A study on the colorectal mucosal immunopathology of inflammatory colorectal polyps in dogs — analyses of innate immunity and epithelial barrier

（炎症性結直腸ポリープの犬における結直腸粘膜の免疫病態に関する研究 — 自然免疫と上皮バリアの解析）

Nozomu Yokoyama

Laboratory of Veterinary Internal Medicine

Department of Veterinary Clinical Sciences

Graduate School of Veterinary Medicine

Hokkaido University

2017. June

GENERAL ABBREVIATIONS

AJ	Adherence junction
HE	Hematoxylin and eosin
IBD	Inflammatory bowel disease
ISH	*In situ* hybridization
ICRP	Inflammatory colorectal polyps
IEC	Intestinal epithelial cell
LPS	Lipopolysaccharide
MDs	Miniature dachshunds
NF-κB	Nuclear factor-κB
PPRs	Pattern recognition receptors
qPCR	Quantitative reverse transcription PCR
TJ	Tight junction
TLR	Toll-like receptor
WSAVA	World Small Animal Veterinary Association

TABLE OF CONTENTS

CHAPTER 3

EXPRESSION OF APICAL JUNCTION COMPLEX PROTEINS IN COLORECTAL MUCOSA OF MINIATURE DACHSHUNDS WITH INFLAMMATORY COLORECTAL POLYPS

GENERAL INTRODUCTION

Intestinal polyps are clinically defined as pedunculated intraluminal growths in small or large intestine. Due to recent advances in veterinary medicine, colorectal polyps are detected with increasing frequency during endoscopic examinations. The major histopathological subtypes of colorectal polyps include nonneoplastic (hyperplastic) polyps, papillary adenoma, and papillary adenocarcinoma.[1] Although there have been some reports on canine colorectal tumors, inflammatory polyps has not been described in details.

Inflammatory colorectal polyps (ICRPs) are recently recognized as a major cause of large bowel diarrhea in miniature dachshunds (MDs) in Japan.[2] ICRPs are characterized by the formation of multiple small polyps around the large bowel mucosa, almost invariably restricted to the descending colon and rectum, sometimes accompanied by space-occupying solitary large polyp formation. Histopathological findings of ICRPs are proliferative changes of epithelium without cellular atypia, increased numbers of goblet cells with dilated crypts, infiltration of inflammatory cells (predominantly by neutrophils and macrophages) and proliferation of granulation tissue.[2, 3] MDs with ICRPs demonstrate a good response for immunosuppressive drugs, such as prednisolone, cyclosporine, and leflunomide.[2, 4] Based on the existence of idiopathic inflammation and effectiveness of immunosuppressive therapy, ICRPs are thought to be a novel form of breed-specific canine inflammatory bowel disease (IBD) in MDs.[5]

One of the etiologies of human and canine IBD is hypothesized to result from the dysregulation of the mucosal immune response toward commensal bacterial flora together with genetic and environmental factors.[6, 7] Evidence available at present suggests that both dysregulated innate and adaptive immune systems contribute to the aberrant intestinal inflammatory response in patients with IBD. Among these immune systems, it is generally believed that the innate immune system plays a pivotal role in the early

inductions of chronic inflammation following the exposure to commensal bacterial flora. The innate immune system prevents pathogens from entering and spreading within the body and is mediated by a variety of different cell types, such as neutrophils, monocytes, macrophages and dendritic cells, as well as non-immune cells, such as intestinal epithelial cells. In particular, the intestinal epithelium forms the first line of defense to bacterial invasion. The intestinal epithelium is not only a physical barrier but also senses the intestinal microbiota and responds to conserved structural motifs on microorganisms. Recent results from genome-wide association studies and immunological studies have suggested that the dysfunction of innate microbial sensing and intestinal epithelial barrier contribute to the pathogenesis of IBD in terms of the induction and the persistence of chronic inflammation.[8]

Sensor activation of innate immune cells provides a primary host response and triggers the downstream effector and regulatory functions. This innate immune response is achieved through the recognition of microbial agents by pattern recognition receptors (PRRs), which include toll-like receptors (TLRs). PRRs are sensing receptors of microbial antigens of the innate immune system in the gastrointestinal mucosa that induce pro-inflammatory and immunomodulatory responses. TLRs are the most characterized PRRs, capable of potently activating different cell types, and they could be highly expressed on most of immune cells, as well as other cell types, including epithelial cells.[9] Regulation of the expression levels of TLRs on the surface of intestinal epithelial cells (IECs) is thought to be crucial for the maintenance of intestinal mucosal tolerance to luminal bacteria. The increased expression of some TLRs in IECs may be an important mechanism for the development of intestinal inflammation.[10] In human IBD, many studies have reported the upregulation of TLR2 and TLR4 in the IECs.[11-13] Moreover, dysregulated responses to TLRs at the intestinal mucosa are assumed to be related with intestinal inflammation in human IBD.[14-16]

Intestinal epithelial barrier is the first physical barrier that intestinal bacteria and food antigens encounter on the mucosal surface. Intestinal epithelial barrier is established by a single layer of

epithelial cells, and the space between these cells is sealed by tight junction (TJ) and adherence junction (AJ), which regulate the permeability of the intestinal barrier.[17, 18] Differential expressions and properties of TJ and AJ proteins are believed to determine tissue-specific variations in electrical resistance and paracellular ionic selectivity among epithelia. In human and canine IBD, alterations in the expression and localization of TJ and AJ proteins expression and localization were thought to compromise the integrity of the epithelial barrier leading to increased permeability and infiltration of pathogens.[19]

In previous research on the pathogenesis of ICRPs, Ohta *et al.* assessed the gene expression of cytokines of T cell subsets (Th1, Th2, and Th17) in the colorectal mucosa from dogs with ICRPs.[5] Moreover, Tamura *et al.* analyzed mRNA expressions of several pro-inflammatory cytokines (*IL-1ß*, *IL-6*, *TNF-ß*, *IL-8*) in colorectal polyps in ICRP dogs by quantitative reverse transcription PCR (qPCR).[20] These results suggested that the dysregulation of the mucosal immune response plays an important role in the pathogenesis of ICRPs. However, it is still unclear whether the dysregulation of innate immune system, especially in innate microbial sensing and epithelial barrier integrity, are associated with the pathogenesis of ICRPs.

Thus, the aim of this thesis was to clarify the involvement of mucosal immunity in the pathogenesis of ICRPs. This thesis especially focused on two parts: innate microbial sensing (Chapters 1 and 2) and epithelial barrier integrity (Chapter 3). In Chapter 1, the localization of TLR2 and TLR4 mRNA in the colorectal mucosa of MDs with ICRPs were evaluated. In Chapter 2, the effects of bacterial antigen-associated TLR ligands (TLR2, TLR4, TLR5 and TLR9) on pro-inflammatory cytokine (*IL-1β*, *IL-6*, *IL-8*, and *TNF-α*) mRNA expression were investigated in *ex vivo* cultured canine colorectal samples. In Chapter 3, the expression and distribution of various TJ proteins (claudin-1, -2, -3, -4, -5, -7, and -8) and AJ proteins (E-cadherin and β-catenin) were analyzed in the colorectal mucosa obtained from MDs with ICRPs.

CHAPTER 1

LOCALIZATION OF TLR2 AND TLR4 mRNA IN THE COLORECTAL MUCOSA OF MINIATURE DACHSHUNDS WITH INFLAMMATORY COLORECTAL POLYPS

1. INTRODUCTION

TLRs are members of the PRR family and play a central role in the initiation of innate cellular responses and subsequent adaptive immune responses to a variety of pathogens.[9] TLRs are the most characterized PRRs, capable of potently activating different cell types, and they can be highly expressed on most of immune cells, as well as other cell types, including epithelial cells. In canine IBD, *TLR4* was shown to be upregulated and *TLR5* downregulated at mRNA levels in the duodenal mucosa from German shepherd dogs with IBD.[21] Furthermore, several non-synonymous single nucleotide polymorphisms in the TLR4 and TLR5 genes are significantly associated with IBD in German shepherd dogs.[22] These results implicated that TLRs play an important role in the pathogenesis of canine IBD.

A recent study reported that mRNA expressions of several *TLRs*, including *TLR2* and *TLR4*, are upregulated mRNA expression in inflamed colorectal mucosa from MDs with ICRPs using qPCR.[23] These findings suggest that the increased expression of TLRs might play an important role in the pathogenesis of ICRPs. However, in this study, the localization of TLR mRNAs in inflamed colorectal mucosa was not determined. The RNAscope technique is a chromogenic *in situ* hybridization (ISH) method which uses a streamlined immunohistochemistry-like procedure.[24, 25] RNAscope provides single-molecule sensitivity and resolution, enabling the digital quantification of transcripts in single cells of interest. This technique has made it possible to determine the localization of *TLR2* and *TLR4* mRNA and quantify the *in situ* expression of *TLR2* and *TLR4* in the colonic epithelium.

The aim of Chapter 1 was to evaluate the localization of *TLR2* and *TLR4* mRNA in the colorectal mucosa of MDs with ICRPs. Furthermore, to compare the expression levels of *TLR2* and *TLR4* mRNAs in the colonic epithelium between MDs with ICRPs and healthy controls, *TLR2*- and *TLR4*-positive hybridization signals in the colonic epithelium were quantified.

2. MATERIALS AND METHODS

2.1 Animals and sample collection

A total of 10 unrelated dogs diagnosed with ICRPs based on the histological findings of a previous report[2, 3] were used in this study. For the analysis of inflamed colorectal mucosa, the paraffin-embedded tissues of large polyp lesions collected from 5 MDs with ICRPs were used. Rectal pull-through excision was performed at referral hospitals, and large polyp tissues were submitted to the Department of Diagnostic Pathology of Graduate School of Veterinary Medicine, Hokkaido University for histopathological examination. The median age of these dogs was 10.0 years (range, 6.0-12.0 years), with 2 intact females and 3 males (1 intact and 2 neutered). For the analysis of the non-inflamed colonic lesions, macroscopically normal colonic mucosa was collected from the descending colon of another 5 MDs with ICRPs. These dogs were referred to Hokkaido University Veterinary Teaching Hospital for the investigation of hematochezia, tenesmus, and an increased frequency of defecation. The median age of these dogs was 10.0 years (range, 9.1-14.7 years), with 1 intact female and 4 males (2 intact and 2 neutered). These cases presented between May 2012 and August 2014. All 5 dogs underwent colonoscopy under general anesthesia. Biopsy samples were collected endoscopically from the colorectal inflamed mucosa and macroscopically normal mucosa in the descending colon apart from the inflamed mucosa. Informed consent was obtained from the owners of the MDs prior to the procedures. The biopsy specimens were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin wax. All samples from MDs with ICRPs were sectioned (3 μm) and stained with hematoxylin and eosin (HE) for histological examination. Biopsy specimens from inflamed mucosa were diagnosed with ICRPs. In contrast, biopsy specimens from the macroscopically normal descending colon showed no abnormality based on World Small Animal Veterinary Association (WSAVA) guidelines.[26]

Control dogs consisted of 5 unrelated healthy beagles. Full-thickness colorectal tissue samples were obtained from the 5 healthy beagles (5 males; median age: 0.6 years, range: 0.6-0.7 years) used in another unrelated study, at necropsy immediately after euthanasia. These dogs had no clinical signs of gastrointestinal disease and showed no abnormalities, as determined by a blood test, fecal examination, and abdominal ultrasound. These experimental procedures were approved by the Laboratory Animal Experimentation Committee, Graduate school of Veterinary Medicine, Hokkaido University (Accession number, 13-0142). All samples were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (3 μm) and stained with HE. These control biopsy specimens showed no abnormality based on WSAVA guidelines.

2.2 Staging of inflamed mucosal lesions in dogs with ICRPs

Inflamed mucosal lesions were staged (I-III) according to criteria adapted from those described previously.[3] The area of the epithelial component of each lesion was measured in a randomly selected field under a ×40 object lens. In each field, the epithelial area was marked manually on the computer screen; then, the percentage of the total area was measured with a digital imaging analyzer (BZ-H3C, Keyence, Osaka, Japan). Lesions in which >15%, 5% – 15%, and <5% of the tissue consisting of epithelial cells were defined as stage I, stage II, and stage III lesions, respectively. Stage I was characterized by a thickened mucosa containing hyperplastic goblet cells. Stage II was characterized by dilated crypts filled with a large amount of mucus, and mild lymphocyte and macrophage infiltration with the proliferation of granulation tissue. Stages III was characterized by more severe neutrophil infiltration, interstitial mucus accumulation and granulation tissue (Figure 1).

2.3 *in situ* hybridization for *TLR2* and *TLR4*

For the detection of *TLR2* and *TLR4* mRNA expressions, RNAscope FFPE Assay Kit (Advanced

Cell Diagnostics, Inc., Hayward, California, USA) was used according to previous reports[24, 27] and the manufacturer's instructions, with a few modifications. RNAscope target probes were designed by Advanced Cell Diagnostics (Hayward, California, USA) using custom software as described previously.[24] The software automatically selects the target sequence from a series of oligonucleotides which have an average length of 25 bases and uniform melting temperature and do not cross-hybridize with any off-target sequence in the canine transcriptome under the RNAscope assay conditions. GenBank accession numbers, the number of probe pairs, and probe regions were: TLR2, NM_001005264.3, 15 pairs, 1061-1881; TLR4, NM_001002950.2, 20 pairs, 1131-2175. Briefly, sections (5 μm) were heated for 60 minutes at 60ºC in a hybridization oven (MHS-200e, Eyela Corporation, Tokyo, Japan). Tissues were dewaxed in xylene followed by dehydration in an ethanol series and air-dried for 5 minutes. Tissue sections were incubated with pretreatment 1 solution (endogenous peroxidase block) for 10 minutes at room temperature. Slides were rinsed by immersion in filtered deionized distilled water, followed by immersion in pretreatment 2 solution (antigen retrieval citrate buffer) for 15 minutes at 100ºC (boiling). Slides were washed in deionized distilled water, and pretreatment 3 (protease) was applied for 30 minutes at 40ºC. Slides were washed in deionized distilled water and target probes for the *TLR2* and *TLR4* genes, the housekeeping gene *POLR2* (positive control), or the bacterial gene *DapB* (negative control) were applied with incubation at 40ºC for 2 hours followed by rinsing in wash buffer. Signal amplification reagents 1 to 6 were applied sequentially for 30, 15, 30, 15, 30, and 15 minutes, respectively. Slides were rinsed in wash buffer for 2 minutes between amplification reagents. Incubations with amplifier reagents 1 to 4 were at 40ºC, while incubations with amplifier reagents 5 and 6 were at room temperature. A positive signal was visualized using diaminobenzidine with Mayer's hematoxylin counterstain. The images were recorded by digital microscopy (BZ-X710, Keyence).

2.4 Quantitative image analysis

To quantify the expression levels of *TLR2* and *TLR4* mRNAs in the colorectal epithelium, the positive hybridization signals of *TLR2* and *TLR4* were quantified by counting the number of brown dots in the crypts of each section. Microscopic pictures were captured in 10 well-oriented crypts whose entire length could be analyzed in each individual dog by digital microscopy (BZ-X710, Keyence) with a ×40 object lens. The brown pixel content of the digitized images was detected as a positive hybridization signal and the number of brown dots in each crypt was measured with a digital imaging analyzer (BZ-H3C, Keyence). The mean number of positive dots in a crypt was calculated from 10 crypts of each individual dog. The mean numbers of *TLR2*- and *TLR4*- positive dots were compared among inflamed lesions of MDs with ICRPs, non-inflamed colonic of MDs with ICRPs, and the colonic mucosa of healthy beagles.

2.5 Statistical analysis

Statistical analyses were performed using the computer program JMP 11 (SAS Institute, Cary, North Carolina, USA). Normality distribution was assessed by the Shapiro-Wilk *W* test. The Kruskal-Wallis test was used to test for overall differences in the mean numbers of *TLR2*- and *TLR4*-positive dots among inflamed colorectal mucosa of MDs with ICRPs, non-inflamed colonic mucosa of MDs with ICRPs, and the colonic mucosa of healthy beagles. The Steel-Dwass test was used to test for the between-group difference. A value of $P < 0.05$ was considered significant.

3. RESULTS

Localization of *TLR2* and *TLR4* mRNA in healthy beagles and MDs with ICRPs

In healthy beagles, the expression of *TLR2* mRNA was localized in the epithelium of the colonic crypt (Figures 2A and 2B). Similar to the results for *TLR2*, the expression of *TLR4* mRNA was found in the colonic epithelium (Figures 2A and 2C). The expressions of *TLR2* and *TLR4* were distributed from the crypt base to luminal surface in the crypts. The *TLR2* and *TLR4* expressions were barely detectable in the colorectal epithelium because of the low number of dots within a single cell and small size of single dots. The negative control probe generated no signals, whereas the positive control probe revealed strong staining (Figure 3).

In MDs with ICRPs, the expression of *TLR2* mRNA was found in the colonic epithelium of non-inflamed mucosa (Figures 2D and 2E), stage I (Figures 2G and 2H), and stage II (Figures 2J and 2K) of inflamed mucosa. *TLR2* mRNA expression was also observed in inflammatory cells and fibroblasts in stages II (Figures 2J and 2K) and III (Figures 2M and 2N) of inflamed mucosa. The localization of *TLR4* mRNA was similar to that of *TLR2*. *TLR4*-positive staining was found in the colonic epithelium of non-inflamed mucosa (Figures 2D and 2F), stage I (Figures 2G and 2I), and stage II (Figures 2J and 2L) of inflamed mucosa. In stage II (Figures 2J and 2L) and III (Figures 2M and 2O), *TLR4*-positive staining was detected in inflammatory cells and fibroblasts in granulation tissue. In stage I, the expressions of *TLR2* and *TLR4* were distributed from the crypt base to luminal surface in the crypts similar to that of healthy beagles, although the numbers of *TLR2*- and *TLR4*-positive dots in the crypt base were more than those in the luminal surface. The size of a single dot of *TLR*2 and *TLR4* in the inflamed mucosa was larger than that of the non-inflamed mucosa of ICRPs and of the colonic mucosa of healthy beagles. In addition, in the inflamed colorectal mucosa, several *TLR2*- and *TLR4*-positive dots were located within a single cell, and those dots were sometimes fused

with each other.

Quantitative analysis of *TLR2*- and *TLR4*-positive hybridization signals in colorectal epithelium of healthy beagles and MDs with ICRPs

To examine the *TLR2* and *TLR4* mRNA expression levels in the colorectal epithelium, but not in the granulation tissue, the positive hybridization signals in the colorectal epithelium of the colonic mucosa of healthy beagles, non-inflamed colonic mucosa of MDs with ICRPs, and stage I of inflamed lesions of MDs with ICRPs were quantified. There was a significant overall intergroup difference in the mean numbers of the *TLR2*-positive dots ($P < 0.01$, Figure 4). The mean numbers of the *TLR2*-positive dots were significantly higher in stage I inflamed mucosa of MDs with ICRPs (median: 202, range: 106–329; $P < 0.05$) compared with the colonic mucosa of healthy beagles (median: 24, range: 12–44) and non-inflamed mucosa of MDs with ICRPs (median: 48, range: 36-57). Similarly, there was a significant overall intergroup difference in the mean numbers of the *TLR4*-positive dots ($P < 0.005$, Figure 4). The mean numbers of the *TLR4*-positive dots showed a significant increase in stage I of the inflamed mucosa of MDs (median 281, range 150–424; $P < 0.05$) with ICRPs compared with the colonic mucosa of healthy beagles (median 28, range 8–48) and non-inflamed mucosa of MDs with ICRPs (median 27, range 18–35).

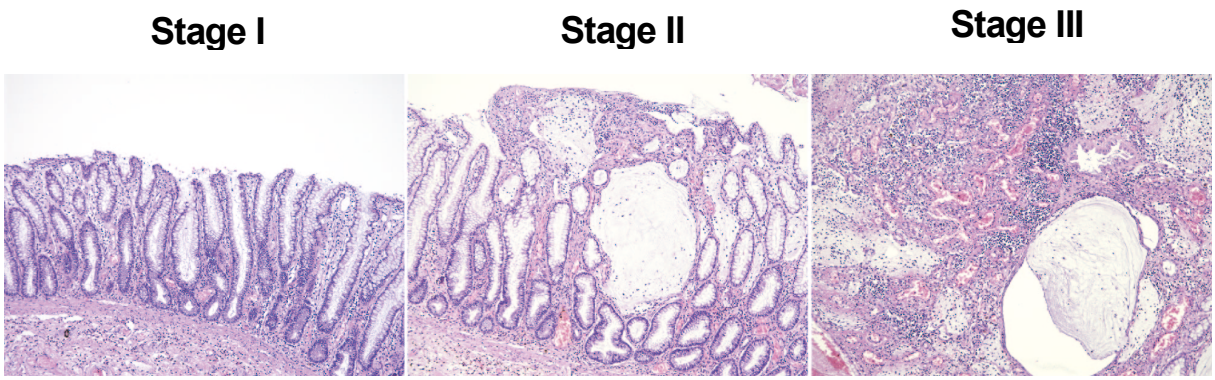

Figure 1. Staging of inflamed lesions in MDs with ICRPs. Histopathological appearance of stage I, stage II, and stage III. Stage I, thickened mucosa composed of hyperplastic goblet cells. Stage II, granulation tissue formation in the apical region of the mucosa and dilated crypts containing abundant mucus. Stage III, excessive production of granulation tissue, abundant neovascularization, and marked inflammatory changes. HE. Bars, 200 μm.

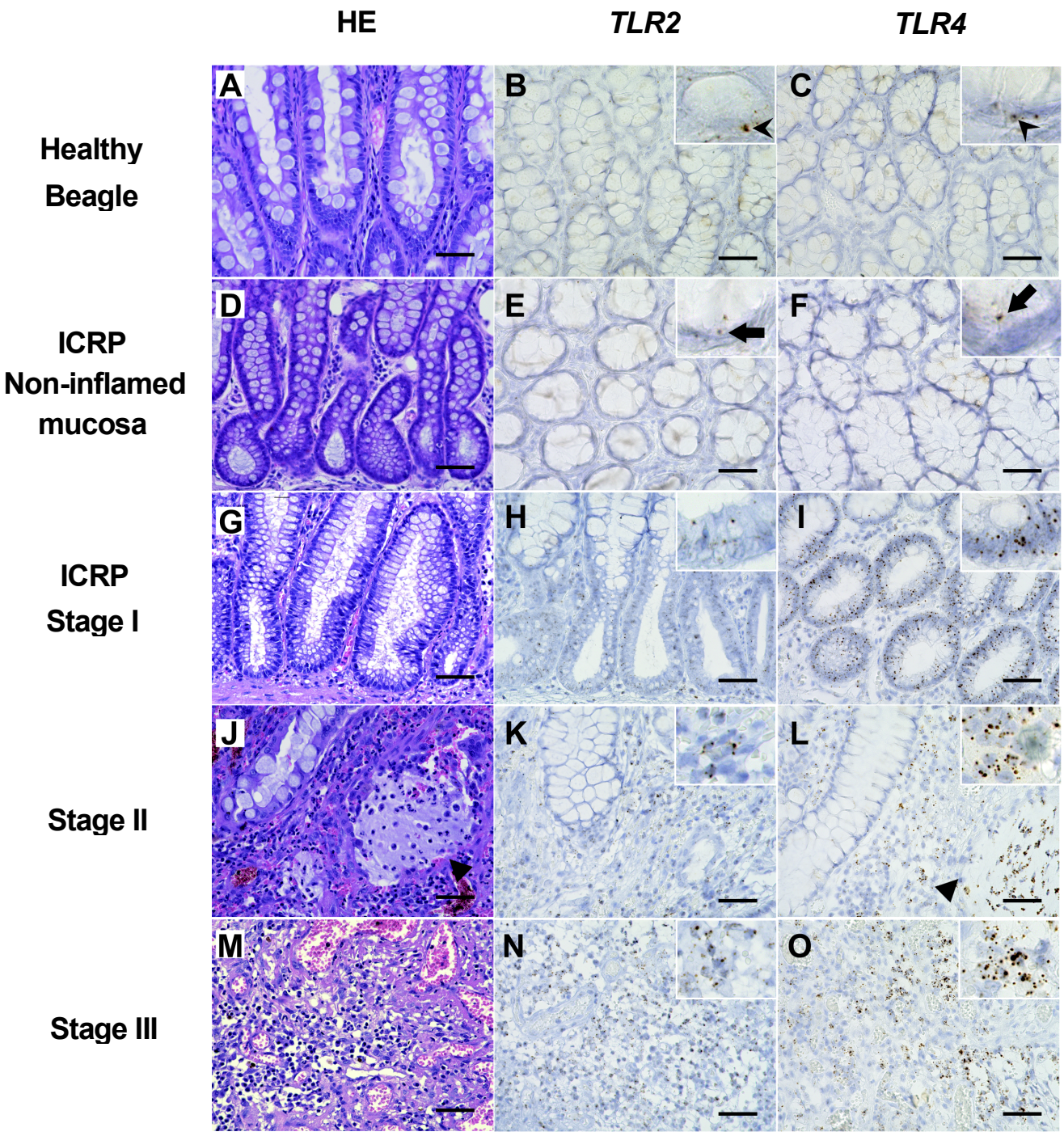

Figure 2. Representative photomicrographs of HE stain (left column) and *in situ* hybridization for *TLR2* (middle column) and *TLR4* (right column) in the colonic mucosa of healthy beagles (A-C) and MDs with ICRPs (D-F: non-inflamed mucosa; G-I: stage I; J-L: stage II; M-O: stage III). Labelling of mRNA transcripts was seen as brown punctate dots. (A-C) In healthy beagles, *TLR2* (B) and *TLR4* (C) were sparsely expressed in colonic epithelium (arrowheads). (D-F) In non-inflamed mucosa of MDs

with ICRPs, *TLR2* (E) and *TLR4* (F) were also sparsely expressed in colonic epithelium (arrows). (G-I) In stage I of MDs with ICRPs, the expressions of *TLR2* (H) and *TLR4* (I) mRNA were mainly localized in the colorectal epithelium. Note abundant dots in the colonic epithelium (Insets). (J-L) In stage II of MDs with ICRPs, *TLR2* (K) and *TLR4* (L) mRNAs were detected in both the colorectal epithelium and granulation tissue (Insets). The flat arrowhead indicates dilated crypts filled with mucinous material and cell debris. (M-O) In stage III of MDs with ICRPs, the expressions of *TLR2* (N) and *TLR4* (O) mRNA were observed in the inflammatory cells and fibroblasts (Insets). Insets: high magnification of positive staining. Left column: HE. Middle and right columns: ISH. Bars, 50 μm.

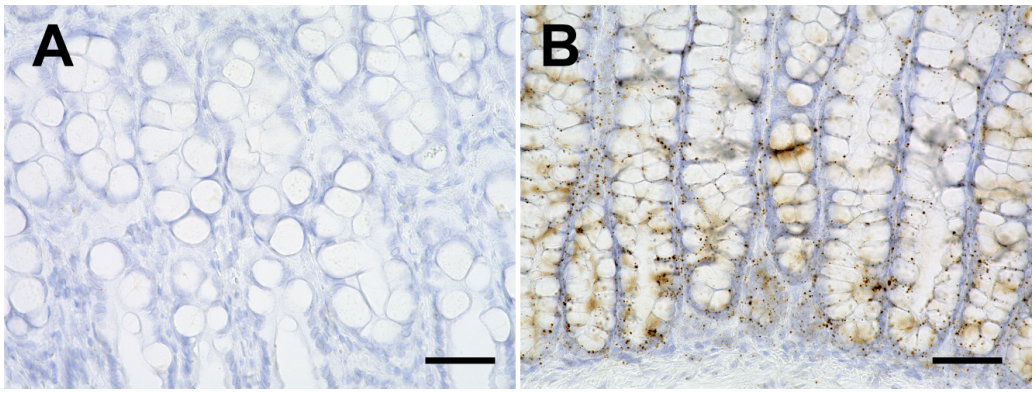

Figure 3. Representative photomicrographs of *in situ* hybridization for negative (*DapB*) and positive control targets (*POLR2*) in the colorectal mucosa of healthy beagles (A and B). The negative control probe (A) generated no signals, whereas the positive control probe (B) revealed numerous brown dots. ISH. Bars, 50 μm.

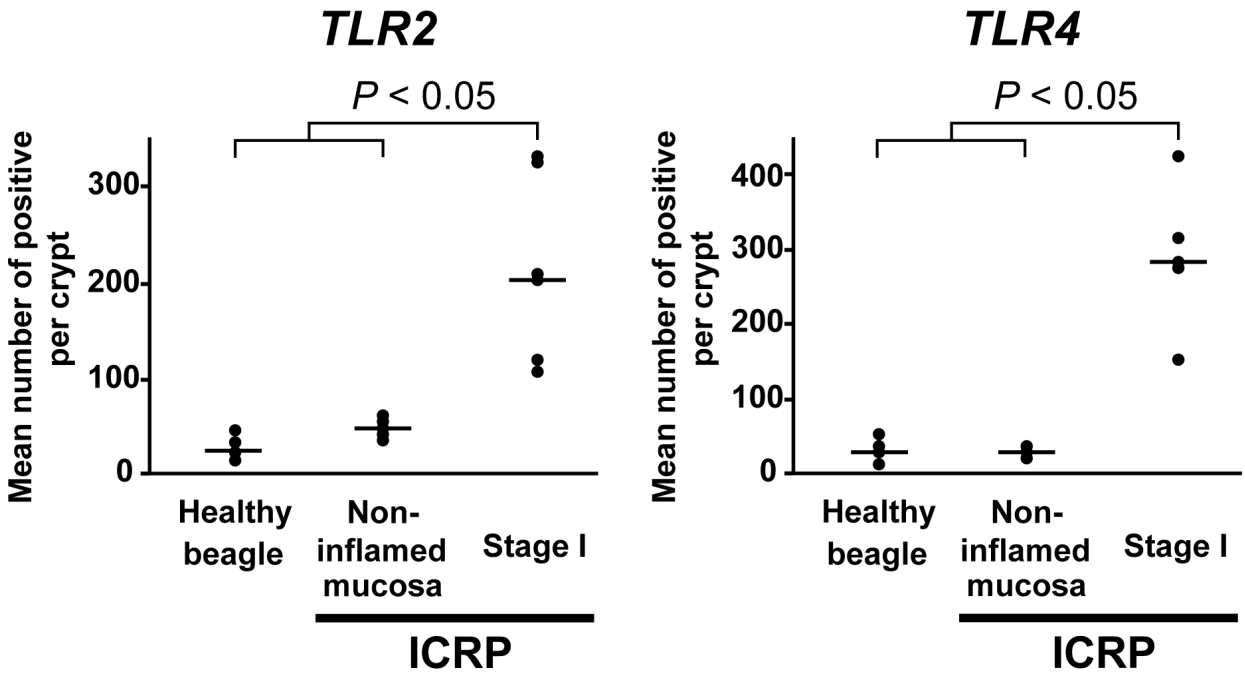

Figure 4. Scatter plots of the mean numbers of *TLR2*- and *TLR4*- positive dots in the colorectal mucosa of healthy beagles (n = 5) and MDs with ICRPs (non-inflamed mucosa, n = 5; stage I, n = 5). The horizontal line for each group represents the median. Asterisks indicate a significant difference.

4. DISCUSSION

In the present study, the localizations of *TLR2* and *TLR4* mRNA in the colorectal mucosa of dogs with ICRPs were demonstrated by the ISH method. It was found that TLR2 and TLR4 mRNAs were mainly localized in the colorectal epithelium of stage I and in the inflammatory cells and fibroblasts in granulation tissues in stage III. In stage II, *TLR2* and *TLR4* mRNAs were localized both in the epithelium and granulation tissues. Moreover, this study also demonstrated that the expressions of *TLR2* and *TLR4* mRNAs in the colonic epithelium were significantly increased in the colorectal epithelium in the inflamed mucosa of ICRPs (stage I) compared with the non-inflamed mucosa of MDs with ICRPs and the colonic mucosa of healthy beagles.

In a previous report, *TLR2* and *TLR4* mRNA expressions were upregulated in the inflamed mucosa of ICRPs compared with the non-inflamed mucosa of ICRP dogs and colonic mucosa of heathy beagles.[23] In this study, *TLR2* and *TLR4* mRNAs were localized both in the colorectal epithelium and inflammatory cells in the inflamed mucosa of MDs with ICRPs. These results suggested that the increased expression of *TLR2* and *TLR4* mRNAs in the inflamed mucosa results from not only inflammatory cell infiltration but also the upregulation of TLR2 and TLR4 mRNA in the colonic epithelium, which is consistent with the findings of human IBD.[11-13]

TLR2 and *TLR4* mRNAs were expressed in the colonic epithelium of healthy beagles. However, *TLR2* and *TLR4* mRNA expressions were limited. In human colonic mucosa, *TLR2* and *TLR4* mRNA are expressed at low levels by colonic epithelium on the basis of the expression analysis of immunohistochemistry, enzymatic separation of IECs, and laser capture microdissection of the intestinal epithelium.[11, 28, 29] The low levels of *TLR2* and *TLR4* expression in a basal condition have been suggested to suppress any unnecessary inflammation induced by commensal bacteria.[11] These findings are consistent with the results of this study. Similar to human colonic mucosa, the limited

expression levels of *TLR2* and *TLR4* in the canine colonic epithelium may prevent harmful hyper-responsiveness in response to commensal bacteria.

No significant difference was demonstrated in *TLR2* and *TLR4* mRNA expression levels in the colorectal epithelium between the non-inflamed mucosa of MDs with ICRPs and healthy beagles. In contrast, the upregulations of *TLR2* and *TLR4* mRNA were found in the colorectal epithelium of ICRPs (stage I). These findings suggested that *TLR2* and *TLR4* mRNA expressions were focally upregulated in the colorectal epithelium of the inflamed mucosa of ICRPs. In human IBD, it is thought that the upregulations of *TLR2* and *TLR4* in the colonic epithelium are induced by intestinal inflammation. In the normal human colonic mucosa, the expressions of *TLR2* and *TLR4* are regulated by suppressive factors, such as Toll inhibitory protein (Tollip) and T-helper 2 cytokines.[30, 31] On the other hand, it is thought that the upregulations of *TLR2* and *TLR4* in colonic epithelial cells are induced by IFN-γ and TNF-α secreted by T-helper 1 cells.[28, 31, 32] Indeed, a previous report showed that IFN-γ and TNF-α mRNA expressions were significantly increased in the inflamed mucosa of ICRPs.[5, 20] Therefore, it is possible that the upregulation of *TLR2* and *TLR4* in the colorectal epithelium of stage I was induced by pro-inflammatory cytokines secreted by inflammatory cells in stages II and III.

There are several limitations in the current study. First, this study did not enroll age- and breed-matched healthy controls. Therefore, the results in this chapter did not exclude the possibilities of the effect of age or breed difference on the expressions of *TLR2* and *TLR4* in the colonic epithelium. Second, the specific positive control sections for canine *TLR2* and *TLR4* mRNAs staining were not used. The specificity of probes for canine *TLR2* and *TLR4* mRNAs need to be confirmed by specific positive control sections such as canine TLR2 or TLR4 gene-transfected cultured cells. Finally, cell types of *TLR2* and *TLR4* mRNA-positive cells were identified solely based on the morphology of HE-stained sections. Thus, it is still unknown which types of cells express *TLR2* and *TLR4* mRNAs. Further studies using dual staining for cell-surface antigens of inflammatory cells and TLRs are

needed.

In conclusion, this study demonstrated that *TLR2* and *TLR4* mRNA were mainly localized in the colorectal epithelium of stage I and in the inflammatory cells and fibroblasts in stage III. These results suggested that the increased expressions of *TLR2* and *TLR4* mRNA in the inflamed colorectal mucosa result from not only inflammatory cell infiltration but also the upregulations of *TLR2* and *TLR4* mRNA in the colorectal epithelium. However, it remains unresolved whether increased *TLR2* and *TLR4* mRNA expressions in the colorectal epithelium are the cause or the consequence of inflammation. In future studies, the functional analysis of *TLR2* and *TLR4* in the colorectal epithelial cells from MDs with ICRPs and investigations of the corresponding genetic background are needed.

5. SUMMARY

TLR2 and *TLR4* mRNA localizations were examined by ISH using an RNAscope assay in samples of inflamed and non-inflamed mucosa of MDs with ICRPs and colonic mucosa of healthy beagles. As a result, *TLR2*- and *TLR4*-positive hybridization signals were localized in the colorectal epithelium, inflammatory cells and fibroblasts in the inflamed colorectal mucosa of ICRPs. In addition, *TLR2*- and *TLR4*-positive hybridization signals were significantly increased in the inflamed colorectal epithelium compared with non-inflamed colonic mucosa of MDs with ICRPs and healthy beagles. These results suggested that the increasing expressions of *TLR2* and *TLR4* mRNA in the inflamed colorectal mucosa result from not only inflammatory cell infiltration but also the upregulation of *TLR2* and *TLR4* mRNA in the colonic epithelium. The results also indicated that excessive TLR signaling in the colonic mucosa play an important role in the pathogenesis of ICRPs.

CHAPTER 2

STIMULATION OF COLORECTAL BIOPSIES FROM MINIATURE DACHSHUNDS WITH INFLAMMATORY COLORECTAL POLYPS WITH TOLL-LIKE RECEPTOR LIGANDS

1. INTRODUCTION

TLRs are the most characterized PRR family expressed by various cells in the gastrointestinal tract, including IEC and resident immune cells in the lamina propria.[33] The most important for bacterial recognition are TLR2, TLR4, TLR5 and TLR9, which recognize peptidoglycans from gram-positive bacteria, lipopolysacharide (LPS) from gram-negative bacteria, flagellin which is a main compartment of bacterial fragella, and unmethylated CpG-containing bacterial DNA, respectively.[33] Activation of these receptors initiates intracellular signalling pathways that promote the production of different pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α through activation of nuclear factor κB (NF-κB).[16]

In a previous study, Igarashi *et al.* demonstrated that several *TLR* mRNA expressions were upregulated in inflamed mucosa from MDs with ICRPs.[23] In Chapter 1, it was shown that *TLR2* and *TLR4* were localized in the colorectal epithelium and inflammatory cells of inflamed mucosa of ICRPs using the ISH method.[34] Moreover, Tamura *et al.* reported that the mRNA expressions of pro-inflammatory cytokines (*IL-1β*, *IL-6*, *IL-8*, *TNF-α*) were upregulated in the inflamed mucosa of ICRPs.[20] These results raised the hypothesis that the excessive production of pro-inflammatory cytokines via aberrant TLR signaling results in chronic inflammation in the colonic mucosa of ICRPs, as reported in colonic mucosa of human IBD.[14-16] Functional analysis of some PRRs in peripheral blood-derived monocytes have revealed that MDs with ICRPs exhibit altered responses to TLR2 ligands.[35] However, functional analysis of TLRs in the colorectal mucosa has not been performed.

The aim of the Chapter 2 was to investigate the effects of bacterial antigens as TLR ligands on pro-inflammatory cytokine (*IL-1β*, *IL-6*, *IL-8*, and *TNF-α*) mRNA expression in *ex vivo* cultured canine colorectal samples.

2. MATERIAL AND METHODS

2.1 Animals

Four MDs with ICRPs were used in this study. MDs with ICRPs were referred to Hokkaido University Veterinary Teaching Hospital between July 2015 and June 2016 for the investigation of hematochezia, tenesmus and increased frequency of defecation. ICRPs was diagnosed based on clinical and histopathological findings from the previous reports.[2, 3] There were three neutered males and one neutered female, and the median age of these dogs was 9 years (range, 6 to 11 years). These dogs had not been received any immunosuppressive drugs for 1 week prior to colonoscopy. Informed consent was obtained from the owner prior to inclusion of dogs in this study.

As healthy controls, colonic biopsies were obtained from four healthy MDs and five healthy beagles. The MDs consisting of two neutered males and two neutered females were owned by a volunteer. The median age of these dogs was 7 years (range, 6 to 8 years). The beagles were from a research colony and comprised of two intact males and three intact females. The median age of these dogs was 2 years (range 2 to 5 years). All controls had no clinical signs of gastrointestinal disease and showed no abnormalities, determined by a blood test, fecal examination, and abdominal ultrasound. All procedures for these dogs were performed with the approval of the Animal Care and Use Committee, Graduate School of Veterinary Medicine, Hokkaido University (Approval no. 13-0142).

2.2 Sample collection

Colonoscopy was performed under general anesthesia using a VQ-8143B flexible video endoscope (AVS, Tokyo, Japan). Multiple mucosal biopsies weighing 5–10 mg each were taken for diagnosis and *ex vivo* culture using VQ-143Q-B53 biopsy forceps (AVS). For *ex vivo* culture, colonic biopsies from ICRP dogs were obtained from the lesion, within 10 cm of macroscopically inflamed mucosa to

analyze the colonic mucosa in the marginal zone between the inflamed and non-inflamed mucosa of ICRPs. In the controls, colorectal mucosa samples were obtained from the lesion within 10–15 cm of the anal verge. Colonic samples for *ex vivo* culture were immediately placed in ice-cold transfer medium (Dulbecco's Modified Eagles Medium supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin). Samples for histopathology were placed in 10% neutral buffered formalin and embedded in paraffin, and hematoxylin and eosin-stained sections were prepared. These biopsy specimens were assessed by a board-certified pathologist (YK) according to WSAVA guidelines.[26] The biopsy specimens from the same lesion as the colorectal mucosa for *ex vivo* culture showed no abnormalities based on these guidelines.

2.3 *Ex vivo* culture of colonic biopsies

Ex vivo culture of colonic biopsies was performed using previously described protocols with some modification.[36, 37] *Ex vivo* culture was performed within 30 min of endoscopy. The colonic pinch biopsies from each dog obtained during colonoscopy were transferred into culture medium based on Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum, 2 mM GlutaMAX™ (Invitrogen, Carlsbad, CA, USA), 50 U/ml penicillin and 50 μg/ml streptomycin and rinsed by decanting three times to reduce the adherent microbiota. The baseline colonic mucosal samples were placed in RNAlater Solution (Ambion Inc., Austin, TX, USA), kept at 4 °C for 24 hours and then transferred to -80°C. The other samples were transferred to 24-well flat-bottomed plates and the following TLR ligands were added, based on the previous reports[35, 37]: synthetic bacterial lipoprotein (Pam3CSK4; TLR1/2 ligand; final concentration, 500 *n*g/m*l*), 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). Culture medium was used as an unstimulated control.

Each culture was performed in duplicate. The preliminary experiments revealed that the colonic biopsies remain viable for 4 hours, with intact colonic architecture based on the morphology of HE-stained section, according to a previous report.[38] Then, the plates were incubated for 4 hours at 37°C in 5% CO_2. After incubation, the biopsies were preserved in RNAlater Solution in the same manner as the baseline sample.

2.4 RNA extraction and cDNA synthesis

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

2.5 Transcription analysis of TLRs and cytokine mRNA by real-time RT-PCR

qPCR was performed for *TLR2*, *TLR4*, *TLR5*, *TLR9*, *IL-1β*, *IL-6*, *IL-8*, and *TNF-α*. For accurate quantification, hydroxymethyl-bilane synthase (*HMBS*), ribosomal protein L32 (*RPL32*), and ribosomal protein S18 (*RPS18*) were chosen as the reference gene as described previously.[23] The sequences of primer pairs used for qPCR were obtained from the previous reports[20, 23] (Table 1). Each qPCR reaction was performed in 20 μl, containing 500 nM of each primer and 1 μl cDNA in addition to FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) using a LightCycler Nano (Roche Diagnostics). The amplification conditions were 95°C for 10 min and 45 cycles PCR (95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec), followed by dissociation (95°C for 10 sec, 65°C for 60 sec, and 95°C for 1 sec). All samples were examined in duplicate, and each PCR reaction included a non-template control. Reaction efficiency was determined for each primer set, using 10-fold dilutions (10^7 molecules μl^{-1} to 10^1 molecules μl^{-1}) of plasmids ligated with each TLR,

cytokine and reference gene, and the correlation coefficient was>0.98. Cycle threshold (Ct) values that indicated the point where the threshold intersected with amplification curves of PCR reaction were determined using LightCycler Nano Software 1.0 (Roche Diagnostics). Melting curve analysis did not show misprinting in any of the reactions.

Gene expression was quantified by averaging the duplicate absolute gene copy number for each sample, followed by normalization of the expression of each target gene to the geometric mean of the three reference genes. In addition, the fold change in gene expression between stimulated and unstimulated samples was calculated as the relative ratio of the expression level in the stimulated samples to the expression level in the unstimulated samples.

2.6 Statistical analysis

Statistical analyses were performed using JMP 12 (SAS Institute Inc., Cary, NC, USA). Normality of distribution was assessed by the Shapiro-Wilk W test. The Mann-Whitney *U* test was used to compare the results of the expressions of TLRs and pro-inflammatory cytokines at baseline and the relative ratio of pro-inflammatory cytokines between case and control dogs. A value of $P < 0.05$ was considered significant.

Table 1 Sequences of the oligonucleotide primers used for quantitative real-time PCR

Primer set		Primer sequence (5′ – 3′)	Position	GenBank accession number
TLR2	Forward	TCGAGAAGAGCCACAAAACC	2085-2176	NM_001005264.2
	Reverse	CGAAAATGGGAGAAGTCCAG		
TLR4	Forward	GTGCTTCATGGTTTCTCTGGT	1521-1667	NM_001002950.1
	Reverse	CCAGTCTTCATCCTGGCTTG		
TLR5	Forward	TCGTGTTGACAGACGGTTATTT	421-564	EU551146.1
	Reverse	TCCGGTTGAGGGAAAAGTC		
TLR9	Forward	ACTGGCTGTTCCTCAAGTCC	289-393	NM_001002998.1
	Reverse	AGTCATGGAGGTGGTGGATG		
IL-1β	Forward	GAGGTTCCAATGTGAAGTGC	83–373	NM_001037971
	Reverse	CCTGTAACTTGCAGTCCACC		
IL-6	Forward	GAACTCCCTCTCCACAAGC	60–385	NM_001003301
	Reverse	TTCTTGTCAAGCAGGTCTCC		
IL-8	Forward	ACTTCCAAGCTGGCTGTTGC	10–181	NM_001003200
	Reverse	GGCCACTGTCAATCACTCTC		
TNF-α	Forward	ACTCTTCTGCCTGCTGCACTTTGG	138–504	NM_001003244
	Reverse	GTTGACCTTTGTCTGGTAGGAGACGG		
HMBS	Forward	TCACCATCGGAGCCATCT	455-566	XM_546491
	Reverse	GTTCCCACCACGCTCTTCT		
RPL32	Forward	TGGTTACAGGAGCAACAAGAAA	220-318	XM_848016
	Reverse	CACATCAGCAGCACTTCA		
RPS18	Forward	TGCTCATGTGGTATTGAGGAA	171-286	XM_532106
	Reverse	TCTTATACTGGCGTGGATTCTG		

HMBS, hydroxymethyl-bilane synthase; RPL32, ribosomal protein L32; RPS18, ribosomal protein S18

3. RESULTS

Gene expressions of TLRs and pro-inflammatory cytokines at baseline samples

No significant difference in the mRNA expression levels of *TLRs* and pro-inflammatory cytokines was observed between case and control dogs (Figure 5).

Relative ratios of the expression level of pro-inflammatory cytokines in the TLR-stimulated biopsies

In TLR4-stimulated biopsies, the relative ratio of *IL-6* (median 3.2; range 1.2–5.3) and *TNF-α* (median 2.2; range 1.4–3.0) of cases was significantly higher compared with *IL-6* (median 0.9; range 0.1–1.4; $P = 0.01$) and *TNF-α* (median 0.5; range: 0.06–2.7; $P = 0.03$) of control dogs (Figure 6). Moreover, in TLR9-stimulated biopsies, the relative ratio of *IL-8* (median 2.0; range 0.9–2.4) of cases was also significantly higher than those of control dogs (median 0.6; range 0.1–1.7; $P = 0.03$). There were no significant differences in the relative ratio of all pro-inflammatory cytokines in TLR2- and TLR5-stimulated biopsies from cases compared with controls.

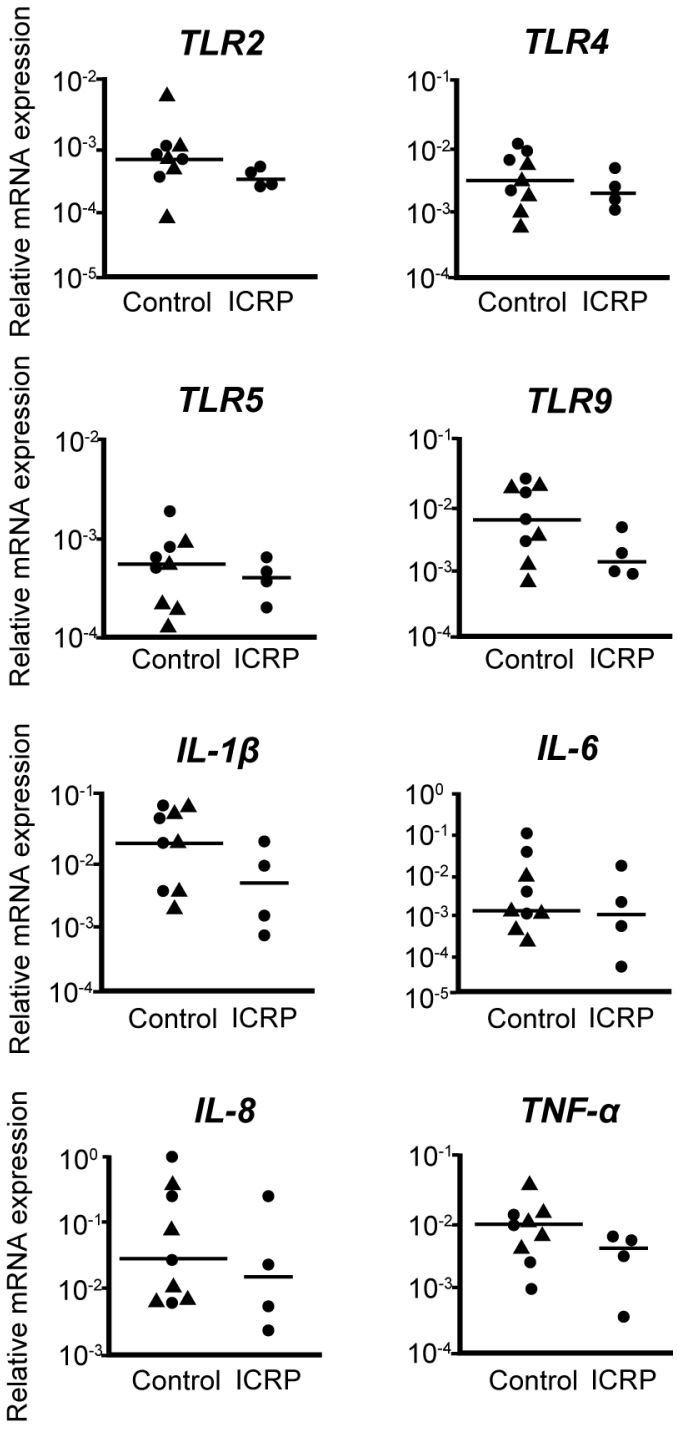

Figure 5. Relative transcription levels of mRNAs of toll-like receptors (*TLR2*, *TLR4*, *TLR5* and *TLR9*) and pro-inflammatory cytokines (*IL-1β*, *IL-6*, *IL-8* and *TNF-α*) in the colonic mucosa of miniature dachshunds (MDs) with inflammatory colorectal polyps (ICRPs) (n = 4) and control dogs (n = 9) at baseline. Black circles and triangles represent MDs and beagles, respectively. Hydroxymethyl-bilane synthase (*HMBS*), ribosomal protein L32 (*RPL32*), and ribosomal protein S18 (*RPS18*) were used as an internal control. The horizontal lines represent the median value.

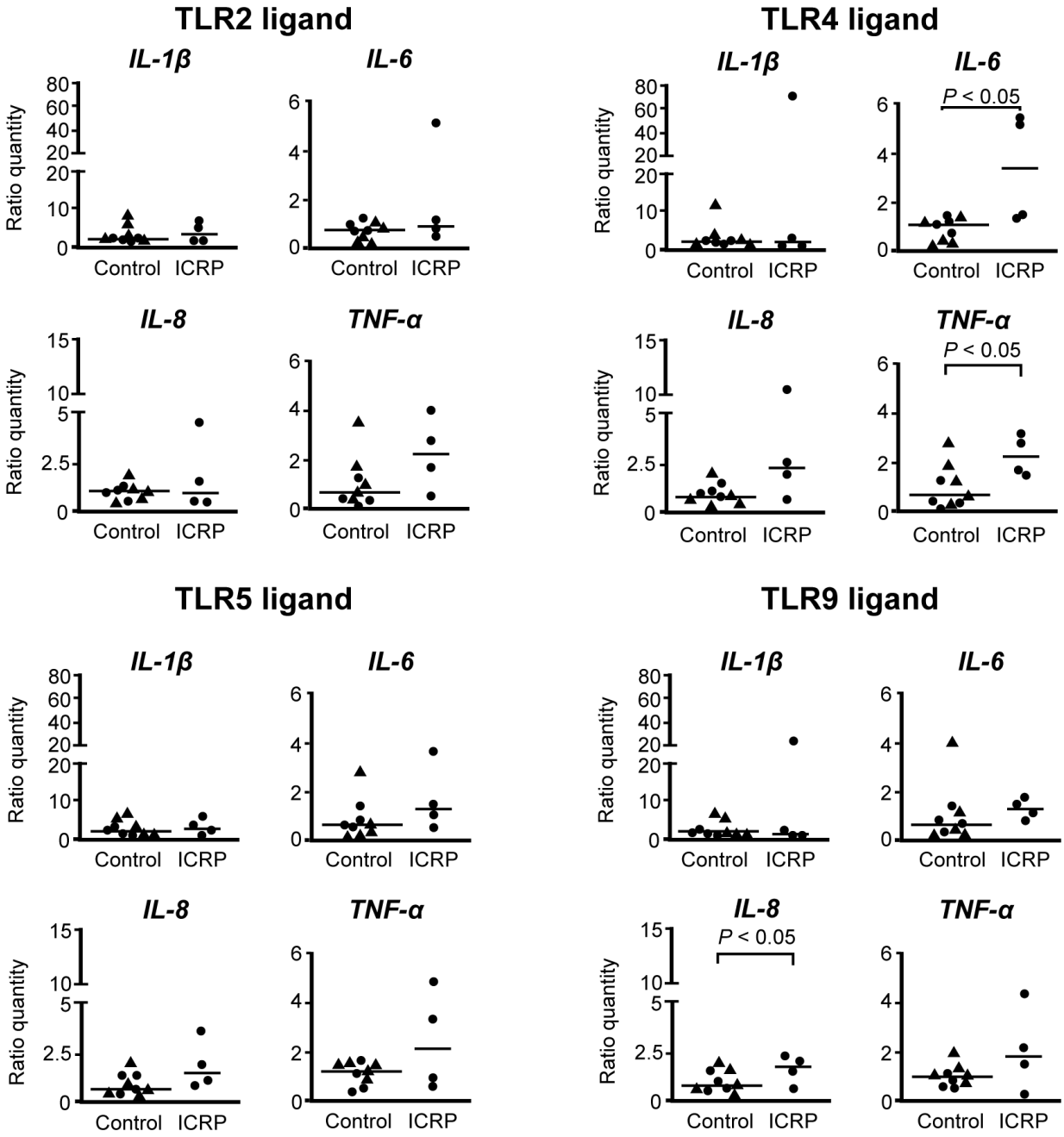

Figure 6. Effects of TLR (TLR2, TLR4, TLR5 and TLR9) stimulations on the mRNA expression levels of pro-inflammatory cytokines (*IL-1β*, *IL-6*, *IL-8* and *TNF-α*) in *ex vivo*-cultured tissues from MDs with ICRPs (n = 4) and healthy dogs (n = 9). Black circles and triangles represent MDs and beagles, respectively. *HMBS*, *RPL32*, and *RPS18* were used as an internal control. The data represents the relative ratio of the expression levels of the stimulated samples to those of the unstimulated samples. The horizontal lines represent the median.

4. DISCUSSION

In this chapter, the functional analysis of TLRs commonly associated with bacterial recognition was performed on *ex vivo*-cultured canine colorectal samples from MDs with ICPRs. The results of this study demonstrated that the relative ratios of IL-6 and TNF-α were elevated in TLR4-stimulated biopsies of MDs with ICRP compared to healthy controls. Moreover, the relative ratios of IL-8 were elevated in TLR9-stimulated biopsies of MDs with ICRP compared to healthy controls. These findings suggested that the response to TLR4 and TLR9 ligands might be enhanced in the colorectal mucosa of ICRPs.

In human IBD, functional alteration of TLR4 in the intestinal mucosa has been implicated as a cause of chronic inflammation.[11-13, 37] In addition, the endoscopic intestinal biopsies of patients with Crohn's disease, one of the major forms of human IBD, exhibited increased IL-8 gene expression in response to bacterial DNA from *Salmonella Dublin*.[39] These results suggest that the aberrant response to TLR ligands results from the dysregulated suppression mechanisms of the TLR-signaling pathway. Dysregulated suppression mechanisms of TLRs activation in the intestinal mucosa were mainly divided into two parts: (1) increased cell surface expression of TLRs which enhance the frontline recognition of bacterial antigen and induce activation of the downstream TLR-signaling pathway; and (2) hyper-responsiveness of TLRs to its ligand by gene polymorphism.[40] The colorectal mucosa from MDs with ICRP used in this study showed no significant increase in *TLR* mRNA expressions at baseline, compared with those of healthy controls. This indicated the possibility of altered TLR4 and TLR9 reactivity as the cause of increased expression of pro-inflammatory cytokines in the colorectal mucosa of ICRPs. In further studies, analysis of single nucleotide polymorphism within the TLR4 and TLR9 gene is needed to provide the evidence of genetic background of this breed for their increased susceptibility to ICRPs.

In the present study, the relative ratios of *IL-6* and *TNF-α* or *IL-8* in colorectal mucosa of ICRPs were elevated by the stimulation of TLR4 or TLR9 with their ligands. These pro-inflammatory cytokines are involved in the activation of the inflammatory response in human IBD.[41] IL-6 and TNF-α induce the pro-inflammatory response of multiple target cells such as antigen-presenting cells and T cells. IL-8 is known as chemokine CXCL8, which is a potent chemoattractant for neutrophils. Similarly, a previous report demonstrated that these pro-inflammatory cytokine mRNA expressions were increased in the inflamed mucosa of ICRPs, suggesting important roles in the induction and persistence of local inflammation.[20] Although the mechanism of enhanced *IL-6*, *TNF-α*, and *IL-8* mRNA expression was not addressed in this study, the hyperreactivity of colorectal mucosa to TLR4 and TLR9 ligands might be associated with the induction of colorectal inflammation of ICRPs.

In the present study, there was no significant difference in the relative ratio of pro-inflammatory cytokines under the stimulation of TLR2 and TLR5, among MDs with ICRPs and control dogs. However, in functional studies in dogs and humans with IBD, animal models and cell lines have revealed that TLR2 and TLR5 play a significant role in the overproduction of pro-inflammatory cytokines via NF-κB activation.[22, 42-44] There are several possibilities for this discrepancy. First, the present study had a small sample size, resulting in a low statistical power to detect true differences. Second, the concentrations of the TLR ligands in this study might not be appropriate for the activation of the TLR2 and TLR5. The physiologically relevant concentrations of native TLR ligands in canine colonic lumen remain unknown. The effects of clinically relevant concentrations of native TLRs ligands on the production of pro-inflammatory cytokines need to be clarified in future studies. Third, the sole cell, such as colonic epithelial cell and tissue-resident mononuclear cells in colorectal mucosa, was not stimulated with the TLR ligands. The stimulation of a heterogeneous cell population with the TLR ligands might have resulted in the large variations in results. Further research is needed to perform

the functional analysis of TLRs in the sole cell population using intestinal epithelial primary culture and the mononuclear cells isolated from colonic mucosa.[45, 46]

In conclusion, the present study demonstrated that the stimulation of the colorectal mucosa of ICRPs with TLR4 ligand induced increased expressions of *IL-6* and *TNF-α* mRNA. Moreover, the colorectal mucosa of ICRPs responded to TLR9 ligands enhanced *IL-8* mRNA gene expression. These results suggest that reactivity against TLR4 and TLR9 ligand is enhanced in the colorectal mucosa of ICRPs. Hyperreactivity against TLR4 and TLR9 ligands in the colorectal mucosa might play an important role in the induction of the inflammation of ICRPs.

5. SUMMARY

In this chapter, the effects of different TLR (TLR2, TLR4, TLR5 and TLR9) ligands on pro-inflammatory cytokines (*IL-1β*, *IL-6*, *IL-8* and *TNF-α*) gene expression were investigated in *ex vivo*-cultured colorectal samples from four MDs with ICRPs and nine healthy dogs. The relative ratios of *IL-6* and *TNF-α* mRNA expression were higher in the TLR4-stimulated colorectal samples from cases compared with healthy controls. Moreover, the relative ratios of *IL-8* mRNA expression were higher in the TLR9-stimulated colorectal samples from cases compared with healthy controls. These results indicated that reactivity against TLR4 or TLR9 ligand in the production of pro-inflammatory cytokines upon the stimulation with TLR4 or TLR9 ligands is enhanced in the colorectal mucosa of ICRPs.

CHAPTER 3

EXPRESSION OF APICAL JUNCTION COMPLEX PROTEINS IN COLORECTAL MUCOSA OF MINIATURE DACHSHUNDS WITH INFLAMMATORY COLORECTAL POLYPS

1. INTRODUCTION

The intestinal epithelial barrier is established by a single layer of epithelial cells, and the space between these cells is sealed by tight junctions (TJs) and adherence junctions (AJs), which regulate the permeability of the intestinal barrier. TJs are complex protein structures comprised of transmembrane proteins. A major component of TJ strands are the integral proteins, claudins. Claudins are transmembrane proteins and there are 27 members of the claudin family.[17] Differential expressions and properties of claudins are believed to determine tissue-specific variations in electrical resistance and paracellular ionic selectivity among epithelia. AJs are composed of the transmembrane protein, E-cadherin, and the associated cytoplasmic proteins, the catenins; these are important in the formation of TJs.[18] Alterations in the expression and localization of TJ and AJ protein were reported in human and canine IBD.[47] In previous reports, increased expression of claudin-2 and decreased expression of claudin-3, -4, -7 and -8 were demonstrated in the colonic mucosa of human IBD.[48, 49] In a previous study, E-cadherin expression was significantly decreased in the villus epithelium in duodenal mucosa samples obtained from dogs with IBD.[50]

Previously, Ohta *et al.* determined the protein expression and distribution of several claudins, E-cadherin and β-catenin, in the colonic mucosa of healthy beagles.[51] However, there is no information regarding the expression of these TJ and AJ proteins in the colorectal mucosa of MDs with ICRPs. The objective of the Chapter 3 was to determine the expression and distribution of various TJ proteins (claudin-1, -2, -3, -4, -5, -7 and -8) and AJ proteins (E-cadherin and β-catenin) in the colorectal mucosa obtained from MDs with ICRPs.

2. MATERIALS AND METHODS

2.1 Animals

For immunoblot analysis, 8 MDs with ICRPs and 8 control dogs were used in this study. The 8 MDs with ICRPs had been referred to Hokkaido University Veterinary Teaching Hospital for the investigation of hematochezia, tenesmus, and an increased frequency of defecation and diagnosed with ICRPs based on clinical and histopathological findings of a previous report.[2, 3] They consisted of 5 males (2 intact and 3 neutered) and 3 females (2 intact and 1 neutered), and the median age of these dogs was 10 years (range, 9 to 14 years). Written owner consent was obtained for the inclusion of dogs in this study. Control tissue samples were obtained from 8 healthy dogs in a research colony (4 beagles and 4 mixed-breed dogs). These healthy dogs consisted of 5 intact males and 3 intact females, and their median age was 2 years (range, 1 to 9 years). None of these dogs had clinical signs of weight loss for more than 1 year before sample collection. Hematologic, serum biochemical, fecal, and abdominal ultrasonographic examinations were performed for all dogs.

For immunohistochemistry analysis, the paraffin-embedded tissues of large inflamed lesions collected from another 5 MDs with ICRPs were used. Rectal pull-through excision was performed at private veterinary hospitals, and large inflamed tissues were submitted to the Department of Diagnostic Pathology of Graduate School of Veterinary Medicine, Hokkaido University for histopathological examination. These 5 MDs consisted of 2 neutered males and 3 females (1 intact and 2 neutered), and the median age of these dogs was 9 years (range, 8 to 11 years). Inflamed mucosal lesions were staged (I-III) according to criteria adopted in Chapter 1 (Figure 1). As a control, full-thickness colorectal tissue samples were obtained from 5 healthy intact male beagles (median age, 0.6 years; range, 0.6-0.7 years), which were used in another unrelated study, at necropsy immediately after euthanasia by an intravenously administered overdose of pentobarbital. These dogs had no clinical signs of

gastrointestinal disease and showed no abnormalities, as determined by hematologic, serum biochemical, fecal, and abdominal ultrasonographic examinations. The samples were immediately fixed in 10% phosphate-buffered formalin, and embedded in paraffin. These control biopsy specimens showed no abnormality based on WSAVA guidelines.[26]

All experimental procedures in this study were approved by the Animal Care and Use Committee and the Laboratory Animal Experimentation Committee, Graduate school of Veterinary Medicine, Hokkaido University (Accession numbers, 13-0142 and 14-0031, respectively).

2.2 Sample collection

Colorectal mucosa biopsy samples were endoscopically obtained. Colonoscopy was performed during anesthesia with a flexible video endoscope (VQ-8143A, AVS, Tokyo, Japan), and multiple (6 to 8) mucosal biopsy samples of inflamed lesions of 8 MDs with ICRPs were collected using serrated biopsy forceps (FB-53Q-1, AVS). In addition, mucosal biopsy samples of the descending colonic mucosa without macroscopic lesions (non-inflamed mucosa) was also collected from these MDs with ICRPs. Control samples were obtained from descending colonic mucosa of 8 healthy dogs in a research colony. Biopsy samples for histologic examination were fixed in neutral buffered 10% formalin, and then embedded in paraffin wax. Biopsy samples were obtained from each dog for immunoblot analysis, and these samples were frozen and stored at –80C°. Biopsy specimens from the descending colonic mucosa of 8 MDs with ICRPs and 8 healthy dogs showed no abnormality based on WSAVA guidelines.[26]

2.3 Immunoblot analysis

Immunoblot analysis was performed as previously described with some modification.[51] One biopsy sample of the colonic mucosa for each dog was homogenized with a plastic pestle in lysis buffer (50

μL per 1 mg of biopsy sample) containing 50 mM Tris-HCl (pH, 7.4), 1 mM EDTA, 2% SDS, and a protease inhibitor cocktail (Complete Mini EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany). Then, lysates were incubated for 30 min on ice and passed through a biopolymer-shredding system (QIAshredder, QIAGEN, Hilden, Germany). The protein concentration was determined by the Lowry method with a protein assay kit (Bio-Rad DC Protein Assay, Bio-Rad Laboratories Inc., Hercules, CA, USA) that included bovine serum ALB as a standard. Aliquots (approx 20 μg) of proteins were separated with PAGE and transferred to a polyvinylidene difluoride filter (Trans-Blot Turbo Mini PVDF Transfer Pack, Bio-Rad Laboratories Inc.), followed by blocking with 5% nonfat milk, incubation with rabbit polyclonal antibodies against claudin-1, -2, -3, -5, -7 and -8 (polyclonal antibody designations: JAY.8, MH44, Z23.JM, Z43.JK, ZMD.241 and ZMD.446, respectively; Zymed Laboratories, Carlsbad, CA, USA.; final concentration, 0.08 μg/mL diluted in 5% nonfat milk), a mouse monoclonal antibody against claudin-4 (clone 3E2C1; Zymed Laboratories; 0.17 μg/mL), mouse monoclonal antibody against E-cadherin (clone 36/E-cadherin; BD Biosciences, San Jose, CA, USA.; 0.04 μg/mL), mouse monoclonal antibody against β-catenin (clone 14/Beta-catenin; BD Biosciences; 0.06 μg/mL) or mouse monoclonal antibody against β-actin (clone AC- 74; Sigma-Aldrich, Saint Louis, MO, USA.; 0.02 μg/mL) for 2 hr. Membranes were then incubated with horseradish peroxidase–conjugated swine anti-rabbit IgG polyclonal antibody (DakoCytomation, Tokyo, Japan; 0.11 μg/mL) or goat anti-mouse IgG polyclonal antibody (Rockland, Gilbertsville, PA, USA.; 0.5 μg/mL) for 1 hr. Signals were detected with a chemiluminescent detection reagent (ECL Prime Western Blotting Detection Reagent, GE Healthcare, Buckinghamshire, U.K.). Densitometric analyses of immunoblot images were performed with software (LumiVision Analyzer 2.0, Aisin Seiki Co., Aichi, Japan). Canine renal cortex and medulla were used as positive control tissue for claudin-1 and -8 as previously reported.[51] The intensity of the signals of each TJ and AJ proteins was normalized to the corresponding β-actin signal.

2.4 Immunohistochemical analysis

Immunohistochemical analysis was conducted on 3-μm-thick paraffin embedded sections of colorectal samples obtained from 5 MDs with ICRPs and 5 healthy beagles. Heat-induced antigen retrieval was performed by autoclaving the sections for 15 min at 105°C in buffer solution for epitope retrieval (Target Retrieval Solution, pH 9, DakoCytomation). Endogenous peroxidase activity was blocked by the incubation with H_2O_2 solutions at room temperature for 30 min. Sections were blocked with 10% goat- or rabbit-serum (Histofine SAB-PO Kit, Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. The primary antibodies against claudin-2, -3, -5 and -7 (1.2 μg/mL) and monoclonal antibodies against claudin-4 (1.2 μg/mL), E-cadherin, and β-catenin (0.8 μg/mL) were added, and the slides were incubated overnight at 4°C. Negative controls were incubated with control rabbit and mouse IgG (1.2 and 0.8 μg/mL, respectively). The slides were washed 3 times with PBS and incubated with biotinylated secondary antibody (Histofine SAB-PO Kit, Nichirei Biosciences Inc.) for 1 hr and then rinsed. To detect the complex formed by the primary and secondary antibodies, slides were incubated with streptavidin (Histofine SAB-PO Kit, Nichirei Biosciences Inc.) for 30 min and rinsed in PBS for 10 min. Thereafter, 3,3′-diaminobenzidine was added to the slides for 5 min, followed by washing in water, counterstaining with Mayer's hematoxylin, dehydration, and mounting with aqueous permanent medium. Images were obtained with a microscope equipped with a camera (BZ-X710, Keyence, Osaka, Japan). Stained tissue sections were evaluated at ×400 magnification. Immunostaining of TJ and AJ proteins were evaluated along the length of colonic crypt base to luminal surface in areas of intact luminal epithelium. Stain intensity was subjectively graded as absent (-) or positive (+) based on staining results of negative control IgG. For evaluation, colonic epithelium was divided into luminal surface and crypt regions.

2.5 Statistical analysis

Statistical analyses were performed using a computer program (JMP 11, SAS Institute Inc., Cary, NC, USA). The normality of the distribution was assessed with the Shapiro-Wilk W test; some data were determined to be non-normally distributed. Expression levels of each protein were compared between control dogs and MDs with ICRPs using the Mann-Whitney *U* test. Values of $P < 0.05$ were considered significant.

3. RESULTS

TJ and AJ protein expression in colorectal mucosa samples

In colonic mucosa from control dogs and non-inflamed mucosa of MDs with ICRPs, expressions of claudin-2, -3, -4, -5, -7; E-cadherin; β-catenin were observed (Figure 7). Expressions of claudin-1 and -8 were not detected in colorectal mucosa samples in any dogs, although anti-claudin-1 and -8 antibodies used in this study detected these claudins in the renal cortex and medulla of dog kidney (Figure 8). In the inflamed mucosa of ICRPs, expressions of E-cadherin and β-catenin were observed. On the other hand, no expressions of any claudins were detected. In addition, β-actin was only slightly and heterogeneously detected. Thus, densitometry analysis of the expression of TJ and AJ proteins could not be performed in the inflamed mucosa of ICRPs. Densitometry analysis of the expression of TJ and AJ proteins in colonic mucosa from control dogs and non-inflamed mucosa of MDs with ICRPs revealed that there were no significant differences in expression levels of claudin-2, -3, -4, -5 or -7, E-cadherin or β-catenin between MDs with ICRPs and control dogs (Figure 9).

Immunostaining of TJ and AJ proteins in colorectal mucosa samples

Next, the expression and distribution of TJ and AJ proteins were examined by immunohistochemistry using the paraffin-embedded tissues of surgically excised inflamed lesions that consisted of sequential histological changes from stage I to stages II lesions. Immunostaining was performed for the TJ and AJ proteins detected by immunoblot analysis. In control dogs, the expressions of claudin-3, -4, -5 and -7, E-cadherin, and β-catenin were observed in the colonic epithelium, and seemed to be uniform along the crypt-to-luminal surface axis (Figures 10 and 11). Claudin-2 was readily detectable only in the colonic epithelium of the crypt base, decreasing in intensity from the distal to proximal crypt and becoming barely detectable at the luminal surface of the colon. The specific labeling of TJ and AJ

proteins were detected in the intestinal epithelium but not in the lamina propria. In the non-inflamed mucosa of MDs with ICRPs, the expression and distribution of TJ and AJ proteins were the same as in colonic mucosa of control dogs (data not shown). In the inflamed lesions of MDs with ICRPs, similar to the results of control dogs, expressions of claudin-2, -3, -4, -5, and -7, E-cadherin, and β-catenin were detected in the colonic epithelium within stages I and II (Figures 10 and 11). Meanwhile, the expression of TJ and AJ proteins was almost abolished in granulation tissue (stage III) because of a limited amount of the remaining epithelial component (data not shown). Negative control staining with rabbit and mouse IgG incubation showed no positive reaction in the colorectal samples of control dogs and MDs with ICRPs (Figure 12).

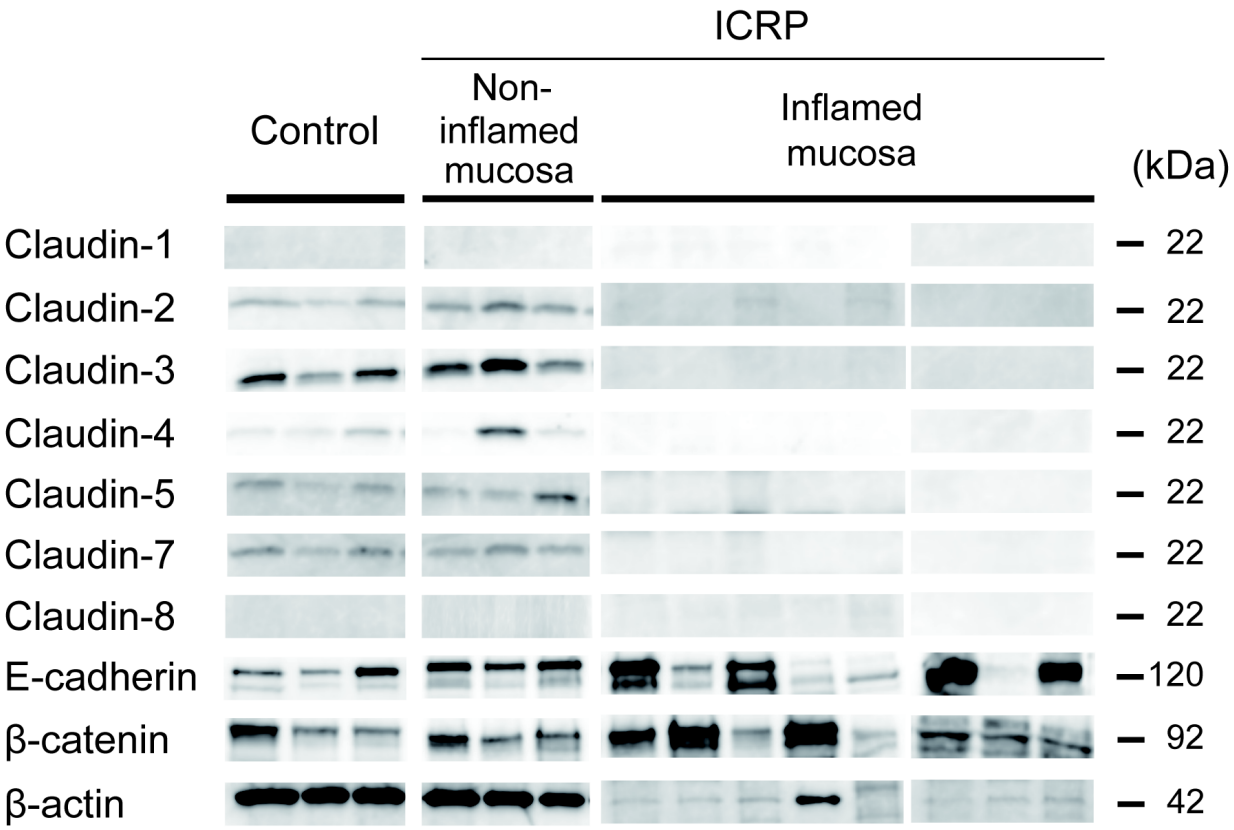

Figure 7. Photographs of immunoblots that indicate expressions of various tight junction and adherence junction proteins in colorectal mucosa biopsy samples obtained from control dogs, and non-inflamed mucosa and inflamed mucosa of MDs with ICRPs. The photograph of control dogs and non-inflamed mucosa of MDs with ICRPs show the representative immunoblots of each group. The photograph of inflamed mucosa of MDs with ICRPs show the results of all of the 8 dogs. β-actin is included as a loading control. Values on the right side indicate the apparent molecular mass (in kilodaltons) of each protein.

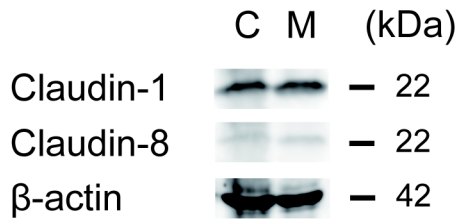

Figure 8. Photographs of immunoblots that indicate expressions of claudin-1 and -8 in canine renal cortex (C) and medulla (M). β-actin is included as a loading control. Values on the right side indicate the apparent molecular mass (in kilodaltons) of each protein.

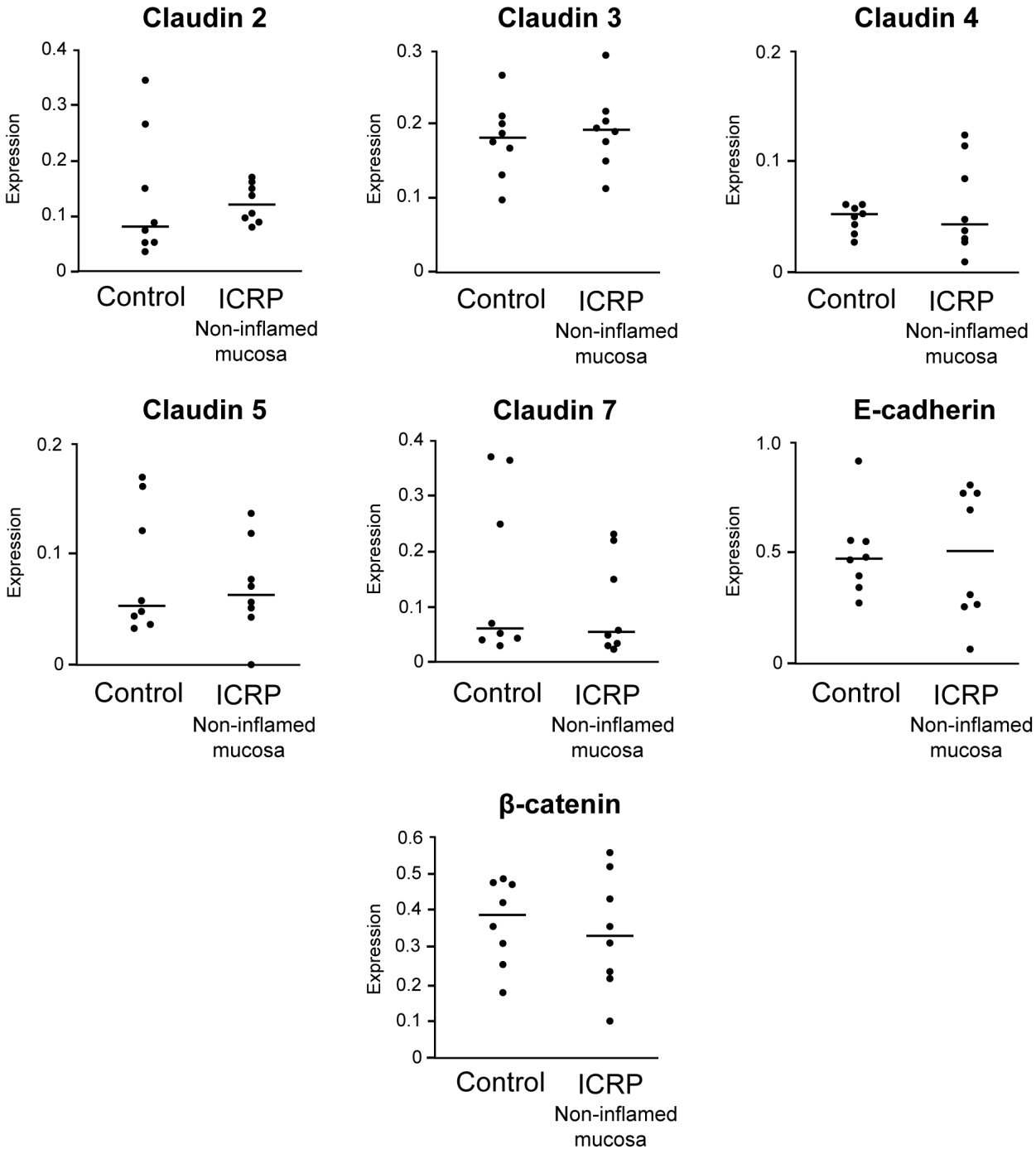

Figure 9. Scatterplots of the expressions of claudin-2, -3, -4, -5, and -7 and E-cadherin; β-catenin in colorectal mucosa samples of control dogs (n = 8) and non-inflamed mucosa of MDs with ICRPs (n = 8). The horizontal line for each group represents the median. Expression units for each protein relative to β-actin expression.

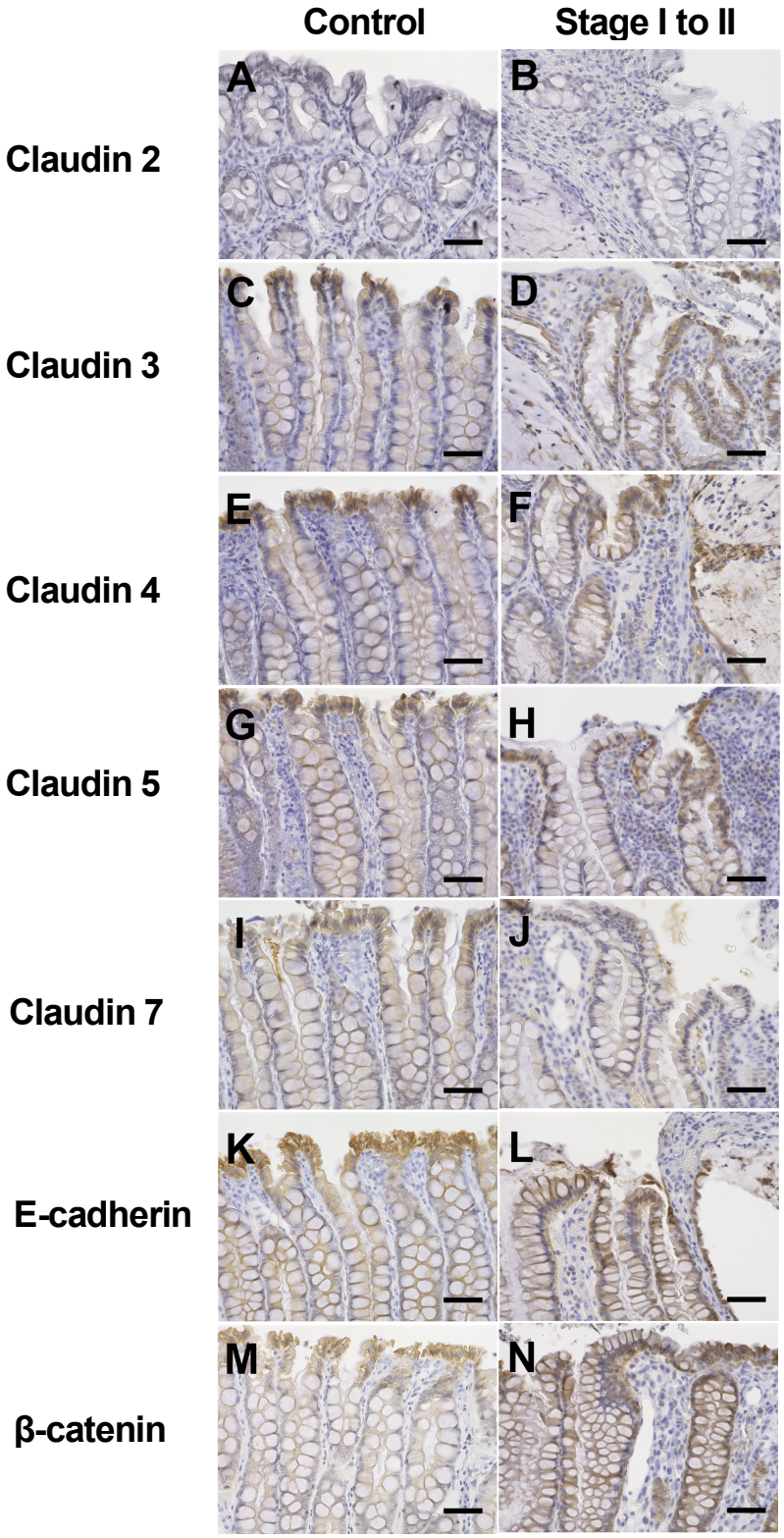

Figure 10. Photomicrographs of representative immunostaining results of claudin-2 (A and B), -3 (C and D), -4 (E and F), -5(G and H), and -7 (I and J) and E-cadherin (K and L); β-catenin (M and N) in the luminal surface of colonic mucosa from control dogs (left column) and stage I to stage II inflamed mucosa (right column) of ICRPs. Bars = 50 μm.

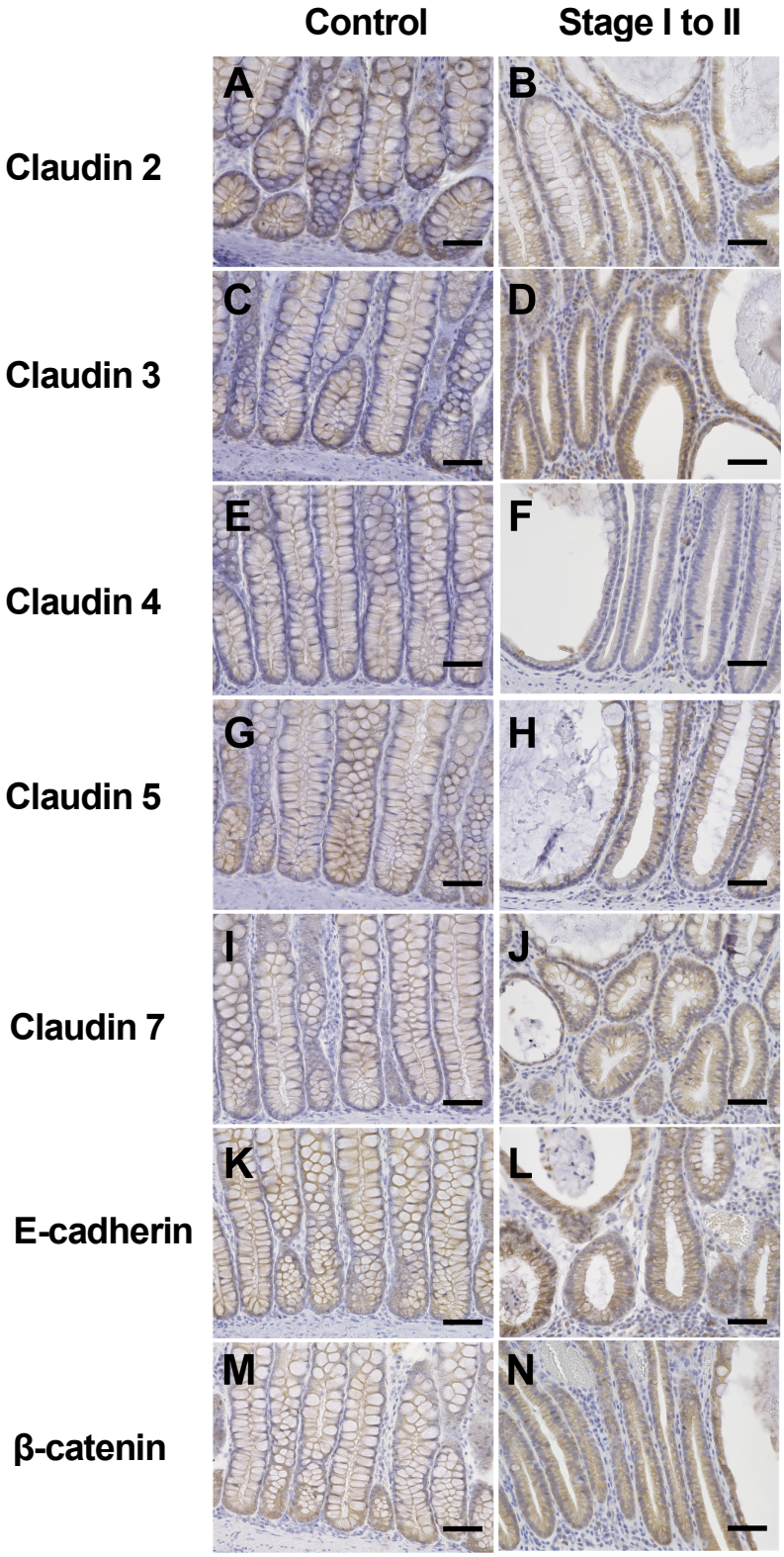

Figure 11. Photomicrographs of representative immunostaining results of claudin-2 (A and B), -3 (C and D), -4 (E and F), -5(G and H) and -7 (I and J), and E-cadherin (K and L); β-catenin (M and N) in the crypt base of colonic mucosa from control dogs (left column) and stage I to stage II inflamed mucosa (right column) of ICRPs. Bars = 50 μm.

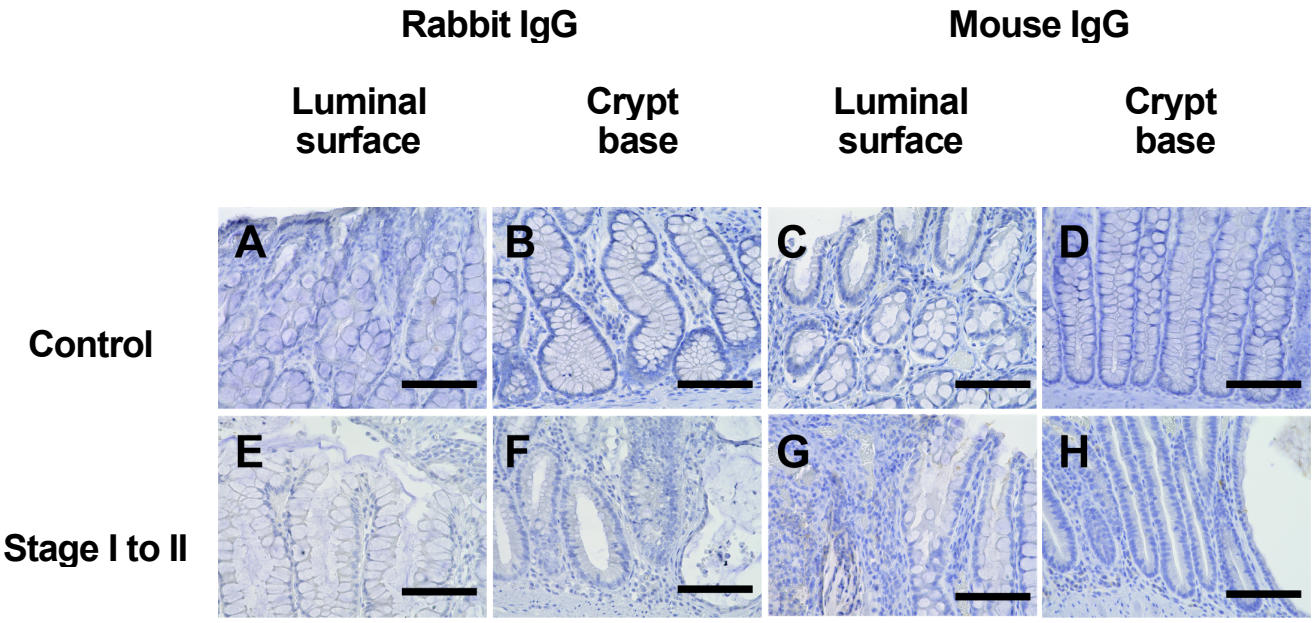

Figure 12. Photomicrographs of representative immunostaining results of control rabbit (A, B, E and F) and mouse (C, D, G and H) IgG as negative control antibodies in the luminal surface (A, C, E and G) and the crypt base (B, D, F and H) of control dogs (A-D) and stage I to stage II inflamed mucosa of ICRPs (E-H). Bars = 50 μm.

4. DISCUSSION

In the present study, the expression and distribution of TJ and AJ proteins were examined in the colorectal mucosa of MDs with ICRPs. The results demonstrated that no alteration in the expression and distribution of TJ and AJ proteins was observed in the colorectal epithelium within the inflamed lesions of MDs with ICRPs.

By immunoblotting, claudin-2, -3, -4, -5 and -7 expressions were detected in the non-inflamed mucosa of MDs with ICRPs and colonic mucosa of control dogs. In addition, there were no significant differences in the expression levels of claudin-2, -3, -4, -5 or -7 between the non-inflamed mucosa of MDs with ICRPs and those of control dogs. In contrast, all claudin proteins were undetectable in the inflamed lesions of ICRPs. A possible reason for these results is that the endoscopic biopsy samples were obtained from the center of inflamed lesions, which was mainly composed of granulation tissue, and a scant amount of the epithelial component remained. Basically, claudin proteins are expressed on the epithelium in the intestinal mucosa.[49, 50] Thus, undetectable levels of expression of claudin proteins in the inflamed lesion might simply reflect a reduced amount of the epithelial component. It is difficult to macroscopically differentiate stage I and II lesions from stage III lesions during the endoscopic procedure. Thus, it was thought that immunoblotting was not a suitable method for the analysis of claudin protein expressions in the inflamed mucosa because of a large amount of granulation tissue in the inflamed lesion, especially in stage III.

In contrast, E-cadherin and β-catenin proteins were detected in inflamed lesions of ICRP. The reason for the detectable levels of E-cadherin and β-catenin proteins, but not claudin proteins, is unknown, but it may be due to the high sensitivity of antibodies for these proteins used in this study. These antibodies could detect E-cadherin and β-catenin proteins on the scant amount of remaining epithelium in the inflamed lesion. However, β-actin expression was barely detectable in the inflamed lesions of ICRPs.

Thus, densitometric analysis of E-cadherin and β-catenin protein expressions could not be performed. It was also unclear why β-actin expression was barely detectable. A possible explanation for this result was that the biopsy samples from inflamed mucosa might not contain sufficient amount of cells for detecting β-actin expression, although the fixed amount of proteins (approx 20 μg) were used for immunoblot analysis. The inflamed mucosa of ICRPs was composed of heterogeneous tissue architecture, including dilated crypts with abundant mucus material and inflammatory cell debris as shown in Figure 1. Indeed, β-actin protein showed heterogeneous expression in the inflamed mucosa of ICRPs as shown in Figure 7. Moreover, the validation experiment was performed by using anti-glyceraldehyde 3-phosphate dehydrogenase monoclonal antibody as an internal control for protein expression, but the result was the same as that of the anti-β-actin antibody (data not shown). From these results, it is thought to be difficult to examine the expression of TJ and AJ proteins in the inflamed mucosa of ICRPs by immunoblotting.

To further compare the expressions of the TJ and AJ proteins between the inflamed lesions of ICRPs and colorectal mucosa of control dogs, immunohistochemical analysis was performed, which enabled us to examine the expression of TJ and AJ proteins on the epithelium of stage I and stage II lesions of ICRPs. As a result, there was no alteration in the expression and distribution of TJ and AJ proteins even in the epithelium of the stage II lesions of ICRPs. These findings suggest that significant changes in apical junction complex proteins expression might not occur in the colorectal epithelium of ICRPs. In human IBD, barrier integrity of the intestinal mucosa was evaluated by not only the expression analysis of TJ and AJ proteins, but also the assessment of barrier functions.[48, 52] In the future study, the evaluation of the barrier function, such as transepithelial electrical resistance and flux assays of molecular tracers, in the colorectal mucosa of MDs with ICRPs is needed.

On the other hand, in human IBD, several previous studies reported that claudin-2 protein expression was upregulated in the colonic epithelium of affected lesions.[48, 53] Furthermore, it has been reported

that alterations of claudin-1, -3, -4, -7 and -8 expressions were observed in the colonic mucosa of human IBD.[49, 53] In human IBD, the alterations in the expressions of claudins were reported to be induced by pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-6) and Th2 cytokine (IL-13).[48, 54, 55] In MDs with ICRPs, similar to human IBD, IFN-γ and TNF-α were upregulated in inflamed mucosa.[5, 20] However, the present study demonstrated no alteration in the expression or distribution of several TJ proteins in MDs with ICRPs. There were several possibilities for these results. Firstly, it is possible that the results could have failed to detect real differences in the expression of TJ and AJ proteins between MDs with ICRPs and control dogs because the sample size of each group was small. Secondly, there is a possibility of changes in the expression of the other TJ proteins, such as occludin and Zona occludens-1[19], in inflamed lesions of MDs with ICRPs. This study examined only seven claudin proteins and two AJ proteins. However, the TJ and AJ consist of a number of transmembrane proteins, including occludin, junctional adhesion molecule, tricelluin, p-cadherin, and α-catenin which might play important roles in forming the TJ and AJ.[52, 56] Further study is needed to examine the expressions of the other TJ and AJ proteins in inflamed lesions of MDs with ICRPs.

In conclusion, the results of the present study indicate that there was no clear change in the expressions of several TJ and AJ proteins in the colorectal epithelium of ICRPs. Further study is needed to examine the other TJ and AJ proteins expression, and to evaluate the barrier function of colorectal mucosal epithelium from MDs with ICRPs.

5. SUMMARY

In this chapter, the expression analysis of TJ and AJ proteins was performed in the colorectal mucosa of MDs with ICRPs. The expression patterns of claudin-1, -2, -3, -4, -5, -7, and -8, E-cadherin, and β-catenin were analyzed in the non-inflamed mucosa and inflamed mucosa of ICRPs and colorectal mucosa of control dogs by immunoblotting. In addition, the localization of these proteins in the inflamed lesions was analyzed by immunohistochemistry. The expressions of each of claudins, E-cadherin, and β-catenin were not significantly different between control dogs and non-inflamed colonic mucosa from MDs with ICRPs. In contrast, only E-cadherin and β-catenin were detected in the inflamed lesions of MDs with ICRPs. By immunohistochemistry, claudin-2, -3, -4, -5, and -7, E-cadherin, and β-catenin were expressed in the colorectal epithelium within the inflamed mucosa, but not in granulation tissue. Distributions of claudin-2, -3, -4, -5, and -7, E-cadherin, and β-catenin in the colonic epithelium were not different between MDs with ICRPs and control dogs. These results indicated that no significant alteration was detected in the expression of several TJ or AJ proteins in the colorectal epithelium of ICRPs.

GENERAL CONCLUSION

The goal of this study was to clarify the involvement of mucosal immunity, especially innate microbial sensing and epithelial barrier integrity, in the pathogenesis of ICRPs. For this purpose, the expression and localization of TLR mRNAs and AJC proteins in the colorectal mucosa of MDs with ICRPs were evaluated. Moreover, the functional analysis of bacterial antigen-associated TLRs were performed in colorectal samples from MDs with ICRPs.

In Chapter 1, the localization of *TLR2* and *TLR4* mRNAs in the colorectal mucosa of MDs with ICRPs were evaluated. The results obtained in this chapter demonstrated that the *TLR2*- and *TLR4*-mRNA expressions were localized in the colorectal epithelium, inflammatory cells, and fibroblasts in the inflamed colorectal mucosa of ICRPs. Moreover, the expressions of *TLR2* and *TLR4* mRNAs in the colonic epithelium were significantly increased in the colorectal epithelium in the inflamed mucosa of ICRPs compared with the non-inflamed mucosa of MDs with ICRPs and the colonic mucosa of healthy beagles. These findings indicated that excessive TLR signaling in the colonic mucosa play an important role in the pathogenesis of ICRPs.

In Chapter 2, the effects of bacterial antigen-associated TLR ligands (TLR2, TLR4, TLR5 and TLR9) on pro-inflammatory cytokine (*IL-1β*, *IL-6*, *IL-8*, and *TNF-α*) mRNA expressions were assessed by *ex vivo* cultured canine colorectal samples. As a result, the relative ratios of *IL-6* and *TNF-α* mRNA expression were higher in the TLR4- or TLR9-stimulated colorectal samples of MDs with ICRPs than those of healthy dogs. Moreover, the relative ratios of *IL-8* mRNA expression were higher in the TLR9-stimulated colorectal samples of MDs with ICRPs than those of healthy dogs. These results indicated that the reactivity against TLR4 or TLR9 ligands on the production on pro-inflammatory cytokines is enhanced in the colorectal mucosa of ICRPs.

In Chapter 3, the expression and distribution of various TJ proteins (claudin-1, -2, -3, -4, -5, -7, and

-8) and AJ proteins (E-cadherin and β-catenin) were evaluated in the colorectal mucosa obtained from MDs with ICRPs. The results obtained in this chapter demonstrated that no alteration in the expression and distribution of TJ and AJ proteins was observed in the colorectal epithelium within the inflamed lesions of MDs with ICRPs. Further study is needed to examine the expressions of other TJ and AJ proteins expression, and to evaluate the barrier function of colorectal mucosal epithelium from MDs with ICRPs.

In conclusion, aberrant expression and function of TLRs in the colonic mucosa could be associated with the induction of colorectal inflammation of ICRPs. On the other hand, the result in the present study did not support the possibility of the alteration of intestinal epithelial barrier in the colorectal mucosa of MDs with ICRPs.

REFERENCES

1. Seiler, R. J. 1979. Colorectal polyps of the dog: a clinicopathologic study of 17 cases. *J. Am. Vet. Med. Assoc.*, **174**: 72-75.

2. 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. *J. Vet. Med. Sci.*, **74**: 59-64.

3. Uchida, E., Chambers, J. K., Nakashima, K., Saito, T., Ohno, K., Tsujimoto, H., Nakayama, H., Uchida, K. 2016. Pathologic Features of Colorectal Inflammatory Polyps in Miniature Dachshunds. *Vet. Pathol.*, **53**: 833-839.

4. Fukushima, K., Eguchi, N., Ohno, K., Kanemoto, H., Takahashi, M., Igarashi, H., Ohmi, A., Nakashima, K., Tsujimoto, H. 2016. Efficacy of leflunomide for treatment of refractory inflammatory colorectal polyps in 15 Miniature Dachshunds. *J. Vet. Med. Sci.*, **78**: 265-269.

5. 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. *Vet. Immunol. Immunopathol.*, **155**: 259-263.

6. 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 J. Gastroenterol.*, **16**: 1050-1056.

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

8. de Souza, H. S., Fiocchi, C. 2016. Immunopathogenesis of IBD: current state of the art. *Nat. Rev. Gastroenterol. Hepatol.*, **13**: 13-27.

9. Kawai, T., Akira, S. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.*, **11**: 373-384.

10. Harris, G., KuoLee, R., Chen, W. 2006. Role of Toll-like receptors in health and diseases of gastrointestinal tract. *World J. Gastroenterol.*, **12**: 2149-2160.

11. 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. *Infect. Immun.*, **68**: 7010-7017.

12. 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. *J. Histochem. Cytochem.*, **56**: 267-274.

13. Szebeni, B., Veres, G., Dezsofi, A., Rusai, K., Vannay, A., Mraz, M., Majorova, E., Arato, A. 2008. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin. Exp. Immunol.*, **151**: 34-41.

14. Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T., Arditi, M. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J. Immunol.*, **167**: 1609-1616.

15. Gribar, S. C., Richardson, W. M., Sodhi, C. P., Hackam, D. J. 2008. No longer an innocent bystander: epithelial toll-like receptor signaling in the development of mucosal inflammation. *Mol. Med.*, **14**: 645-659.

16. Walsh, D., McCarthy, J., O'Driscoll, C., Melgar, S. 2013. Pattern recognition receptors--molecular orchestrators of inflammation in inflammatory bowel disease. *Cytokine Growth Factor Rev.*, **24**: 91-104.

17. Capaldo, C. T., Nusrat, A. 2015. Claudin switching: Physiological plasticity of the Tight Junction. *Semin. Cell Dev. Biol.*, **42**: 22-29.

18. Cereijido, M., Shoshani, L., Contreras, R. G. 2000. Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. *Am. J. Physiol. Gastrointest. Liver Physiol.*, **279**: G477-482.

19. Landy, J., Ronde, E., English, N., Clark, S. K., Hart, A. L., Knight, S. C., Ciclitira, P. J., Al-Hassi, H. O. 2016. Tight junctions in inflammatory bowel diseases and inflammatory bowel disease associated colorectal cancer. *World J. Gastroenterol.*, **22**: 3117-3126.

20. 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. *Vet. Immunol. Immunopathol.*, **156**: 32-42.

21. 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. *Vet. Microbiol.*, **146**: 326-335.

22. 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.

23. Igarashi, H., Ohno, K., Maeda, S., Kanemoto, H., Fukushima, K., Uchida, K., Tsujimoto, H. 2014. Expression profiling of pattern recognition receptors and selected cytokines in miniature dachshunds with inflammatory colorectal polyps. *Vet. Immunol. Immunopathol.*, **159**: 1-10.

24. Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo, H. T., Ma, X. J., Luo, Y. 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed,

paraffin-embedded tissues. *J. Mol. Diagn.*, **14**: 22-29.

25. Wang, Z., Portier, B. P., Gruver, A. M., Bui, S., Wang, H., Su, N., Vo, H. T., Ma, X. J., Luo, Y., Budd, G. T., Tubbs, R. R. 2013. Automated quantitative RNA in situ hybridization for resolution of equivocal and heterogeneous ERBB2 (HER2) status in invasive breast carcinoma. *J. Mol. Diagn.*, **15**: 210-219.

26. Day, M. J., Bilzer, T., Mansell, J., Wilcock, B., Hall, E. J., Jergens, A., Minami, T., Willard, M., Washabau, R. 2008. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. *J. Comp. Pathol.*, **138 Suppl 1**: S1-43.

27. Tubbs, R. R., Wang, H., Wang, Z., Minca, E. C., Portier, B. P., Gruver, A. M., Lanigan, C., Luo, Y., Cook, J. R., Ma, X. J. 2013. Ultrasensitive RNA in situ hybridization for detection of restricted clonal expression of low-abundance immunoglobulin light chain mRNA in B-cell lymphoproliferative disorders. *Am. J. Clin. Pathol.*, **140**: 736-746.

28. Abreu, M. T., Arnold, E. T., Thomas, L. S., Gonsky, R., Zhou, Y., Hu, B., Arditi, M. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J. Biol. Chem.*, **277**: 20431-20437.

29. Otte, J. M., Cario, E., Podolsky, D. K. 2004. Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology*, **126**: 1054-1070.

30. Melmed, G., Thomas, L. S., Lee, N., Tesfay, S. Y., Lukasek, K., Michelsen, K. S., Zhou, Y., Hu, B., Arditi, M., Abreu, M. T. 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host-microbial interactions in the gut. *J. Immunol.*, **170**: 1406-1415.

31. Mueller, T., Terada, T., Rosenberg, I. M., Shibolet, O., Podolsky, D. K. 2006. Th2 cytokines down-regulate TLR expression and function in human intestinal epithelial cells. *J. Immunol.*, **176**: 5805-5814.

32. Suzuki, M., Hisamatsu, T., Podolsky, D. K. 2003. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular Toll-like receptor 4-MD-2 complex. *Infect. Immun.*, **71**: 3503-3511.

33. Abreu, M. T. 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat. Rev. Immunol.*, **10**: 131-144.

34. Yokoyama, N., Ohta, H., Yamazaki, J., Kagawa, Y., Ichii, O., Nisa, K., Morita, T., Osuga, T., Lim, S. Y., Sasaki, N., Morishita, K., Nakamura, K., Takiguchi, M. 2016. Localization of TLR2 and TLR4 mRNA in the colorectal mucosa of miniature dachshunds with inflammatory colorectal polyps. *J. Comp. Pathol.*, in press.

35. Igarashi, H., Ohno, K., Fujiwara-Igarashi, A., Kanemoto, H., Fukushima, K., Goto-Koshino, Y.,

Uchida, K., Tsujimoto, H. 2015. Functional analysis of pattern recognition receptors in miniature dachshunds with inflammatory colorectal polyps. *J. Vet. Med. Sci.*, **77**: 439-447.

36. Mapes, B., Chase, M., Hong, E., Ludvik, A., Ceryes, K., Huang, Y., Kupfer, S. S. 2014. Ex vivo culture of primary human colonic tissue for studying transcriptional responses to 1alpha,25(OH)2 and 25(OH) vitamin D. *Physiol. Genomics*, **46**: 302-308.

37. Swerdlow, M. P., Kennedy, D. R., Kennedy, J. S., Washabau, R. J., Henthorn, P. S., Moore, P. F., Carding, S. R., Felsburg, P. J. 2006. Expression and function of TLR2, TLR4, and Nod2 in primary canine colonic epithelial cells. *Vet. Immunol. Immunopathol.*, **114**: 313-319.

38. German, A. J., Hall, E. J., Day, M. J. 2000. Relative deficiency in IgA production by duodenal explants from German shepherd dogs with small intestinal disease. *Vet. Immunol. Immunopathol.*, **76**: 25-43.

39. Hotte, N. S., Salim, S. Y., Tso, R. H., Albert, E. J., Bach, P., Walker, J., Dieleman, L. A., Fedorak, R. N., Madsen, K. L. 2012. Patients with inflammatory bowel disease exhibit dysregulated responses to microbial DNA. *PLoS One*, **7**: e37932.

40. Cario, E. 2010. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm. Bowel Dis.*, **16**: 1583-1597.

41. Francescone, R., Hou, V., Grivennikov, S. I. 2015. Cytokines, IBD, and colitis-associated cancer. *Inflamm. Bowel Dis.*, **21**: 409-418.

42. Furi, I., Sipos, F., Germann, T. M., Kalmar, A., Tulassay, Z., Molnar, B., Muzes, G. 2013. Epithelial toll-like receptor 9 signaling in colorectal inflammation and cancer: clinico-pathogenic aspects. *World J. Gastroenterol.*, **19**: 4119-4126.

43. Hawn, T. R., Verbon, A., Lettinga, K. D., Zhao, L. P., Li, S. S., Laws, R. J., Skerrett, S. J., Beutler, B., Schroeder, L., Nachman, A., Ozinsky, A., Smith, K. D., Aderem, A. 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J. Exp. Med.*, **198**: 1563-1572.

44. Mukherjee, S., Biswas, T. 2014. Activation of TOLLIP by porin prevents TLR2-associated IFN-gamma and TNF-alpha-induced apoptosis of intestinal epithelial cells. *Cell. Signal.*, **26**: 2674-2682.

45. Dedhia, P. H., Bertaux-Skeirik, N., Zavros, Y., Spence, J. R. 2016. Organoid Models of Human Gastrointestinal Development and Disease. *Gastroenterology*, **150**: 1098-1112.

46. Weigmann, B., Tubbe, I., Seidel, D., Nicolaev, A., Becker, C., Neurath, M. F. 2007. Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat. Protoc.*, **2**: 2307-2311.

47. Laukoetter, M.-G. 2008. Role of the intestinal barrier in inflammatory bowel disease. *World J. Gastroenterol.*, **14**: 401.

48. Zeissig, S., Burgel, N., Gunzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., Kroesen, A. J., Zeitz, M., Fromm, M., Schulzke, J. D. 2007. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. *Gut*, **56**: 61-72.

49. Barmeyer, C., Schulzke, J. D., Fromm, M. 2015. Claudin-related intestinal diseases. *Semin. Cell Dev. Biol.*, **42**: 30-38.

50. Ohta, H., Sunden, Y., Yokoyama, N., Osuga, T., Lim, S. Y., Tamura, Y., Morishita, K., Nakamura, K., Yamasaki, M., Takiguchi, M. 2014. Expression of apical junction complex proteins in duodenal mucosa of dogs with inflammatory bowel disease. *Am. J. Vet. Res.*, **75**: 746-751.

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

52. Mehta, S., Nijhuis, A., Kumagai, T., Lindsay, J., Silver, A. 2015. Defects in the adherens junction complex (E-cadherin/ beta-catenin) in inflammatory bowel disease. *Cell Tissue Res.*, **360**: 749-760.

53. Weber, C. R., Nalle, S. C., Tretiakova, M., Rubin, D. T., Turner, J. R. 2008. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. *Lab. Invest.*, **88**: 1110-1120.

54. Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A. H., Burgel, N., Fromm, M., Zeitz, M., Fuss, I., Strober, W., Schulzke, J. D. 2005. Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology*, **129**: 550-564.

55. Suzuki, T., Yoshinaga, N., Tanabe, S. 2011. Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J. Biol. Chem.*, **286**: 31263-31271.

56. Tsukita, S., Furuse, M., Itoh, M. 2001. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.*, **2**: 285-293.

JAPANESE SUMMARY　（要旨）

A study on the colorectal mucosal immunopathology of inflammatory colorectal polyps in dogs ― analyses of innate immunity and epithelial barrier

（炎症性結直腸ポリープの犬における結直腸粘膜の免疫病態に関する研究 ― 自然免疫と上皮バリアの解析）

炎症性結直腸ポリープ（Inflammatory colorectal polyps, ICRP）は、国内のミニチュア・ダックスフンドに好発する大腸の炎症性ポリープ病変である。ICRPは肉眼的に結腸から直腸領域に多発性に形成され、病理組織学的には陰窩の拡張を伴った結腸上皮細胞および杯細胞の過形成、好中球、マクロファージを主体とした炎症細胞の浸潤、肉芽組織の増生を特徴とする。いまだその発症原因は不明であるが、その病理組織学的特徴や好発犬種の存在、また免疫抑制剤に対して良好な治療反応性を示すことから、近年、新たな型の犬種特異的な炎症性腸疾患（Inflammatory bowel disease, IBD）として認識されつつある。

ヒトや犬のIBDの発症仮説では、腸内細菌叢とそれを認識する宿主の粘膜免疫との過剰な相互作用が病因として考えられている。粘膜免疫の中でも自然免疫系の異常は、IBD発症初期に腸管の過剰な炎症を誘導し、さらに慢性化と増悪にも関与すると考えられている。腸管の自然免疫系は、好中球、マクロファージといった免疫細胞と腸管上皮など腸管粘膜を構成する非免疫細胞によって構成されており、とりわけ腸管上皮は腸管の物理的なバリアとしての役割のみならず、消化管内の病原体を認識し、腸管に適切な免疫反応を誘導する役割を担っている。これら腸管上皮の機能異常は、消化管内抗原の

体内への侵入や消化管の異常な抗原認識を引き起こし、腸管に過剰な慢性炎症を引き起こすと考えられている。

そこで本研究では、ICRP の病態における粘膜免疫の役割を明らかにすることを目的に、細菌抗原の認識を担う受容体である Toll-like receptor (TLR)と上皮バリアを構成する Apical junction complex (AJC)の発現および局在解析を行った。さらに TLR については、細菌抗原のリガンド刺激による炎症性サイトカイン合成能を測定し、機能解析を行った。

第 1 章では、*in situ* hybridization 法により *TLR2* と *TLR4* の局在解析を行い、加えてポリープ病変内の上皮細胞における発現量の定量を行った。その結果、*TLR2*、*TLR4* は、ポリープ病変部内の結腸上皮、炎症細胞、線維芽細胞に発現が認められた。特にポリープ病変部の結腸上皮では、健常犬と比較して、*TLR2* と *TLR4* の mRNA 発現量の増加が認められ、ICRP の病態への関与が示唆された。

続いて第 2 章では、ICRP 症例の正常結直腸粘膜を用いて、細菌抗原を認識する TLR のリガンド刺激試験を行い、TLR の機能のひとつである炎症性サイトカインの発現誘導について解析した。ICRP と診断された症例のポリープ病変部より 10cm 以内の肉眼的に正常な結直腸粘膜を内視鏡下で採取し、各種 TLR リガンド（TLR1/2, TLR4, TLR5, TLR9）を培養液に添加して、37℃、4 時間培養した。また対照群として、健常犬の結直腸粘膜を用いて、同様の処置を行った。培養後の組織から cDNA を合成し、定量的リアルタイム PCR により炎症性サイトカイン(*IL-1β*, *IL-6*, *IL-8*, *TNF-α*)ならびに *TLR2*, *TLR4*, *TLR5*, *TLR9* の mRNA 発現量を定量し、各リガンド刺激後の mRNA 発現量は、培養液のみで 4 時間培養したサンプルにおける発現量に対する相対値として算出した。その結果、培養 0 時間の ICRP 結直腸粘膜では、いずれの TLR、炎症性

サイトカインの相対発現量も健常群と比較して有意な差は認められなかった。その一方で、培養 4 時間後の ICRP 結直腸粘膜では、TLR4 刺激下における *IL-6* と *TNF-α*、TLR9 刺激下における *IL-8* の発現比が、健常群と比較して有意に上昇していた。以上の結果から、ICRP 症例のポリープ病変部境界の結直腸粘膜では、TLR4 ならびに TLR9 のリガンド刺激によって、炎症性サイトカインの発現が誘導しやすい可能性が示唆された。

第 3 章では、AJC を構成する Tight junction (TJ)と Apical junction (AJ)の発現、局在解析を行った。ICRP 症例犬の病変部ならびに肉眼的に正常な下行結腸粘膜を内視鏡下で採取し、対照として健常犬下行結腸粘膜を用いた。採取した組織から蛋白質の抽出を行い、イムノブロット法にて claudin-1、-2、-3、-4、-5、-7、-8、E-cadherin、β-catenin の発現解析を行った。またイムノブロット法によって発現を認めた claudin-2、-3、-4、-5、-7、E-cadherin、β-catenin について酵素抗体法により局在解析を行った。その結果、ICRP 症例犬と健常犬において、いずれの TJ と AJ も発現、局在に変化は認められなかった。

本研究の結果から、ICRP 症例の結直腸粘膜の TLR は発現、機能ともに変化が認められ、結直腸の過剰な炎症誘導への関与が示唆された。その一方で、AJC の蛋白発現には変化が認められず、病態への関与は示唆されなかった。

ACKNOWLEDGEMENT

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

First and foremost, I wish to thank the person who made it all possible, my supervisor, Prof. Mitsuyoshi Takiguchi (Graduate School of Veterinary Medicine, Hokkaido University) for giving me the opportunities to achieve my goals and aspirations, and for his constant encouragement and guidance. Without him, I would have never learnt and experienced as much as I did.

I wish to also thank Dr. Hiroshi Ohta (Graduate School of Veterinary Medicine, Hokkaido University), Profs. Kazuhiro Ohashi (Graduate School of Veterinary Medicine, Hokkaido University), and Yumiko Kagawa (Graduate School of Veterinary Medicine, Hokkaido University) for their time and effort in providing priceless advice and critical comments into making this thesis a better one. I am greatly indebted to my mentor Dr. Ohta, who had taken his time and effort to critically evaluate my research, papers, and presentations.

I wish to thank Drs. Kensuke Nakamura (Graduate School of Veterinary Medicine, Hokkaido University), Keitaro Morishita (Graduate School of Veterinary Medicine, Hokkaido University), and Noboru Sasaki (Graduate School of Veterinary Medicine, Hokkaido University) for their constant support and assistance, Drs. Osamu Ichii (Graduate School of Veterinary Medicine, Hokkaido University), Jumpei Yamazaki (Graduate School of Veterinary Medicine, Hokkaido University) for technical support and advise. I also wish to thank my fellow comrades; Drs. Sue Yee Lim, Tatsuyuki Osuga, and Tomoya Morita for their priceless advice and support. In addition, I am also thankful to all members of the Laboratory of Veterinary Internal Medicine and Veterinary Teaching Hospital for this support, encouragements, and great times.

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

encouragements.