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Functional analysis of pattern recognition receptors in miniature dachshunds with inflammatory colorectal polyps

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

KEY WORDS: inflammatory colorectal polyp, innate immunity, miniature dachshund, pattern recognition receptor, pro-inflammatory cytokine

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Colorectal polyps are relatively common in dogs, and most of these polyps are neoplastic [37, 44]. On the contrary, miniature dachshunds (MDs) are commonly affected by inflammatory colorectal polyps (ICRPs), suggesting a genetic predisposition [31]. MDs with ICRPs typically develop multiple polyps in the colorectal region, show severe inflammatory infiltration (predominantly with neutrophils and macrophages) and respond relatively well to immunosuppressive treatment [31]. ICRPs are considered as a novel form of breed-specific inflammatory bowel disease (IBD) in dogs [32], but no report has investigated their genetic background.

The etiology of human IBD has been described as multifactorial and including the interplay of environment, gut microbiota, mucosal immune system and genetic background, which also have roles in canine IBD [6, 7, 48]. To date, many studies of human and canine IBD have reported genetic predispositions, including genetic disorders of pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs), induce cell signaling via activation of nuclear factor-kappa B (NF-κB) and subsequently increase the upregulation of immune response gene expression involving pro-inflammatory cytokines and co-stimulatory molecules [25]. Therefore, the genetic backgrounds of PRRs are of interest, because they play crucial roles in the interaction between luminal antigens and host immunity. A meta-analysis of genome-wide association studies revealed 163 risk-associated loci for human IBD [16]. Genetic variations associated with human IBD have been reported in a variety of PRR genes including toll-like receptors (TLRs; e.g., TLR1, TLR2, TLR4, TLR5, TLR6 and TLR9) and nucleotide-oligomerization domain (NOD)-like receptors including NOD2 [6, 8, 29]. Polymorphisms of TLR4, TLR5 and NOD2 are also associated with canine IBD [18–20].

A defect in PRRs is considered to influence ligand recognition, mucosal immune tolerance and commensal composition, leading to innate or adaptive immune hypo- or hyperreactivity [6]. Many risk-associated genetic variations for human IBD in PRRs have been shown to confer functional disorder [3, 11, 42]; for example, a D299G mutation in human TLR4 gene results in conformational change and hyporesponsiveness to bacterial LPS, but is considered to induce a signaling disequilibrium of other TLRs, leading to intestinal inflammation [6]. Furthermore, an IBD risk-associated haplotype of canine TLR5 reportedly includes hyperresponsiveness to bacterial flagellin [17].

We hypothesized that functional disorder related to

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genetic background would also exist in MDs with ICRPs. Recently, Tamura *et al.* [41] have suggested that macrophages in the colorectal area of ICRP-affected MDs play a key role in neutrophil recruitment via production of pro-inflammatory cytokines. Therefore, this study aimed to evaluate the reactivity of PRRs in ICRP-affected MDs using peripheral blood-derived monocytes to narrow the candidate PRR genes responsible for development of the condition.

MATERIALS AND METHODS

Animals: ICRP-affected MDs evaluated at the Veterinary Medical Center of the University of Tokyo between April 2012 and November 2013 were recruited for the study. The diagnosis of ICRP was determined based on the colonoscopic and histopathological findings as characterized in a previous study [31]. As controls, MDs owned by veterinarians or veterinary technicians were also recruited. These control MDs were confirmed as having no inflammatory, infectious or immune-mediated diseases with a health examination. All procedures were conducted according to the animal experimentation guidelines of the University of Tokyo, and informed consent was obtained from the owners of the MDs.

Cell preparation and culture: Approximately 10 m*l* of whole blood in ethylenediaminetetraacetic acid (EDTA) was collected from each dog. One milliliter was used for routine blood tests including complete blood count (CBC), and the remainder was used to obtain peripheral blood-derived monocytes modifying the methods as described in previous studies [4, 12]. Peripheral blood mononuclear cells (PBMCs) were obtained from the EDTA blood via Ficoll-Paque Plus (GE Healthcare Ltd., Buckinghamshire, U.K.) density gradient centrifugation. The PBMCs were resuspended in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, U.S.A.) containing 10% fetal bovine serum (Biowest, *Nuaillé*, France) supplemented with penicillin and streptomycin (Sigma–Aldrich). PBMCs were seeded into 9 wells of a 12-well plate at a concentration of 1×10^6 cells/well and 9 wells of a 48-well plate at 2.5×10^5 cells/well and cultured at 37°C in 5% CO_2 overnight. To obtain monocytes, we washed the cells twice with Hank's balanced salt solution (Sigma–Aldrich) to remove non-adherent cells, and the adherent cells were then cultured for an additional 6 days and used for experiments.

The viability and purity of peripheral blood-derived monocytes were preliminary assessed using 4 healthy laboratory beagles. The use of laboratory beagles was approved by the Animal Care Committee of the University of Tokyo (Approval No. P13-774). Cell viability was assessed by trypan blue staining, and 92–97% of the cells were regarded as viable (100 cells counted per dog, in duplicate). The morphology of these cells was assessed by Wright–Giemsa staining; these cells presented variable cell size,<1.0 of N:C ratios, irregularly round-shaped nuclei with diffuse chromatins, vacuolated cytoplasms and sometimes multinucleated. Non-specific esterase staining was performed using a commercially available kit (Muto pure chemicals, Tokyo, Japan) according to the manufacturer's manual; 90–96% of the cells were positively stained (100 cells counted per dog, in duplicate).

Stimulating cells with PAMPs: The monocytes were washed twice with culture medium and cultured in 500 μl of culture medium for the 12-well plate and 200 μl for the 48-well plate with stimulation by the following PAMPs: peptidoglycan-like molecule (iE-DAP; NOD1 ligand, 10 μg/m*l*), muramyl dipeptide (MDP; NOD2 ligand, 10 μg/m*l*), synthetic bacterial lipoprotein (Pam3CSK4; TLR1/2 ligand, 500 *n*g/m*l*), peptidoglycan from *Escherichia coli K12*(PGN-EK; TLR2 ligand, 20 μg/m*l*), synthetic diacylated lipoprotein (FSL-1; TLR2/6 ligand, 50 *n*g/m*l*), ultrapure LPS from *E. coli K12*(LPS-EK Ultrapure; TLR4 ligand, 10 μg/m*l*), purified flagellin from *Salmonella typhimurium*(FLA-ST Ultrapure; TLR5 ligand, 100 *n*g/m*l*), CpG oligonucleotide (ODN2006; TLR9 ligand, 5 μM) (all from Invivogen, San Diego, CA, U.S.A.) and culture medium only (negative control). The stimulation lasted 6 hr for monocytes in the 12-well plate and 24 hr for those in the 48-well plate. Each cell-free supernatant of culture media was collected and stored at −80°C for further analysis. The cells in the 12-well plate were washed twice with Hank's balanced salt solution and subsequently lysed to extract their total RNA using a commercially available kit (RNAspin Mini RNA Isolation Kit, GE Healthcare) according to the manufacturer's instructions and stored at −80°C for further analysis.

Quantification of PRR and cytokine messenger RNA (mRNA) expression with quantitative PCR: Reverse transcription was performed using a PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan) to synthesize complementary DNA from total RNA according to the manufacturer's instructions. Subsequently, quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Takara Bio) and a Thermal Cycler Dice Real Time System (Takara Bio). The amplification conditions were as follows: 95°C for 10 sec, 40 cycles of PCR (95°C for 15 sec and 60°C for 30 sec) and dissociation (95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec). Nuclease-free water and non-reverse transcription controls were used as negative controls. A sample with a known cycle threshold (Ct) value (as a positive control) was included with all sample runs to control for run-to-run Ct variation. The real-time data were analyzed using Thermal Cycler Dice Real Time System software version 4.01A (Takara Bio). Ct values were determined with second derivative maximum cycles.

The primers used in our analyses are detailed in Table 1. The primer sequences were obtained from previous studies [24, 26, 33]. The most stably expressed reference genes were preliminarily determined using 50 *n*g total RNA derived from monocytes of 7 ICRP-affected MDs and 7 control MDs via assessment of 10 candidate genes: β-2 microglobulin, CG14980-PB, glyceraldehyde-3-phosphate dehydrogenase, hydroxymethylbilane synthase, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, ribosomal protein L32, ribosomal protein S18, succinate dehydrogenase complex subunit A and TATA box binding protein [23, 33]. Hydroxymethylbilane synthase, succinate dehydrogenase complex subunit A and TATA box binding protein were selected as the most stable reference genes in the current study

Table 1. Primer sequences of pattern recognition receptors and proinflammatory cytokines used in the study

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

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

using the GeNorm, NormFinder and BestKeeper programs (data not shown) [2, 34, 45].

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

Quantification of cytokine production by ELISA: The concentrations of IL-1β protein in culture media stimulated with PAMPs for 24 hr were determined using a commercially available ELISA kit (Canine IL-1β VetSet^{TM} ELISA Development Kit, Kingfisher Biotech, St. Paul, MN, U.S.A.) according to the manufacturer's instructions. Each assay was performed in duplicate.

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

RESULTS

Animals: Twenty-six MDs with ICRPs were included in the current study: their median age was 125.5 months (range, 68–153 months), and the group included 16 males (4 intact and 12 neutered) and 10 females (3 intact and 7 spayed). Eight of 26 ICRP-affected MDs had clinical histories of other inflammatory disease, most predominantly with chronic gastroenteritis (Table 2). EDTA blood was collected at initial diagnosis in 17 dogs and at clinical remission in 9 dogs. Ten of 26 ICRP-affected MDs had received immunosuppressive therapy including prednisolone, cyclosporine and/or leflunomide within 3 weeks prior to blood collection, while 16 ICRP-affected MDs had not. In addition, 16 control MDs—4 males (1 intact and 3 neutered) and 12 females (5 intact and 7 spayed)—were included, with a median age of 103 months (range, 60–171 months). Thirteen control MDs were clinically healthy, while 2 had obsolete vertebral fracture resulted from a traffic accident, and 1 had mitral insufficiency. No control dogs had received any immunomodulatory therapy at the sample collection. The numbers of white blood cells and monocytes in the CBCs were not significantly different between the groups (white blood cells: 10,438 ± 4,836/*μl* vs.

Table 2. History in 26 dogs with inflammatory colorectal polyps (ICRPs)

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

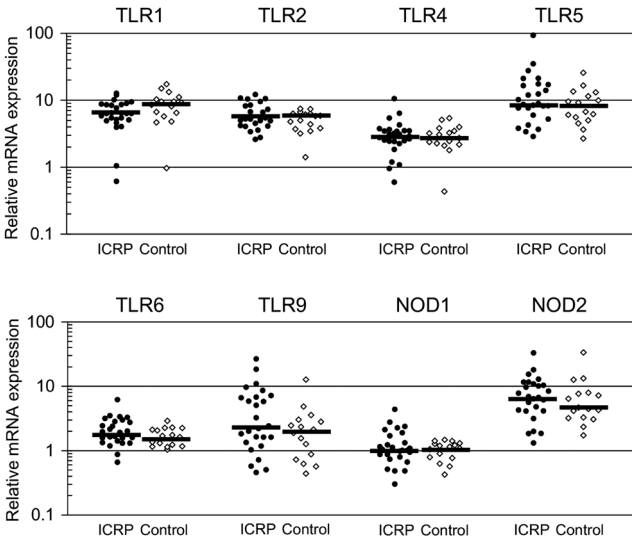

Fig. 1. Relative transcription levels of pattern recognition receptor messenger RNAs (mRNAs) in non-stimulated monocytes in miniature dachshunds (MDs) with inflammatory colorectal polyps (ICRPs; n=26) and control MDs (n=16). Data are expressed relative to the geometric mean of three reference genes (Hydroxymethylbilane synthase, succinate dehydrogenase complex subunit A and TATA box binding protein). The horizontal lines represent the median value of that group.

10,719 ± 4,595/μl, P=0.698; monocytes: 640 ± 289/μl vs. 786 ± 487/μl, P=0.521; data represent mean ± SD).

Quantification of mRNA expression levels and protein production of PRRs and pro-inflammatory cytokines in unstimulated canine monocytes: As shown in Figs. 1 and 2, no significant difference in the mRNA expression levels of any of the PRRs or pro-inflammatory cytokines investigated was observed between the groups. Subsequently, we selected IL-1β as an indicator of PRR reactivity, since it showed relatively small inter-dog and inter-group variance (Fig. 2). The protein secretion of IL-1β in the unstimulated monocytes was equivalent between the groups (Fig. 2).

Responses of canine monocytes to PAMPs: The IL-1β mRNA expression level in monocytes from ICRP-affected MDs was significantly higher than that from control MDs when stimulated with MDP (Fig. 3). By contrast, no significant difference in the response of monocytes to stimulation with iE-DAP, Pam3CSK4, PGN-EK, FSL-1, LPS-EK, FLA-ST or ODN2006 was observed between the MD groups (Fig. 3). Furthermore, IL-1β protein production after stimulation with MDP, Pam3CSK4, PGN-EK and FSL-1 in monocytes from ICRP-affected MDs was greater than that in monocytes from control MDs (Fig. 4). Conversely, no significant difference in monocyte response was observed between the MD groups after stimulation with iE-DAP, LPS-EK, FLA-ST or ODN2006 (Fig. 4).

In addition, there was no significant difference in all mRNA expression and protein production levels investigated in this study between the ICRP-affected MDs which had received immunosuppressive therapy and those had not (data not shown). Furthermore, the IL-1β mRNA expression and protein production after stimulation with MDP in ICRP-affected MDs without immunosuppression were also significantly greater than those in control MDs (Table 3), while no significant difference of IL-1β mRNA expression or protein production was observed between them when stimulated with other ligands including Pam3CSK4, PGN-EK and FSL-1

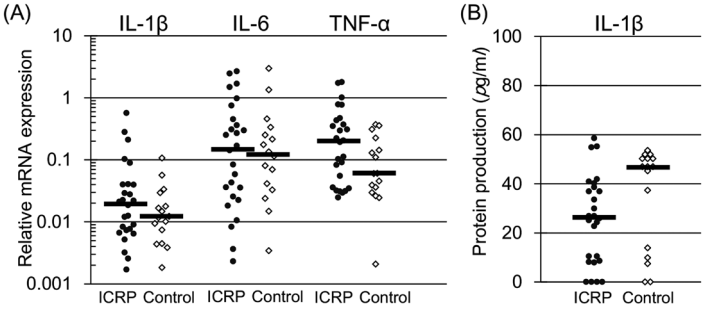

Fig. 2. Relative transcription levels of proinflammatory cytokine mRNAs (A) and protein production of IL-1β (B) in non-stimulated monocytes in MDs with ICRPs (n=26) and control MDs (n=16). Data of mRNAs are expressed relative to the geometric mean of three reference genes. Samples below the limit of detection (5.9 *p*g/m*l*) have been assigned a value of zero. The horizontal lines represent the median value of that group.

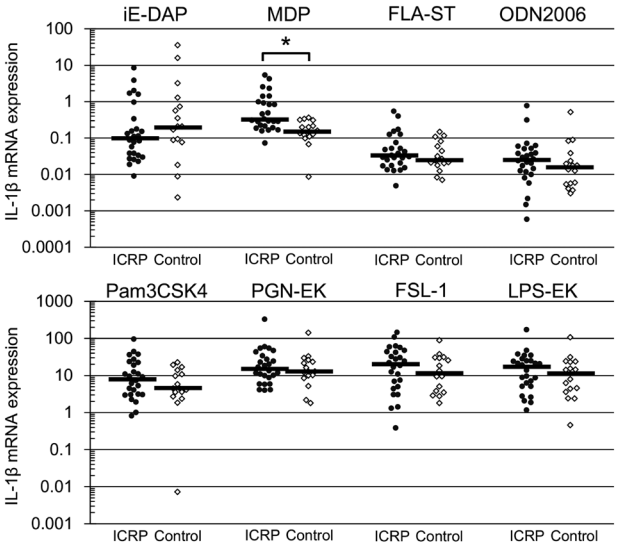

Fig. 3. Relative transcription levels of IL-1β mRNAs in monocytes stimulated with pathogen-associated molecular patterns (PAMPs) for 6 hr in ICRP-affected (n=26) and control (n=16) MDs. The horizontal lines represent the median value of that group. Data are expressed relative to the geometric mean of three reference genes. Asterisks indicate statistically significant differences (P<0.05). iE-DAP, peptidoglycan-like molecule (NOD1 ligand); FLA-ST, flagellin (TLR5 ligand); MDP, muramyl dipeptide (NOD2 ligand); FSL-1, synthetic diacylated lipoprotein (TLR2/6 ligand); LPS-EK, LPS (TLR4 ligand); ODN2006, CpG oligonucleotide (TLR9 ligand); Pam3CSK4, synthetic bacterial lipoprotein (TLR1/2 ligand); PGN-EK, peptidoglycan (TLR2 ligand).

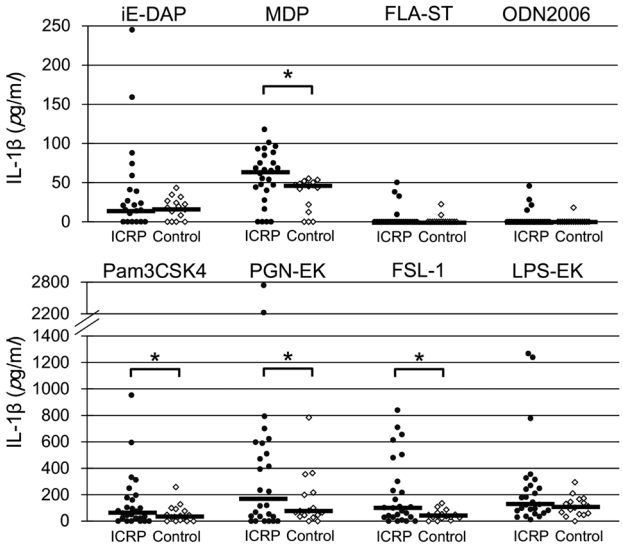

Fig. 4. Secretion of IL-1β protein from monocytes stimulated with PAMPs for 24 hr in ICRP-affected (n=26) and control (n=16) MDs. Samples below the limit of detection (5.9 *p*g/m*l*) have been assigned a value of zero. The horizontal lines represent the median value of that group. Asterisks indicate statistically significant differences (P<0.05).

(Table 3). Moreover, no difference was observed in IL-1β mRNA expression after stimulation with every ligand investigated between ICRP-affected MDs at initial diagnosis and those at clinical remission, whereas greater IL-1β protein productions were observed in ICRP-affected MDs at initial diagnosis after stimulation with Pam3CSK4, PGN-EK or FSL-1 compared with those at clinical remission (Table 4).

DISCUSSION

The current study compared the PRR responses to specific PAMP stimulation in monocytes from ICRP-affected and control MDs. The study aimed to determine candidate PRRs responsible for disease development that might result from genetic predispositions in MDs. We found hyperreactivity of certain PRRs, including NOD2, TLR1/2, TLR2 and TLR2/6. Since it has been reported that the number of macrophage is increased in the polypoid lesion [31, 41], the hyperreactivity of these PRRs in monocytes would be involved in the pathogenesis of ICRPs in MDs.

MDP, a ligand of NOD2, induced mRNA upregulation and IL-1β protein production, and the inductions were greater in ICRP-affected MDs than in control MDs. These findings indicate a hyperreactivity of NOD2 in MDs with ICRPs. NOD2 recognizes peptides derived from bacterial peptidoglycan and induces pro-inflammatory cytokine production through NF-κB activation [30]. Constitutive activation of NF-κB and/or hyperresponsiveness to MDP stimulation, which is due to mutations of the NOD2 gene [42], are considered to associate with Blau syndrome (BS) in humans [38]; this is partly consistent with the findings of the present study. We did not evaluate NF-κB activity in this study; however, baseline IL-1β mRNA expression and protein production levels were equivalent between the groups. BS is characterized as a systemic granulomatous inflammation including uveitis, dermatitis and symmetric arthritis. Interestingly, miniature dachshund also commonly develops a granulomatous disease including sterile panniculitis [49]; however, no ICRP-affected MDs included in this study did not have a history or complication of such disease (Table 2). Moreover, inflammation in BS is thought to be independent of bacterial participation [27]. By contrast, NOD2 hyperreactivity seems to play a crucial role in the development of ICRP inflammation via response against fecal antigens, because the large intestine has the highest density of bacteria in the gut [14]. Therefore, the significance of functional disorders of NOD2 in ICRPs in MDs seems to differ from those of BS in humans. Further analyses of the genetic background of the NOD2 gene in MDs with ICRPs are warranted.

Monocytes derived from ICRP-affected MDs also showed overproduction of IL-1β protein in response to stimulation with Pam3CSK4, PGN-EK and FSL-1, the ligands of TLR1/2, TLR2 and TLR2/6, respectively. TLR2 recognizes bacterial peptidoglycan and induces pro-inflammatory and immunomodulatory cytokines via NF-κB activation, whereas TLR1 and TLR6 form dimers with TLR2 and alter TLR2 ligand specificity [1, 28]. The activation of NF-κB

Table 3. Comparison of IL-1β mRNA expression and protein production between ICRP-affected miniature dachshunds (MDs) without immunosuppressive therapy (n=16) and control MDs (n=16)

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

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

Table 4. Comparison of IL-1β mRNA expression and protein production between ICRP-affected MDs at initial diagnosis (n=17) and those at clinical remission (n=9)

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

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

by these TLRs is induced through a myeloid differentiation factor 88-dependent pathway that is also involved in TLR4-, TLR5- and TLR9-induced pathways [28]. Therefore, the overproduction of IL-1β observed in this study might be the result of functional disorders of TLRs, especially TLR2, but not from disorders in lower signaling pathways. Some polymorphisms of TLR1, TLR2 and TLR6 in humans have also been associated with an increased risk of IBD [6], although their effects on reactivity have not been well characterized. Therefore, further investigation of the genetic background of the TLRs contributing to the hyperreactivity and development of ICRPs in MDs is also needed.

Despite the increased production of IL-1β protein in response to TLR1/2, TLR2 and TLR2/6 stimulation, the mRNA expression level of IL-1β in ICRP-affected MDs and control MDs was not significantly different (Fig. 3). We have two explanations for this result. First, greater negative feedback on signaling pathway, such as the NF-κB pathways, may be induced by these TLRs, because they usually elicit greater cellular responses compared with those elicited by NOD2 [21, 35, 46], which is also consistent with the results of the current study (Figs. 3 and 4). We chose a 6-hr duration of stimulation, because it resulted in the highest IL-1β mRNA expression in a preliminary study (data not shown). However, the 6-hr duration of stimulation may induce negative feedback and interfere with mRNA expression. Sequential evaluation of mRNA expression levels after shorter or longer stimulations may confirm the existence of the negative feedback interference; however, we did not extract mRNA from the monocytes stimulated for 24 hr. Furthermore, response against lower-dose stimulation might also help determine whether the negative feedback interference occurs. These follow-up studies need to be performed in the future. Second, an inflammasome disorder may exist, because inflammasomes activate the conversion of IL-1β from pro-IL-1β, which is synthesized from IL-1β mRNA [27]. However, we believe this explanation is unlikely, because protein production with or without the stimulation of other PRRs was not significantly different between the groups.

Although the hyperreactivity of NOD2, TLR1/2, TLR2 and TLR2/6 might reasonably lead to the overproduction of inflammatory cytokines in ICRP lesions, several contradictions remain. The primary role of NOD2 in the gastrointestinal tract appears to be the modulation of TLR signaling

through the induction of interferon regulatory factor 4 and the mediation of tolerance to bacterial antigens [13, 46, 47]. A frameshift mutation of NOD2 gene, the most influential mutation for Crohn's disease, leads to the hypo-reactivity to MDP [13]; the consequent deficiency of the induction of TLR tolerance is regarded as one of the important etiology of Crohn's disease [39]. In addition, TLR2 stimulation leads to the induction of pro-inflammatory and immunomodulatory cytokines [5, 10]. Moreover, a recent study revealed that the responses to PRR stimulation differed in duodenal biopsy specimens and whole blood, which indicates that responses to bacterial stimuli differ by location [36]. Because only monocytes derived from peripheral blood were used in the present study, we are uncertain whether the observed PRR hyperreactivity can be applied in the same manner to the colorectal mucosa. However, we speculate that a genetic disorder affecting the response of monocytes derived from peripheral blood interferes with the function of macrophages in the colorectal mucosa. Further analyses using colorectal tissue cultures or mucosal macrophages and evaluating both pro-inflammatory and immunomodulatory responses must be performed. In addition, interactions between NOD2 and TLRs, particularly as they relate to interferon regulatory factor 4 in both control and ICRP-affected MDs, are of interest.

The protein production of IL-1β was decreased in both ICRP-affected and control MDs when stimulated with FLA-ST and ODN2006, the ligands of TLR5 and TLR9, respectively (see Figs. 2 and 4). These TLRs in the gut have been described that they do not directly induce pro-inflammatory reactions like TLR2 and TLR4, but contribute to homeostasis via inducing the differentiation of anti-inflammatory responses and IgA production [9, 43]. House *et al.* reported that these PAMPs hardly induce pro-inflammatory cytokine mRNA expression and protein production in canine PBMC-derived monocytes [15], which is consistent with the results of IL-1β mRNA expression in this study. The difference in the stimulating duration might explain the reduction of IL-1β protein production observed in this study. House *et al.* stimulated cells for 4 hr [15], while 24 hr-stimulation was performed in this study; we selected the duration to obtain detectable amount of IL-1β protein in a preliminary study (data not shown). The chronic or repetitive stimulation with PAMPs including ligands of TLR5 and TLR9 induces a TLR tolerance, which is characterized as the unresponsiveness of lower signaling pathways and reduced induction of pro-inflammatory cytokines by further TLR stimulations [22, 40]. The induction of TLR tolerance and little pro-inflammatory induction might result in the reduction of IL-1β protein production. Further analyses whether other pro-inflammatory or anti-inflammatory cytokines show similar reduction via stimulation of TLR5 or TLR9 and the TLR tolerance induced by them would confirm these findings.

This study evaluated only pro-inflammatory cytokine as an indicator of PRR reactivity. Since it has been reported that the alteration of anti-inflammatory response including IL-10 is not consistent with that of pro-inflammatory cytokines in various diseases, such as canine chronic enteropathy [36], a possibility that dysregulation of anti-inflammatory cytokine induction could not be excluded, which cannot be detected in this study design. In addition, Tamura *et al.* reported the marked up-regulation of IL-8 in macrophage at polypoid lesion [41], which suggests the importance of IL-8 over-production from macrophage in the pathogenesis of ICRPs in MDs. Unfortunately, experiments could not be repeated, because of the limited amount of monocytes obtained.

The limited yields of monocytes resulted in another limitation of this study. The cell purity is crucial in this study design. We checked the morphology of adherent cells obtained from ICRP-affected and control MDs and regarded them as the monocyte lineage. However, we did not perform immunocytochemistry, flow cytometry or other staining on the plate-adherent cells of ICRP-affected and control MDs, because of the limited number of cells. In addition, the cell viability was not evaluated in ICRP-affected and control MDs in this study. However, the non-adherent cells were removed before and after cell stimulation at each assay, and thus, we consider that the most of dead cells were not remained in the experimental system.

Our study had further limitations. Although the control group was composed of age-matched MDs, their genetic backgrounds were not clarified; in other words, MDs enrolled in the control group in this study might develop ICRPs in future. However, the prevalence of ICRPs in MDs is approximately 1.1%, according to a previous retrospective study [31]; therefore, we believe that this limitation does not affect the results of our study.

Another limitation was that inflammatory status and treatment with anti-inflammatory agents including prednisolone, cyclosporine and/or leflunomide at the time of sampling might affect the reactivity of monocytes. Although we cultured monocytes for 7 days to reduce their interference and confirmed that no significant differences in baseline PRR and pro-inflammatory cytokine mRNA expression and protein production levels occurred between the groups, some non-statistically significant differences in unstimulated cells might affected the results of stimulated monocytes. Thus, we further divided the ICRP-affected MD group into two groups by disease status (whether the dogs were at initial diagnosis or at clinical remission) or immunosuppression status (whether dogs received immunosuppressive therapy or not). Consequently, the ICRP-affected MDs at initial diagnosis showed a greater reactivity of TLR1/2, TLR2 or TLR2/6 (Table 4). Furthermore, the reactivity of TLR1/2, TLR2 or TLR2/6 could not show significant difference when compared between ICRP-affected MDs without immunosuppression and control MDs (Table 3), although the difference of immunosuppression status within ICRP-affected MDs did not result in any significant difference. Therefore, the hyperreactivity of TLR1/2, TLR2 and TLR2/6 should be interpreted with caution that the disease or immunosuppression status may interfere with the result; in other words, the hyperreactivity of NOD2 in ICRP-affected MDs might be true. Confirming this conclusion requires further investigations of repeatability using pre- and post-treatment specimens from MDs with ICRPs.

In conclusion, we demonstrated that the reactivity of

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

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