Table 9). EDTA blood was collected at initial diagnosis in 17 dogs and at clinical remission in nine dogs. Ten of 26 ICRP-affected MDs had received immunosuppressive therapy including prednisolone, cyclosporine, and/or leflunomide within 3 weeks prior to blood collection, while 16 ICRP-affected MDs had not. In addition, 16 control MDs—four males (one intact and three neutered) and 12 females (five intact and seven spayed)—were included, with a median age of 103 months (range, 60–171 months). Thirteen control MDs were clinically healthy, while two had obsolete vertebral fracture resulted from a traffic accident, and one had mitral insufficiency. No control dogs had received any immunomodulatory therapy at the sample collection. The numbers of white blood cells and monocytes in the CBCs were not significantly different between the groups (white blood cells: 10,438 ± 4,836/μl vs. 10,719 ± 4,595/μl, P = 0.698; monocytes: 640 ± 289/μl vs. 786 ± 487/μl, P = 0.521; data represent mean ± SD).

3.2. Quantification of mRNA expression levels and protein production of PRRs and proinflammatory cytokines in unstimulated canine monocytes

As shown in Figures 9 and 10, no significant difference in the mRNA expression levels of any of the PRRs or proinflammatory cytokines investigated was observed between the groups. Subsequently, I selected IL-1β as an indicator of PRR reactivity,

since it showed relatively small inter-dog and inter-group variance (Figure 10). The protein secretion of IL-1β in the unstimulated monocytes was equivalent between the groups (Figure 10).

3.3. Responses of canine monocytes to PAMPs

The IL-1β mRNA expression level in monocytes from ICRP-affected MDs was significantly higher than that from control MDs when stimulated with MDP (Figure 11). By contrast, no significant difference in the response of monocytes to stimulation with iE-DAP, Pam3CSK4, PGN-EK, FSL-1, LPS-EK, FLA-ST, or ODN2006 was observed between the MD groups (Figure 11). Furthermore, IL-1β protein production after stimulation with MDP, Pam3CSK4, PGN-EK, and FSL-1 in monocytes from ICRP-affected MDs was greater than that in monocytes from control MDs (Figure 12). Conversely, no significant difference in monocyte response was observed between the MD groups after stimulation with iE-DAP, LPS-EK, FLA-ST, or ODN2006 (Figure 12).

In addition, there was no significant difference in all mRNA expression and protein production levels investigated in this study between the ICRP-affected MDs which had received immunosuppressive therapy and those had not (data not shown). Furthermore, the IL-1β mRNA expression and protein production after stimulation with MDP in ICRP-affected MDs without immunosuppression were also significantly greater than those in control MDs (Table 10), while no significant difference of IL-1β mRNA expression or protein production was observed between them when stimulated with other ligands including Pam3CSK4, PGN-EK, and FSL-1 (Table 10). Moreover, no difference was observed in IL-1β mRNA expression after stimulation with every ligand investigated between ICRP-affected MDs at initial diagnosis and those at clinical remission, whereas

greater IL-1β protein productions were observed in ICRP-affected MDs at initial diagnosis after stimulation with Pam3CSK4, PGN-EK, or FSL-1 compared with those at clinical remission (Table 11).

4. Discussion

In this Chapter, I compared the PRR responses to specific PAMP stimulation in monocytes from ICRP-affected and control MDs. The study aimed to determine candidate PRRs responsible for disease development that might result from genetic predispositions in MDs. I found hyperreactivity of certain PRRs, including NOD2, TLR1/2, TLR2, and TLR2/6. Since it has been reported that the number of macrophage is increased in the polypoid lesion (Ohmi et al., 2012; Tamura et al., 2013), the hyperreactivity of these PRRs in monocytes would be involved in the pathogenesis of ICRPs in MDs.

MDP, a ligand of NOD2, induced mRNA upregulation and IL-1β protein production, and the inductions were greater in ICRP-affected MDs than in control MDs. These findings indicate a hyperreactivity of NOD2 in MDs with ICRPs. NOD2 recognizes peptides derived from bacterial peptidoglycan and induces proinflammatory cytokine production through NF-κB activation (Ogura et al., 2001b). Constitutive activation of NF-κB and/or hyperresponsiveness to MDP stimulation, which is due to mutations of the NOD2 gene (Tanabe et al., 2004), are considered to associate with Blau syndrome in humans (Sfriso et al., 2012); this is partly consistent with the findings of the present study. I did not evaluate NF-κB activity in this study; however, baseline IL-1β mRNA expression and protein production levels were equivalent between the groups. Blau syndrome is characterized as a systemic granulomatous inflammation including uveitis, dermatitis, and symmetric arthritis. Interestingly, Miniature Dachshund also commonly develops a granulomatous disease including sterile panniculitis (Yamagishi et al., 2007); however, no ICRP-affected MDs included in this study did not have a history or complication of such disease (Table 9). Moreover, inflammation in Blau syndrome is thought to be independent of bacterial participation (Meylan et al., 2006). By contrast, NOD2

hyperreactivity seems to play a crucial role in the development of ICRP inflammation via response against fecal antigens, because the large intestine has the highest density of bacteria in the gut (Hooda et al., 2012). Therefore, the significance of functional disorders of NOD2 in ICRPs in MDs seems to differ from those of Blau syndrome in humans. Further analyses of the genetic background of the NOD2 gene in MDs with ICRPs are warranted.

Monocytes derived from ICRP-affected MDs also showed overproduction of IL-1β protein in response to stimulation with Pam3CSK4, PGN-EK, and FSL-1, the ligands of TLR1/2, TLR2, and TLR2/6, respectively. TLR2 recognizes bacterial peptidoglycan and induces proinflammatory and immunomodulatory cytokines via NF-κB activation, whereas TLR1 and TLR6 form dimers with TLR2 and alter TLR2 ligand specificity (Abreu, 2010; O'Neill and Bowie, 2007). The activation of NF-κB by these TLRs is induced through a myeloid differentiation factor 88-dependent pathway that is also involved in TLR4-, TLR5-, and TLR9-induced pathways (O'Neill and Bowie, 2007). Therefore, the overproduction of IL-1β observed in this study might be the result of functional disorders of TLRs, especially TLR2, but not from disorders in lower signaling pathways. Some polymorphisms of TLR1, TLR2, and TLR6 in humans have also been associated with an increased risk of IBD (Cario, 2010), although their effects on reactivity have not been well characterized. Therefore, further investigation of the genetic background of the TLRs contributing to the hyperreactivity and development of ICRPs in MDs is also needed.

Despite the increased production of IL-1β protein in response to TLR1/2, TLR2, and TLR2/6 stimulation, the mRNA expression level of IL-1β in ICRP-affected MDs and control MDs was not significantly different (Figure 11). I have two explanations for this

result. First, greater negative feedback on signaling pathway, such as the NF-κB pathways, may be induced by these TLRs because they usually elicit greater cellular responses compared with those elicited by NOD2 (Kullberg et al., 2008; Ruland, 2011; Watanabe et al., 2008), which is also consistent with the results of the current study (Figures 11 and 12). I chose a 6-hr duration of stimulation because it resulted in the highest IL-1β mRNA expression in a preliminary study (data not shown). However, the 6-hr duration of stimulation may induce negative feedback and interfere with mRNA expression. Sequential evaluation of mRNA expression levels after shorter or longer stimulations may confirm the existence of the negative feedback interference; however, I did not extract mRNA from the monocytes stimulated for 24 hr. Furthermore, response against lower-dose stimulation might also help determine whether the negative feedback interference occurs. These follow-up studies need to be performed in the future. Second, an inflammasome disorder may exist, because inflammasomes activate the conversion of IL-1β from pro-IL-1β, which is synthesized from IL-1β mRNA (Meylan et al., 2006). However, I believe this explanation is unlikely because protein production with or without the stimulation of other PRRs was not significantly different between the groups.

Although the hyperreactivity of NOD2, TLR1/2, TLR2, and TLR2/6 might reasonably lead to the overproduction of inflammatory cytokines in ICRP lesions, several contradictions remain. The primary role of NOD2 in the gastrointestinal tract appears to be the modulation of TLR signaling through the induction of interferon regulatory factor 4 and the mediation of tolerance to bacterial antigens (Hedl et al., 2007; Watanabe et al., 2008, 2004). A frameshift mutation of NOD2 gene, the most influential mutation for Crohn's disease, leads to the hypo-reactivity to MDP (Hedl et al., 2007); the consequent deficiency of the induction of TLR tolerance is regarded as one of the important etiology

of Crohn's disease (Strober et al., 2008). In addition, TLR2 stimulation leads to the induction of proinflammatory and immunomodulatory cytokines (Cantó et al., 2006; Dillon et al., 2004). Moreover, a recent study revealed that the responses to PRR stimulation differed in duodenal biopsy specimens and whole blood, which indicates that responses to bacterial stimuli differ by location (Schmitz et al., 2014). Because only monocytes derived from peripheral blood were used in the present study, I am uncertain whether the observed PRR hyperreactivity can be applied in the same manner to the colorectal mucosa. However, I speculate that a genetic disorder affecting the response of monocytes derived from peripheral blood interferes with the function of macrophages in the colorectal mucosa. Further analyses using colorectal tissue cultures or mucosal macrophages and evaluating both proinflammatory and immunomodulatory responses must be performed. In addition, interactions between NOD2 and TLRs, particularly as they relate to interferon regulatory factor 4 in both control and ICRP-affected MDs, are of interest.

The protein production of IL-1β was decreased in both ICRP-affected and control MDs when stimulated with FLA-ST and ODN2006, the ligands of TLR5 and TLR9, respectively (see Figures 10 and 12). These TLRs in the gut have been described that they do not directly induce proinflammatory reactions like TLR2 and TLR4, but contribute to homeostasis via inducing the differentiation of anti-inflammatory responses and immunoglobulin A production (de Kivit et al., 2014; Uematsu and Akira, 2009). House et al. (2008) reported that these PAMPs hardly induce proinflammatory cytokine mRNA expression and protein production in canine PBMC-derived monocytes, which is consistent with the results of IL-1β mRNA expression in this study. The difference in the stimulating duration might explain the reduction of IL-1β protein production observed in

this study. House et al. (2008) stimulated cells for 4 hr, while 24-hr stimulation was performed in this study; I selected the duration to obtain detectable amount of IL-1β protein in a preliminary study (data not shown). The chronic or repetitive stimulation with PAMPs including ligands of TLR5 and TLR9 induces a TLR tolerance, which is characterized as the unresponsiveness of lower signaling pathways and reduced induction of proinflammatory cytokines by further TLR stimulations (Lee et al., 2006; Sun et al., 2007). The induction of TLR tolerance and little proinflammatory induction might result in the reduction of IL-1β protein production. Further analyses whether other proinflammatory or anti-inflammatory cytokines show similar reduction via stimulation of TLR5 or TLR9 and the TLR tolerance induced by them would confirm these findings.

This study evaluated only proinflammatory cytokine as an indicator of PRR reactivity. Since it has been reported that the alteration of anti-inflammatory response including IL-10 is not consistent with that of proinflammatory cytokines in various diseases, such as canine chronic enteropathy (Schmitz et al., 2014), a possibility that dysregulation of anti-inflammatory cytokine induction could not be excluded, which cannot be detected in this study design. In addition, Tamura et al. (2013) reported the marked up-regulation of IL-8/CXCL8 in macrophage at polypoid lesion, which suggests the importance of IL-8/CXCL8 overproduction from macrophage in the pathogenesis of ICRPs in MDs. Unfortunately, experiments could not be repeated because of the limited amount of monocytes obtained.

The limited yields of monocytes resulted in another limitation of this study. The cell purity is crucial in this study design. I checked the morphology of adherent cells obtained from ICRP-affected and control MDs, and regarded them as the monocyte lineage. However, I did not perform immunocytochemistry, flow cytometry, or other staining on

the plate-adherent cells of ICRP-affected and control MDs because of the limited number of cells. In addition, the cell viability was not evaluated in ICRP-affected and control MDs in this study. However, the non-adherent cells were removed before and after cell stimulation at each assay, and thus, I consider that the most of dead cells were not remained in the experimental system.

My study had further limitations. Although the control group was composed of age-matched MDs, their genetic backgrounds were not clarified; in other words, MDs enrolled in the control group in this study might develop ICRPs in future. However, the prevalence of ICRPs in MDs is approximately 1.1%, according to a previous retrospective study (Ohmi et al., 2012); therefore, I believe that this limitation does not affect the results of my study.

Another limitation was that inflammatory status and treatment with anti-inflammatory agents including prednisolone, cyclosporine, and/or leflunomide at the time of sampling might affect the reactivity of monocytes. Although I cultured monocytes for 7 days to reduce their interference and confirmed that no significant differences in baseline PRR and proinflammatory cytokine mRNA expression and protein production levels occurred between the groups, some non-statistically significant differences in unstimulated cells might affected the results of stimulated monocytes. Thus, I further divided the ICRP-affected MD group into two groups by disease status (whether the dogs were at initial diagnosis or at clinical remission) or immunosuppression status (whether dogs received immunosuppressive therapy or not). Consequently, the ICRP-affected MDs at initial diagnosis showed a greater reactivity of TLR1/2, TLR2, or TLR2/6 (Table 11). Furthermore, the reactivity of TLR1/2, TLR2 or TLR2/6 could not show significant difference when compared between ICRP-affected MDs without immunosuppression and

control MDs (Table 10), although the difference of immunosuppression status within ICRP-affected MDs did not result in any significant difference. Therefore, the hyperreactivity of TLR1/2, TLR2, and TLR2/6 should be interpreted with caution that the disease or immunosuppression status may interfere with the result; in other words, the hyperreactivity of NOD2 in ICRP-affected MDs might be true. Confirming this conclusion requires further investigations of repeatability using pre- and post-treatment specimens from MDs with ICRPs.

In conclusion, I demonstrated that the reactivity of NOD2, TLR1/2, TLR2, and TLR2/6 in MDs with ICRPs was greater than that in control MDs. The results indicate that the ICRP-affected dogs have PPRs with genetic backgrounds that predispose them to ICRP development. Further investigations of the corresponding genetic backgrounds and the significance of hyperreactivity on the development of ICRPs are warranted.

Table 8

Primer sequences of pattern recognition receptors, proinflammatory cytokines, and reference genes used in Chapter 1-2.

Gene	Primer sequences (5'–3')		Product length (bp)	GenBank accession number
NOD1	Forward	GTCACTCACATCCGCAACAC	84	JF681170
	Reverse	CCACGATCTCCGCATCTT		
NOD2	Forward	GCACATCACCTTCCAGTGTTT	98	JF681171
	Reverse	GGCCCATGACAAATGAAGA		
TLR1	Forward	GCCATCCTACCGTGAACCT	114	NM_001146143.1
	Reverse	GCACTCAACCCCAGAAACTC		
TLR2	Forward	TCGAGAAGAGCCACAAAACC	90	NM_001005264.2
	Reverse	CGAAAATGGGAGAAGTCCAG		
TLR4	Forward	GTGCTTCATGGTTTCTCTGGT	146	NM_001002950.1
	Reverse	CCAGTCTTCATCCTGGCTTG		
TLR5	Forward	TCGTGTTGACAGACGGTTATTT	143	EU551146.1
	Reverse	TCCGGTTGAGGGAAAAGTC		
TLR6	Forward	TCAAGCATTTAGACCTCTCATTCA	109	EU551147.1
	Reverse	CCGTAACTTTGTAGCACTTAAACCT		
TLR9	Forward	ACTGGCTGTTCCTCAAGTCC	104	NM_001002998.1
	Reverse	AGTCATGGAGGTGGTGGATG		
IL-1β	Forward	ACCCGAACTCACCAGTGAAATG	110	NM_001037971
	Reverse	GGTTCAGGTCTTGGCAGCAG		
IL-6	Forward	TCTGTGCACATGAGTACCAAGATCC	125	NM_001003301
	Reverse	TCCTGCGACTGCAAGATAGCC		
TNF-α	Forward	CCCAAGTGACAAGCCAGTAGCTC	146	NM_001003244
	Reverse	ACAACCCATCTGACGGCACTATC		
HMBS	Forward	TCACCATCGGAGCCATCT	112	XM_546491
	Reverse	GTTCCCACCACGCTCTTCT		
SDHA	Forward	GCCTTGGATCTCTTGATGGA	92	XM_535807
	Reverse	TTCTTGGCTCTTATGCGATG		
TBP	Forward	CTATTTCTTGGTGTGCATGAGG	96	XM_849432
	Reverse	CCTCGGCATTCAGTCTTTTC		

HMBS, hydroxymethylbilane synthase; IL, interleukin; NOD, nucleotide-binding oligomerization domain; SDHA, succinate dehydrogenase complex subunit A; TBP, TATA box binding protein; TLR, toll-like receptor; TNF, tumor necrosis factor.

Table 9

History in 26 dogs with inflammatory colorectal polyps (ICRPs).

Inflammatory disease	Cases	Non-inflammatory disease	Cases
Chronic enteritis	5	Lipomatosis	3
Chronic rhinitis	2	Mammary grand tumor	3
Pancreatitis	2	Corneal dystrophy	2
Anal sacculitis	1	Cryptorchidism	2
Dermatitis	1	Inguinal hernia	2
Otitis externa	1	Alimentary Lymphoma	1
		Diaphragmatic hernia	1
		Intervertebral disk disease	1
		Progressive retinal atrophy	1
		Prostatic cyst	1
		Prostatic hypertrophy	1
		Renal Lymphoma	1
		Sudden acquired retinal degeneration	1

Table 10
Comparison of IL-1β messenger RNA (mRNA) expression and protein production between ICRP-affected miniature dachshunds (MDs) (n = 16)* and control MDs (n = 16).

PAMPs	IL-1β mRNA expression			IL-1β protein production (pg/ml)		
	ICRP-affected	Control	*P*-value**	ICRP-affected	Control	*P*-value**
unstimulated	0.03 (0.00–0.57)	0.01 (0.00–0.11)	0.291	24.7 (0.0–55.2)	46.9 (0.0–53.6)	0.082
iE-DAP	0.10 (0.01–3.84)	0.21 (0.00–35.79)	0.243	0.0 (0.0–159.0)	16.9 (0.0–43.3)	0.230
MDP	0.59 (0.07–5.35)	0.15 (0.01–0.36)	**0.002**	63.5 (0.0–117.8)	46.0 (0.0–55.2)	**0.026**
FLA-ST	0.04 (0.00–0.54)	0.03 (0.01–0.15)	0.547	0.0 (0.0–38.0)	0.0 (0.0–22.4)	0.551
ODN2006	0.03 (0.00–0.77)	0.02 (0.00–0.52)	0.142	0.0 (0.0–21.3)	0.0 (0.0–17.9)	0.551
Pam3CSK4	9.37 (0.82–44.23)	4.47 (0.01–22.63)	0.187	76.4 (0.0–594.7)	36.6 (0.0–258.0)	0.304
PGN-EK	14.32 (5.77–59.97)	12.93 (1.82–143.07)	0.763	229.9 (0.0–2223.1)	72.4 (0.0–783.9)	0.426
FSL-1	23.37 (1.42–62.57)	11.52 (1.82–88.99)	0.228	104.6 (0.0–839.5)	38.3 (0.0–135.1)	0.089
LPS-EK	19.96 (1.86–37.96)	11.07 (0.46–108.34)	0.243	124.4 (12.0–1267.9)	107.6 (0.0–294.3)	0.522

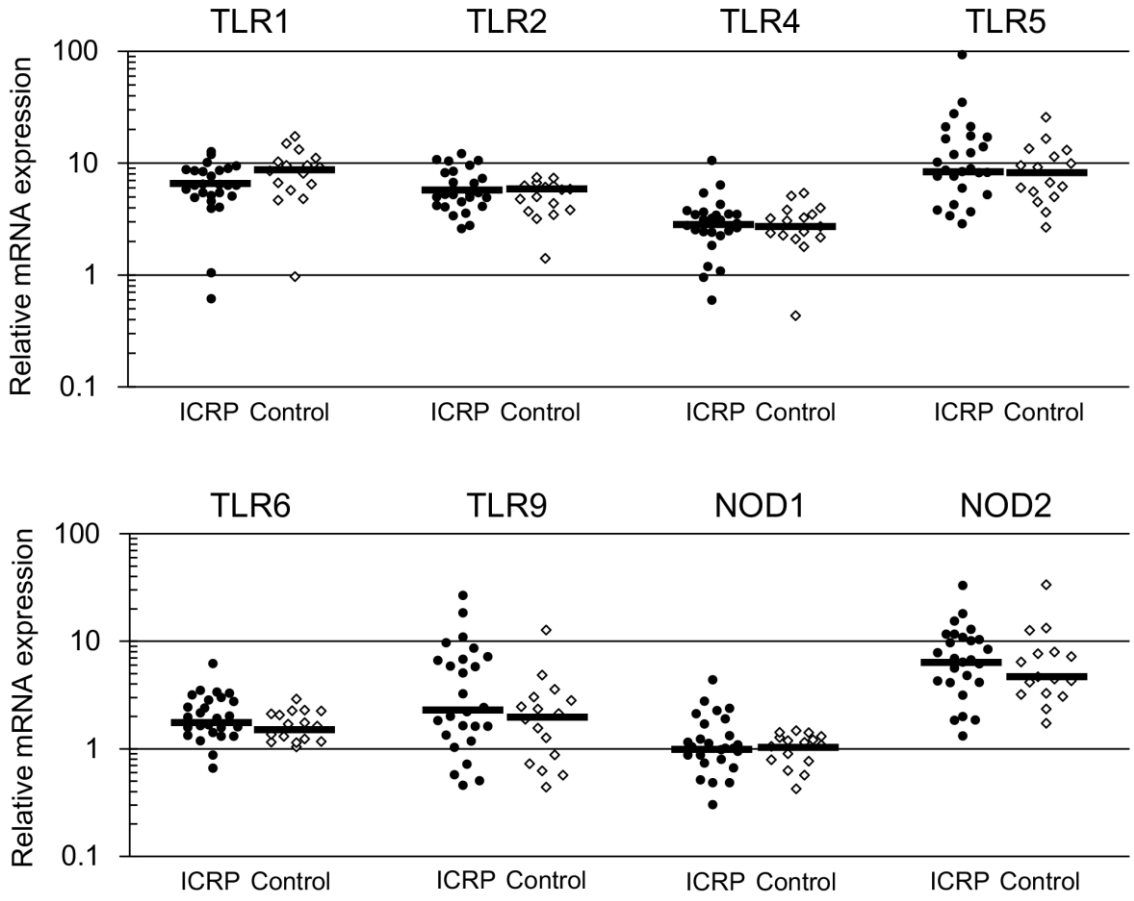
Data are represented in median (range).
*Data of ICRP-affected MDs that had received immunosuppressive therapy were excluded.
**Mann–Whitney *U*–test.
iE-DAP, peptidoglycan-like molecule (NOD1 ligand); FLA-ST, flagellin (TLR5 ligand); FSL-1, synthetic diacylated lipoprotein (TLR2/6 ligand); LPS-EK, LPS (TLR4 ligand); MDP, muramyl dipeptide (NOD2 ligand); ODN2006, CpG oligonucleotide (TLR9 ligand); PAMP, pathogen-associated molecular pattern; Pam3CSK4, synthetic bacterial lipoprotein (TLR1/2 ligand); PGN-EK, peptidoglycan (TLR2 ligand).

Table 11
Comparison of IL-1β mRNA expression and protein production between ICRP-affected MDs at initial diagnosis (n = 17) and those at clinical remission (n = 9).

PAMPs	IL-1β mRNA expression			IL-1β protein production (pg/ml)		
	Initial diagnosis	Clinical remission	*P*-value*	Initial diagnosis	Clinical remission	*P*-value*
unstimulated	0.02 (0.00–0.57)	0.01 (0.00–0.10)	0.501	24.3 (0.0–55.2)	33.5 (0.0–58.5)	0.130
iE-DAP	0.13 (0.01–8.50)	0.06 (0.03–1.59)	0.346	15.3 (0.0–244.9)	0.0 (0.0–74.2)	0.228
MDP	0.33 (0.07–0.54)	0.31 (0.17–2.55)	0.726	66.3 (0.0–101.2)	61.9 (0.0–117.8)	0.935
FLA-ST	0.05 (0.00–0.54)	0.03 (0.01–0.04)	0.058	0.0 (0.0–50.2)	0.0 (0.0–0.0)	0.123
ODN2006	0.04 (0.00–0.77)	0.01 (0.00–0.05)	0.090	0.0 (0.0–28.3)	0.0 (0.0–45.8)	0.764
Pam3CSK4	10.91 (0.82–95.79)	4.10 (1.01–37.53)	0.169	97.8 (0.0–953.0)	21.8 (0.0–249.4)	**0.009**
PGN-EK	20.26 (4.00–328.87)	10.93 (4.12–54.42)	0.153	413.7 (0.0–2741.1)	33.7 (0.0–597.1)	**0.007**
FSL-1	27.71 (1.42–146.09)	7.51 (0.39–109.24)	0.169	164.3 (0.0–839.5)	31.4 (0.0–479.6)	**0.049**
LPS-EK	21.25 (1.17–172.59)	8.70 (2.07–47.87)	0.435	178.4 (29.6–1267.9)	98.5 (12.0–354.9)	0.346

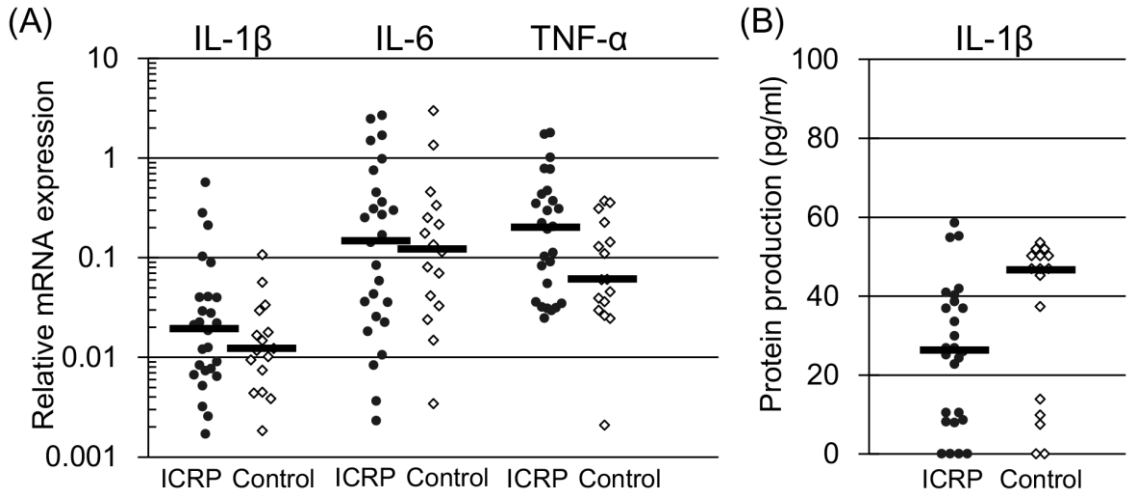
Data are represented in median (range).
*Mann–Whitney *U*–test.

Figure 9



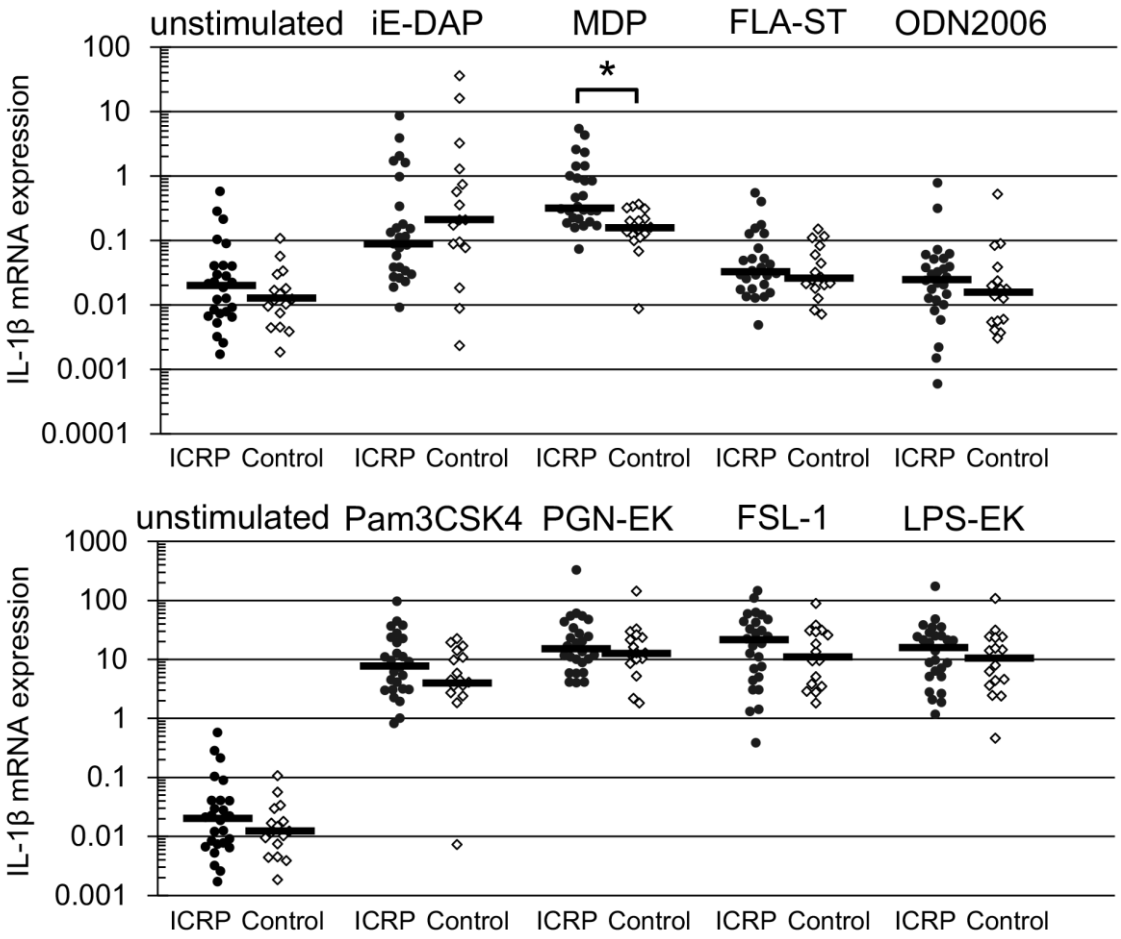
Relative transcription levels of pattern recognition receptor messenger RNAs (mRNAs) in non-stimulated monocytes in Miniature Dachshunds (MDs) with inflammatory colorectal polyps (ICRPs; n = 26) and control MDs (n = 16). Data are expressed relative to the geometric mean of three reference genes (Hydroxymethylbilane synthase, succinate dehydrogenase complex subunit A and TATA box binding protein). The horizontal lines represent the median value of that group. NOD, nucleotide-binding oligomerization domain; TLR, toll-like receptor.

Figure 10



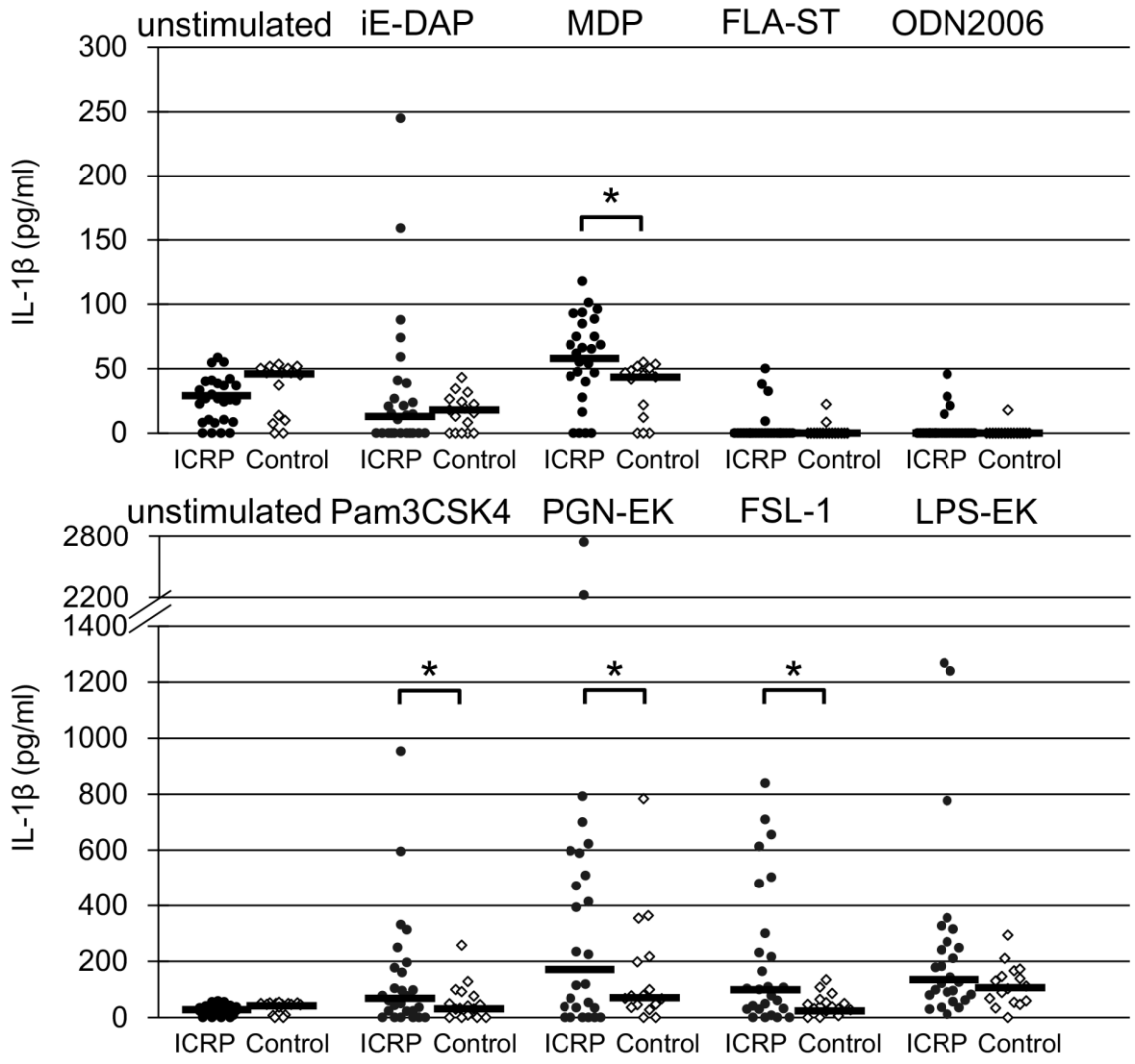
Relative transcription levels of proinflammatory cytokine mRNAs (A) and protein production of IL-1β (B) in non-stimulated monocytes in MDs with ICRPs (n = 26) and control MDs (n = 16). Data of mRNAs are expressed relative to the geometric mean of three reference genes. Samples below the limit of detection (5.9 pg/ml) have been assigned a value of zero. The horizontal lines represent the median value of that group.

Figure 11



Relative transcription levels of IL-1β mRNAs in monocytes stimulated with pathogen-associated molecular patterns (PAMPs) for 6 hr in ICRP-affected (n = 26) and control (n = 16) MDs. The horizontal lines represent the median value of that group. Data are expressed relative to the geometric mean of three reference genes. Asterisks indicate statistically significant differences ($P < 0.05$). iE-DAP, peptidoglycan-like molecule (NOD1 ligand); FLA-ST, flagellin (TLR5 ligand); MDP, muramyl dipeptide (NOD2 ligand); FSL-1, synthetic diacylated lipoprotein (TLR2/6 ligand); LPS-EK, LPS (TLR4 ligand); ODN2006, CpG oligonucleotide (TLR9 ligand); Pam3CSK4, synthetic bacterial lipoprotein (TLR1/2 ligand); PGN-EK, peptidoglycan (TLR2 ligand).

Figure 12



Secretion of IL-1β protein from monocytes stimulated with PAMPs for 24 hr in ICRP-affected (n = 26) and control (n = 16) MDs. Samples below the limit of detection (5.9 pg/ml) have been assigned a value of zero. The horizontal lines represent the median value of that group. Asterisks indicate statistically significant differences ($P < 0.05$).

Chapter 1-3

Polymorphisms of nucleotide-binding oligomerization domain 2 (NOD2) gene and their association with inflammatory colorectal polyps in Miniature Dachshunds

Abstract

Pattern recognition receptors (PRRs) play an important role in the differentiation of pathogens from commensal bacteria and food antigens, and polymorphisms of various PRRs have been shown to be associated with human and canine inflammatory bowel disease. In Chapter 1-2, I revealed that the reactivity of nucleotide-binding oligomerization domain 2 (NOD2), toll-like receptor (TLR) 1/2, TLR2, and TLR2/6 are greater in inflammatory colorectal polyp (ICRP)-affected Miniature Dachshunds (MDs) than that in controls. Therefore, this Chapter was aimed to investigate single nucleotide polymorphisms (SNPs) of PRRs associated with ICRPs in MDs. Mutational analysis of canine NOD2, TLR1, TLR2, and TLR6 genes was performed with six ICRP-affected MDs, five control MDs, and five healthy beagles. The mutational analysis identified 13 non-synonymous SNPs in NOD2, TLR1, TLR2, and TLR6 genes, of which six SNPs in NOD2 exon 3 were further analyzed in an association study using 63 ICRP-affected MDs, 82 control MDs, and 237 control dogs of various breeds. Four of the SNPs (A1532G, T1573C, C1688G, and G1880A of the NOD2 gene) were in Hardy–Weinberg equilibrium and in complete linkage disequilibrium in MDs, and their minor allele frequencies were significantly lower in ICRP-affected MDs than in control MDs (0.016 vs. 0.140, P = 0.0002). The calculated inheritance model was an additive model (odds ratio = 0.10, 95% confidence interval = 0.02–0.45, P = 0.0001), which indicates that the haplotype with minor alleles in these SNPs (A, T, C, and G in A1532G, T1573C, C1688G, and G1880A) possess a protective effect regarding the development of ICRPs. However, these SNPs were not specific for MDs, although the minor allele frequencies of these SNPs in control MDs were significantly lower than in other breed dogs. These results suggest that the identified four SNPs (A1532G, T1573C, C1688G, and G1880A in the NOD2 gene) may

play a role in the pathogenesis of ICRPs in MDs. Because the majority of MDs and other breed dogs do not have the protective alleles, their absence may not be a specific cause of ICRPs in MDs but rather contribute to the development of inflammation.

1. Introduction

To date, a number of gene variations have been reported to be associated with human inflammatory bowel disease (IBD). A meta-analysis of genome-wide association studies revealed 163 risk-associated loci for human IBD (Jostins et al., 2012). Pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors, are well-studied genes because they play a central role in the maintenance of mucosal immunity by distinguishing the pathogens from commensal bacteria and food antigens (Abreu, 2010; Cario, 2010; Magalhaes et al., 2007). Dysfunction of PRRs resulting from genetic background is involved in the pathogenesis of IBD in humans, although the etiology of IBD is considered multifactorial (Cario, 2010; Xavier and Podolsky, 2007). Polymorphisms of NOD2 have been characterized to encompass the genetic susceptibility to the Crohn's disease, a major form of human IBD (Hugot et al., 2001; Ogura et al., 2001a). In addition, polymorphisms of other PRRs including NOD1, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR9 also have been reported to be associated with the development of human IBD (Cario, 2010; Lu et al., 2010). Furthermore, associations between polymorphisms of TLR4, TLR5, and NOD2 genes and IBD in German shepherd breed have also been reported (Kathrani et al., 2010, 2014).

Due to the breed specificity, inflammatory colorectal polyp (ICRP) in Miniature Dachshunds (MDs) is speculated to involve a genetic susceptibility, but there are no reports on the genetic background. In Chapter 1-2, I revealed that the reactivity of NOD2, TLR1/2, TLR2, and TLR2/6 is greater in ICRP-affected MDs than those in healthy MDs using peripheral blood-derived monocytes. Therefore, I hypothesized that genetic variations in the NOD2, TLR1, TLR2, and/or TLR6 are associated with the development

of ICRPs in MDs. For this purpose, I first performed molecular cloning of canine NOD2 gene since its reference sequence (GenBank accession number: NM_001287039.1) was not supported by experimental evidence. Second, I performed a mutational analysis of these genes using six ICRP-affected MDs, five control MDs, and five healthy beagles to detect candidate single nucleotide polymorphisms (SNPs) responsible for ICRPs. Finally, the allele frequencies of the SNPs and their association with the development of ICRPs were analyzed using 63 ICRP-affected MDs, 82 control MDs, and 237 control other breed dogs.

2. Materials and methods

2.1. Animals

In total, 63 unrelated MDs with ICRPs were included. Sixty-one of them were diagnosed with ICRPs at the Veterinary Medical Center of the University of Tokyo (VMC-UT) based on the presence of solitary or multiple polyps restricted to the colorectal mucosa and histopathological findings (severe inflammatory infiltration predominantly by neutrophils, without neoplastic changes). The other two MDs were diagnosed at reference hospitals based on similar findings.

In total, 324 unrelated control dogs were also included in the present study. Client-owned 82 MDs were recruited as a non-ICRP control, and client-owned 237 dogs with other breeds were also recruited to examine the effect of breed differences; both groups were presented to the reference hospitals for the periodic health examination. The other five healthy dogs were laboratory-owned beagles at VMC-UT and used for molecular cloning of NOD2 gene and mutational analysis of PRR genes. All control dogs did not have inflammatory, infectious, or immune system-mediated diseases, and showed no clinical signs of gastrointestinal disorders. The use of control dogs was approved by the Animal Care Committee of the University of Tokyo (Approval No. P13-774).

2.2. Molecular cloning of NOD2 gene

Total RNA of three healthy laboratory-owned beagles extracted from colonic mucosa in Chapter 1-1 was used. Genomic DNA was removed from the samples with a TURBO DNA-free Kit (Applied Biosystems, Foster City, CA, USA). Reverse-transcription was performed to synthesize complementary DNA (cDNA) using PrimeScript RT Reagent Kit (Takara Bio Inc., Shiga, Japan). Oligonucleotide primers were designed based on the

predicted messenger RNA (mRNA) sequence of canine NOD2 (NM_001287039.1), such that the primers overlapped with each fragment (Table 12). PCR was performed using AmpliTaq Gold 360 (Applied Biosystems) with the following conditions: 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 3 s), annealing (58–60°C for 30 s), and extension (72°C for 75–90 s), and then a final long extension (72°C for 7 min). The resulting PCR products were electrophoresed on a 1.5% agarose gel and purified from the gel by using a commercially available kit (Wizard SV Gel and PCR Clean-Up System; Promega Corp., Madison, WI, USA). Purified PCR products were subcloned into the pGEM-T easy vector (Promega Corp.) and transfected into competent cells (Competent high DH5α; TOYOBO, Tokyo, Japan). Plasmid DNA was extracted using a plasmid purification kit (Nucleospin Plasmid QuickPure; Takara Bio Inc.), and subsequently subjected to sequencing using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and two primers complementary to T7 and SP6 promoter sites of the vector. The reaction conditions were as follows: 96°C for 1 min, followed by 25 cycles of denaturation (96°C for 10 s), annealing (50°C for 5 s), and extension (60°C for 4 min). Following the sequencing reaction, DNA was purified by ethanol/ ethylenediaminetetraacetic acid (EDTA) precipitation and dried up (as recommended for ABI prism BigDye Terminator v3.1 Cycle Sequencing kit by Applied Biosystems). DNA was subsequently resuspended in Hi–Di Formamide (Applied Biosystems) and subsequently denatured by heating 95°C for 2 min followed by rapidly cooling on ice. Nucleotide sequences were analyzed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Five independent clones per dog were sequenced (for each fragment) to prevent errors in sequence analysis.

2.3. Mutational analysis of NOD2, TLR1, TLR2, and TLR6 exons

This analysis was performed using six ICRP-affected MDs (all diagnosed at VMC-UT), five control MDs, and five laboratory-owned beagles to investigate the presence and frequency of non-synonymous SNPs in the coding region of the genes.

Genomic DNA was extracted from the EDTA-stabilized blood sample of each dog using a commercially available kit (QIAmp DNA Mini kit; Qiagen, Hilden, Germany). Primers for full-length canine NOD2, TLR1, TLR2, and TLR6 genes are shown in Table 13. Primer sequences for canine NOD2 gene were designed based on the full sequences for canine chromosome 2 (NC_006584.3). Primer sequences for canine TLR1, TLR2, and TLR6 were described in previous studies (House et al., 2009; Kathrani et al., 2010). PCR was performed using AmpliTaq Gold 360 (Applied Biosystems) using the following conditions: 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 3 s), annealing (55–65°C for 30 s), and extension (72°C for 40–180 s), and then a final long extension (72°C for 7 min). The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega), and directly sequenced using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) with sequencing primers shown in Table 14. The conditions of sequencing reaction were as follows: 96°C for 1 min, followed by 25 cycles of denaturation (96°C for 10 s), annealing (54–60°C for 5 s), and extension (60°C for 4 min). The analysis was performed in duplicate, and matched sequences were accepted. Sequence data from the NOD2, TLR1, TLR2, and TLR6 genes were compared to those in the database (NM_001287039.1, EU551145.1, EU487534.1, and NM_005618633.1, respectively) to identify the non-synonymous SNPs using CLC DNA Workbench version 7.0.2 (CLC Bio, Hilden, Germany).

2.4. Genotyping of NOD2 SNPs and association study

The allele frequency and association between non-synonymous SNPs and disease status or breed were further analyzed. Six SNPs identified in exon 3 of canine NOD2 gene was evaluated in this analysis.

Genomic DNA was extracted using QIAmp DNA Mini kit (Qiagen) from EDTA blood obtained from further 32 MDs who were diagnosed with ICRPs at VMC-UT between August 2011 and October 2014, and two ICRP-affected MDs diagnosed at reference hospitals, and all 324 control dogs. In addition, paraffin sections of polypoid tissue obtained from another 23 ICRP-affected MDs diagnosed at VMC-UT between April 2008 and July 2011 were included in the analysis, and the genomic DNA was extracted using QIAmp DNA Mini kit (Qiagen) according to the manufacturer's protocol.

PCR was carried out on all samples using AmpliTaq Gold 360 (Applied Biosystems) with the primers designed for the genotyping analysis to cover all six SNPs (Forward: 5'-TGTGCCACCTTCCTGTTGTC-3'; Reverse: 5'-GTACAGGCTCCGGATGAGCC-3'; 1,364–2,163 bp of canine NOD2 coding sequences [NM_001287039.1]) under following conditions: 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 3 s), annealing (65°C for 30 s), and extension (72°C for 60 s), and then a final long extension (72°C for 7 min). The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega), and directly sequenced using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) with same primers. The conditions of sequencing reaction were as follows: 96°C for 1 min, followed by 25 cycles of denaturation (96°C for 10 s), annealing (65°C for 5 s), and extension (65°C for 4 min). The genotype in all

six SNPs of NOD2 exon 3 were evaluated by both forward and reverse primers, and the analysis was carried out in duplicate.

2.5. Statistical analysis

All raw sequence data of cDNA and genomic DNA were analyzed using Sequence Scanner Software version 2.0 (Applied Biosystems), and the nucleotide sequence, presence of SNPs, and their genotype were determined. Allele frequency, association and linkage disequilibrium for SNPs under study were statistically assessed using the Haploview software package version 4.2 (http://www.broad.mit.edu/personal/jcbarret/haplo/). The calculation of the Hardy–Weinberg equilibrium and additional genotype and haplotype associations for SNPs of NOD2 exon 3 were performed using the SNPSTATS software (http://bioinfo.iconcologia.net/index.php?module=Snpstats). Statistical significance of the differences was assumed at $P < 0.05$. In addition, the impact of all non-synonymous SNPs on the each protein function were assessed by using PROVEAN (http://provean.jcvi.org/index.php) and SIFT programs (http://sift.jcvi.org/) (Choi et al., 2012; Kumar et al., 2009). The PROVEAN score <–2.500 and SIFT score <0.05 were considered deleterious.

3. Results

3.1. Molecular cloning of canine NOD2 gene

By combining the sequences of four overlapping DNA fragments, a linear sequence corresponding to canine NOD2 was obtained. This sequence includes the entire open reading frame of 3,042 bp encoding 1,013 amino acid residues (NM_001287039.1). The homology of NOD2 cDNA sequences between dogs and humans (AF178930.1), pigs (NM_001105295.1), cattle (NM_001002889.1), or mice (NM_145857.2) were 85.6, 85.5, 85.0, and 78.4%, respectively; those of deduced NOD2 amino acid sequences were 83.4, 82.8, 82.5, and 75.9%, respectively (Figure 13). A transcript variant ideal for the predicted sequence (XM_005617571.1) was also identified, which contains a 106-bp deletion in the region of exon 2–3 (as a result, this variant has a 195-amino acid deletion at the N-terminus). In addition, two other splicing variants were also detected with a deletion of exon 7 or exon 10 region (both were not reported previously).

3.2. Mutational analysis of canine NOD2, TLR1, TLR2, and TLR6 genes

This analysis revealed eight non-synonymous SNPs in the NOD2 gene (Figure 13): C178T (not reported in the canine genome database), A1532G (GenBank accession number: rs8647661), T1573C (rs8647662), C1688G (rs8647663), G1690A (not reported in the canine genome database but described by House et al. [2009]), G1880A (rs8647665), G1990A (not reported previously), and G2564A (rs22792936), one non-synonymous SNP in the TLR1 gene: G83T (rs23585044), two non-synonymous SNPs in the TLR2 gene: G599A (not reported previously) and G1117A (not reported previously), and two non-synonymous SNPs in the TLR6 gene: C1369T (not reported previously) and G1537A (not reported in the canine genome database but described by House et al.

[2009]; Table 15). To predict the functional effect of each SNP, the PROVEAN and SIFT programs were used. Both programs predicted the C1369T in TLR6 as deleterious; furthermore, the SIFT software also regarded four SNPs—C178T in NOD2 gene, G83T in TLR1 gene, and G599A and G1117A in TLR2 gene—as deleterious (Table 15). In addition, 18 synonymous SNPs in the PRR genes under study were also detected; they are depicted in Table 16 and excluded from further analyses. Other types of genetic variation including in-frame insertion, deletion, or replacement were not identified in this analysis.

3.3. Genotyping of SNPs in NOD2 exon 3 and association study

A total of 63 MDs with ICRPs were included in this analysis; 61 were diagnosed with ICRPs at VMC-UT and two diagnosed with ICRPs at reference hospitals. Their median age was 114 months (range, 48–171 months) with 38 males and 25 females. The control MDs group consisted of 82 dogs; their median age was 84 months (7–214 months) with 37 males and 45 females. Seventeen of 82 the control MDs were diagnosed with a non-inflammatory disease; the most common diagnosis among them was intervertebral disk disease (Table 17). In addition, another control group with 237 dogs was also recruited; the median age of dogs in this control group was 60 months (6–182 months) with 127 males, 109 females, and one hermaphroditism. They consisted of 40 breeds predominantly with Toy poodle (n = 40) and mixed breed dogs (n = 36) (Table 18). Forty-four of the 237 control dogs of other breeds were diagnosed with a non-inflammatory disease (Table 19).

The minor allele frequencies of six non-synonymous SNPs in NOD2 exon 3 (A1532G, T1573C, C1688G, G1690A, G1880A, and G1990A) were determined. All six SNPs were

in the Hardy–Weinberg equilibrium in each of the three groups ($P > 0.05$). Significant association was observed in the minor allele frequency of SNPs including A1532G, T1573C, C1688G, and G1880A when I compared ICRP-affected MDs with control MDs ($P = 0.0002$) (Table 20). These four SNPs were in complete linkage disequilibrium among MDs; there was also evidence of linkage disequilibrium between these four SNPs and G1990A (LOD > 3) (Figure 14). To evaluate the significance of the genotype of the four SNPs (A1532G, T1573C, C1688G, and G1880A) on the disease status in MDs, five inheritance models (co-dominant, dominant, recessive, over-dominant, and additive) were applied to statistical analysis; the best inheritance model was assessed using the Akaike information criteria (AIC) and Bayesian information criteria (BIC); the model with the lowest values was assumed to be the most valid model. According these values, the additive model was determined as being the best fit, and I found that the minor allele of each SNP (A, T, C, and G at A1532G, T1573C, C1688G, and G1880A) has a protective effect regarding the development of ICRPs in MDs (odds ratio: 0.10 [95% confidence interval: 0.02–0.45], $P = 0.0001$) (Table 21). In addition, age and gender were not significantly associated with SNP outcomes based on univariate analysis.

Furthermore, the minor allele frequencies of six SNPs in NOD2 exon 3 in control MDs were further compared with those in control dogs of other breeds; the minor allele frequencies of A1532G, T1573C, C1688G, and G1880A were significantly or relatively lower in control MDs than in control other breed dogs ($P = 0.0017$, 0.0017, 0.0689, and 0.0017, respectively), while that of G1990A was significantly higher in control MDs than in control dogs of other breeds ($P = 0.0013$) (Table 22). The linkage disequilibrium of these SNPs in control dogs of other breeds was analyzed; there was evidence of linkage disequilibrium among A1532G, T1573C, C1688G, and G1880A, where the A1532G,

T1573C, and G1880A showed complete linkage disequilibrium (data not shown).

4. Discussion

In this Chapter, I identified an association between four SNPs of the NOD2 gene including A1532G, T1573C, C1688G, and G1880A, which are in complete linkage disequilibrium in MDs, and the development of ICRPs in MDs. Genetic variations in NOD2 have been described to be an important factor in the etiology of several inflammatory diseases including Crohn's disease and Blau syndrome in humans (Corridoni et al., 2014; Sfriso et al., 2012). Therefore, the observed SNPs in this study may have a significance in the pathogenesis of ICRPs in MDs.

Interestingly, these SNPs associating with ICRPs were completely identical with those associated with IBD in German shepherd dogs (Kathrani et al., 2014). However, there was a critical difference: the inheritance model of these SNPs was over-dominant and increased heterozygous haplotype of these four SNPs in German shepherd dogs with IBD (Kathrani et al., 2014), while the additive model seems to be the best fit and the haplotype identical to the database shows a significant protective effect in this study. Although ICRPs in MDs are thought as a novel form of canine IBD based on the presence of idiopathic inflammation and clinical response to immunosuppressive therapy (Ohta et al., 2013), the histological findings and localization of the lesion are different; lymphocytic-plasmacytic enteritis in the small and/or large intestine is common in canine IBD (Allenspach et al., 2007; Craven et al., 2004; Jergens et al., 2010, 2003), while the severe infiltration of neutrophils and macrophages restricted to the colorectal region is characteristic of ICRPs in MDs (Ohmi et al., 2012; Tamura et al., 2013). Therefore, these differences indicate that the pathogenesis of mucosal inflammation is different between canine IBD and ICRPs in MDs, which may lead to the inconsistent results.

The identified four SNPs that are associated with the development of ICRPs are

located in the NACHT region of the NOD2 gene or around it (Figure 13), which is more similar to Blau syndrome than Crohn's disease in humans; the mutations conferring susceptibility to Blau syndrome occur in the NACHT region, while the most common mutation that confers susceptibility to Crohn's disease is a frameshift mutation in the LRR region (Borzutzky et al., 2010; Sfriso et al., 2012; Zhong et al., 2013) (Figure 13). Blau syndrome is an autosomal dominantly inherited disorder, characterized by chronic inflammation including granulomatous dermatitis, symmetric arthritis, and recurrent uveitis (Sfriso et al., 2012). The causative SNPs of Blau syndrome lead to increased NF-κB activation with or without ligand stimulation (Sfriso et al., 2012; Tanabe et al., 2004). Therefore, this gain-of-function mutation of the NOD2 gene and the resulting NF-κB activation are believed to play a key role in the etiology of Blau syndrome. Interestingly, this notion is consistent with the result of Chapter 1-2 in which the reactivity of peripheral monocytes to the NOD2 ligand was enhanced in ICRP-affected MDs compared to control MDs. However, unlike in Blau syndrome, the coded amino acid residues from the identified four SNPs in this study were not conserved residues (Figure 13), which may result in non-significant scores in the PROVEAN and SIFT programs (Table 15). Therefore, the functional impact of these SNPs may be small, but I hypothesize that the late onset of ICRPs in MDs might explain this situation. A recent study suggests that an elevated basal NF-κB activity may affect disease progression rather than disease onset because the patients with mutated NOD2 with low NF-κB activity tend to experience the complications at a later age (Okafuji et al., 2009). ICRPs in MDs typically develop at an old age (at least 4 years, median is about 9 years in the present and previous studies) (Ohmi et al., 2012; Tamura et al., 2013); whereas Blau syndrome is characterized by early onset, typically at ages before 3–4 years (by teenage at the latest) (Sfriso et al., 2012).

Therefore, the observed four SNPs associated with ICRPs in MDs might induce a weak predisposition. How these SNPs affect the basal NF-κB activity and the reactivity to the ligand stimulation remains unknown, which needs to be investigated in the future. Furthermore, the presence of transcript variants of NOD2 (Leung et al., 2007), which was also identified in this study, should be taken into account. One of the variants has been reported to be preferentially expressed in the human colon, and to downregulate the NOD2-induced NF-κB activity (Rosenstiel et al., 2006). I described that the mRNA expression of NOD2 is upregulated in the polypoid lesion of ICRP-affected MDs; however, their expression pattern of transcript variants was not investigated. Although their role in the pathogenesis of Blau syndrome or Crohn's disease has not been studied, that of full-length NOD2 and its transcript variant observed in this study, and the effects of SNPs need to be analyzed further.

Unfortunately, the majority of the control MDs had the ICRP risk-associated haplotype (G, C, G, and A at A1532G, T1573C, C1688G, and G1880A) of the NOD2 gene homozygously. This result shows that these SNPs do not induce ICRPs in MDs independently without any other factors. It was not surprising because the chronic intestinal inflammation is considered a multifactorial disease (Cerquetella et al., 2010; German et al., 2003); these SNPs may be only a genetic factor predisposing MDs to ICRPs. Furthermore, most of control dogs other than MDs also have the risk-associated haplotype in the present study. The minor allele frequencies of these SNPs in dogs with various breeds was lower in this study compared with those reported in a previous study (Kathrani et al., 2014). However, this phenotype can be modified by the breed disposition because dog breeds have developed into close breeding populations representing isolated genetic pools. As shown in Table 23, inter-breed variation of the minor allele frequency

was observed in this study. The minor allele frequencies of these four SNPs appears to be relatively low in MDs but not the lowest. Associations between these four SNPs predisposing ICRPs in MDs and other various inflammatory or immune-mediated disease are a subject of an upcoming project.

Although the minor allele frequencies of SNPs in NOD2 exon 3 do not seem to differ between ICRP-affected MDs and control MDs in the mutational analysis, they are significantly different in the subsequent association analysis; this contradiction may be due to the limited number of dogs included in the analysis and suggests that the significance of other SNPs in NOD2 and other TLRs identified in this mutational analysis for ICRPs in MDs cannot be ruled out. Moreover, the absence of breed-specific SNPs responsible for ICRPs indicates that there might be another genetic variation specific for MDs because of the breed-specificity of the disease (Ohmi et al., 2012). Genome-wide association studies in MDs with ICRPs may help to identify additional genetic background associated with the development of ICRPs.

Another possible limitation was that the age of control groups was younger than that of ICRP-affected MDs. Since ICRPs in MDs commonly develop at middle and advanced age, the younger control dogs may develop inflammatory disorders in the future. However, I believe that this limitation does not affect the results of association between SNPs and the disease phenotype because there was a significant difference when the minor allele frequencies of four SNPs in NOD2 exon 3 (A1532G, T1573C, C1688G, and G1880A) were compared between ICRP-affected MDs (n = 32) and control MDs (n = 29) older than 114 months, a median age of the ICRP-affected MDs in this study (0.000 vs. 0.121, $P = 0.0042$, respectively).

In summary, I first cloned the canine NOD2 gene and detected three transcript variants.

Second, a mutational analysis was performed and revealed 13 non-synonymous SNPs in canine NOD2, TLR1, TLR2, and TLR6 genes. Finally, an association analysis was performed and revealed that four SNPs (A1532G, T1573C, C1688G, and G1880A in NOD2 gene) are associated with the development of ICRPs in MDs. These four SNPs predispose MDs to ICRPs, but are not sufficient for the development of the disease. Further functional analyses of these SNPs should confirm their role and significance in the pathogenesis of ICRPs, which might increase our understanding of inflammatory disorders such as Blau syndrome in humans.

Table 12

Sequences of primers used for molecular cloning of canine nucleotide-binding oligomerization domain 2 (NOD2) gene.

	Primer sequence (5'–3')		Primer position	Annealing (°C)	Extension (sec)
Fragment 1	Forward	TCTCCTCCCCAGGTTATGAA	–20	60	75
	Reverse	CCAGCACAGTGTCTGCATCT	808		
Fragment 2	Forward	GGAGATCCGAACAGAAATGG	690	60	90
	Reverse	CTTGGCATGCACCAGGAAG	1686		
Fragment 3	Forward	TGTGCTGCTACGTGTTCTCC	1598	60	90
	Reverse	GCATGCTCGATGAGCTTACA	2435		
Fragment 4	Forward	CTGGACCACAACTCTGTTGG	2308	58	90
	Reverse	ACTGAGATAAATGCTGGCCC	3069		

Primer positions were represented by the numbers within the coding region of NOD2 gene (GenBank accession number: NM_001287039.1).

Table 13

PCR primers used in mutational analysis of NOD2, toll-like receptor (TLR) 1, TLR2, and TLR6.

	Primer sequence (5'–3')		Primer position	Annealing (°C)	Extension (sec)
NOD2					
exon 1	Forward	GTAGATCCATGGCCTCTTCAG	64,667,386	60	60
	Reverse	CCCAAGACAGAAGTGCCTTAC	64,666,745		
exon 2	Forward	ATCGGTAGAAACCTTCCCAC	64,659,352	60	45
	Reverse	TCCCTATAGCCTTTCTTCCC	64,659,107		
exon 3	Forward	ACCATCCTACCTCATTGGCC	64,656,695	65	130
	Reverse	CACTGCCCTGCGACACTCAC	64,654,822		
exon 4	Forward	CAGATGCTGGCACATAGGGC	64,650,615	63	45
	Reverse	CATGACCTGGAGAGTGGCTG	64,650,357		
exon 5	Forward	ACTGACTGCTGTGCTCTGTC	64,650,253	65	40
	Reverse	CTGCGCAGAAGACAGCTTCC	64,650,073		
exon 6	Forward	TCACTGACTTGGTCTCCTGC	64,647,857	65	40
	Reverse	TGCCCCAGAATCCAACCTAC	64,647,722		
exon 7	Forward	ACTGACCCAATTTGTTGGCC	64,645,919	65	40
	Reverse	CCAAACAGACCCAGAAGCTT	64,645,787		
exon 8	Forward	ATCTCTGAGGACCCTCTGTG	64,645,094	65	40
	Reverse	TGTGAATCCCCCCAACTCAC	64,644,937		
exon 9	Forward	GGTGCAAGCAGGTACATTCT	64,643,513	56	45
	Reverse	CCTGCTTATAGGCTCCTTAC	64,643,316		
exon 10	Forward	CTTGAAGGCTCACCTGTCCA	64,639,879	65	40
	Reverse	AGAGAGGTAAAGAGGCCTGC	64,639,730		
exon 11	Forward	TGGCTCAGTGCTCATCGAAA	64,638,283	65	40
	Reverse	ACTGAGATAAATGCTGGCCC	64,638,139		
TLR1	Forward	GATCTTTACCCGAATTGCGA	73,542,185	56	180
	Reverse	GGTGAACTGGAGAGCCTGAA	73,544,787		
TLR2	Forward	GGACAATGTCACGTGTTTTG	51,462,879	55	180
	Reverse	CGAATCTAGGATTTTATTGCTGT	51,465,245		
TLR6	Forward	CAACAACCCTTTGGGGAATA	73,520,672	55	180
	Reverse	TCTGCGTTATTGTTTTCAGCA	73,523,089		

Primer positions of NOD2, TLR1, TLR2, and TLR6 were represented by the numbers within the chromosome 2 (NC_006584.3), chromosome 3 (NC_006585.3), chromosome 15 (NC_006597.3), and chromosome 3 (NC_006585.3), respectively.

Table 14

Sequence primers used in mutational analysis of NOD2, TLR1, TLR2, and TLR6.

	Primer sequence (5'–3')		Primer position	Annealing (°C)
NOD2				
exon 1	Forward	GTAGATCCATGGCCTCTTCAG	64,667,386	60
	Internal Forward	TGAAATGTGCACACAGGAGG	64,667,228	60
	Internal Reverse	TAGTCTTCCCAGGAGAGGAC	64,667,094	60
	Reverse	CCCAAGACAGAAGTGCCTTAC	64,666,745	60
exon 2	Forward	ATCGGTAGAAACCTTCCCAC	64,659,352	60
	Reverse	TCCCTATAGCCTTTCTTCCC	64,659,107	60
exon 3	5' region Forward	ACCATCCTACCTCATTGGCC	64,656,695	60
	5' region Reverse	CAAAGCCATCGAAGGTTAAG	64,656,160	60
	Central region Forward	CTCTTTGAACACTGCTGTTG	64,656,259	60
	Central region Reverse	GCACGTTGTGGGCAGAAGCC	64,655,360	60
	3' region Forward	TGTGCTGCTACGTGTTCTCC	64,655,625	60
	3' region Reverse	CACTGCCCTGCGACACTCAC	64,654,822	60
exon 4	Forward	CAGATGCTGGCACATAGGGC	64,650,615	60
	Reverse	CATGACCTGGAGAGTGGCTG	64,650,357	60
exon 5	Forward	ACTGACTGCTGTGCTCTGTC	64,650,253	60
	Reverse	CTGCGCAGAAGACAGCTTCC	64,650,073	60
exon 6	Forward	TCACTGACTTGGTCTCCTGC	64,647,857	60
	Reverse	TGCCCCAGAATCCAACCTAC	64,647,722	60
exon 7	Forward	ACTGACCCAATTTGTTGGCC	64,645,919	60
	Reverse	CCAAACAGACCCAGAAGCTT	64,645,787	60
exon 8	Forward	ATCTCTGAGGACCCTCTGTG	64,645,094	60
	Reverse	TGTGAATCCCCCCAACTCAC	64,644,937	60
exon 9	Forward	GGTGCAAGCAGGTACATTCT	64,643,513	54
	Reverse	CCTGCTTATAGGCTCCTTAC	64,643,316	54
exon 10	Forward	CCTTGAAGCTCACCTGTCCA	64,639,879	60
	Reverse	AGAGAGGTAAAGAGGCCTGC	64,639,730	60
exon 11	Forward	TGGCTCAGTGCTCATCGAAA	64,638,283	60
	Reverse	ACTGAGATAAATGCTGGCCC	64,638,139	60
TLR1	Forward	GATCTTTACCCGAATTGCGA	73,542,185	60
	Internal Forward	ATGCATTCAATTTGCCACAA	73,543,192	60
	Internal Reverse	AATTTGAGATGGGCAAACCA	73,543,304	60
	Reverse	GGTGAACTGGAGAGCCTGAA	73,544,787	60
TLR2	Forward	GGACAATGTCACGTGTTTTG	51,462,879	60
	Internal Forward	CATTTGGACACTTTCCAC	51,463,582	60
	Internal Reverse	ATGTCAAAATCACCGAGCCC	51,463,776	60
	Reverse	GAATCTAGGATTTTATTGCTGT	51,465,245	60
TLR6	Forward	CAACAACCCTTTGGGGAATA	73,520,672	60
	Internal Forward	TGCACTTGGGTTGGGAGTAT	73,521,975	60
	Internal Reverse	CACCTTGACCTTGGGAGGTA	73,522,067	60
	Reverse	TCTGCGTTATTGTTTTCAGCA	73,523,089	60

Primer positions of NOD2, TLR1, TLR2, and TLR6 were represented by the numbers within the chromosome 2 (NC_006584.3), chromosome 3 (NC_006585.3), chromosome 15 (NC_006597.3), and chromosome 3 (NC_006585.3), respectively.

Table 15
Distribution of non-synonymous single nucleotide polymorphisms (SNPs) in pattern recognition receptor genes investigated in this study.

Group		ICRP-affected MD						Control MD					Laboratory Beagle					PRO-VEAN*	SIFT**
Case		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
SNP	Resultant amino acid																		
NOD2																			
C178T	R60C	–/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–1.789	0.04
A1532G	H511R	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+	–0.185	0.20
T1573C	W525R	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+	4.713	1.00
C1688G	T563S	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+	0.703	0.85
G1690A	V564M	+/–	–/–	–/–	+/–	–/–	–/–	+/–	+/+	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	–0.094	0.30
G1880A	R627H	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+	0.238	0.14
G1990A	A664T	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–1.164	0.41
G2564A	R855H	–/–	+/+	+/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/–	1.354	1.00
TLR1																			
G83T	S28I	+/–	+/+	–/–	–/–	+/–	+/+	+/–	+/+	–/–	+/–	+/+	+/–	+/–	+/+	+/+	–/–	–2.250	0.04
TLR2																			
G599A	S200N	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	1.178	0.00
G1117A	V373M	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	1.784	0.01
TLR6																			
C1369T	L457F	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	–/–	+/+	+/–	–/–	–3.849	0.00
G1537A	D513N	–/–	+/–	–/–	–/–	–/–	+/+	–/–	+/–	–/–	–/–	+/–	+/–	+/–	+/+	+/–	–/–	–1.976	0.08

Nucleotides identical to the database (NOD2; NM_001287039.1, TLR1; EU551145.1, TLR2; EU487534.1, and TLR6; NM_005618633.1, respectively) were represented as –.
*Score of < –2.500 was considered deleterious.
**Score of < 0.05 was considered deleterious.
ICRP, inflammatory colorectal polyp; MD, miniature dachshund.

Table 16

Distribution of synonymous SNPs in pattern recognition receptor genes investigated in this study.

Group	ICRP-affected MD						Control MD					Laboratory Beagle				
Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SNP																
NOD2																
A1869G	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+
C1992T	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+
C2121T	+/+	+/+	+/+	+/+	+/–	+/+	+/–	+/+	+/+	+/+	+/+	+/–	+/+	–/–	–/–	+/+
C2130T	+/–	+/–	+/+	+/–	+/–	+/+	–/–	–/–	+/+	+/+	–/–	+/–	+/–	–/–	–/–	+/+
C2235T	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
C2256T	+/–	+/–	+/+	–/–	+/–	+/–	–/–	–/–	+/–	+/+	–/–	–/–	–/–	–/–	–/–	+/+
C2427T	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
G2511A	–/–	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
T2544G	+/–	+/+	+/+	–/–	–/–	–/–	–/–	–/–	+/–	+/+	–/–	–/–	+/–	–/–	–/–	+/+
T2574C	+/+	+/+	+/+	+/+	–/–	–/–	+/–	+/+	–/–	+/+	+/+	–/–	–/–	–/–	–/–	+/+
C2595T	–/–	+/+	+/–	–/–	–/–	–/–	–/–	–/–	–/–	+/+	–/–	–/–	–/–	–/–	–/–	+/–
C2718T	–/–	–/–	–/–	+/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/–	+/–	+/–	+/–	+/–
TLR1																
T1662C	–/–	+/–	+/+	–/–	+/–	+/+	+/+	+/+	–/–	+/–	+/+	–/–	+/–	+/+	+/–	+/–
C1773T	–/–	+/–	+/+	–/–	+/–	+/+	+/+	+/+	–/–	+/–	+/+	–/–	+/–	+/+	+/–	+/–
TLR2																
T1371C	–/–	+/–	+/–	–/–	+/–	–/–	+/+	+/–	–/–	+/–	–/–	–/–	+/–	–/–	+/–	–/–
A2130G	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
TLR6																
G507A	–/–	–/–	+/+	–/–	–/–	+/–	–/–	–/–	–/–	–/–	+/–	–/–	–/–	+/–	–/–	+/–
A633C	–/–	+/–	+/+	–/–	+/–	+/+	+/+	+/+	–/–	+/–	+/+	–/–	+/–	+/+	+/–	+/–

Nucleotides identical to the database (NOD2; NM_001287039.1, TLR1; EU551145.1, TLR2; EU487534.1, and TLR6; NM_005618633.1, respectively) were represented as –.

Table 17

Disease phenotype of control MDs.

Diagnosis		Case
Neurological	Intervertebral disk disease	5
	Idiopathic epilepsy	1
Gastrointestinal	Cholelithiasis	2
Neoplasia	Complex mammary adenoma	1
Cardiovascular	Mitral valve regurgitation	3
	Dilated cardiomyopathy	2
Respiratory	Diaphragmatic hernia	1
Urogenital	Chronic renal failure	1
	Prostatic hypertrophy	1
Healthy*		65

*Dogs without any diagnosis or clinical signs were regarded as healthy.

Table 18

Breed distribution of other control breed dogs.

Breed	Case
Toy poodle	40
Mixed breed	36
Chihuahua	22
Shiba inu	15
Shetland Sheepdog	13
Yorkshire terrier	12
Golden Retriever	10
Miniature Schnauzer	10
Labrador Retriever	8
Shih Tzu	8
Beagle	6
French bulldog	6
Pug	5
Papillon	4
Border Collie	3
English Cocker Spaniel	3
Miniature Pinscher	3
Pomeranian	3
Pembroke Welsh Corgi	3
American Cocker Spaniel	2
German shepherd	2
Italian Greyhound	2
Jack Russell Terrier	2
Norwich Terrier	2
Pekinese	2
Cairn Terrier	1
Cavalier King Charles Spaniel	1
Chinese Crested Dog	1
Dalmatian	1
Great Pyrenees	1
Irish Setter	1
Irish Soft Coated Wheaten Terrier	1
Japanese Chin	1
Kai Ken	1
Maltese	1
Miniature poodle	1
Samoyed	1
Weimaraner	1
West Highland White Terrier	1
Wire-haired Fox Terrier	1

Table 19

Disease phenotype of other control breed dogs.

Diagnosis		Case
Neurological	Intervertebral disk disease	2
	Hydrocephalus	1
	Idiopathic vestibular dysfunction	1
Musculoskeletal	Medial patellar luxation	3
	Hip dysplasia	1
	Inguinal hernia	1
	Perineal hernia	1
	Radioulnar fracture	1
Gastrointestinal	Cholelithiasis	1
Neoplasia	Cutaneous histiocytoma	1
	Laryngeal tumor	1
	Lipomatosis	1
	Mammary gland tumor	1
	Meibomian gland adenoma	1
	Sebaceous adenoma	1
Cardiovascular	Mitral valve regurgitation	5
Respiratory	Laryngeal paralysis	1
Urogenital	Urolithiasis	6
	Cryptorchidism	4
	Chronic renal failure	1
	Hermaphroditism	1
	Prostatic hypertrophy	1
Ophthalmological	Dermoid	1
	Keratoconjunctivitis sicca	1
Other	Diabetes mellitus	1
	Hyperlipidemia	2
	Seborrhea	1
	Splenic Mass	1
Healthy*		193

*Dogs without any diagnosis or clinical signs were regarded as healthy.

Table 20

Association of six SNPs in NOD2 exon 3 with ICRPs in MDs.

SNP	Associated allele	Minor allele frequency		*P*-value
		Control MD	ICRP-affected MD	
A1532G	G	0.140	0.016	0.0002
T1573C	C	0.140	0.016	0.0002
C1688G	G	0.140	0.016	0.0002
G1690A	A	0.110	0.135	0.5144
G1880A	A	0.140	0.016	0.0002
G1990A	G	0.037	0.008	0.1151

Table 21

Association between four SNPs in NOD2 exon 3 and ICRPs in MDs.

NOD2 A1532G SNP association with ICRPs								
Model	Genotype	Control MD		ICRP-affected MD		OR (95% CI)	*P*-value	AIC	BIC
Codominant	G/G	60	(73.2%)	61	(96.8%)	1.00	0.0004	187.7	199.6
	A/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.46)			
	A/A	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Dominant	G/G	6	(73.2%)	61	(96.8%)	1.00	0.0001	185.9	194.8
	A/G-A/A	22	(26.8%)	2	(3.2%)	0.10 (0.02–0.43)			
Recessive	G/G-A/G	81	(98.8%)	63	(100%)	1.00	0.23	199.8	208.7
	A/A	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Overdominant	G/G-A/A	61	(74.4%)	61	(96.8%)	1.00	0.0002	187.3	196.2
	A/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.47)			
Log-additive	-					0.10 (0.02–0.45)	0.0001	185.7	194.6
NOD2 T1573C association with ICRPs								
Model	Genotype	Control MD		ICRP-affected MD		OR (95% CI)	*P*-value	AIC	BIC
Codominant	C/C	60	(73.2%)	61	(96.8%)	1.00	0.0004	187.7	199.6
	C/T	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.46)			
	T/T	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Dominant	C/C	6	(73.2%)	61	(96.8%)	1.00	0.0001	185.9	194.8
	C/T-T/T	22	(26.8%)	2	(3.2%)	0.10 (0.02–0.43)			
Recessive	C/C-C/T	81	(98.8%)	63	(100%)	1.00	0.23	199.8	208.7
	T/T	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Overdominant	C/C-T/T	61	(74.4%)	61	(96.8%)	1.00	0.0002	187.3	196.2
	C/T	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.47)			
Log-additive	-					0.10 (0.02–0.45)	0.0001	185.7	194.6
NOD2 C1688G SNP association with ICRPs								
Model	Genotype	Control MD		ICRP-affected MD		OR (95% CI)	*P*-value	AIC	BIC
Codominant	G/G	60	(73.2%)	61	(96.8%)	1.00	0.0004	187.7	199.6
	C/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.46)			
	C/C	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Dominant	G/G	6	(73.2%)	61	(96.8%)	1.00	0.0001	185.9	194.8
	C/G-C/C	22	(26.8%)	2	(3.2%)	0.10 (0.02–0.43)			
Recessive	G/G-C/G	81	(98.8%)	63	(100%)	1.00	0.23	199.8	208.7
	C/C	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Overdominant	G/G-C/C	61	(74.4%)	61	(96.8%)	1.00	0.0002	187.3	196.2
	C/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.47)			
Log-additive	-					0.10 (0.02–0.45)	0.0001	185.7	194.6
NOD2 G1880A SNP association with ICRPs								
Model	Genotype	Control MD		ICRP-affected MD		OR (95% CI)	*P*-value	AIC	BIC
Codominant	A/A	60	(73.2%)	61	(96.8%)	1.00	0.0004	187.7	199.6
	A/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.46)			
	G/G	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Dominant	A/A	6	(73.2%)	61	(96.8%)	1.00	0.0001	185.9	194.8
	A/G-G/G	22	(26.8%)	2	(3.2%)	0.10 (0.02–0.43)			
Recessive	A/A-A/G	81	(98.8%)	63	(100%)	1.00	0.23	199.8	208.7
	G/G	1	(1.2%)	0	(0%)	0.00 (0.00–NA)			
Overdominant	A/A-G/G	61	(74.4%)	61	(96.8%)	1.00	0.0002	187.3	196.2
	A/G	21	(25.6%)	2	(3.2%)	0.10 (0.02–0.47)			
Log-additive	-					0.10 (0.02–0.45)	0.0001	185.7	194.6

OR, odds ratio; CI, confidence interval; AIC, Akaike information criteria; BIC, Bayesian information criteria.

Table 22

Association of six SNPs in NOD2 exon 3 with canine breeds.

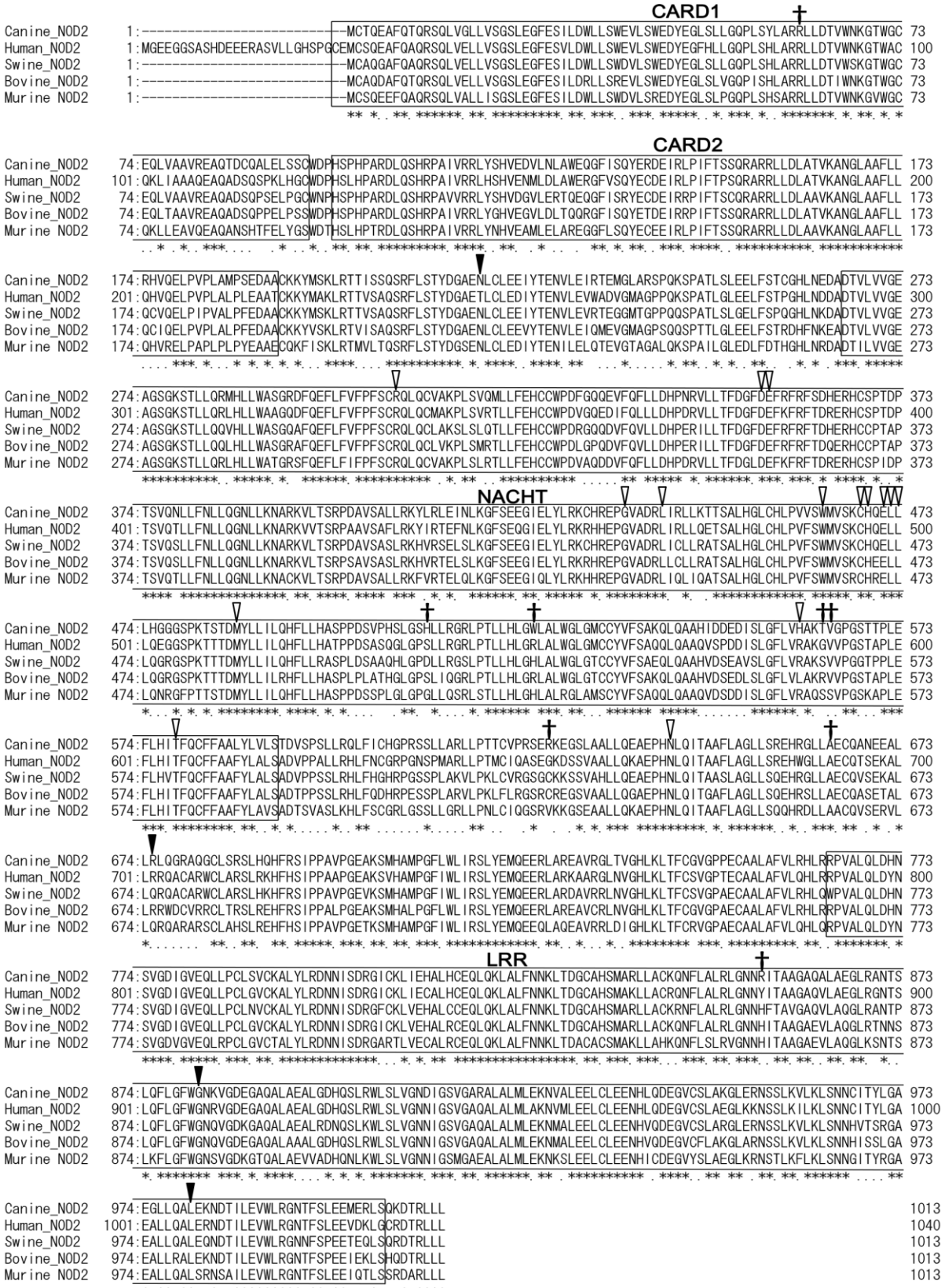
SNP	Associated allele	Minor allele frequency		*P*-value
		Control other breeds	Control MD	
A1532G	G	0.259	0.140	0.0017
T1573C	C	0.259	0.140	0.0017
C1688G	G	0.205	0.140	0.0689
G1690A	G	0.162	0.110	0.1023
G1880A	A	0.259	0.140	0.0017
G1990A	A	0.004	0.037	0.0013

Table 23

Minor allele frequencies of SNPs in NOD2 exon 3 in control dogs of predominant breeds.

Breed	Case	SNP					
		A1532G	T1573C	C1688G	G1690A	G1880A	G1990A
Miniature Dachshund	82	0.140	0.140	0.140	0.110	0.140	0.037
Toy poodle	40	0.213	0.213	0.213	0.100	0.213	0.000
Mixed breed	36	0.319	0.319	0.208	0.208	0.319	0.000
Chihuahua	22	0.114	0.114	0.091	0.227	0.114	0.045
Shiba inu	15	0.467	0.467	0.000	0.167	0.467	0.000
Shetland Sheepdog	13	0.000	0.000	0.000	0.462	0.000	0.000
Yorkshire terrier	12	0.167	0.167	0.167	0.208	0.167	0.000
Golden Retriever	10	0.100	0.100	0.100	0.000	0.100	0.000
Miniature Schnauzer	10	0.050	0.050	0.050	0.000	0.050	0.000

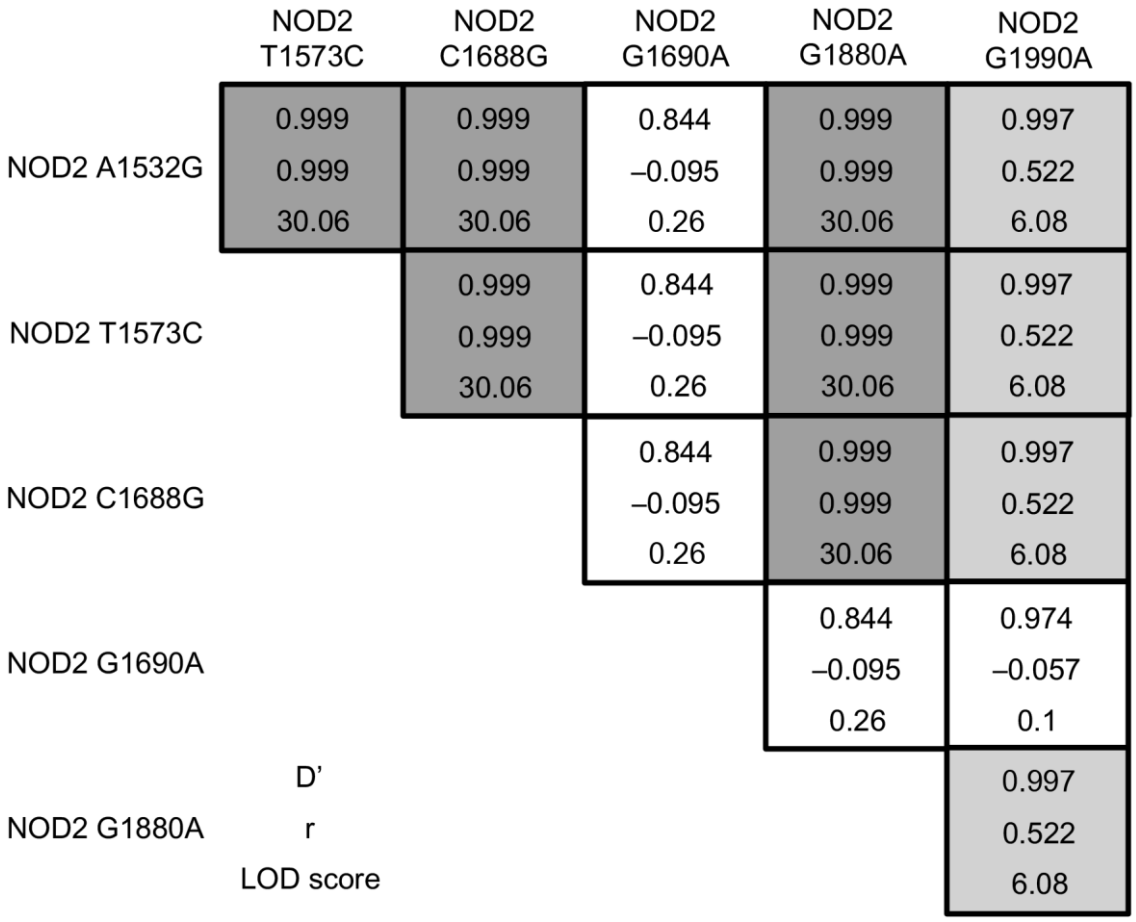
Figure 13


CARD1
CARD2
NACHT
LRR
Canine_NOD2
Human_NOD2
Swine_NOD2
Bovine_NOD2
Murine NOD2

Legend for Figure 13.

Comparison of the deduced amino acid sequence of canine nucleotide-binding oligomerization domain 2 (NOD2) with those of human (GenBank accession number: AAG33677.1), pig (NP_001098765.1), cattle (NP_001002889.1), and mouse (NP_665856.2) homologs. Asterisks and colons indicate identical and conserved amino acids, respectively. Each structural domain of NOD2 protein is boxed with a solid line (data obtained from http://www.uniprot.org/uniprot/Q9HC29). Daggers indicate the site of single nucleotide polymorphisms (SNPs) in the present study (R60C, H511R, W525R, T563S, V564M, R627H, A664T, and R855H; see Table 15). Black arrows represent the site of SNPs associated with Crohn's disease in humans (T245M, R702W, G908R, and L1007fsinsC). White arrows represent the site of SNPs associated with Blau syndrome in humans (R334W/R334Q, D382E, E383K/E383G, G464W, L469F, W490L, C495Y, H496L, E498_L500delinsV, M513R/M513T, R587C, T605N, and N670K). CARD, caspase recruitment domain; NACHT, neuronal apoptosis inhibitor proteins (NAIPs), class II transactivator (CIITA), incompatibility locus protein from *Podospora anserine* (HET-E), and telomerase-associated protein (TP-1); LRR, leucine-rich repeat.

Figure 14



Linkage disequilibrium of single nucleotide polymorphisms SNPs in NOD2 gene exon 3 in Miniature Dachshunds. A1532G, T1573C, C1688G, and G1880A showed complete linkage disequilibrium. There was also evidence of linkage disequilibrium between these four SNPs and G1990A. LOD, logarithm of odds.

Chapter 2

Analyses on the fecal microbiota in Miniature Dachshunds with inflammatory colorectal polyps

Chapter 2-1

Fecal dysbiosis in Miniature Dachshunds with inflammatory colorectal polyps

Abstract

The gastrointestinal microbiota plays an important role in the etiology of various gastrointestinal disorders including inflammatory bowel disease (IBD). Inflammatory colorectal polyp (ICRP) in Miniature Dachshunds (MDs) is thought to be a novel form of canine IBD, but the involvement of gut microbiota in the pathogenesis of ICRP is unknown. The objective of this Chapter was to compare the fecal microbiota in ICRP-affected MDs with that of healthy MDs. High-throughput sequencing of amplicons derived from the V3–V4 region of the 16S rRNA gene was applied to characterize fecal microbiota of six ICRP-affected MDs and 12 healthy MDs using the Illumina MiSeq system. In addition, paired samples were obtained from five MDs with ICRPs during clinical remission. Principal coordinates analysis of unweighted UniFrac distances revealed that fecal microbiota of ICRP-affected MDs was significantly altered compared with that of healthy MDs (ANOSIM; R = 0.302, P = 0.003). Proportions of Fusobacteriaceae, Helicobacteraceae, Porphyromonadaceae, and Turicibacteraceae were significantly more abundant in ICRP-affected MDs, while those of Lachnospiraceae were significantly less abundant in ICRP-affected MDs compared with healthy MDs. Furthermore, the microbiota of ICRP-affected MDs tended to change in composition to be similar to that of healthy MDs when clinical remission was achieved. These results suggest that the microbiota play a role in the development of ICRPs and have a potential as a therapeutic target though further investigations are needed.

1. Introduction

The pathogenesis of human and canine IBD is multifactorial, and consists of aberrant interactions between luminal antigens and mucosal immunity, which lead to the development of intestinal inflammation (Cerquetella et al., 2010; Wallace et al., 2014; Xavier and Podolsky, 2007). Gut microbiota play a crucial role in the maintenance of gastrointestinal health in humans, as well as in dogs (Hooda et al., 2012; Hooper et al., 2001; Wallace et al., 2014). Gut microbiota are a component of the mucosal barrier that defends against pathogen invasion, induce mucosal immune responses, support digestion, and provide nutritional support including short chain fatty acids (SCFAs) for enterocytes (Hooda et al., 2012; Kanauchi et al., 2005; Suchodolski, 2011). To date, many molecular studies have revealed the dysbiosis in the gastrointestinal tract in human IBD, predominantly with the decrease of Clostridiales including *Faecalibacterium prausnitzii* and *Clostridium* clusters XIVa and IV, and the increase of Proteobacteria (Frank et al., 2007; Packey and Sartor, 2009; Sokol et al., 2008). Similarly, the dysbiosis and the proportional changes of these bacterial groups have also been described in canine IBD patients (Deng and Swanson, 2014; Honneffer et al., 2014).

Since the large intestine has the highest density and diversity of bacteria (Hooda et al., 2012), it is thought that the luminal microbiota play an important role in the pathogenesis of inflammatory colorectal polyps (ICRPs). However, no studies have investigated the composition of microbiota in Miniature Dachshunds (MDs) with ICRPs, although several have revealed disturbed mucosal immune condition at the sites of polypoid lesions, such as the expression of proinflammatory cytokines (Tamura et al., 2013), CD4+ T cell cytokines (Ohta et al., 2013), and pattern recognition receptors as described in Chapter 1-1. Therefore, the study of Chapter 2-1 was performed to test the hypothesis that the fecal

dysbiosis is associated with the disease status of ICRPs in MDs.

2. Materials and methods

2.1. Animals and sample collection

All procedures were conducted according to the animal experimentation guidelines of the Animal Care Committee of the University of Tokyo, and written informed consent was obtained from the owners of each dog.

MDs that were referred to the Veterinary Medical Center of the University of Tokyo for investigation of chronic hematochezia and/or tenesmus and diagnosed as ICRPs between July 2011 and November 2012 were included in this study (active-ICRP group). The diagnosis of ICRP were determined based on the colonoscopic and histopathological findings as characterized in a previous study (Ohmi et al., 2012). MDs that had received antibiotic treatment within 4 weeks of the clinical examination were excluded from this study. As healthy controls, 12 MDs were also recruited (healthy group), which had no clinical signs of gastrointestinal disease, and showed no abnormalities as determined by fecal examination and rectal palpation. All ICRP-affected and healthy MDs enrolled were privately owned in diverse environments. None of the control MDs received antibiotic treatment within the 3 months leading up to the study. Naturally passed feces were collected from each dog and frozen within a few hours of defecation at –80°C where they were stored until further analysis. In addition, paired samples were obtained from five MDs with ICRPs during clinical remission based on clinical responses (i.e., resolution of hematochezia and tenesmus) and endoscopic evaluation (controlled-ICRP group). The time between fecal sample collections ranged from 44 to 494 days. Detailed descriptions of all samples taken from dogs enrolled in the present study are listed in Table 24.

2.2. DNA extraction

Fecal samples (20 mg) were suspended in 450-µl extraction buffer (100 mM Tris/HCl, 40 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0), and 50-µl 10% SDS. Glass beads (300 mg, 0.1 mm diameter) and 500-µl buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 30 s using a FastPrep FP 100A (MP Biomedicals, LLC, Santa Ana, CA, USA) at a power level of five. After centrifugation at 14,000 × *g* for 5 min, 400-µl of the supernatant was extracted using phenol/chloroform, and 250-µl of supernatant was precipitated with propan-2-ol. Purified DNA was washed with 300-µl 70% ethanol, and then suspended in 200-µl Tris/EDTA buffer (pH 8.0).

2.3. 16S rRNA gene sequencing

The V3–V4 region of the bacterial 16S rRNA gene was amplified using TaKaRa Ex Taq HS (Takara Bio Inc., Shiga, Japan) using the following primer sequences: Tru357F (Forward, 5′-CGCTCTTCCGATCTCTGTACGGRAGGCAGCAG-3′) and Tru806R (Reverse, 5′-CGCTCTTCCGATCTGACGGACTACHVGGGTWTCTAAT-3′) under the following cycling conditions: 94°C for 3 min, 25 cycles of PCR (94°C for 30 s, 50°C for 1 min, and 72°C for 1 min), and a final elongation step of 72°C for 10 min. Subsequently, dual barcoded amplicons were generated using TaKaRa Ex Taq HS (Takara Bio Inc.) with fusion barcoded primers depicted in Table 25 under the amplification conditions as follows: 94°C for 3 min, 15 cycles of PCR (94°C for 30 s, 50°C for 1 min, and 72°C for 1 min), and a final elongation step of 72°C for 10 min. The amplicons were pooled at equimolar concentrations and sequenced with an Illumina MiSeq platform using MiSeq Reagent Kit v2 (Illumina, Inc., San Diego, CA, USA).

Raw 250-bp paired-end sequence reads were combined using the script fastq-join (ea-utils-1.1.2-301.x86_64.rtp: https://code.google.com/p/ea-utils/downloads/list) with the default settings. Further data processing included filtering and denoising by clustering similar sequences with less than 3% dissimilarity using USEARCH v5.2.32 (http://drive5.com/usearch/) (Edgar, 2010), and *de novo* chimera detection and removal in UCHIME (http://drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011). 16S rRNA operational taxonomic units (OTUs) were selected from the combined reads using a de-novo OTU picking protocol clustered at 97% identity through the Quantitative Insights Into Microbial Ecology (QIIME) pipeline software version 1.6.0 (http://qiime.org) (Caporaso et al., 2012), with USEARCH against the Greengenes database (http://greengenes.secondgenome.com/downloads/database/12_10; Oct. 2012 release). The representative sequences for each OTU were compared with those in the Greengenes database for taxonomy assignment. To account for unequal sequencing depth across samples, subsequent analyses were performed on a randomly selected subset of 56,270 sequences per sample.

2.4. Statistical analysis

To estimate the bacterial diversity in each sample, three indices—number of OTUs, Shannon index, and Chao1—were calculated, and rarefraction curves were depicted using QIIME (Chao, 1987; Shannon, 1948). Differences in microbial communities among samples were investigated using a phylogeny-based unweighted UniFrac distance matrix, which was calculated using the Greengenes reference tree. Principal coordinates analysis (PCoA) was performed using QIIME. Differences in microbiota composition between groups were tested using the one-way analysis of similarity (ANOSIM) function in the

statistical software package PRIMER 6 (PRIMER-E Ltd., Luton, UK).

Differences in age, gender, bacterial diversity indices, and the proportions of bacterial taxa between the groups were determined using Kruskal–Wallis test with Dunn's post hoc test or Chi-squared test where appropriate (JMP Pro version 10.0.2, SAS Institute, Cary, NC, USA). Only bacterial taxa that were present in at least 50% of dogs (either active-ICRP, controlled-ICRP, or healthy) were included in the analysis. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Animals

Six ICRP-affected MDs were included in the present study. The median age of dogs with ICRPs at initial diagnosis was 117.5 months (range, 68–144 months) and consisted of two intact females and four neutered males. Their median body weight was 6.35 kg (range, 4.80–7.40 kg). Three out of six MDs with ICRPs had received corticosteroid and/or cyclosporine treatment prior to initial diagnosis, while the other three dogs had not received any medication. In addition, with the exception of one ICRP-affected MD who received probiotics (MitoMax SUPER; Imagilin technology LLC, MD, USA) and was withdrawn 2 weeks prior to the initial sample collection (Active-ICRP 3 in Table 24), no dogs had received any probiotics during the 4 weeks leading up to the clinical examination.

The median age of control dogs was 84 months (range, 60–168 months) with six females (one intact and five neutered) and six males (four intact and two neutered). Their median body weight was 6.80 kg (range, 3.90–7.50 kg). No significant difference in age gender, or body weight was observed (Table 26).

3.2. Sequence analysis

A total of 2,165,353 sequence reads (mean ± SD, 94,146 ± 19,498 reads/sample) were analyzed across all fecal samples. Sequences were classified into ten bacterial phyla across all samples. The major bacterial phyla were Firmicutes (70.6% of all sequences), Bacteroidetes (14.2%), Fusobacteria (10.0%), Proteobacteria (2.6%), and Actinobacteria (2.0%). The phyla Deferribacteres, Spirochaetes, Tenericutes, TM7, and Verrucomicrobia each accounted for <0.1% of all obtained sequencing tags (Figure 15).

3.3. Comparison of fecal microbiota between controls and ICRP-affected MDs

Rarefraction curves of each group indicated a relatively good depth of coverage, with an initial steep increase of identified OTUs per read for each sample, and subsequent leveling of the curve by approximately 40,000 reads (Figure 16). The Chao1 scores of MDs with active-ICRPs were significantly higher than the scores of healthy MDs, while other bacterial indices showed no significant differences between the groups (Table 26).

PCoA plots (Figure 17) revealed a significant difference between active-ICRP and healthy dogs (ANOSIM; global R = 0.166, $P = 0.031$; active-ICRP vs. healthy, R = 0.302, $P = 0.003$). In contrast, no significant difference was observed between active-ICRP and controlled-ICRP (ANOSIM; R = 0.059, $P = 0.340$), or controlled-ICRP and healthy dogs (ANOSIM; R = 0.067, $P = 0.331$).

The relative proportions of the predominant bacterial taxa at the phylum level are shown in Table 27, and those at the class, order, family, or genus level are shown in Tables 28–31, respectively. The proportion of Firmicutes was significantly decreased in MDs with active-ICRPs compared with that in MDs with controlled-ICRPs and healthy controls ($P = 0.0413$ and 0.0100, respectively; Figure 15 and Table 27). In contrast, levels of bacteria of the phyla Actinobacteria and Fusobacteria were significantly increased in MDs with active ICRPs compared with healthy controls ($P = 0.0465$ and 0.0028, respectively; Figure 15 and Table 27).

Within the phylum Actinobacteria, the major difference was observed in the family Bifidobacteriaceae (genus *Bifidobacterium*), which were present at higher levels in the group with active ICRP ($P = 0.056$), although there was no difference in taxa at the family level. Within Firmicutes; Lachnospiraceae, belonging to the class Clostridiales, was the

most predominant family and was significantly decreased in MDs with active-ICRPs compared with healthy MDs (P = 0.0406; Table 30). Within the phylum Fusobacteria, all taxa belonged to Fusobacteriaceae and were significantly increased in MDs that had active-ICRPs compared with healthy MDs (P = 0.0028; Table 30).

4. Discussion

In this Chapter, I evaluated differences between the fecal microbiome of ICRP-affected MDs and healthy MDs, and found that there are significant alterations in microbiota composition in the diseased group. These results suggest that the gastrointestinal microbiota is involved in the pathogenesis of ICRPs in MDs. Alterations in some bacterial taxa were consistent with those observed in human and/or canine IBD.

The proportion of Firmicutes, mainly Lachnospiraceae family, was significantly decreased in MDs with active-ICRPs. Lachnospiraceae is one of the most abundant autochthonous bacterial families in human, mouse, and canine colonic mucosa (Hooda et al., 2012; Nava and Stappenbeck, 2011). This family is a member of Clostridium clusters IV or XIVa which is an important SCFA producer, and decreased level of Lachnospiraceae has been reported in human and canine IBD (Collins et al., 1994; Packey and Sartor, 2009; Suchodolski et al., 2012a, 2010). SCFAs have anti-inflammatory properties *in vitro* and *in vivo* (Cook and Sellin, 1998; Furusawa et al., 2013; Hamer et al., 2008; Tedelind et al., 2007). Recently, Furusawa et al. revealed that SCFAs, especially butyrate, induce the differentiation of colonic regulatory T cells through enhanced histone H3 acetylation in the promoter and conserved non-coding sequence region of the FOXP3 gene (Furusawa et al., 2013). Since the reduction of SCFA-producing bacteria and insufficiency of SCFAs have been implicated in the pathogenesis of human IBD (Frank et al., 2007; Huda-fanjan et al., 2010; Takaishi et al., 2008), the reduction of SCFAs might be involved in the development of ICRPs. Further analyses of the association between luminal SCFA concentrations or disease status of ICRPs and the reduction of Lachnospiraceae are warranted.

The proportion of Fusobacteria, mainly Fusobacteriaceae family, was significantly

increased in MDs with active-ICRPs. A similar increase of Fusobacteria has been reported in dogs with acute hemorrhagic diarrhea, whereas no increase was observed in dogs with non-hemorrhagic diarrhea (Suchodolski et al., 2012b). Since MDs with ICRPs commonly present chronic hematochezia, this increase might be due to the contamination of the feces with blood. Comparison of the Fusobacteria composition between patients with and without hematochezia in various gastrointestinal disorders may confirm the theory. However, it is difficult to determine how the increased level of Fusobacteriaceae may interfere with mucosal inflammation, since the Fusobacteriaceae had consist of heterogeneous species with different pathogenicities (Allen-Vercoe et al., 2011). Interestingly, *Fusobacterium nucleatum* and other *Fusobacterium* species have been associated with IBD and the development of colorectal cancer in humans (Allen-Vercoe et al., 2011; Tahara et al., 2014). ICRPs in MDs occasionally show neoplastic progression (Igarashi et al., 2013); therefore, it is possible that the presence of Fusobacteriaceae might play a role in tumorigenesis. Further investigations are needed to determine which species are associated with the development of ICRPs in MDs.

Several contradicting changes were observed in the present study. The significant increase in the phylum Actinobacteria, predominantly of *Bifidobacterium*, was observed in MDs with active-ICRPs. Since *Bifidobacterium* can lower intestinal pH by increasing fermentation products and by modulating the intestinal immune system (Jiang and Savaiano, 1997; Tanabe et al., 2008), it is commonly prescribed as a probiotic in both human and veterinary medicine (Chrzastowska et al., 2009; Vieira et al., 2013). Furthermore, one bacterial diversity index (i.e., Chao1) was significantly increased in MDs with active-ICRPs compared with that in healthy controls. The reason for this was unclear, because bacterial diversity is commonly decreased in some gastrointestinal

disorders including human and canine IBD (Suchodolski et al., 2012b; Walker et al., 2011). Several limitations of the present study, including dietary differences, the use of medication prior to sampling, or the small number of dogs used might explain these contradictions. Dietary fiber is beneficial for gastrointestinal health and affects the gastrointestinal microbiota (Galvez et al., 2005; Hooda et al., 2012; Viladomiu et al., 2013). As shown in Table 24, most dogs were fed a general diet, but two ICRP-affected MDs (ICRP-4 and ICRP-6) were fed with a commercial high-fiber diet (Hill's prescription diet w/d and r/d, Hill's Pet Nutrition, Inc., Kansas, USA). However, several studies have reported that supplementation with dietary fiber generally induced a significant increase in Firmicutes and significant decreases in Actinobacteria and Fusobacteria, whereas it did not significantly affect to bacterial diversity indices (Middelbos et al., 2010; Panasevich et al., 2014); which is not consistent with the findings of the present study. In addition, each dog remained on the high-fiber diet until clinical remission was confirmed. Therefore, it is unlikely that the dietary fiber content affected the present results. On the other hand, three out of six MDs with active-ICRPs received prednisolone and/or cyclosporine. Although there were significant difference in the proportions of some minor bacterial taxa including Desulfovibrionaceae and Peptococcaceae between MDs with active-ICRPs received immunosuppressive agents and those without immunosuppression (see Table 32), the bacterial composition was not significantly different (ANOSIM: R = 0.000, P = 0.600). In addition, each dog received similar immunomodulatory therapy throughout the study period (Table 24). Furthermore, prednisolone administration have been reported to have no direct effect on fecal microbiota (Igarashi et al., 2014). Thus, previous use of prednisolone does not seem to have affected the present result. The fact that one ICRP-affected MD ceased taking

probiotics 2 weeks before recruitment might have affected the results. Garcia-Mazcorro et al. reported that oral administration of multi-species symbiotic did show the increase of probiotic bacteria in the feces, whereas did not alter the composition of major bacterial phyla or bacterial diversity (Garcia-Mazcorro et al., 2011). The fecal content of *Bifidobacterium* in the MD that had received a probiotic, only accounted for 0.07% in the present study (ICRP-3 in Tables 24 and 32). Therefore, I do not believe that would have interfered with the present results. As large inter-individual and intra-individual temporal variations in fecal microbiota have been reported (Garcia-Mazcorro et al., 2012), a larger sample size of dogs should be investigated to determine whether the observed increase of *Bifidobacterium* and bacterial diversity are consistent and of clinical significance.

Interestingly, the bacterial composition in the feces from MDs with controlled-ICRPs was intermediate between that from MDs with active-ICRPs and that from healthy MDs. Although the cause and effect relationship between microbiota and mucosal inflammation is not well clarified in human and canine IBD, nor in the present study, these results suggest that the microbiota may be important in the management of ICRPs in MDs. To date, immunosuppressive therapy, endoscopic polypectomy, argon plasma coagulation, and/or surgical excision by the rectal pull-through technique have been performed to treat ICRPs in MDs (Ohmi et al., 2012; Tsukamoto et al., 2012). However, the clinical efficacy of antibiotics, pre- or probiotics, and food therapy has not been investigated in this disease. Further investigations evaluating their effects on the microbiota and clinical outcome would determine the usefulness of this therapy and help to establish appropriate therapeutic protocols for the treatment of ICRPs in MDs. A possible limitation for this finding was the lack of clinical scoring system, which would provide more information of the association between specific bacterial taxa and disease status.

In conclusion, I have shown that the composition of the fecal microbiota in MDs with active-ICRPs was significantly different from that in healthy MDs. Furthermore, my study also revealed that the microbiota of ICRP-affected MDs tended to change in composition to be similar to that of healthy MDs when clinical remission was achieved with immunosuppressive therapy. These results suggest that the microbiota play a key role in the development of ICRPs and may have a potential use as a therapeutic target. Since limited number of ICRP-affected MDs were included, the findings observed in this Chapter are preliminary; thus further follow-up study should be performed. Further studies preparing a gnotobiotic mouse as an ICRP model, and the evaluation of fecal SCFA concentrations might confirm the association of microbiota in the pathogenesis of ICRPs in MDs.

Table 24
Baseline characteristics of miniature dachshunds enrolled in Chapter 2-1.

Disease status	Age (year)	Sex*	Diet	Received drugs prior to 4 weeks	Days after initial sample collection
Active-ICRP 1	9	mn	Royal canin selected protein	None	-
Active-ICRP 2	11	mn	Hill's Science diet adult	prednisolone, cyclosporine	-
Active-ICRP 3	12	f	Hill's Science diet adult	none	-
Active-ICRP 4	10.6	mn	Hill's Prescription w/d	none	-
Active-ICRP 5	5.7	f	Novartis Dr.'s diet senior	prednisolone	-
Active-ICRP 6	9	mn	Hill's Prescription r/d	prednisolone	-
Controlled-ICRP 1	9.2	mn	Royal canin selected protein	prednisolone	68
Controlled-ICRP 2	12.3	mn	Hill's Science diet adult	prednisolone, cyclosporine	494
Controlled-ICRP 4	10.8	mn	Hill's Prescription w/d	prednisolone, famotidine	51
Controlled-ICRP 5	5.8	f	Novartis Dr.'s diet senior	prednisolone, tranexamic acid, ursodeoxycholic acid	44
Controlled-ICRP 6	9.6	mn	Hill's Prescription w/d	cyclosporine	231
Healthy 1	6	m	Unknown	none	-
Healthy 2	6	m	Unknown	none	-
Healthy 3	11	m	Royal canin hypo hydrolyzed protein	none	-
Healthy 4	5	fs	Unknown	none	-
Healthy 5	5	f	Unknown	none	-
Healthy 6	12	fs	Unknown	none	-
Healthy 7	8	m	Hill's Prescription d/d	none	-
Healthy 8	10	fs	Unknown	none	-
Healthy 9	7	fs	Unknown	none	-
Healthy 10	16	mn	Eukanuba lamb and rice	none	-
Healthy 11	7	mn	Hill's Science diet Pro light	none	-
Healthy 12	7	fs	Hill's Science diet senior	none	-

*m = male intact; f = female intact; mn = male neutered; fs = female spayed.
ICRP, inflammatory colorectal polyp.

Table 25
Fusion barcoded primers used for the present study.

Sample	Direction	Primer sequence (5'–3')
Active-ICRP 1	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATATTACTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Active-ICRP 2	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCCGGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Active-ICRP 3	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCGCTCATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Active-ICRP 4	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATATTCAGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Active-ICRP 5	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Active-ICRP 6	Forward	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Controlled-ICRP 1	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCTGAAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Controlled-ICRP 2	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTAATGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Controlled-ICRP 4	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCGGCTATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Controlled-ICRP 5	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCCGCGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Controlled-ICRP 6	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCTCGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC

Table 25
Cont.

Sample	Direction	Primer sequence (5'–3')
Healthy 1	Forward	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATAGCGATAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 2	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATATTACTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 3	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCCGGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 4	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCGCTCATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 5	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATGAGATTCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 6	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATATTCAGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 7	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATGAATTCGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 8	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCTGAAGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 9	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTAATGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 10	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATCGGCTATGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 11	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCCGCGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
Healthy 12	Forward	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
	Reverse	CAAGCAGAAGACGGCATACGAGATTCTCGCGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC

Table 26

Characteristics and bacterial diversity indices of dogs enrolled in Chapter 2-1.

	Active-ICRP	Controlled-ICRP	Healthy	*P*-value*
Sex				
male	4	4	6	0.301
female	2	1	6	
Age (months)	117.5 (68–144)	115 (70–148)	84 (60–168)	0.638
Body weight (kg)	6.35 (4.80–7.40)	5.95 (4.75–6.70)	6.80 (3.90–7.50)	0.606
Bacterial diversity indices				
OTU	2119 (1431–2726)	2026 (1711–2138)	1597 (1330–2430)	0.0610
Shannon Index	6.88 (5.33–7.65)	6.68 (6.04–7.07)	6.00 (4.89–7.58)	0.349
Chao1	3133[a] (2162–3951)	2929[a,b] (2529–3274)	2385[b] (2036–3901)	**0.0312**

Age, body weight, and bacterial diversity indices are represented by the median value (range).

Medians not sharing a common superscript are significantly different (Dunn's multiple comparison, $P < 0.05$).

*Kruskal–Wallis test or Chi–square test. Statistically significant values ($P < 0.05$) are highlighted in bold.

ICRP, inflammatory colorectal polyp; OTU, operational taxonomic unit.

Table 27

Relative proportions of the predominant bacterial phyla.

	Medians % (min.–max. %) of sequences*			
	Active-ICRP	Controlled-ICRP	Healthy	*P*-value**
Actinobacteria	4.50[a] (0.08–20.42)	0.06[a,b] (0.04–0.25)	0.07[b] (0.01–1.76)	**0.041**
Bacteroidetes	15.52 (5.34–36.45)	6.40 (0.37–38.90)	8.50 (0.63–38.05)	0.441
Firmicutes	43.26[a] (27.68–60.32)	89.20[b] (28.48–98.85)	82.68[b] (57.72–98.58)	**0.007**
Fusobacteria	25.59[a] (14.68–33.74)	3.67[a,b] (0.27–27.77)	0.97[b] (0.13–13.24)	**0.004**
Proteobacteria	3.83 (0.74–14.63)	1.87(0.23–3.76)	0.61 (0.13–6.06)	0.053
Tenericutes	0.00 (0.00–0.74)	0.00 (0.00–0.02)	0.00 (0.00–0.02)	0.067
TM7	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.410

Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Medians not sharing a common superscript are significantly different (Dunn's multiple comparison, $P < 0.05$).

**Kruskal–Wallis test. Statistically significant values ($P < 0.05$) are highlighted in bold.

Table 28

Relative proportions of the predominant bacterial classes.

	Medians % (min.–max. %) of sequences*			
	Active-ICRP	Controlled-ICRP	Healthy	*P*-value**
Actinobacteria	4.50[a] (0.08–20.42)	0.06[a,b] (0.03–0.25)	0.07[b] (0.01–1.75)	**0.041**
Bacilli	2.40 (0.50–33.69)	7.78 (0.96–83.05)	1.49 (0.37–72.91)	0.291
Bacteroidia	15.52 (5.34–36.45)	6.40 (0.37–38.90)	8.50 (0.63–38.05)	0.441
Betaproteobacteria	0.72 (0.03–5.47)	0.23 (0.01–1.83)	0.06 (0.01–1.19)	0.107
Clostridia	33.01 (25.33–54.12)	27.31 (5.32–77.43)	65.90 (23.12–88.96)	0.055
Deltaproteobacteria	0.00 (0.00–0.07)	0.01 (0.00–0.34)	0.00 (0.00–0.01)	0.093
Epsilonproteobacteria	0.61[a] (0.17–8.93)	0.17[a,b] (0.02–1.30)	0.02[b] (0.00–0.78)	**0.004**
Erysipelotrichi	0.36 (0.20–0.93)	1.51 (0.03–3.70)	0.45 (0.05–5.99)	0.751
Fusobacteria	25.59[a] (14.68–33.74)	3.67[a,b] (0.27–27.77)	0.97[b] (0.13–13.24)	**0.004**
Gammaproteobacteria	1.38 (0.05–7.23)	0.29 (0.04–2.20)	0.21 (0.04–4.56)	0.616
Mollicutes	0.00 (0.00–0.74)	0.00 (0.00–0.02)	0.00 (0.00–0.02)	0.067
Nitriliruptoria	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.189
TM7-3	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.410

Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Medians not sharing a common superscript are significantly different (Dunn's multiple comparison, $P < 0.05$).

**Kruskal–Wallis test. Statistically significant values ($P < 0.05$) are highlighted in bold.

Table 29

Relative proportions of the predominant bacterial orders.

	Medians % (min.–max. %) of sequences*			
	Active-ICRP	Controlled-ICRP	Healthy	*P*-value**
Actinomycetales	0.04 (0.00–1.54)	0.01 (0.00–0.13)	0.02 (0.00–0.10)	0.792
Aeromonadales	0.00 (0.00–0.62)	0.01 (0.00–0.24)	0.00 (0.00–3.96)	0.501
Anaeroplasmatales	0.00 (0.00–0.74)	0.00 (0.00–0.02)	0.00 (0.00–0.02)	0.067
Bacillales	0.00 (0.00–1.74)	0.00 (0.00–0.01)	0.00 (0.00–0.05)	0.736
Bacteroidales	15.52 (5.34–36.45)	6.40 (0.37–38.90)	8.50 (0.63–38.05)	0.441
Bifidobacteriales	3.74 (0.04–20.41)	0.06 (0.03–0.15)	0.03 (0.00–1.72)	0.056
Burkholderiales	0.69 (0.02–5.40)	0.22 (0.01–1.79)	0.06 (0.01–1.03)	0.159
Campylobacterales	0.61[a] (0.17–8.93)	0.17[a,b] (0.02–1.30)	0.02[b] (0.00–0.78)	**0.004**
Clostridiales	32.30 (23.80–53.59)	26.82 (5.20–76.12)	65.22 (22.72–88.78)	0.055
Coriobacteriales	0.07 (0.00–1.04)	0.00 (0.00–0.02)	0.00 (0.00–0.55)	0.309
Desulfovibrionales	0.00 (0.00–0.07)	0.01 (0.00–0.34)	0.00 (0.00–0.01)	0.093
Enterobacteriales	0.92 (0.04–6.48)	0.14 (0.01–2.19)	0.13 (0.03–4.53)	0.377
Erysipelotrichales	0.36 (0.20–0.93)	1.51 (0.03–3.70)	0.45 (0.05–5.99)	0.751
Euzebyales	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.189
Fusobacteriales	25.59[a] (14.68–33.74)	3.67[a,b] (0.27–27.77)	0.97[b] (0.13–13.24)	**0.004**
Gemellales	0.01 (0.00–0.13)	0.00 (0.00–0.08)	0.00 (0.00–0.02)	0.818
Lactobacillales	1.15 (0.35–33.43)	7.71 (0.17–69.80)	0.90 (0.36–72.90)	0.714
Pasteurellales	0.07 (0.00–0.74)	0.00 (0.00–0.15)	0.00 (0.00–0.01)	0.762
Pseudomonadales	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.02)	0.415
Turicibacterales	0.14[a] (0.05–6.89)	0.06[a] (0.05–23.92)	0.02[b] (0.00–1.09)	**0.002**

Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Medians not sharing a common superscript are significantly different (Dunn's multiple comparison, $P < 0.05$).

**Kruskal–Wallis test. Statistically significant values ($P < 0.05$) are highlighted in bold.

Table 30

Relative proportions of the predominant bacterial families.

	Medians % (min.–max. %) of sequences*			
	Active-ICRP	Controlled-ICRP	Healthy	*P*-value**
Actinomycetaceae	0.02 (0.00–1.51)	0.00 (0.00–0.01)	0.01 (0.00–0.05)	0.272
Alcaligenaceae	0.67 (0.02–5.29)	0.22 (0.01–1.76)	0.06 (0.01–1.02)	0.159
Anaeroplasmataceae	0.00 (0.00–0.74)	0.00 (0.00–0.02)	0.00 (0.00–0.02)	0.067
Bacteroidaceae	11.52 (5.19–36.17)	1.87 (0.28–38.57)	8.34 (0.26–37.98)	0.296
Bifidobacteriaceae	3.74 (0.04–20.39)	0.06 (0.03–0.15)	0.03 (0.00–1.72)	0.056
Campylobacteraceae	0.00 (0.00–0.18)	0.00 (0.00–0.26)	0.00 (0.00–0.12)	0.278
Clostridiaceae	10.97 (0.90–19.78)	10.86 (0.72–28.87)	5.05 (0.33–54.87)	0.761
Coprobacillaceae	0.28 (0.12–0.42)	0.12 (0.02–3.67)	0.31 (0.02–2.84)	0.518
Coriobacteriaceae	0.07 (0.00–1.04)	0.00 (0.00–0.02)	0.00 (0.00–0.55)	0.309
Corynebacteriaceae	0.00 (0.00–0.06)	0.01 (0.00–0.11)	0.00 (0.00–0.04)	0.573
Desulfovibrionaceae	0.00 (0.00–0.07)	0.01 (0.00–0.34)	0.00 (0.00–0.01)	0.093
Enterobacteriaceae	0.92 (0.04–6.48)	0.14 (0.01–2.19)	0.13 (0.03–4.53)	0.377
Enterococcaceae	0.04 (0.01–1.90)	0.31 (0.03–0.60)	0.05 (0.01–10.21)	0.342
Erysipelotrichaceae	0.05 (0.02–0.65)	0.05 (0.01–2.06)	0.06 (0.01–3.15)	0.983
Eubacteriaceae	0.23 (0.08–2.01)	0.19 (0.08–3.15)	1.44 (0.05–7.35)	0.064
Euzebyaceae	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.189
Fusobacteriaceae	25.59[a] (14.68–33.72)	3.67[a,b] (0.27–27.77)	0.97[b] (0.13–13.24)	**0.004**
Gemellaceae	0.01 (0.00–0.13)	0.00 (0.00–0.08)	0.00 (0.00–0.02)	0.818
Helicobacteraceae	0.61[a] (0.05–8.93)	0.17[a,b] (0.02–1.30)	0.01[b] (0.00–0.77)	**0.008**
Lachnospiraceae	8.14[a] (5.71–32.33)	11.87[a,b] (1.20–41.05)	31.36[b] (6.58–81.10)	**0.029**
Lactobacillaceae	0.13 (0.07–28.82)	0.21 (0.08–59.32)	0.08 (0.02–38.72)	0.211
Microbacteriaceae	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.03)	0.446
Paraprevotellaceae	0.00 (0.00–1.74)	0.00 (0.00–0.32)	0.00 (0.00–0.21)	0.205
Pasteurellaceae	0.07 (0.00–0.74)	0.00 (0.00–0.15)	0.00 (0.00–0.01)	0.762
Peptococcaceae	0.01 (0.00–0.24)	0.00 (0.00–0.29)	0.00 (0.00–2.93)	0.997
Peptostreptococcaceae	2.24 (0.31–4.35)	0.49 (0.09–12.28)	0.36 (0.04–5.68)	0.152
Porphyromonadaceae	0.07[a] (0.02–0.17)	0.02[a,b] (0.00–0.15)	0.00[b] (0.00–0.41)	**0.022**
Prevotellaceae	0.77 (0.03–10.06)	2.78 (0.04–4.44)	0.04 (0.01–14.41)	0.126
Ruminococcaceae	4.71 (2.76–18.62)	7.98 (0.94–13.40)	8.12 (0.97–36.41)	0.520
Staphylococcaceae	0.00 (0.00–1.74)	0.00 (0.00–0.01)	0.00 (0.00–0.04)	0.802
Streptococcaceae	0.76 (0.10–2.31)	7.01 (0.05–30.29)	0.41 (0.22–72.81)	0.533
Succinivibrionaceae	0.00 (0.00–0.62)	0.01 (0.00–0.24)	0.00 (0.00–3.96)	0.501
S24-7	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.119
Turicibacteraceae	0.14[a] (0.05–6.89)	0.06[a] (0.05–23.92)	0.02[b] (0.00–1.09)	**0.002**
Veillonellaceae	0.79 (0.01–1.74)	0.69 (0.07–2.81)	0.29 (0.00–0.99)	0.097

Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Medians not sharing a common superscript are significantly different (Dunn's multiple comparison,

$P < 0.05$).

**Kruskal–Wallis test. Statistically significant values ($P < 0.05$) are highlighted in bold.

Table 31

Relative proportions of the predominant bacterial genera.

	Medians % (min.–max. %) of sequences*			
	Active ICRP	Controlled ICRP	Healthy	*P*-value**
Actinomyces	0.01 (0.00–1.46)	0.00 (0.00–0.00)	0.01 (0.00–0.04)	0.191
Allobaculum	0.02 (0.00–0.65)	0.05 (0.01–2.05)	0.05 (0.00–3.06)	0.714
Anaerobiospirillum	0.00 (0.00–0.62)	0.00 (0.00–0.24)	0.00 (0.00–3.92)	0.465
Anaeroplasma	0.00 (0.00–0.74)	0.00 (0.00–0.02)	0.00 (0.00–0.02)	0.067
Anaerotruncus	0.00[a,b] (0.00–0.02)	0.00[a] (0.00–0.01)	0.00[b] (0.00–0.00)	**0.028**
Bacteroides	11.42 (5.18–36.17)	1.85 (0.28–38.47)	8.30 (0.25–37.92)	0.296
Bifidobacterium	3.74 (0.04–20.39)	0.06 (0.03–0.15)	0.03 (0.00–1.69)	0.056
Bilophila	0.00 (0.00–0.07)	0.00 (0.00–0.34)	0.00 (0.00–0.00)	0.216
Blautia	4.42 (2.86–16.97)	7.71 (0.53–17.87)	15.15 (0.94–59.80)	0.115
Bulleidia	0.00 (0.00–0.04)	0.00 (0.00–0.02)	0.00 (0.00–0.04)	0.451
Campylobacter	0.00 (0.00–0.18)	0.00 (0.00–0.26)	0.00 (0.00–0.12)	0.278
Catenibacterium	0.09 (0.00–0.24)	0.06 (0.00–3.61)	0.01 (0.00–2.66)	0.956
Clostridium	7.15 (0.87–19.52)	10.80 (0.70–28.80)	5.02 (0.29–54.27)	0.914
Collinsella	0.01 (0.00–0.97)	0.00 (0.00–0.02)	0.00 (0.00–0.55)	0.363
Coprococcus	0.00 (0.00–0.01)	0.00 (0.00–0.01)	0.00 (0.00–0.90)	0.109
Corynebacterium	0.00 (0.00–0.06)	0.01 (0.00–0.11)	0.00 (0.00–0.04)	0.573
Dorea	0.04 (0.01–1.54)	0.07 (0.01–0.38)	0.13 (0.02–5.40)	0.232
Enterococcus	0.03 (0.01–1.70)	0.23 (0.02–0.59)	0.04 (0.01–9.19)	0.310
Escherichia	0.65 (0.03–5.64)	0.11 (0.01–1.91)	0.10 (0.03–3.54)	0.409
Eubacterium	0.23 (0.08–2.01)	0.19 (0.08–3.15)	1.44 (0.05–7.35)	0.064
Euzebya	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.189
Faecalibacterium	0.02 (0.01–2.51)	0.06 (0.01–2.61)	0.01 (0.00–1.85)	0.181
Fusobacterium	0.04 (0.00–27.22)	0.02 (0.01–0.03)	0.01 (0.01–0.07)	0.225
Gemella	0.01 (0.00–0.13)	0.00 (0.00–0.08)	0.00 (0.00–0.02)	0.720
Helicobacter	0.60[a] (0.05–8.87)	0.17[a,b] (0.02–1.29)	0.01[b] (0.00–0.77)	**0.008**
J2-29	5.17 (0.08–10.93)	1.56 (0.03–11.26)	0.09 (0.02–5.26)	0.108
Lactobacillus	0.04 (0.02–1.02)	0.08 (0.03–25.14)	0.05 (0.00–13.43)	0.380
Lactococcus	0.00 (0.00–0.11)	0.00 (0.00–1.83)	0.00 (0.00–0.02)	0.478
Leucobacter	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.00 (0.00–0.03)	0.443
Megamonas	0.31 (0.01–0.79)	0.05 (0.01–1.60)	0.09 (0.00–0.72)	0.578
Megasphaera	0.00 (0.00–0.06)	0.00 (0.00–0.05)	0.00 (0.00–0.05)	0.167
Oscillospira	0.03 (0.00–0.33)	0.09 (0.00–0.64)	0.01 (0.00–0.33)	0.306
Parabacteroides	0.02 (0.00–0.16)	0.02 (0.00–0.14)	0.00 (0.00–0.41)	0.268
Pasteurella	0.00 (0.00–0.71)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.760
Pediococcus	0.00 (0.00–0.03)	0.00 (0.00–0.86)	0.00 (0.00–0.02)	0.313
Peptococcus	0.01 (0.00–0.24)	0.00 (0.00–0.29)	0.00 (0.00–2.93)	0.997
Peptostreptococcus	0.51 (0.01–3.13)	0.01 (0.01–2.04)	0.01 (0.00–0.07)	0.115
Phascolarctobacterium	0.01 (0.00–1.14)	0.04 (0.00–1.16)	0.04 (0.00–0.34)	0.899
Porphyromonas	0.01 (0.00–0.16)	0.00 (0.00–0.06)	0.00 (0.00–0.03)	0.289
Prevotella	0.77 (0.03–10.06)	2.78 (0.04–4.44)	0.04 (0.01–14.41)	0.126
Proteus	0.00[a,b] (0.00–0.10)	0.00[a] (0.00–0.00)	0.00[b] (0.00–0.01)	**0.007**
Roseburia	0.02 (0.00–0.43)	0.00 (0.00–0.17)	0.00 (0.00–0.08)	0.078
Ruminococcus	4.03 (1.72–18.25)	4.45 (0.83–12.57)	8.03 (0.50–36.37)	0.499

Table 31

Cont.

	Medians % (min.–max. %) of sequences*			
	Active ICRP	Controlled ICRP	Healthy	*P*-value**
Sarcina	0.00 (0.00–0.33)	0.00 (0.00–0.01)	0.00 (0.00–0.00)	0.104
Slackia	0.00 (0.00–0.07)	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.126
Staphylococcus	0.00 (0.00–0.05)	0.00 (0.00–0.00)	0.00 (0.00–0.01)	0.182
Streptococcus	0.76 (0.10–2.05)	5.41 (0.04–28.44)	0.39 (0.18–70.19)	0.533
Sutterella	0.67 (0.02–5.29)	0.22 (0.01–1.76)	0.05 (0.01–1.02)	0.159
Turicibacter	0.14[a] (0.05–6.89)	0.06[a] (0.05–23.92)	0.02[b] (0.00–1.09)	**0.002**
Veillonella	0.00 (0.00–0.67)	0.01 (0.00–0.63)	0.00 (0.00–0.33)	0.313

Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Medians not sharing a common superscript are significantly different (Dunn's multiple comparison, $P < 0.05$).

**Kruskal–Wallis test. Statistically significant values ($P < 0.05$) are highlighted in bold.

Table 32

Percent of sequences of active-ICRP samples with or without immunosuppressive therapy.

	Without immunosuppression			With immunosuppression			*P*-value*
	ICRP-1	ICRP-3**	ICRP-4***	ICRP-2	ICRP-5	ICRP-6***	
Actinomycetaceae	1.51	0.01	0.00	0.08	0.03	0.00	0.827
Alcaligenaceae	0.73	0.02	0.44	0.69	0.64	5.29	0.275
Anaeroplasmataceae	0.74	0.00	0.00	0.00	0.00	0.47	0.827
Bacteroidaceae	12.33	36.17	10.71	5.19	18.52	5.61	0.275
Bifidobacteriaceae	0.04	0.07	20.39	14.02	0.05	7.42	0.827
Campylobacteraceae	0.00	0.18	0.00	0.02	0.00	0.00	0.513
Clostridiaceae	6.81	12.36	15.69	9.57	0.90	19.78	0.827
Coprobacillaceae	0.28	0.12	0.33	0.28	0.42	0.22	0.827
Coriobacteriaceae	0.12	0.00	1.04	0.89	0.00	0.02	0.513
Corynebacteriaceae	0.00	0.00	0.01	0.06	0.02	0.00	0.127
Desulfovibrionaceae	0.00	0.00	0.00	0.07	0.07	0.00	**0.046**
Enterobacteriaceae	1.90	6.48	0.04	1.47	0.37	0.20	0.513
Enterococcaceae	0.01	0.05	0.03	1.90	0.02	0.06	0.275
Erysipelotrichaceae	0.65	0.08	0.03	0.07	0.03	0.02	0.275
Eubacteriaceae	0.29	0.16	2.01	0.18	0.08	0.37	0.513
Euzebyaceae	0.00	0.00	0.00	0.02	0.00	0.00	0.121
Fusobacteriaceae	33.72	27.65	14.86	14.68	23.52	30.66	0.513
Gemellaceae	0.13	0.00	0.00	0.01	0.09	0.00	0.817
Helicobacteraceae	0.75	0.05	0.17	0.46	0.77	8.93	0.127
Lachnospiraceae	13.63	6.17	9.54	5.71	32.33	6.75	0.827
Lactobacillaceae	0.07	0.14	0.13	28.82	0.08	0.73	0.275
Microbacteriaceae	0.00	0.00	0.00	0.00	0.01	0.00	0.246
Paraprevotellaceae	1.74	0.00	0.00	0.00	0.00	0.00	0.817
Pasteurellaceae	0.00	0.72	0.00	0.74	0.14	0.00	0.658
Peptococcaceae	0.00	0.00	0.00	0.11	0.02	0.24	**0.049**
Peptostreptococcaceae	1.47	0.31	4.35	3.36	1.58	2.89	0.513
Porphyromonadaceae	0.17	0.17	0.02	0.09	0.03	0.05	0.513
Prevotellaceae	10.06	0.03	1.49	0.05	0.04	2.59	0.827
Ruminococcaceae	6.87	5.17	4.25	2.95	18.62	2.76	0.513
Staphylococcaceae	0.00	0.00	0.00	0.00	0.01	1.74	0.246
Streptococcaceae	1.30	1.78	0.21	2.31	0.23	0.10	0.827
Succinivibrionaceae	0.62	0.00	0.00	0.00	0.00	0.00	0.658
S24-7	0.00	0.00	0.00	0.00	0.01	0.01	0.246
Turicibacteraceae	0.25	0.08	6.89	0.19	0.05	0.10	0.275
Veillonellaceae	1.74	0.71	0.86	1.66	0.01	0.57	0.275

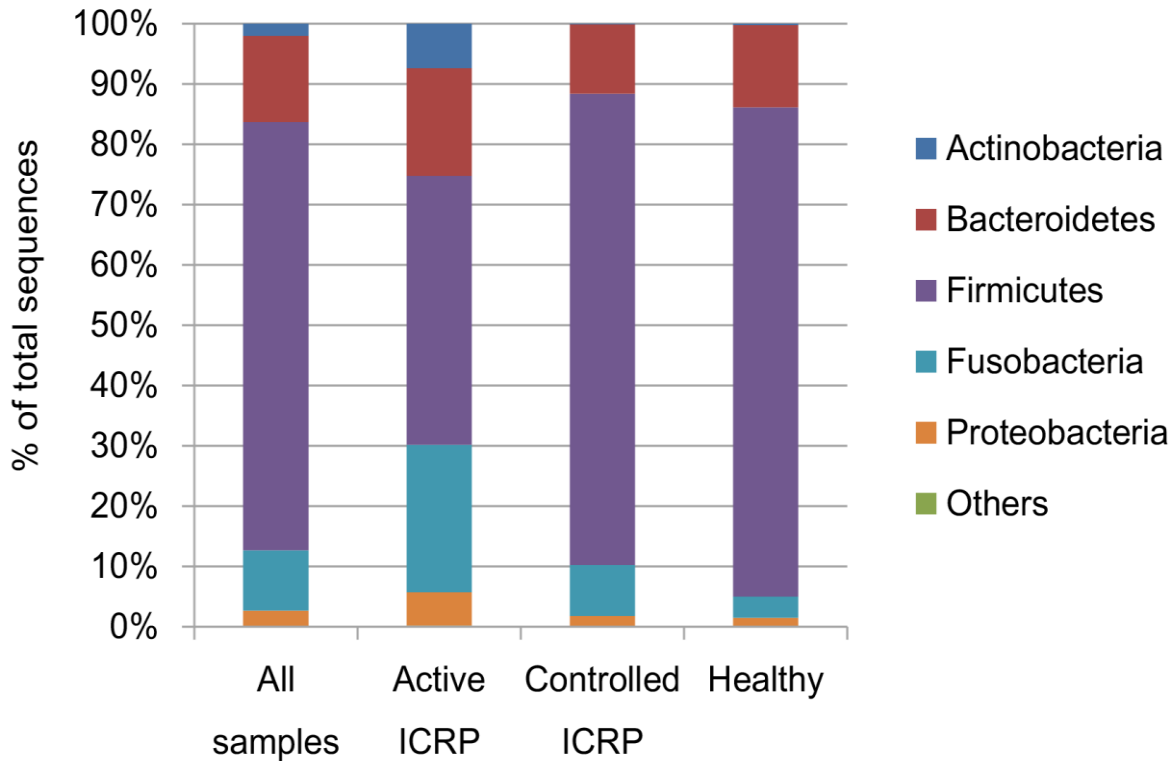
Taxa identified in at least 50% of dogs (either ICRP-affected or healthy) were included in analysis.

*Mann–Whitney *U* test between dogs with and without immunosuppression. Statistically significant values ($P < 0.05$) are highlighted in bold.

**Dog ceased taking probiotics 2 weeks before recruitment.

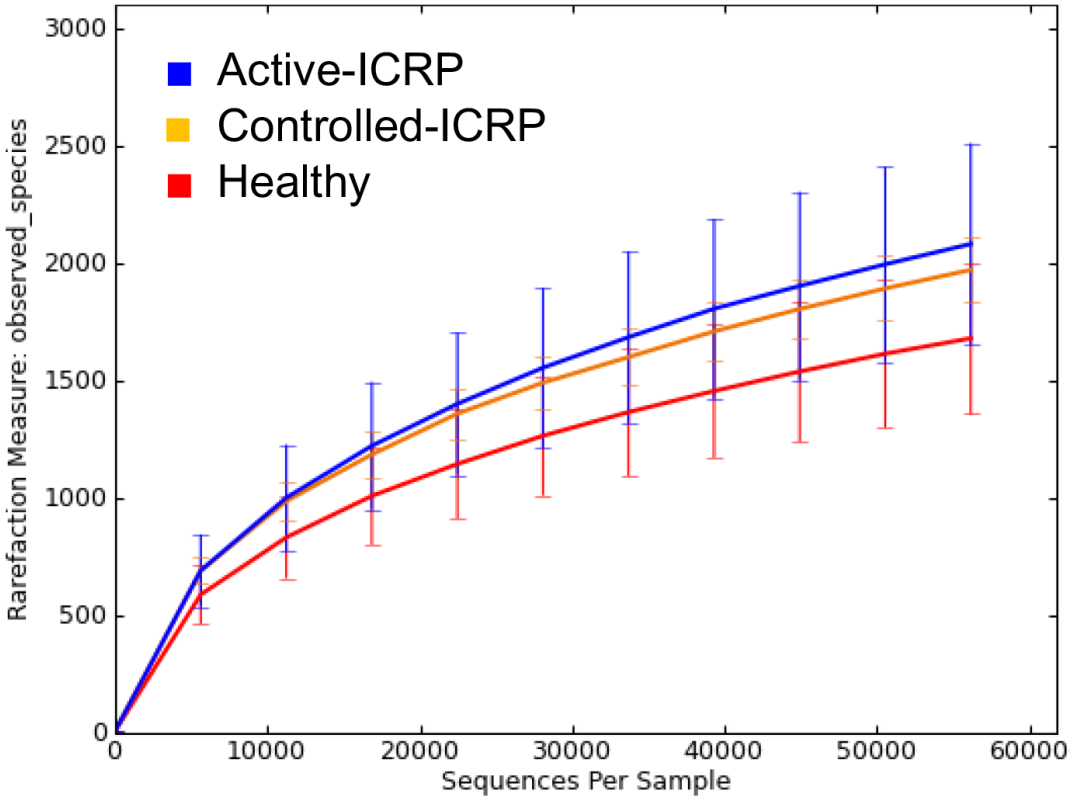
***Dogs fed with high-fiber diets.

Figure 15



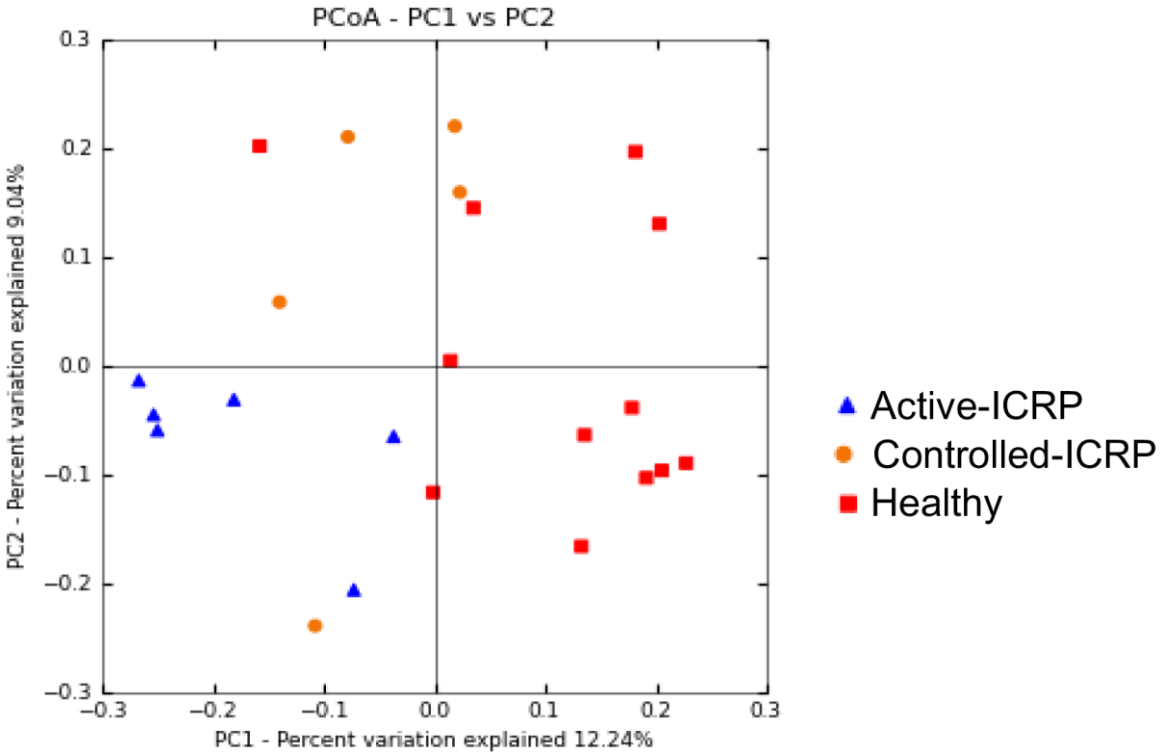
Distributions of major bacterial groups at the phylum level. The bars “Others” includes the minor phyla Deferribacteres, Spirochaetes, Tenericutes, TM7, and Verrucomicrobia. ICRP, inflammatory colorectal polyp.

Figure 16



Rarefraction analysis of V3–V4 16S rRNA gene sequences obtained from fecal samples. Lines represent the mean of each group, while error bars represent standard deviation. This analysis was performed using a randomly selected subset of 56,270 sequences per sample.

Figure 17



Principal coordinates analysis (PCoA) generated using the unweighted UniFrac distance metric of 16S rRNA genes. Samples of dogs with active-ICRP were separated from those of healthy dogs (ANOSIM; R = 0.302, *P* = 0.003). By contrast, samples of dogs with controlled-ICRP were not separated from the other group.

Chapter 2-2

Decreased concentrations of fecal short chain fatty acids in Miniature Dachshunds with inflammatory colorectal polyps

Abstract

Short chain fatty acids (SCFAs) play an important role in the maintenance of colonic homeostasis, and their reduction has been reported in various gastrointestinal disorders including inflammatory bowel disease (IBD) in humans. This study was performed based on the hypothesis that a reduced SCFA concentration is associated with the development of inflammatory colorectal polyps (ICRPs). A total of 19 ICRP-affected and 25 control Miniature Dachshunds (MDs) were recruited for the study. Fecal concentrations of SCFAs including acetic, propionic, butyric, isobutyric, lactic, valeric, and isovaleric acids were measured using high performance liquid chromatography. Fecal concentrations of total SCFAs, and acetic and propionic acids were significantly decreased in ICRP-affected compared to control MDs. These results indicate that SCFAs are a factor in the pathogenesis of ICRPs in MDs. Further investigations are needed to determine if promoting SCFAs using prebiotics or SCFA enemas would be therapeutically beneficial.

1. Introduction

Gut microbiota are part of the mucosal barrier that defends against pathogen invasion, induces mucosal immune responses, supports digestion, and provides nutritional support, including short chain fatty acids (SCFAs) for enterocytes (Suchodolski, 2011). SCFAs are produced by the fermentation of carbohydrates, peptides, and glycoprotein precursors (Cummings et al., 2001; Garcia et al., 2008). SCFAs, predominantly composed of acetate, propionate, and butyrate, play an essential role in maintaining colonic homeostasis. Butyrate is the most effective SCFA in inhibiting colonic inflammation, carcinogenesis, and oxidative stress; improving the colonic defense barrier; promoting satiety; and providing a primary energy substrate for colonocytes (Hamer et al., 2008). The anti-inflammatory mechanism of SCFAs involves the suppression of nuclear factor-kappa B reporter activity, inflammation-related gene expression, and cytokine release (Hamer et al., 2008). A recent study revealed that SCFAs, particularly butyrate, induce the differentiation of colonic regulatory T (Treg) cells via upregulation of histone acetylation in the promoter and enhancer region of the FOXP3 gene (Furusawa et al., 2013). Several studies have reported decreased SCFAs in human inflammatory bowel disease (IBD) (Huda-Faujan et al., 2010; Takaishi et al., 2008; Vernia et al., 1988).

In Chapter 2-1, I performed a 16S rRNA gene sequencing analysis of fecal microbiota in Miniature Dachshunds (MDs) with inflammatory colorectal polyps (ICRPs), and revealed a reduction in the composition of SCFA-producing bacterial groups including Lachnospiraceae. However, it has been reported that alteration in the microbiota composition does not directly correlate with functional microbiota change (Turnbaugh et al., 2009). The primary objective of this study was to compare the SCFA concentrations in the feces of ICRP-affected and control MDs. A recent report showed that the oral

administration of metronidazole, which is often prescribed for ICRP-affected MDs, promotes SCFA-producing bacterial groups including *Bifidobacterium* and Lactobacillales (Igarashi et al., 2014). I also compared the SCFA concentrations between untreated ICRP-affected MDs and those that received metronidazole.

2. Materials and methods

2.1. Animals and sample collection

MDs that were referred to the Veterinary Medical Center of the University of Tokyo for investigation of chronic hematochezia and/or tenesmus and diagnosed with ICRPs between October 2012 and October 2014 were included in this study. The diagnosis of ICRP was confirmed by colonoscopic and histopathological findings as described previously (Ohmi et al., 2012). Dogs that received antibiotics other than metronidazole 2 weeks before sampling were excluded. Data comparing metronidazole treated and untreated ICRP-affected MDs were analyzed separately (see below). As controls, MDs presented to the reference hospital for routine examinations and with no clinical signs of gastrointestinal disease or abnormalities as determined by fecal examination and rectal palpation were recruited. Control MDs had not received any antibiotics within 1 year prior to the study. Detailed descriptions of all samples collected from ICRP-affected and control MDs are listed in Tables 33 and 34, respectively. Naturally passed feces were collected from each dog into sterile plastic tubes, and frozen at –20°C within an hour of defecation until further analysis.

2.2. Sample Preparation

Fecal pH was measured by inserting the glass electrode of an H-7 HP pH meter (Horiba Seisakusho Co. Ltd., Tokyo, Japan) directly into the feces. The fecal moisture content was determined by overnight oven-drying of each sample at 103°C, and mean data were calculated from three different sites in each sample.

Approximately 0.3 g of feces were diluted at a ratio 1:4 to 1:10 (w/v) in distilled water, vortexed for 1 min, centrifuged at 2,000 × *g* for 5 min, and the supernatant filtered using

a 0.45 μm syringe filter. The ammonia concentration of the supernatant was measured using an automated chemistry analyzer (FUJI DRI-CHEM 7000, Fujifilm Medical Co., Tokyo, Japan). The SCFA concentration of the supernatant was measured using high performance liquid chromatography (HPLC) (Miwa et al., 1985, 1987). A mixture of 100 μl of the supernatant and 200 μl of crotonic acid (0.5 mM), an internal standard, was pre-labelled with 2-nitrophenylhydrazide using a Short- and Long-Chain Fatty Acid Analysis Kit (YMC Co. Ltd., Kyoto, Japan). The SCFA derivatives were extracted with n-hexane and diethyl ether, followed by evaporation to dryness. The residue was reconstituted with methanol, filtered through a 0.2 μm syringe filter, and 10 μl injected into the HPLC system with a YMC-Pack FA column (250 × 6.0 mm; YMC Co. Ltd.).

2.3. HPLC analysis

The HPLC system (JASCO, Tokyo, Japan) consisted of two pumps (PU-980), a column oven (CO-965), an autosampler (AS-950), a UV-VIS detector (UV-970), and an integrator (LCSS-905) and was used under the following conditions. The column oven temperature was 50°C, the mobile phase consisted of acetonitrile-methanol-water (30:16:54 v/v, pH 4–5 adjusted by 0.01 N HCl), the flow rate was 1.2 ml/min, and the absorbance of eluates was simultaneously monitored at a wavelength of 230 nm.

To construct calibration curves for each SCFA, eight calibration standards, including acetic, propionic, butyric, isobutyric, lactic, valeric, isovaleric, and crotonic acids, were prepared at six concentration levels ranging from 0.1 mM to 5.0 mM (0.1, 0.2, 0.5, 1.0, 2.0, and 5.0). The typical chromatogram of standard solutions and a fecal sample from a control dog are shown in supplementary Figure 18. The correlation coefficient of the calibration curves ranged from 0.9922 to 0.9984 (Table 35). Recovery tests were

performed by adding known amounts (10 µmol) of each SCFA to 10 ml of calibration standard solution (1.0 mM); the recovery ranged from 95.9% to 118.0% (Table 35). All SCFA concentration analyses were performed in duplicate.

2.4. Statistical analysis

Statistical analyses were performed using commercially available software (JMP Pro version 11.0.0, SAS Institute, Cary, NC, USA). The normality of data was checked using the Shapiro-Wilk test. Gender differences were tested using the Chi-squared test. Differences in age, body weight, and fecal parameters between ICRP-affected MDs with and without metronidazole administration, and control MDs were determined using ANOVA and Tukey's test or the Kruskal–Wallis test with Dunn's post-hoc test where appropriate.

3. Results

3.1. Animals

Of the 25 ICRP-affected MDs evaluated, eight had been treated with metronidazole within 2 weeks before recruitment (Met-ICRP group) and 11 were untreated (nonMet-ICRP group). The remaining six ICRP-affected MDs were excluded from the current study because they had received other antibiotics including ampicillin, amoxicillin, tylosin, orbifloxacin, or enrofloxacin. As controls, 25 MDs were further included (Control group). None of the dogs had undergone abdominal surgery in the year prior to the study. Baseline characteristics, including gender, median age, body weight, and body condition score were not significantly different between the groups (Table 36).

3.2. Fecal characteristics

The fecal dry matter content and pH were significantly lower in the nonMet-ICRP compared with the control group (P = 0.0042 and 0.0217, respectively; Table 36). In addition, the Met-ICRP group had a relatively lower fecal dry matter content (P = 0.0937; Table 36). No significant difference was observed in the fecal ammonia concentration between the groups (Table 36).

3.3. Fecal SCFA concentrations

Slight concentrations of isobutyric, valeric, and isovaleric acids were detected in only one to two dogs, and there were no significant differences between the groups. The total SCFA concentration in the nonMet-ICRP group was significantly lower than in the Control group (P = 0.0291; Table 36). In particular, significantly lower acetic and propionic acids concentrations were observed in the nonMet-ICRP group compared with

controls (P = 0.0406 and 0.0357, respectively; Table 36). No significant difference was observed in the concentrations of butyric and lactic acids between the groups. The Met-ICRP group showed a relatively lower propionic acid concentration compared with the Control group (P = 0.0504; Table 36), while no other parameters showed significant difference compared with other two groups.

4. Discussion

This Chapter revealed decreased fecal SCFA concentrations (predominantly acetic and propionic acids) in ICRP-affected MDs. The fecal butyrate concentration did not differ significantly between the groups, although the median values in ICRP-affected MDs were lower than in controls (Table 36). In contrast, butyric and propionic acids are likely to decrease in human IBD patients (Huda-Faujan et al., 2010; Takaishi et al., 2008). The differences in the pattern of reduced SCFAs between humans and MDs may be due to species or disease pathogenesis (including dysbiosis) differences.

Although the anti-inflammatory effect of butyrate had been described as the most effective (Hamer et al., 2008), propionate and acetate have also been reported to show anti-inflammatory activity with the rank order of potency butyrate > propionate > acetate (Tedelind et al., 2007). Therefore, the decrease of propionic and acetic acids in MDs with ICRPs may contribute to the development of inflammation.

The major products of SCFAs are acetic, propionic, and butyric acids, which are commonly found in proportions of approximately 60:20:20 (acetic: propionic: butyric) in humans (Garcia et al., 2008; Wong et al., 2006). In contrast, the butyrate concentration in healthy dogs is lower (approximately 10% of the total of acetic, propionic, and butyric acids) (Hang et al., 2013; Patra, 2011; Strompfová et al., 2014; Yogo et al., 2011), which is consistent with the present study. A low fecal butyrate ratio seems to be characteristic for dogs, which might result in the absence of a statistically significant difference in the fecal butyrate concentration.

The fecal dry matter content was also significantly lower in the nonMet-ICRP than in the Control group, which may have resulted from excessive mucus secretion commonly observed in MDs with ICRPs (Ohmi et al., 2012; Tamura et al., 2013). This might lead to

the dilution of fecal SCFAs. Since I did not investigate the proportion of bacteria in each fecal sample, in this study, I cannot conclude the exact cause of the SCFA reduction. The nonMet-ICRP group showed a higher fecal pH than the Control group, which may due to the decreased SCFAs. A lower pH reduces the growth and activity of potential pathogens (Hooda et al., 2012). The composition of microbiota in the nonMet-ICRP group would therefore be altered compared with the Control group.

The median fecal concentrations of total SCFAs, particularly acetic and lactic acids, in the Met-ICRP group were relatively higher than in the nonMet-ICRP group but lower than controls; however, these values were not statistically significant. A recent report revealed that metronidazole administration to healthy dogs increases the fecal bacterial composition of *Bifidobacterium* and Lactobacillales (Igarashi et al., 2014), which have been described to predominantly produce acetic and/or lactic acid (Lidbeck and Nord, 1993; Strompfová et al., 2014). In addition, metronidazole administration reduced Bacteroidetes, Clostridiaceae, Lachnospiraceae, Ruminococcaceae, and Veillonellaceae (Igarashi et al., 2014), which are major contributors to the production of propionic and butyric acids in humans (Reichardt et al., 2014). This might explain the relatively equivalent concentrations of propionic and butyric acids between the Met-ICRP and nonMet-ICRP groups. Further studies comparing the fecal SCFA concentrations and bacterial populations pre- and post- metronidazole treatment in healthy and diseased dogs would confirm the findings and the prebiotic effect.

Commensal microbe-derived SCFAs, particularly butyric and propionic acids, can induce differentiation of colonic Treg cells (Furusawa et al., 2013) which have a central role in suppression of the inflammatory response. Since the total fecal SCFAs and propionic acid concentrations in ICRP-affected MDs were decreased in the present study,

I suspect that the colorectal Treg cells in ICRP-affected MDs are reduced. However, one report showed an elevated mRNA expression of IL-10 in the polypoid lesion of ICRP-affected MDs (Ohta et al., 2013), which is a well-known anti-inflammatory cytokine produced by mononuclear cells, including Treg cells (Saraiva and O'Garra, 2010). Further studies investigating the cellular source of IL-10 in ICRPs and the distribution of Treg cells in polypoid lesions are warranted. In addition, the association between colonic SCFAs and induction of Treg cells in dogs needs to be investigated.

Enemas with SCFAs have been described as a treatment for ulcerative colitis patients, a major form of human IBD (Cummings, 1997; Hamer et al., 2008). Because of species differences, which may result in a different SCFA colonic molar ratio, regimens may need to be modified to identify the most effective enema composition for dogs. This method might be a novel therapeutic option for ICRPs in MDs since the lesions are commonly restricted to the colorectal mucosa (Ohmi et al., 2012).

SCFAs have been described as having anti-carcinogenic effects (Hamer et al., 2008). Since ICRPs in MDs occasionally develop into colorectal neoplasia (Igarashi et al., 2013), further investigations regarding the association between SCFA concentrations and colorectal neoplasia in dogs might help to explain ICRP tumorigenesis.

The heterogeneously- diseased and control dogs used in this study may have been a possible imitation. Several dogs in each group had received probiotics and immunosuppressive drugs, predominantly prednisolone (Tables 33 and 34). A recent report described that the oral administration of prednisolone did not directly affect the microbiota (Igarashi et al., 2014). There were no significant differences in SCFA concentrations between ICRP-affected MDs that had received immunosuppressive treatment and those that were untreated (Figure 19). Only five ICRP-affected MDs and

no control MDs had received probiotics in the present study. Therefore, drug administration was not considered a factor in the SCFA levels I observed. Dietary content, particularly dietary fiber (Panasevich et al., 2013; Patra, 2011), may have affected the results, however, information regarding diet was not available for several dogs. Only four ICRP-affected MDs had received a high-fiber diet (Hill's prescription diet w/d, Hill's Pet Nutrition, Inc., Kansas, USA; Royal Canin GI Fiber Response, Royal Canin, Aimargues, France) at recruitment. The body weights of nonMet-ICRP and Met-ICRP were likely to be lower than Control group dogs (Table 36). Obesity has been reported to be associated with altered microbiota and increased fecal SCFA concentrations (Ley et al., 2006; Turnbaugh et al., 2006); this might confound the results of the present study. However, the body condition scores were not different between diseased and control dogs. Since only a limited number of cases were recruited, further studies using larger sample sizes should be performed in the future.

In conclusion, this study revealed a significant decrease in fecal SCFA concentrations, predominantly propionic and acetic acids, in ICRP-affected MDs. These results indicate that SCFAs are a factor in the pathogenesis of ICRPs in MDs. The usefulness of SCFAs as a disease monitoring marker and its significance as a therapeutic target should be further investigated.

Table 33
Dogs with inflammatory colorectal polyps (ICRPs) enrolled in Chapter 2-2.

Case	Age (month)	Sex*	Diagnosis	Received medication prior to 2 weeks of sampling	Diet
nonMet-ICRP 1	68	f	ICRP	prednisolone, tranexamic acid, ursodeoxycholic acid	Novartis Dr.'s diet senior
nonMet-ICRP 2	119	f	ICRP, mammary gland adenocarcinoma	none	unknown
nonMet-ICRP 3	123	mn	ICRP	probiotics	Royal canin GI fiber response
nonMet-ICRP 4	74	mn	ICRP	probiotics	unknown
nonMet-ICRP 5	128	mn	ICRP, diaphragmatic hernia, sudden acquired retinal degeneration	prednisolone	Hill's Prescription w/d
nonMet-ICRP 6	124	fs	ICRP	prednisolone, leflunomide	Royal canin hypo hydrolyzed protein
nonMet-ICRP 7	151	fs	ICRP	tranexamic acid	unknown
nonMet-ICRP 8	159	mn	ICRP	prednisolone, cyclosporine, famotidine, ursodeoxycholic acid	unknown
nonMet-ICRP 9	85	fs	ICRP, chronic enteritis	dexamethasone, tranexamic acid	Royal canin GI fiber response
nonMet-ICRP 10	113	fs	ICRP	none	Royal canin selected protein duck and tapioca
nonMet-ICRP 11	48	m	ICRP, chronic rhinitis	tranexamic acid, probiotics	Royal canin urinary SO
Met-ICRP 1	148	fs	ICRP	metronidazole, prednisolone	unknown
Met-ICRP 2	127	mn	ICRP	metronidazole	Hill's Science diet senior
Met-ICRP 3	68	m	ICRP	metronidazole, prednisolone	Hill's Prescription w/d
Met-ICRP 4	136	fs	ICRP	metronidazole, probiotics	unknown
Met-ICRP 5	95	mn	ICRP	metronidazole, prednisolone, cyclosporine	Royal canin GI low fat
Met-ICRP 6	112	mn	ICRP	metronidazole, prednisolone, famotidine	unknown
Met-ICRP 7	97	m	ICRP, cryptorchidism	metronidazole, tranexamic acid	unknown
Met-ICRP 8	84	fs	ICRP	metronidazole, prednisolone, mesalazine, probiotics	Hill's Science diet adult

*m= male intact; f = female intact; mn= male neutered; fs = female spayed.

Table 34

Control dogs enrolled in Chapter 2-2.

Case	Age (month)	Sex*	Diagnosis	Received medication prior to 2 weeks of sampling	Diet
Control 1	103	f	idiopathic epilepsy	none	Hill's Prescription d/d
Control 2	84	fs	healthy	none	Hill's Science diet senior
Control 3	132	fs	mitral valve regurgitation	benazepril	Eukanuba lamb and rice
Control 4	118	m	pituitary-dependent hyperadrenocorticism	trilostane	Royal canin Hepatic
Control 5	121	mn	progressive retinal atrophy	none	unknown
Control 6	95	f	healthy	none	unknown
Control 7	91	m	intervertebral disk disease	prednisolone	unknown
Control 8	25	m	cryptorchidism	none	Royal canin Hepatic
Control 9	125	m	pituitary-dependent hyperadrenocorticism	trilostane, ursodeoxycholic acid	unknown
Control 10	97	mn	idiopathic epilepsy	phenobarbital, potassium bromide	unknown
Control 11	109	mn	progressive retinal atrophy, mitral valve regurgitation	benazepril	Novartis Dr.'s Care amino protect care
Control 12	197	m	mitral valve regurgitation	enalapril	Eukanuba lamb and rice
Control 13	72	m	wandering pacemaker	none	unknown
Control 14	84	fs	healthy	none	Hill's Science diet Pro light
Control 15	84	mn	healthy	none	Hill's Science diet Pro light
Control 16	167	m	healthy	none	unknown
Control 17	114	m	idiopathic epilepsy	none	unknown
Control 18	192	mn	healthy	none	Hill's Prescription h/d
Control 19	48	mn	intervertebral disk disease	none	unknown
Control 20	135	mn	progressive retinal atrophy	none	unknown
Control 21	72	m	intervertebral disk disease	none	unknown
Control 22	60	f	healthy	none	unknown
Control 23	144	fs	healthy	none	unknown
Control 24	24	mn	healthy	none	unknown
Control 25	110	fs	complex mammary adenoma	none	Royal canin selected protein duck and tapioca

*m= male intact; f = female intact; mn= male neutered; fs = female spayed.

Table 35

Correlation coefficient of calibration plots and recovery of short chain fatty acids (SCFAs).

SCFA	r^2	Recovery (%)
Lactic acid	0.9922	107.5
Acetic acid	0.9974	106.9
Propionic acid	0.9984	112.8
Crotonic acid	0.9961	118.0
Isobutyric acid	0.9945	112.7
Butyric acid	0.9976	102.3
Isovaleric acid	0.9963	95.9
Valeric acid	0.9975	97.7

Table 36

Summary statistics for case signalment parameters and evaluated markers.

	nonMet-ICRP (n = 11)	Met-ICRP (n = 8)	Control (n = 25)	*P*-value*
Sex male (neutered)	5 (4)	5 (3)	17 (8)	0.6110
female (spayed)	6 (4)	3 (3)	8 (5)	
Age (months)	119 (48–159)	105 (68–148)	103 (24–197)	0.9429
Body weight (kg)	5.15 (3.85–7.50)	4.78 (3.20–5.80)	6.10 (3.90–7.75)	0.0708
Body condition score**	3 (3–4)	3 (2–3)	3 (2–4)	0.0878
Fecal parameters				
Dry matter (%)	20.32^{a} (9.24–38.37)	$22.88^{a,b}$ (8.39–38.11)	33.22^{b} (15.84–46.18)	**0.0033**
pH	6.80^{a} (5.90–8.65)	$6.78^{a,b}$ (5.70–8.05)	6.40^{b} (5.50–7.40)	**0.0193**
NH_3***	16.02 (4.68–46.10)	13.31 (6.12–49.03)	17.10 (5.97–32.73)	0.8663
Total SCFAs***	19.49^{a} (0.00–101.52)	$59.25^{a,b}$ (7.56–228.56)	81.19^{b} (20.59–125.53)	**0.0167**
Acetic acid***	13.62^{a} (0.00–60.23)	$27.48^{a,b}$ (7.38–101.52)	46.57^{b} (6.68–77.42)	**0.0362**
Propionic acid***	0.00^{a} (0.00–30.10)	$0.12^{a,b}$ (0.00–27.04)	16.83^{b} (0.00–35.57)	**0.0113**
Butyric acid***	0.60 (0.00–10.86)	0.63 (0.00–15.07)	3.17 (0.00–12.66)	0.1697
Isobutyric acid***	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.29)	0.3420
Lactic acid***	0.00 (0.00–20.84)	3.46 (0.00–197.75)	10.07 (0.00–51.86)	0.0967
Valeric acid***	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.02)	0.2298
Isovaleric acid***	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.00 (0.00–0.16)	0.3420

*ANOVA, Kruskal–Wallis, or chi-square test. Statistically significant values ($P < 0.05$) are highlighted in bold.

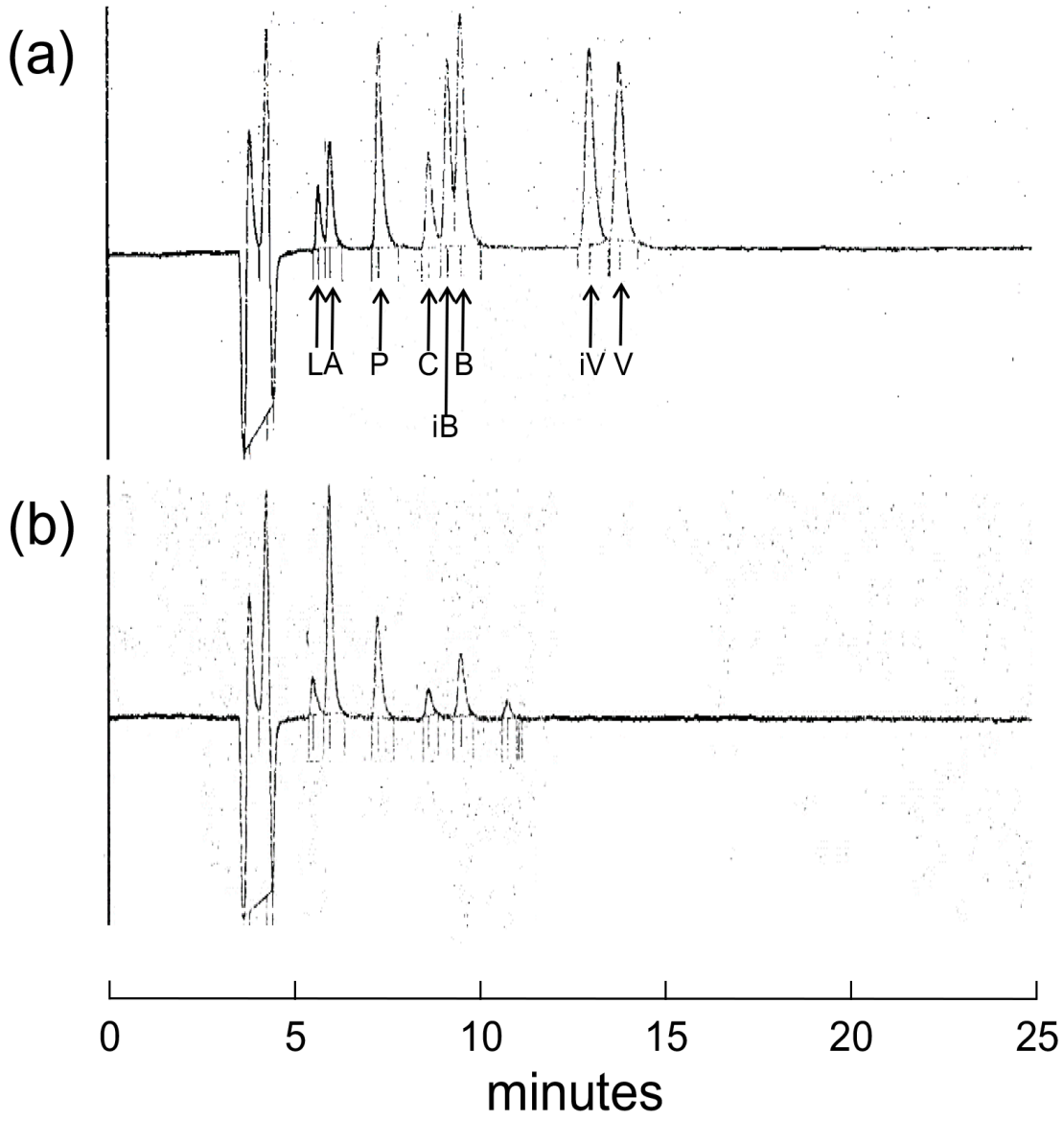
**Represented in 5-point scale (Baldwin et al., 2010).

***Data are shown in µmol/g wet feces.

Data other than sex are presented as the median (range).

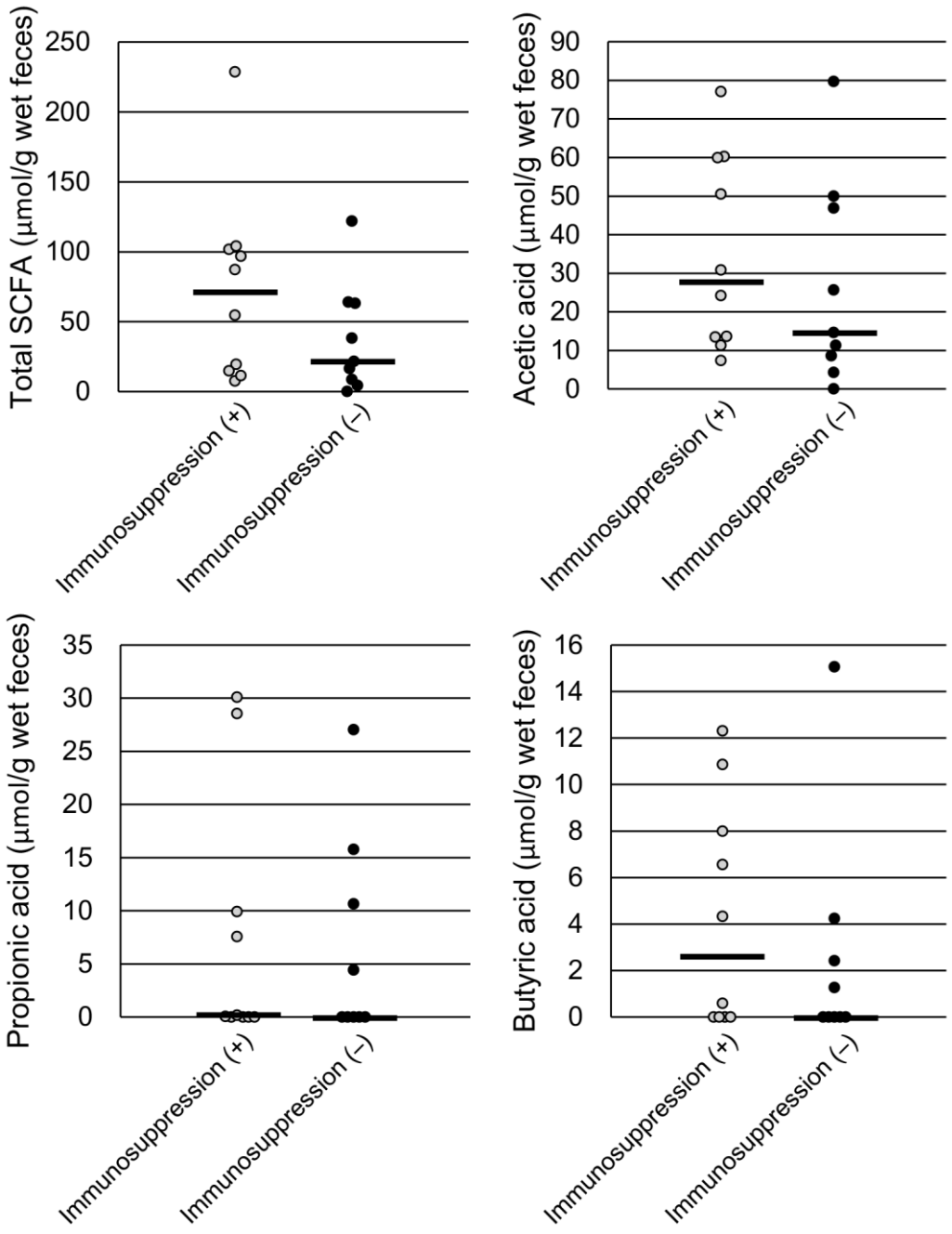
Medians not sharing a common superscript are significantly different ($P < 0.05$ based on a Tukey's test or Dunn's multiple comparison).

Figure 18



Chromatogram patterns of a mixture of standard solutions (5.0 mM each) of eight short chain fatty acids (SCFAs) (a), and a control dog fecal sample (b). L, lactic acid; A, acetic acid; P, propionic acid; C, crotonic acid; iB, isobutyric acid; B, butyric acid; iV, isovaleric acid; V, valeric acid.

Figure 19



Fecal concentrations of total and major SCFAs in inflammatory colorectal polyp (ICRP)-affected dogs treated with and without immunosuppressive drugs. The horizontal lines represent the median value of that group. No statistically significant difference was observed in any SCFA.

Conclusion

Inflammatory colorectal polyps (ICRPs) in Miniature Dachshunds (MDs) have recently been recognized as a disease, known only in Japan, and they are assumed to involve a genetic predisposition. Based on the presence of idiopathic inflammation and the clinical response to immunosuppressive treatment, ICRPs are thought to be a novel form of breed-specific IBD in dogs (Ohta et al., 2013); however, few studies have investigated their pathogenesis. As shown in Chapter 0, ICRPs in MDs show a tendency to develop at the ventral angle of the colorectal mucosa. This led me to propose two factors that may contribute to pathogenesis: (1) anatomical factors, such as distance from regional lymph nodes or the mesocolon, and distribution of nerves or blood supply, and (2) aberrant interactions or mechanical abrasion between the colorectal mucosa and fecal antigens. Since the development of ICRPs in MDs is restricted to the colorectum (Ohmi et al., 2012), I focused on the factors associated with the mucosal-microbiota interaction in this thesis.

The interaction between mucosal innate immunity and gut microbiota plays a central role in the maintenance of mucosal immunological homeostasis (Wallace et al., 2014; Xavier and Podolsky, 2007). The aberrant recognition of commensal microbiota by dysregulation and/or dysfunction of pattern recognition receptors (PRRs) leads to an excessive mucosal immune response (Cario, 2010). In addition, dysbiosis, including a decrease in beneficial bacteria and an increase in pathogenic bacteria, and reduced luminal short chain fatty acid (SCFA) content also contribute to the development of mucosal inflammation (Wallace et al., 2014; Wong et al., 2006). For this thesis, a series of studies was performed to characterize the mucosal immune condition, genetic background, and microbiota in ICRPs in MDs.

In Chapter 1-1, the messenger RNA (mRNA) expression levels of PRRs in polypoid

lesions were investigated and the mRNA expression of several PRRs was found to be upregulated (toll-like receptor 1 [TLR1], TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10, and nucleotide-binding oligomerization domain 2 [NOD2]); in contrast, the mRNA expression of TLR3 and NOD1 was downregulated. The mRNA expression of all upregulated PRRs also showed a positive correlation with the expression of each proinflammatory cytokine. However, most dysregulation of PRR mRNA expression was restricted to the polypoid lesions. These data indicated that the upregulation of PRRs is not the cause but is the consequence of inflammation and may aggravate the disease, as indicated in human inflammatory bowel disease (IBD) (Szebeni et al., 2008).

Next, functional analysis of PRRs was performed. Chapter 1-2 details the evaluation of reactivity to pathogen-associated molecular pattern (PAMP) stimulation using peripheral blood-derived monocytes. Reactive IL-1β induction was enhanced in ICRP-affected MDs when stimulated with muramyl dipeptide (MDP), synthetic bacterial lipoprotein (Pam3CSK4), peptidoglycan from *Escherichia coli K12* (PGN-EK), and synthetic diacylated lipoprotein (FSL-1) (ligands for NOD2, TLR1/2, TLR2, and TLR2/6, respectively). The difference in reactivity to MDP stimulation remained significant when several limiting factors, including disease condition (samples collected at initial diagnosis or at clinical remission) or drug administration (i.e., immunosuppressive agents), were taken into account. Although the reactivity of peripheral monocytes should differ from that of mucosal macrophages in the colorectum (Schmitz et al., 2014), the above-mentioned results indicate that hyperreactivity might contribute to the development of inflammation. Furthermore, these data suggested a genetic contribution, particularly of the NOD2 gene.

Based on the results reported in Chapter 1-2, I explored mutations and single

nucleotide polymorphisms (SNPs) in the canine NOD2, TLR1, TLR2, and TLR6 genes in Chapter 1-3. First, molecular cloning of the canine NOD2 gene was performed, and three novel transcript variants were detected. Second, a mutational analysis was performed using genomic DNA, and a total of 13 non-synonymous SNPs were detected in the canine NOD2, TLR1, TLR2, and TLR6 genes. Third, an association study was performed on the six SNPs detected in exon 3 of the canine NOD2 gene, which revealed that four of the SNPs (A1532G, T1573C, C1688G, and G1880A) were in complete linkage disequilibrium and were associated with the development of ICRPs in MDs. These four SNPs were considered to predispose MDs to ICRPs. Given the results reported in Chapter 1-2, it appears that these SNPs are localized at the NACHT domain and may be associated with NOD2 hyperreactivity, as characterized in Blau syndrome in humans (Sfriso et al., 2012). Further investigations evaluating the relationship between these SNPs and their functional effect should be performed by transfecting each haplotype *in vitro* and/or assessing the reactivity in healthy dogs harboring each haplotype to confirm this theory; these studies may improve our understanding of how dysfunction of NOD2 contributes to the development of inflammatory disorders, including ICRPs in MDs, as well as Blau syndrome in humans. However, these SNPs could not be defined as markers for the development of the disease because the majority of control dogs also harbored risk-associated haplotype. This finding supports the hypothesis that the etiology of ICRPs in MDs is multifactorial, as has been shown in human and canine IBD (Cerquetella et al., 2010; Wallace et al., 2014). Thus, it appears that the risk-associated haplotype of these SNPs gives rise to a predisposition to develop ICRPs in MDs, but is not a specific cause.

In Chapter 2-1, I reported an analysis of the composition of the fecal microbiota in ICRP-affected MDs using high-throughput 16S rRNA gene sequencing and revealed that

dogs with ICRPs showed fecal dysbiosis, predominantly with an increase in Fusobacteria and a decrease in Lachnospiraceae. Furthermore, my study also revealed that the composition of the microbiota of ICRP-affected MDs tended to shift, becoming more similar to that of healthy MDs, when clinical remission was achieved with immunosuppressive therapy. Although the cause-effect relationship could not be determined by this study, these results indicate that the microbiota are involved in the pathogenesis of ICRPs in MDs. The group Fusobacteria contains heterologous bacterial species, including *Fusobacterium nucleatum* and other *Fusobacterium* species that are considered pathogenic, with proinflammatory, invasive, and tumorigenic potential and are present in the colorectum in humans (Allen-Vercoe et al., 2011; Tahara et al., 2014). Since MDs with ICRPs occasionally develop neoplasia (Igarashi et al., 2013), an increase in Fusobacteria might play a part in tumorigenesis as well as in the development of mucosal inflammation. Further studies to analyze microbial proportions at the species level are warranted.

Furthermore, the decrease in Lachnospiraceae reported in Chapter 2-1 indicated a reduction in fecal SCFA concentrations. Therefore, I further investigated the fecal SCFA concentrations by high performance liquid chromatography (HPLC) in Chapter 2-2. The total fecal SCFAs was decreased in ICRP-affected MDs; the major contributors to the decrease were acetic and propionic acids. This result indicates that the reduced SCFA content may contribute to the development of inflammation in this disease.

This series of studies characterized the aberrant condition of host innate immunity and the fecal microbiota in ICRPs in MDs. The findings reported in this thesis, including dysregulation of PRR expression, hyperreactivity of PRRs, genetic factors, fecal dysbiosis, and reduced fecal SCFA concentrations, may be associated with the

development of mucosal inflammation. However, the data remain insufficient to fully determine the etiology and establish appropriate therapeutic protocols for ICRPs in MDs.

Although the studies reported here revealed a genetic background predisposing MDs to ICRPs, the underlying reason that ICRPs commonly develop in MDs remains unknown. Genome-wide association studies would aid in identifying additional mutations critical for the development of ICRPs in MDs, and may also explain why ICRPs in MDs are observed only in Japan. Moreover, it remains unclear how NOD2 hyperreactivity affects the pathogenesis of ICRPs in MDs, and Blau syndrome in humans (Sfriso et al., 2012). Furthermore, this thesis did not evaluate other mucosal immunity factors, including the mucosal barrier, luminal antimicrobial peptides, and immunoglobulin A composition, or adaptive immunity in ICRPs in MDs. The reduced fecal SCFAs indicates a reduction in mucosal regulatory T (Treg) cells, which may contribute to the progression of inflammation; this factor needs to be further investigated in future.

The lack of a disease severity score was a major limitation to all investigations performed as part of this thesis. Ohta et al. (2013) and Tamura et al. (2013) reported that upregulation of inflammatory cytokines was more severe in large polyps than in small polyps. Similar to the CIBDAI scoring system for canine IBD (Jergens et al., 2003), construction of a clinical severity score, including clinical signs, biochemical markers (e.g. C-reactive protein), and subjective macroscopic findings, similar to that presented in Chapter 0 (0, no polyps present; 1, small polyps; 2, medium-sized polyps; and 3, large polyps), may be useful for the management of ICRPs in MDs. Furthermore, several of the objective markers used to evaluate canine IBD, such as S100A12 and calprotectin, might be useful for monitoring and prognostic prediction for ICRPs in MDs (Collins, 2013).

To date, ICRPs in MDs have been treated with immunosuppressive therapy, endoscopic polypectomy, argon plasma coagulation, and/or surgical resection (Ohmi et al., 2012; Tsukamoto et al., 2012). The efficacy of dietary intervention, antibiotics, and pre/probiotics has not been investigated. The alteration of the microbiota and the reduced fecal SCFA concentrations observed in this thesis indicate that these methods may have therapeutic efficacy. Many studies have described the effect of probiotics in dogs, as promoting the growth of beneficial bacteria, including *Bifidobacterium* and Lactobacillales, and increasing fecal SCFA concentrations (Garcia-Mazcorro et al., 2011; Strompfová et al., 2014). Interestingly, a recent report compared the effect of combination therapy using prednisone and metronidazole with probiotics (VSL#3) alone on canine IBD patients; dogs treated with probiotics showed a greater improvement in histopathology and the fecal proportion of beneficial bacteria (*Faecalibacterium*) (Rossi et al., 2014). Similarly, supplementation with prebiotics, such as dietary fiber, in healthy dogs has also been shown to induce an increase in beneficial bacteria and to promote higher fecal SCFA concentrations (Middelbos et al., 2010; Panasevich et al., 2013, 2014; Patra, 2011; Sunvold et al., 1995a, b), although data on their efficacy in dogs suffering from gastrointestinal disorders are limited. Furthermore, SCFA enemas, which harbor a limitation to prolonged use (with regard to compliance) in the treatment of human IBD (Cummings, 1997; Hamer et al., 2008), may also show promise as a novel treatment protocol for ICRPs in MDs as well as chronic colitis in dogs. The effect of probiotics, prebiotics, and/or SCFA supplementation on mucosal immunity, gut microbiota, and/or tumorigenesis of ICRPs is of great interest.

Unlike IBD in dogs, ICRPs in MDs could be considered a homologous disorder; it typically occurs in MDs, shows relatively consistent macroscopic and histopathological

findings, and mostly develops in the colorectum. Thus, it shows promise to become a novel animal model for spontaneous inflammatory disorders. As described above, the etiology of ICRPs in MDs is expected to be multifactorial, similar to IBD in humans and in dogs. This thesis investigated only mucosal innate immunity and the microflora; the contributions of adaptive immunity, the mucosal barrier system, environmental factors, and interactions among these factors remain unclear. Unraveling the pathogenesis of ICRPs in MDs could provide new insights into various idiopathic inflammatory disorders, such as Blau syndrome or IBD in humans.

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References

Abreu, M.T., 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature Reviews Immunology 10, 131–144.

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nature Reviews Immunology 4, 499–511.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Allen-Vercoe, E., Strauss, J., Chadee, K., 2011. *Fusobacterium nucleatum*: an emerging gut pathogen? Gut Microbes 2, 294–298.

Allenspach, K., House, A., Smith, K., McNeill, F.M., Hendricks, A., Elson-Riggins, J., Riddle, A., Steiner, J.M., Werling, D., Garden, O.A., Catchpole, B., Suchodolski, J.S., 2010. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Veterinary Microbiology 146, 326–335.

Allenspach, K., Wieland, B., Gröne, A., Gaschen, F., 2007. Chronic enteropathies in dogs: evaluation of risk factors for negative outcome. Journal of Veterinary Internal Medicine 21, 700–708.

Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245–5250.

Arbour, N.C., Lorenz, E., Schutte, B.C., Zabner, J., Kline, J.N., Jones, M., Frees, K., Watt, J.L., Schwartz, D.A., 2000. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nature Genetics 25, 187–191.

Baldwin, K., Bartges, J., Buffington, T., Freeman, L.M., Grabow, M., Legred, J., Ostwald, D., Jr., 2010. AAHA nutritional assessment guidelines for dogs and cats. Journal of the American Animal Hospital Association 46, 285–296.

Becker, C.E., O'Neill, L.A., 2007. Inflammasomes in inflammatory disorders: the role of TLRs and their interactions with NLRs. Seminars in Immunopathology 29, 239–248.

Bell, M.P., Svingen, P.A., Rahman, M.K., Xiong, Y., Faubion, W.A., Jr., 2007. FOXP3 regulates TLR10 expression in human T regulatory cells. The Journal of Immunology 179, 1893–1900.

Berrebi, D., Maudinas, R., Hugot, J.P., Chamaillard, M., Chareyre, F., De Lagausie, P., Yang, C., Desreumaux, P., Giovannini, M., Cézard, J.P., Zouali, H., Emilie, D., Peuchmaur, M., 2003. Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. Gut 52, 840–846.

Borzutzky, A., Fried, A., Chou, J., Bonilla, F.A., Kim, S., Dedeoglu, F., 2010. NOD2-associated diseases: bridging innate immunity and autoinflammation. Clinical Immunology 134, 251–261.

Bueno, R., Mello, M.N., Menezes, C.A., Dutra, W.O., Santos, R.L., 2005. Phenotypic, functional, and quantitative characterization of canine peripheral blood monocyte-derived macrophages. Memórias do Instituto Oswaldo Cruz 100, 521–524.

Burgener, I.A., König, A., Allenspach, K., Sauter, S.N., Boisclair, J., Doherr, M.G., Jungi, T.W., 2008. Upregulation of toll-like receptors in chronic enteropathies in dogs. Journal of Veterinary Internal Medicine 22, 553–560.

Cantó, E., Ricart, E., Monfort, D., González-Juan, D., Balanzó, J., Rodríguez-Sánchez, J.L., Vidal, S., 2006. TNF alpha production to TLR2 ligands in active IBD patients. Clinical Immunology 119, 156–165.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal 6, 1621–1624.

Cario, E., 2010. Toll-like receptors in inflammatory bowel diseases: a decade later. Inflammatory Bowel Diseases 16, 1583–1597.

Cario, E., Podolsky, D.K., 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infection and Immunity 68, 7010–7017.

Cerquetella, M., Spaterna, A., Laus, F., Tesei, B., Rossi, G., Antonelli, E., Villanacci, V., Bassotti, G., 2010. Inflammatory bowel disease in the dog: differences and similarities with humans. World Journal of Gastroenterology 16, 1050–1056.

Chao, A., 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43, 783–791.

Choi, Y., Sims, G.E., Murphy, S., Miller, J.R., Chan, A.P., 2012. Predicting the functional effect of amino acid substitutions and indels. PLoS ONE 7, e46688.

Chrzastowska, M., Kander, M., Depta, A., 2009. Prospects for the use of probiotic bacteria in the treatment of gastrointestinal diseases in dogs. Polish Journal of Veterinary Sciences 12, 279–284.

Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., Farrow, J.A., 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. International Journal of Systematic Bacteriology 44, 812–826.

Collins, M.T., 2013. Canine inflammatory bowel disease: current and prospective biomarkers for diagnosis and management. Compendium: Continuing Education for Veterinarians 35, E5.

Cook, S.I., Sellin, J.H., 1998. Review article: short chain fatty acids in health and disease. Alimentary Pharmacology and Therapeutics 12, 499–507.

Corridoni, D., Arseneau, K.O., Cifone, M.G., Cominelli, F., 2014. The dual role of nod-like receptors in mucosal innate immunity and chronic intestinal inflammation. Frontiers in Immunology 5, 317.

Craven, M., Simpson, J.W., Ridyard, A.E., Chandler, M.L., 2004. Canine inflammatory bowel disease: retrospective analysis of diagnosis and outcome in 80 cases (1995-2002). Journal of Small Animal Practice 45, 336–342.

Cummings, J.H., 1997. Short-chain fatty acid enemas in the treatment of distal ulcerative colitis. European Journal of Gastroenterology and Hepatology 9, 149–153.

Cummings, J.H., Macfarlane, G.T., Englyst, H.N., 2001. Prebiotic digestion and fermentation. The American Journal of Clinical Nutrition 73, 415S–420S.

Cummings, J.R., Cooney, R.M., Clarke, G., Beckly, J., Geremia, A., Pathan, S., Hancock, L., Guo, C., Cardon, L.R., Jewell, D.P., 2010. The genetics of NOD-like receptors in Crohn's disease. Tissue Antigens 76, 48–56.

de Kivit, S., Tobin, M.C., Forsyth, C.B., Keshavarzian, A., Landay, A.L., 2014. Regulation of intestinal immune responses through TLR activation: implications for pro- and prebiotics. Frontiers in Immunology 5, 60.

Deng, P., Swanson, K.S., 2014. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. British Journal of Nutrition, in press.

Dillon, S., Agrawal, A., Van Dyke, T., Landreth, G., McCauley, L., Koh, A., Maliszewski,

C., Akira, S., Pulendran, B., 2004. A Toll-like receptor 2 ligand stimulates Th2 responses *in vivo*, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. The Journal of Immunology 172, 4733–4743.

Drexler, S.K., Foxwell, B.M., 2010. The role of toll-like receptors in chronic inflammation. The International Journal of Biochemistry & Cell Biology 42, 506–518.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

Franchi, L., Park, J.H., Shaw, M.H., Marina-Garcia, N., Chen, G., Kim, Y.G., Núñez, G., 2008. Intracellular NOD-like receptors in innate immunity, infection and disease. Cellular Microbiology 10, 1–8.

Frank, D.N., St Amand, A.L., Feldman, R.A., Boedeker, E.C., Harpaz, N., Pace, N.R., 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proceedings of the National Academy of Sciences of the United States of America 104, 13780–13785.

Frolova, L., Drastich, P., Rossmann, P., Klimesova, K., Tlaskalova-Hogenova, H., 2008. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. Journal of Histochemistry and Cytochemistry 56, 267–274.

Fukata, M., Arditi, M., 2013. The role of pattern recognition receptors in intestinal inflammation. Mucosal Immunology 6, 451–463.

Funke, B., 2011. Laser microdissection of intestinal epithelial cells and downstream analysis. Methods in Molecular Biology 755, 189–196.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., Takahashi, M., Fukuda, N.N., Murakami, S., Miyauchi, E., Hino, S., Atarashi, K., Onawa, S., Fujimura, Y., Lockett, T., Clarke, J.M., Topping, D.L., Tomita, M., Hori, S., Ohara, O., Morita, T., Koseki, H., Kikuchi, J., Honda, K., Hase, K., Ohno, H., 2013. Commensal microbe-derived

butyrate induces the differentiation of colonic regulatory T cells. Nature 504, 446–450.

Galvez, J., Rodríguez-Cabezas, M.E., Zarzuelo, A., 2005. Effects of dietary fiber on inflammatory bowel disease. Molecular Nutrition and Food Research 49, 601–608.

Garcia-Mazcorro, J.F., Dowd, S.E., Poulsen, J., Steiner, J.M., Suchodolski, J.S., 2012. Abundance and short-term temporal variability of fecal microbiota in healthy dogs. Microbiologyopen 1, 340–347.

Garcia-Mazcorro, J.F., Lanerie, D.J., Dowd, S.E., Paddock, C.G., Grützner, N., Steiner, J.M., Ivanek, R., Suchodolski, J.S., 2011. Effect of a multi-species synbiotic formulation on fecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. FEMS Microbiology Ecology 78, 542–554.

Garcia, A., Olmo, B., Lopez-Gonzalvez, A., Cornejo, L., Rupérez, F.J., Barbas, C., 2008. Capillary electrophoresis for short chain organic acids in faeces: reference values in a Mediterranean elderly population. Journal of Pharmaceutical and Biomedical Analysis 46, 356–361.

German, A.J., Hall, E.J., Day, M.J., 2003. Chronic intestinal inflammation and intestinal disease in dogs. Journal of Veterinary Internal Medicine 17, 8–20.

Gewirtz, A.T., Vijay-Kumar, M., Brant, S.R., Duerr, R.H., Nicolae, D.L., Cho, J.H., 2006. Dominant-negative TLR5 polymorphism reduces adaptive immune response to flagellin and negatively associates with Crohn's disease. American Journal of Physiology: Gastrointestinal and Liver Physiology 290, G1157–G1163.

Ghadimi, D., Vrese, M.D., Heller, K.J., Schrezenmeir, J., 2010. Effect of natural commensal-origin DNA on toll-like receptor 9 (TLR9) signaling cascade, chemokine IL-8 expression, and barrier integritiy of polarized intestinal epithelial cells. Inflammatory Bowel Diseases 16, 410–427.

Goto-Koshino, Y., Ohno, K., Nakajima, M., Mochizuki, H., Kanemoto, H., Tsujimoto, H., 2011. A rapid and simple method to obtain canine peripheral blood-derived macrophages. The Journal of Veterinary Medical Science 73, 773–778.

Hajjar, A.M., O'Mahony, D.S., Ozinsky, A., Underhill, D.M., Aderem, A., Klebanoff, S.J., Wilson, C.B., 2001. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. The Journal of Immunology 166, 15–19.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., Brummer, R.J., 2008. Review article: the role of butyrate on colonic function. Alimentary Pharmacology and Therapeutics 27, 104–119.

Hang, I., Heilmann, R.M., Grützner, N., Suchodolski, J.S., Steiner, J.M., Atroshi, F., Sankari, S., Kettunen, A., de Vos, W.M., Zentek, J., Spillmann, T., 2013. Impact of diets with a high content of greaves-meal protein or carbohydrates on faecal characteristics, volatile fatty acids and faecal calprotectin concentrations in healthy dogs. BMC Veterinary Research 9, 201.

Hayashi, K., Okanishi, H., Kagawa, Y., Asano, K., Watari, T., 2012. The role of endoscopic ultrasound in the evaluation of rectal polypoid lesions in 25 dogs. Japanese Journal of Veterinary Research 60, 185–189.

Hedl, M., Li, J., Cho, J.H., Abraham, C., 2007. Chronic stimulation of Nod2 mediates tolerance to bacterial products. Proceedings of the National Academy of Sciences of the United States of America 104, 19440–19445.

Hisamatsu, T., Suzuki, M., Podolsky, D.K., 2003. Interferon-gamma augments CARD4/NOD1 gene and protein expression through interferon regulatory factor-1 in intestinal epithelial cells. The Journal of Biological Chemistry 278, 32962–32968.

Honneffer, J.B., Minamoto, Y., Suchodolski, J.S., 2014. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. World Journal of Gastroenterology 20, 16489–16497.

Hooda, S., Minamoto, Y., Suchodolski, J.S., Swanson, K.S., 2012. Current state of knowledge: the canine gastrointestinal microbiome. Animal Health Research Reviews 13, 78–88.

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., Gordon, J.I., 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science 291, 881–884.

House, A.K., Binns, M.M., Gregory, S.P., Catchpole, B., 2009. Analysis of NOD1, NOD2, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9 genes in anal furunculosis of German shepherd dogs. Tissue Antigens 73, 250–254.

House, A.K., Gregory, S.P., Catchpole, B., 2008. Pattern-recognition receptor mRNA expression and function in canine monocyte/macrophages and relevance to canine

anal furunuclosis. Veterinary Immunology and Immunopathology 124, 230–240.

Huda-Faujan, N., Abdulamir, A.S., Fatimah, A.B., Muhammad Anas, O., Shuhaimi, M., Yazid, A.M., Loong, Y.Y., 2010. The impact of the level of the intestinal short chain fatty acids in inflammatory bowel disease patients versus healthy subjects. The Open Biochemistry Journal 4, 53–58.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J.F., Sahbatou, M., Thomas, G., 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411, 599–603.

Igarashi, H., Maeda, S., Ohno, K., Horigome, A., Odamaki, T., Tsujimoto, H., 2014. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. PLoS ONE 9, e107909.

Igarashi, H., Ohno, K., Ohmi, A., Tsukamoto, A., Nakashima, K., Fujino, Y., Uchida, K., Tsujimoto, H., 2013. Polypoid adenomas secondary to inflammatory colorectal polyps in 2 miniature dachshunds. The Journal of Veterinary Medical Science 75, 535–538.

Jergens, A.E., Crandell, J., Morrison, J.A., Deitz, K., Pressel, M., Ackermann, M., Suchodolski, J.S., Steiner, J.M., Evans, R., 2010. Comparison of oral prednisone and prednisone combined with metronidazole for induction therapy of canine inflammatory bowel disease: a randomized-controlled trial. Journal of Veterinary Internal Medicine 24, 269–277.

Jergens, A.E., Schreiner, C.A., Frank, D.E., Niyo, Y., Ahrens, F.E., Eckersall, P.D., Benson, T.J., Evans, R., 2003. A scoring index for disease activity in canine inflammatory bowel disease. Journal of Veterinary Internal Medicine 17, 291–297.

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

Jiang, T., Savaiano, D.A., 1997. Modification of colonic fermentation by bifidobacteria and pH *in vitro*. Impact on lactose metabolism, short-chain fatty acid, and lactate production. Digestive Diseases and Sciences 42, 2370–2377.

Jostins, L., Ripke, S., Weersma, R.K., Duerr, R.H., McGovern, D.P., Hui, K.Y., Lee, J.C., Schumm, L.P., Sharma, Y., Anderson, C.A., Essers, J., Mitrovic, M., Ning, K., Cleynen, I., Theatre, E., Spain, S.L., Raychaudhuri, S., Goyette, P., Wei, Z., Abraham, C., Achkar, J.P., Ahmad, T., Amininejad, L., Ananthakrishnan, A.N., Andersen, V., Andrews, J.M., Baidoo, L., Balschun, T., Bampton, P.A., Bitton, A., Boucher, G., Brand, S., Büning, C., Cohain, A., Cichon, S., D'Amato, M., De Jong, D., Devaney, K.L., Dubinsky, M., Edwards, C., Ellinghaus, D., Ferguson, L.R., Franchimont, D., Fransen, K., Gearry, R., Georges, M., Gieger, C., Glas, J., Haritunians, T., Hart, A., Hawkey, C., Hedl, M., Hu, X., Karlsen, T.H., Kupcinskas, L., Kugathasan, S., Latiano, A., Laukens, D., Lawrance, I.C., Lees, C.W., Louis, E., Mahy, G., Mansfield, J., Morgan, A.R., Mowat, C., Newman, W., Palmieri, O., Ponsioen, C.Y., Potocnik, U., Prescott, N.J., Regueiro, M., Rotter, J.I., Russell, R.K., Sanderson, J.D., Sans, M., Satsangi, J., Schreiber, S., Simms, L.A., Sventoraityte, J., Targan, S.R., Taylor, K.D., Tremelling, M., Verspaget, H.W., De Vos, M., Wijmenga, C., Wilson, D.C., Winkelmann, J., Xavier, R.J., Zeissig, S., Zhang, B., Zhang, C.K., Zhao, H.; International IBD Genetics Consortium (IIBDGC), Silverberg, M.S., Annese, V., Hakonarson, H., Brant, S.R., Radford-Smith, G., Mathew, C.G., Rioux, J.D., Schadt, E.E., Daly, M.J., Franke, A., Parkes, M., Vermeire, S., Barrett, J.C., Cho, J.H., 2012. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 491, 119–124.

Kanauchi, O., Matsumoto, Y., Matsumura, M., Fukuoka, M., Bamba, T., 2005. The beneficial effects of microflora, especially obligate anaerobes, and their products on the colonic environment in inflammatory bowel disease. Current Pharmaceutical Design 11, 1047–1053.

Kathrani, A., Holder, A., Catchpole, B., Alvarez, L., Simpson, K., Werling, D., Allenspach, K., 2012. TLR5 risk-associated haplotype for canine inflammatory bowel disease confers hyper-responsiveness to flagellin. PLoS ONE 7, e30117.

Kathrani, A., House, A., Catchpole, B., Murphy, A., German, A., Werling, D., Allenspach, K., 2010. Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. PLoS ONE 5, e15740.

Kathrani, A., House, A., Catchpole, B., Murphy, A., Werling, D., Allenspach, K., 2011.

Breed-independent toll-like receptor 5 polymorphisms show association with canine inflammatory bowel disease. Tissue Antigens 78, 94–101.

Kathrani, A., Lee, H., White, C., Catchpole, B., Murphy, A., German, A., Werling, D., Allenspach, K., 2014. Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease. Veterinary Immunology and Immunopathology 161, 32–41.

Kullberg, B.J., Ferwerda, G., de Jong, D.J., Drenth, J.P., Joosten, L.A., Van der Meer, J.W., Netea, M.G., 2008. Crohn's disease patients homozygous for the 3020insC NOD2 mutation have a defective NOD2/TLR4 cross-tolerance to intestinal stimuli. Immunology 123, 600–605.

Kumar, P., Henikoff, S., Ng, P.C., 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nature Protocols 4, 1073–1081.

Lee, J., Mo, J.H., Katakura, K., Alkalay, I., Rucker, A.N., Liu, Y.T., Lee, H.K., Shen, C., Cojocaru, G., Shenouda, S., Kagnoff, M., Eckmann, L., Ben-Neriah, Y., Raz, E., 2006. Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nature Cell Biology 8, 1327–1336.

Leung, E., Hong, J., Fraser, A., Krissansen, G.W., 2007. Splicing of NOD2 (CARD15) RNA transcripts. Molecular Immunology 44, 284–294.

Ley, R.E., Turnbaugh, P.J., Klein, S., Gordon, J.I., 2006. Human gut microbes associated with obesity. Nature 444, 1022–1023.

Lidbeck, A., Nord, C.E., 1993. Lactobacilli and the normal human anaerobic microflora. Clinical Infectious Diseases 16, S181–S187.

Lu, W.G., Zou, Y.F., Feng, X.L., Yuan, F.L., Gu, Y.L., Li, X., Li, C.W., Jin, C., Li, J.P., 2010. Association of NOD1 (CARD4) insertion/deletion polymorphism with susceptibility to IBD: a meta-analysis. World Journal of Gastroenterology 16, 4348–4356.

Maccoux, L.J., Clements, D.N., Salway, F., Day, P.J., 2007a. Identification of new reference genes for the normalisation of canine osteoarthritic joint tissue transcripts from microarray data. BMC Molecular Biology 8, 62.

Maccoux, L.J., Salway, F., Day, P.J., Clements, D.N., 2007b. Expression profiling of select cytokines in canine osteoarthritis tissues. Veterinary Immunology and

Immunopathology 118, 59–67.

Mackie, R.I., Sghir, A., Gaskins, H.R., 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. The American Journal of Clinical Nutrition 69, 1035S–1045S.

Maeda, S., Maeda, S., Shibata, S., Chimura, N., Fukata, T., 2009. House dust mite major allergen Der f 1 enhances proinflammatory cytokine and chemokine gene expression in a cell line of canine epidermal keratinocytes. Veterinary Immunology and Immunopathology 131, 298–302.

Magalhaes, J.G., Tattoli, I., Girardin, S.E., 2007. The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. Seminars in Immunology 19, 106–115.

McMahon, L.A., House, A.K., Catchpole, B., Elson-Riggins, J., Riddle, A., Smith, K., Werling, D., Burgener, I.A., Allenspach, K., 2010. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. Veterinary Immunology and Immunopathology 135, 158–163.

Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., Jr., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394–397.

Mercier, E., Peters, I.R., Day, M.J., Clercx, C., Peeters, D., 2012. Toll- and NOD-like receptor mRNA expression in canine sino-nasal aspergillosis and idiopathic lymphoplasmacytic rhinitis. Veterinary Immunology and Immunopathology 145, 618–624.

Meylan, E., Tschopp, J., Karin, M., 2006. Intracellular pattern recognition receptors in the host response. Nature 442, 39–44.

Middelbos, I.S., Vester Boler, B.M., Qu, A., White, B.A., Swanson, K.S., Fahey, G.C., Jr., 2010. Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLoS ONE 5, e9768.

Miwa, H., Hiyama, C., Yamamoto, M., 1985. High-performance liquid chromatography of short-and long-chain fatty acids as 2-nitrophenylhydrazides. Journal of Chromatography 321, 165–174.

Miwa, H., Yamamoto, M., Nishida, T., Nunoi, K., Kikuchi, M., 1987. High-performance liquid chromatographic analysis of serum long-chain fatty acids by direct derivatization method. Journal of Chromatography 416, 237–245.

Nava, G.M., Stappenbeck, T.S., 2011. Diversity of the autochthonous colonic microbiota. Gut Microbes 2, 99–104.

Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., Akira, S., 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. The Journal of Immunology 164, 3476–3479.

O'Neill, L.A., Bowie, A.G., 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nature Reviews Immunology 7, 353–364.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nuñez, G., Cho, J.H., 2001a. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411, 603–606.

Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S., Nunez, G., 2001b. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. The Journal of Biological Chemistry 276, 4812–4818.

Ohmi, A., Tsukamoto, A., Ohno, K., Uchida, K., Nishimura, R., Fukushima, K., Takahashi, M., Nakashima, K., Fujino, Y., Tsujimoto, H., 2012. A retrospective study of inflammatory colorectal polyps in miniature dachshunds. The Journal of Veterinary Medical Science 74, 59–64.

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., Takiguchi, M., 2013. Expression of CD4+ T cell cytokine genes in the colorectal mucosa of inflammatory colorectal polyps in miniature dachshunds. Veterinary Immunology and Immunopathology 155, 259–263.

Okafuji, I., Nishikomori, R., Kanazawa, N., Kambe, N., Fujisawa, A., Yamazaki, S., Saito, M., Yoshioka, T., Kawai, T., Sakai, H., Tanizaki, H., Heike, T., Miyachi, Y., Nakahata, T., 2009. Role of the NOD2 genotype in the clinical phenotype of Blau

syndrome and early-onset sarcoidosis. Arthritis and Rheumatology 60, 242–250.

Okanishi, H., Hayashi, K., Sakamoto, Y., Sano, T., Maruyama, H., Kagawa, Y., Watari, T., 2013a. NOD2 mRNA expression and NFkappaB activation in dogs with lymphocytic plasmacytic colitis. Journal of Veterinary Internal Medicine 27, 439–444.

Okanishi, H., Kabeya, H., Maruyama, S., Kagawa, Y., Watari, T., 2013b. Activation of nuclear factor-kappa B and cell adhesion molecule mRNA expression in duodenal mucosa of dogs with lymphocytic-plasmacytic enteritis. Veterinary Immunology and Immunopathology 154, 145–152.

Okumura, S., Yuki, K., Kobayashi, R., Okamura, S., Ohmori, K., Saito, H., Ra, C., Okayama, Y., 2009. Hyperexpression of NOD2 in intestinal mast cells of Crohn's disease patients: preferential expression of inflammatory cell-recruiting molecules via NOD2 in mast cells. Clinical Immunology 130, 175–185.

Ordás, I., Eckmann, L., Talamini, M., Baumgart, D.C., Sandborn, W.J., 2012. Ulcerative colitis. Lancet 380, 1606–1619.

Packey, C.D., Sartor, R.B., 2009. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Current Opinion in Infectious Diseases 22, 292–301.

Panasevich, M.R., Kerr, K.R., Dilger, R.N., Fahey, G.C., Jr., Guérin-Deremaux, L., Lynch, G.L., Wils, D., Suchodolski, J.S., Steiner, J.M., Dowd, S.E., Swanson, K.S., 2014. Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. British Journal of Nutrition 24, 1–9.

Panasevich, M.R., Rossoni Serao, M.C., de Godoy, M.R., Swanson, K.S., Guérin-Deremaux, L., Lynch, G.L., Wils, D., Fahey, G.C., Jr., Dilger, R.N., 2013. Potato fiber as a dietary fiber source in dog foods. Journal of Animal Science 91, 5344–5352.

Patra, A.K., 2011. Responses of feeding prebiotics on nutrient digestibility, faecal microbiota composition and short-chain fatty acid concentrations in dogs: a meta-analysis. Animal 5, 1743–1750.

Peters, I.R., Peeters, D., Helps, C.R., Day, M.J., 2007. Development and application of multiple internal reference (housekeeper) gene assays for accurate normalisation of canine gene expression studies. Veterinary Immunology and Immunopathology

117, 55–66.

Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnology Letters 26, 509–515.

Pierik, M., Joossens, S., Van Steen, K., Van Schuerbeek, N., Vlietinck, R., Rutgeerts, P., Vermeire, S., 2006. Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. Inflammatory Bowel Diseases 12, 1–8.

Pott, J., Stockinger, S., Torow, N., Smoczek, A., Lindner, C., Mclnerney, G., Bäckhed, F., Baumann, U., Pabst, O., Bleich, A., Hornef, M.W., 2012. Age-dependent TLR3 expression of the intestinal epithelium contributes to rotavirus susceptibility. . PLoS Pathogens 8, e1002670.

Reichardt, N., Duncan, S.H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K.P., Flint, H.J., Louis, P., 2014. Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. The ISME Journal 8, 1323–1335.

Rogler, G., Andus, T., 1998. Cytokines in inflammatory bowel disease. World Journal of Surgery 22, 382–389.

Rosenstiel, P., Huse, K., Till, A., Hampe, J., Hellmig, S., Sina, C., Billmann, S., von Kampen, O., Waetzig, G.H., Platzer, M., Seegert, D., Schreiber, S., 2006. A short isoform of NOD2/CARD15, NOD2-S, is an endogenous inhibitor of NOD2/receptor-interacting protein kinase 2-induced signaling pathways. Proceedings of the National Academy of Sciences of the United States of America 103, 3280–3285.

Rossi, G., Pengo, G., Caldin, M., Palumbo Piccionello, A., Steiner, J.M., Cohen, N.D., Jergens, A.E., Suchodolski, J.S., 2014. Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic VSL#3 strains in dogs with idiopathic inflammatory bowel disease. PLoS ONE 9, e94699.

Rubino, S.J., Selvanantham, T., Girardin, S.E., Philpott, D.J., 2012. Nod-like receptors in

the control of intestinal inflammation. Current Opinion in Immunology 24, 398–404.

Ruland, J., 2011. Return to homeostasis: downregulation of NF-κB responses. Nature Immunology 12, 709–714.

Sánchez-Muñoz, F., Fonseca-Camarillo, G., Villeda-Ramírez, M.A., Miranda-Pérez, E., Mendivil, E.J., Barreto-Zúñiga, R., Uribe, M., Bojalil, R., Domínguez-López, A., Yamamoto-Furusho, J.K., 2011. Transcript levels of Toll-Like Receptors 5, 8 and 9 correlate with inflammatory activity in Ulcerative Colitis. BMC Gastroenterology11, 138.

Saraiva, M., O'Garra, A., 2010. The regulation of IL-10 production by immune cells. Nature Reviews Immunology 10, 170–181.

Schmitz, S., Garden, O.A., Werling, D., Allenspach, K., 2012. Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease. Veterinary Immunology and Immunopathology 146, 87–91.

Schmitz, S., Henrich, M., Neiger, R., Werling, D., Allenspach, K., 2014. Stimulation of duodenal biopsies and whole blood from dogs with food-responsive chronic enteropathy and healthy dogs with toll-like receptor ligands and probiotic *Enterococcus faecium*. Scandinavian Journal of Immunology 80, 85–94.

Seiler, R.J., 1979. Colorectal polyps of the dog: a clinicopathologic study of 17 cases. Journal of the American Veterinary Medical Association 174, 72–75.

Sfriso, P., Caso, F., Tognon, S., Galozzi, P., Gava, A., Punzi, L., 2012. Blau syndrome, clinical and genetic aspects. Autoimmunity Reviews 12, 44–51.

Shannon, C.E., 1948. A mathematical theory of communication. The Bell System Technical Journal, 379–423 and 623–656.

Shibolet, O., Podolsky, D.K., 2007. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. American Journal of Physiology: Gastrointestinal and Liver Physiology 292, G1469–G1473.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermúdez-Humarán, L.G., Gratadoux, J.J., Blugeon, S., Bridonneau, C., Furet, J.P., Corthier, G., Grangette, C., Vasquez, N., Pochart, P., Trugnan, G., Thomas, G., Blottière, H.M., Doré, J., Marteau, P., Seksik, P., Langella, P., 2008. *Faecalibacterium prausnitzii* is an anti-

inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proceedings of the National Academy of Sciences of the United States of America 105, 16731–16736.

Steenholdt, C., Andresen, L., Pedersen, G., Hansen, A., Brynskov, J., 2009. Expression and function of toll-like receptor 8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease. Scandinavian Journal of Gastroenterology 44, 195–204.

Strober, W., Kitani, A., Fuss, I., Asano, N., Watanabe, T., 2008. The molecular basis of NOD2 susceptibility mutations in Crohn's disease. Mucosal Immunology 1, S5–S9.

Strompfová, V., Pogány Simonová, M., Gancarčíková, S., Mudroňová, D., Farbáková, J., Mad'ari, A., Lauková, A., 2014. Effect of *Bifidobacterium animalis* B/12 administration in healthy dogs. Anaerobe 28, 37–43.

Suchodolski, J.S., 2011. Companion animals symposium: microbes and gastrointestinal health of dogs and cats. Journal of Animal Science 89, 1520–1530.

Suchodolski, J.S., Dowd, S.E., Wilke, V., Steiner, J.M., Jergens, A.E., 2012a. 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS ONE 7, e39333.

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., Toresson, L., 2012b. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS ONE 7, e51907.

Suchodolski, J.S., Xenoulis, P.G., Paddock, C.G., Steiner, J.M., Jergens, A.E., 2010. Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. Veterinary Microbiology 142, 394–400.

Sun, J., Fegan, P.E., Desai, A.S., Madara, J.L., Hobert, M.E., 2007. Flagellin-induced tolerance of the Toll-like receptor 5 signaling pathway in polarized intestinal epithelial cells. American Journal of Physiology: Gastrointestinal and Liver Physiology 292, G767–778.

Sunvold, G.D., Fahey, G.C., Jr., Merchen, N.R., Reinhart, G.A., 1995a. *In vitro* fermentation of selected fibrous substrates by dog and cat fecal inoculum:

influence of diet composition on substrate organic matter disappearance and short-chain fatty acid production. Journal of Animal Science 73, 1110–1122.

Sunvold, G.D., Fahey, G.C., Jr., Merchen, N.R., Titgemeyer, E.C., Bourquin, L.D., Bauer, L.L., Reinhart, G.A., 1995b. Dietary fiber for dogs: IV. *In vitro* fermentation of selected fiber sources by dog fecal inoculum and *in vivo* digestion and metabolism of fiber-supplemented diets. Journal of Animal Science 73, 1099–1109.

Szebeni, B., Veres, G., Dezsõfi, A., Rusai, K., Vannay, A., Mraz, M., Majorova, E., Arató, A., 2008. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. Clinical and Experimental Immunology 151, 34–41.

Szkaradkiewicz, A., Marciniak, R., Chudzicka-Strugała, I., Wasilewska, A., Drews, M., Majewski, P., Karpiński, T., Zwoździak, B., 2009. Proinflammatory cytokines and IL-10 in inflammatory bowel disease and colorectal cancer patients. Archivum Immunologiae et Therapiae Experimentalis 57, 291–294.

Tahara, T., Yamamoto, E., Suzuki, H., Maruyama, R., Chung, W., Garriga, J., Jelinek, J., Yamano, H.O., Sugai, T., An, B., Shureiqi, I., Toyota, M., Kondo, Y., Estécio, M.R., Issa, J.P., 2014. *Fusobacterium* in colonic flora and molecular features of colorectal carcinoma. Cancer Research 74, 1311–1318.

Takaishi, H., Matsuki, T., Nakazawa, A., Takada, T., Kado, S., Asahara, T., Kamada, N., Sakuraba, A., Yajima, T., Higuchi, H., Inoue, N., Ogata, H., Iwao, Y., Nomoto, K., Tanaka, R., Hibi, T., 2008. Imbalance in intestinal microflora constitution could be involved in the pathogenesis of inflammatory bowel disease. International Journal of Medical Microbiology 298, 463–472.

Tamura, Y., Ohta, H., Torisu, S., Yuki, M., Yokoyama, N., Murakami, M., Lim, S.Y., Osuga, T., Morishita, K., Nakamura, K., Yamasaki, M., Takiguchi, M., 2013. Markedly increased expression of interleukin-8 in the colorectal mucosa of inflammatory colorectal polyps in miniature dachshunds. Veterinary Immunology and Immunopathology 156, 32–42.

Tanabe, S., Kinuta, Y., Saito, Y., 2008. *Bifidobacterium infantis* suppresses proinflammatory interleukin-17 production in murine splenocytes and dextran sodium sulfate-induced intestinal inflammation. International Journal of Molecular Medicine 22, 181–185.

Tanabe, T., Chamaillard, M., Ogura, Y., Zhu, L., Qiu, S., Masumoto, J., Ghosh, P., Moran, A., Predergast, M.M., Tromp, G., Williams, C.J., Inohara, N., Núñez, G., 2004. Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition. The EMBO Journal 23, 1587–1597.

Tedelind, S., Westberg, F., Kjerrulf, M., Vidal, A., 2007. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World Journal of Gastroenterology 13, 2826–2832.

Tsukamoto, A., Ohno, K., Irie, M., Ohmi, A., Maeda, S., Nakashima, K., Fukushima, K., Fujino, Y., Uchida, K., Tsujimoto, H., 2012. A case of canine multiple inflammatory colorectal polyps treated by endoscopic polypectomy and argon plasma coagulation. The Journal of Veterinary Medical Science 74, 503–506.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R., Gordon, J.I., 2009. A core gut microbiome in obese and lean twins. Nature 457, 480–484.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.

Uematsu, S., Akira, S., 2009. Immune responses of TLR5(+) lamina propria dendritic cells in enterobacterial infection. Journal of Gastroenterology 44, 803–811.

Valerius, K.D., Powers, B.E., McPherron, M.A., Hutchison, J.M., Mann, F.A., Withrow, S.J., 1997. Adenomatous polyps and carcinoma in situ of the canine colon and rectum: 34 cases (1982-1994). Journal of the American Animal Hospital Association 33, 156–160.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, RESEARCH0034.

Vernia, P., Gnaedinger, A., Hauck, W., Breuer, R.I., 1988. Organic anions and the diarrhea of inflammatory bowel disease. Digestive Diseases and Sciences 33, 1353–1358.

Vieira, A.T., Teixeira, M.M., Martins, F.S., 2013. The Role of Probiotics and Prebiotics in Inducing Gut Immunity. Frontiers in Immunology 4, 445.

Viladomiu, M., Hontecillas, R., Yuan, L., Lu, P., Bassaganya-Riera, J., 2013. Nutritional protective mechanisms against gut inflammation. The Journal of Nutritional Biochemistry 24, 929–939.

Walker, A.W., Sanderson, J.D., Churcher, C., Parkes, G.C., Hudspith, B.N., Rayment, N., Brostoff, J., Parkhill, J., Dougan, G., Petrovska, L., 2011. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiology 11, 7.

Wallace, K.L., Zheng, L.B., Kanazawa, Y., Shih, D.Q., 2014. Immunopathology of inflammatory bowel disease. World Journal of Gastroenterology 20, 6–21.

Wang, Z.K., Yang, Y.S., Chen, Y., Yuan, J., Sun, G., Peng, L.H., 2014. Intestinal microbiota pathogenesis and fecal microbiota transplantation for inflammatory bowel disease. World Journal of Gastroenterology 20, 14805–14820.

Watanabe, T., Asano, N., Murray, P.J., Ozato, K., Tailor, P., Fuss, I.J., Kitani, A., Strober, W., 2008. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. Journal of Clinical Investigation 118, 545–559.

Watanabe, T., Kitani, A., Murray, P.J., Strober, W., 2004. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nature Immunology 5, 800–808.

Wong, J.M., de Souza, R., Kendall, C.W., Emam, A., Jenkins, D.J., 2006. Colonic health: fermentation and short chain fatty acids. Journal of Clinical Gastroenterology 40, 235–243.

Xavier, R.J., Podolsky, D.K., 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature 448, 427–434.

Yamagishi, C., Momoi, Y., Kobayashi, T., Ide, K., Ohno, K., Tsujimoto, H., Iwasaki, T., 2007. A retrospective study and gene analysis of canine sterile panniculitis. The Journal of Veterinary Medical Science 69, 915–924.

Yogo, T., Ohashi, Y., Terakado, K., Harada, Y., Nezu, Y., Hara, Y., Tagawa, M., Kageyama, H., Fujisawa, T., 2011. Influence of dried okara-tempeh on the composition and metabolites of fecal microbiota in dogs. International Journal of Applied Research in Veterinary Medicine 9, 176–183.

Zarember, K.A., Godowski, P.J., 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. The Journal of Immunology168, 554–561.

Zhong, Y., Kinio, A., Saleh, M., 2013. Functions of NOD-like receptors in human diseases. Frontiers in Immunology 4, 333.