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Ki-67/CD3 ratio in the diagnosis of chronic inflammatory enteropathy in dogs

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Abstract

Background: T cells play a key role in the pathogenesis of chronic inflammatory enteropathy (CIE) in dogs. Cluster of differentiation 3 (CD3) antigen serves as a marker for T cells. In human medicine, Ki-67 is an indicator for cell growth but there are only a few studies in dogs with CIE.

Objective: To investigate Ki-67 in relation to T cells as a marker for CIE in dogs.

Animals: Eleven dogs with CIE and 6 healthy beagle controls (CO).

Methods: Retrospective case-control study. Dogs were clinically assessed by the Canine Chronic Enteropathy Clinical Activity Index (CCECAI). Duodenal mucosal biopsy samples were endoscopically obtained for histopathologic examination by means of the World Small Animal Veterinary Association score. Double-labeled immunofluorescence was used to investigate colocalization of Ki-67 and CD3 in epithelium and lamina propria (LP) of villi and crypts.

Results: Dogs with CIE had significantly higher clinical score (median, 5.0; interquartile range [IQR], 3-7) compared to CO (all 0; $P < .001$). The Ki-67/CD3 double-positive cells were significantly increased in the LP of the crypt region of CIE dogs (0.63 cells/mm^2; IQR, 0-0.54) versus CO (0.08 cells/mm^2; IQR, 0-0.26; $P = .044$). A significant correlation was found between CCECAI and the Ki-67/CD3 ratio in the LP of the crypt region ($r = 0.670$; $P = .012$) in dogs with CIE.

Conclusions and Clinical Importance: The Ki-67/CD3 ratio is upregulated in the LP crypt region of dogs with CIE and it correlates with clinical severity. Therefore, Ki-67/CD3 could be a useful tool for detection of CIE.

KEYWORDS

canine, diagnostic marker, inflammatory bowel disease, intestinal inflammation, T cells

Abbreviations: ARD, antibiotic-responsive diarrhea; CBC, complete blood count; CCECAI, Canine Chronic Enteropathy Clinical Activity Index; CD3, cluster of differentiation 3; CIE, chronic inflammatory enteropathy; CO, control group; FRD, food-responsive diarrhea; GI, gastro-intestinal; IBD, inflammatory bowel disease; IF, immunofluorescence; IL, interleukin; IQR, interquartile range; LP, lamina propria; MD, median; PBS, phosphate-buffered saline; PLE, protein-losing enteropathy; SRD, steroid-responsive diarrhea; WSAVA, World Small Animal Veterinary Association.

1 | INTRODUCTION

Chronic inflammatory enteropathy (CIE) in dogs is a collective term to describe disorders of the gastrointestinal (GI) tract characterized by

persistent or recurrent GI signs such as vomiting, diarrhea, hyporexia, abdominal pain, nausea, and weight loss with histopathologic signs of intestinal inflammation.[1]

The etiology of CIE is not well understood. The current hypothesis is that intestinal inflammation develops in genetically predisposed dogs, through interactions among food components, environmental factors, and intestinal microbiota.[2,3] In dogs, CIE is diagnosed based on exclusion of extra-GI as well as GI disorders of other etiology (eg, mechanical obstruction caused by foreign bodies, intestinal tumors, intussusception).[4] In dogs, the type of CIE often is determined retrospectively by response to treatment and includes food-responsive diarrhea (FRD), antibiotic-responsive diarrhea (ARD), and idiopathic inflammatory bowel disease (IBD), which in most cases is steroid-responsive diarrhea (SRD).[1,5,6]

The cause of idiopathic CIE is unknown, but mounting evidence suggests that hypersensitivity and proliferation of T cells play key roles in the pathogenesis.[7] The cluster of differentiation 3 (CD3) cell infiltrates in the duodenum of dogs with CIE decrease after treatment with cyclosporine, which decreases the expression of interleukin-2 (IL-2) that is necessary for T-cell survival.[8] A multimeric protein complex, CD3 functions as a T-cell coreceptor.[9] The T-cell response is initiated by presenting foreign antigen through the major histocompatibility complex molecule binding to the T-cell receptor-CD3 complex.[10] The CD3 antigen is well known as a T-cell marker in both human as well as veterinary medicine.[7,11]

The Ki-67 antigen is expressed in all active phases of the cell cycle (G_1, S, and G_2) but it is absent in resting cells (G_0 phase).[12] Therefore, Ki-67 is an ideal indicator for cell proliferation. Cells positive for Ki-67 and CD3 thus can be considered proliferating T cells. Proliferating T cells have increased cytokine production, which may contribute to the clinical signs seen in patients with CIE.[13-16]

Our aim was to establish Ki-67/CD3 immunohistochemical double labeling in sections of canine duodenum. The first hypothesis was that a significant difference in Ki-67/CD3 expression would exist between dogs with CIE and healthy control dogs without GI signs (controls [CO]). The second hypothesis was that significant differences would be found in the number of Ki67/CD3 double-positive cells in different areas of the intestinal mucosa (lamina propria [LP] and lamina epithelialis of villi and crypts), thereby indicating areas of proliferation of mucosal T cells.

2 | MATERIALS AND METHODS

Protocols for this study were approved by the Institutional Ethics Committee, the Advisory Committee for Animal Experiments (§12 of Law for Animal Experiments, Tierversuchsgesetz), and the Federal Ministry for Science and Research (reference number: §8 GZ 68.205/0201-II/3b/2010).

2.1 | Study groups

2.1.1 | Control dogs

Six neutered male, clinic-owned beagle dogs 3 to 6 years of age without GI signs were classified as healthy based on history, clinical examination, CBC, biochemistry profile, fecal flotation, and Giardia antigen testing, and served as CO.

2.1.2 | Dogs with chronic inflammatory enteropathy

This retrospective study was performed on dogs seen between 2011 and 2014. Diagnostic evaluation, endoscopic biopsy, and grading were part of a prospective study performed at this time and yielding biopsy samples that were retrospectively used in the present study for special staining and immunofluorescence (IF) microscopy.

Medical records of client-owned dogs in which CIE had been diagnosed at the Clinic for Internal Medicine at the Veterinary University of Vienna were reviewed retrospectively. Criteria for inclusion in the study were clinical signs consistent with CIE (eg, weight loss, vomiting, diarrhea) for longer than 4 weeks. No pretreatment was given 2 weeks before gastroduodenoscopy was performed in dogs included in the study. All CIE patients had histopathological evidence of small intestinal inflammation on endoscopically retrieved biopsy samples. Dogs with metabolic, parasitic, or neoplastic causes of GI signs as well as patients that were not clinically stable for gastroduodenoscopy were excluded from the study.

Eleven dogs met the inclusion criteria. All of the enrolled patients received a routine diagnostic evaluation including history, physical examination, CBC, biochemical profile, basal cortisol concentration, serum cobalamin concentration, fecal flotation, Giardia antigen test as well as abdominal ultrasound examination to exclude infectious, endocrine, or neoplastic diseases as a cause for the GI signs. Gastroduodenoscopy was performed under general anesthesia, and 8 to 10 endoscopic biopsy samples were taken from the duodenum using a flexible endoscope. Control dogs underwent the same endoscopic procedure. All dogs (CIE and CO) were graded according to the Canine Chronic Enteropathy Clinical Activity Index (CCECAI).[5] Biopsy samples were scored by means of the World Small Animal Veterinary Association (WSAVA) guidelines.[17]

2.2 | Tissue preparation and staining

All biopsy samples were fixed in 4% neutral buffered formaldehyde and embedded in paraffin. Serial sections from paraffin tissue blocks were cut for standard Hematoxylin & Eosin staining as well as for IF microscopy. All slides were deparaffinized with xylene and rehydrated through a graded series of alcohols (100, 96, 70%) followed by distilled water at room temperature. Heat-induced epitope retrieval was performed in TRIS-EDTA buffer at pH 9 (Target Retrieval Solution, Ref S2367, Dako, Glostrup, Denmark) with a steamer for 30 minutes. After a cooling phase, the slides were washed 3 times in phosphate-buffered saline (PBS) with 0.025% Tween 20 for 5 minutes each. Protein blocking was performed with 1.5% goat serum (Dako, Glostrup, Denmark) in PBS.

Immunolabeling was performed by a primary antibody mix composed of a polyclonal rabbit anti-CD3 (DAKO, Glostrup, Denmark) at a dilution of 1 : 500 in PBS and monoclonal mouse anti-Ki67 (clone MM1, Novocastra/Leica, Wetzlar, Germany) at a dilution of 1 : 100 in PBS overnight at 4°C followed by 60 minutes at room temperature. The use of both antibodies previously has been validated for dogs.[10,18,19]

After a triple-washing step (5 minutes each) in PBS with 0.025% Tween 20, a secondary antibody mixture composed of goat anti-rabbit labeled with Alexa Fluor 488 and goat anti-mouse labeled with Alexa 568 (Life technologies/Thermo Fisher Scientific, Waltham, MA), both diluted 1 : 150 in PBS, was added and cells were incubated for 60 minutes on the shaker at room temperature. Nuclei were counterstained with Dapi (Sigma-Aldrich, MO) for 10 minutes and covered with Aqua-Poly/Mount (Polysciences Europe GmbH, 69493 Hirschberg an der Bergstrasse, Germany) and a coverslip.

As positive control samples, canine lymph node was used for CD3-staining and canine intestinal mucosa was used for Ki-67-staining. "No primary antibody" controls were used to show that there was no host specific binding of the secondary antibodies.

2.3 | Canine Chronic Enteropathy Clinical Activity Index and histopathology

All dogs were scored by CCECAI, which is a clinical scoring system including attitude, appetite, vomiting, fecal consistency, frequency of defecation, weight loss, serum albumin concentration, ascites, and peripheral edema as well as pruritus.[5] Tissue samples were graded by a single blinded board-certified pathologist (BR) according to WSAVA guidelines.[17]

Four inflammatory histologic variables (intraepithelial lymphocytes, LP lymphocytes and plasma cells, LP eosinophils, and LP neutrophils) were scored in at least 8 specimens per dog and graded as normal = 0, mildly increased = 1, moderately increased = 2, and markedly increased = 3. The arithmetic mean for each variable then was determined for each patient.

2.4 | Fluorescence microscopy

The Ki-67/CD3 ratio was determined by counting double-positive cells in different layers of the intestinal wall (epithelium and LP of villi and crypts) in 15 representative fields of each slide by a single independent observer (S. Karlovits) with Zeiss Imager Z2 (Carl Zeiss Microscopy GmbH, Jena, Germany; Figure 1). Randomly selected slides were double-checked by another investigator (A. Manz) to verify results. Observers were blinded to patient and study group history. Double-positive cell counts were expressed as cells/mm^2.

The areas on the slides without tissue were extracted by 2 different computer programs—ilastik, an interactive learning and segmentation toolkit, as well as Fiji.[20,21]

Not all areas were available on each slide of every patient.

2.5 | Statistical analysis

Data were analyzed by IBM SPSS v24 (IBM Corporation, Armonk, NY). The assumption of normal distribution was tested using the Kolmogorov-Smirnov test. Because data were not normally distributed and because of small sample size, differences in variables such as CCECAI, WSAVA scoring and double-positive cells in the LP, and epithelium of villi and crypts between CIE and CO were analyzed using the nonparametric Mann-Whitney test. Holms correction method was used to adjust *P*-values for multiple testing. Results are given as median (MD) and interquartile range (IQR). Correlation between variables (CCECAI and Ki-67/CD3 ratio; WSAVA and Ki-67/CD3 ratio) was calculated by Spearman's correlation coefficient. For all statistical tests, a *P*-value <.05 was considered significant.

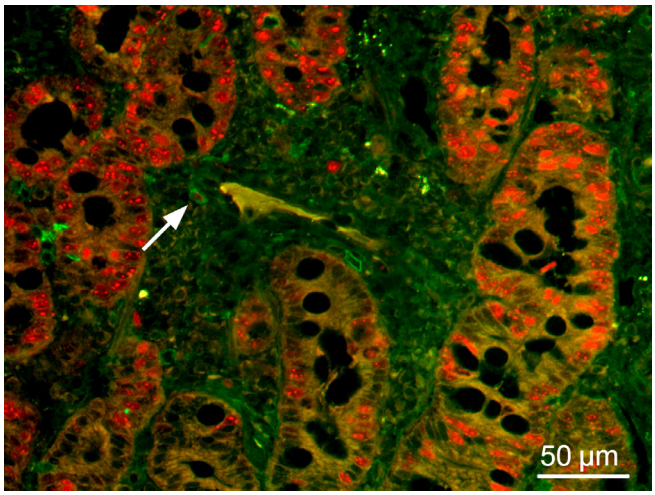

FIGURE 1 Ki-67/cluster of differentiation 3 (CD3) double positive cells. Ki-67 (red)/CD3 (green) double positive cell in the crypt lamina propria in dogs with chronic inflammatory enteropathy—cell with a red nucleus and a light green membranous staining (arrow)

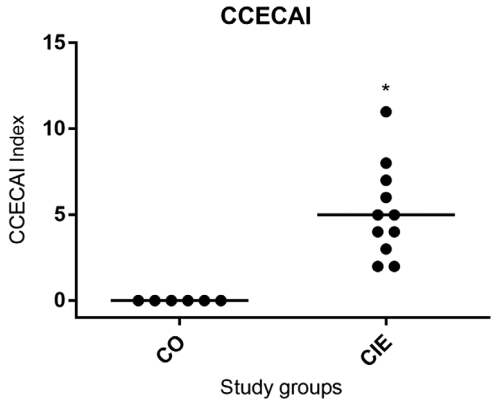

FIGURE 2 Canine Chronic Enteropathy Clinical Activity Index (CCECAI). The CCECAI was significantly upregulated in dogs with chronic inflammatory enteropathy (5.0; interquartile range 3-7) versus control group (all 0; $P < .001$). *$P < .05$

3 | RESULTS

3.1 | Descriptive statistics

The CO (n = 6) consisted of healthy male neutered beagle dogs which were all 3 years of age. Median body weight was 17.0 kg (IQR, 16.9-17.2).

The CIE group (n = 11) consisted of each of the following: American Staffordshire Terrier, Boxer, Cavalier King Charles Spaniel, French Bulldog, German Shepherd, Groenendale, Jack Russell Terrier, Malinois, mixed breed dog, Pit Bull Terrier, and Shar Pei. Only the Shar Pei was clinically classified as having protein-losing enteropathy (PLE) because of panhypoproteinemia with hypoalbuminemia (serum albumin concentration, 15 g/L; reference range, 26-47 g/L). The MD age was 4 years (IQR: 3-5.3) and MD body weight was 17.5 kg (IQR, 10.6-27.4).

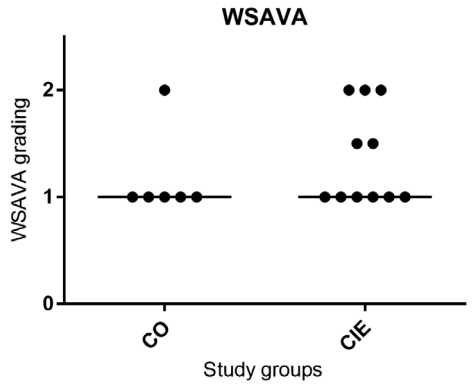

FIGURE 3 World Small Animal Veterinary Association (WSAVA) grading. There was no significant difference in the WSAVA grading between both groups (P = .313)

3.2 | Canine Chronic Enteropathy Clinical Activity Index and histopathology

The CCECAI was significantly higher in CIE (5.0, IQR: 3-7) versus CO (all 0; P < .001; Figure 2).

Both CIE and CO had a MD WSAVA score of 1 (CIE, 1.0, IQR: 1.0-2.0; CO, 1.0; IQR, 1.0-1.3). The WSAVA score was not different between groups (P = .313; Figure 3).

3.3 | Immunofluorescence

The LP of the crypt area had significantly higher expression of Ki-67/CD3 double-positive cells/mm^2 (MD, 0.63; IQR, 0-0.54; P = .044) compared to crypt epithelium, villus epithelium and villus LP (Figure 4A–D). In LP of the crypt area, a significant correlation was found between CCECAI and the Ki-67/CD3 ratio (r = 0.670; P = .012).

4 | DISCUSSION

Currently, the diagnosis of CIE is based upon persistent or recurrent GI signs in combination with histopathologic signs of intestinal inflammation.[1] However, the definitive diagnosis is difficult because often only a

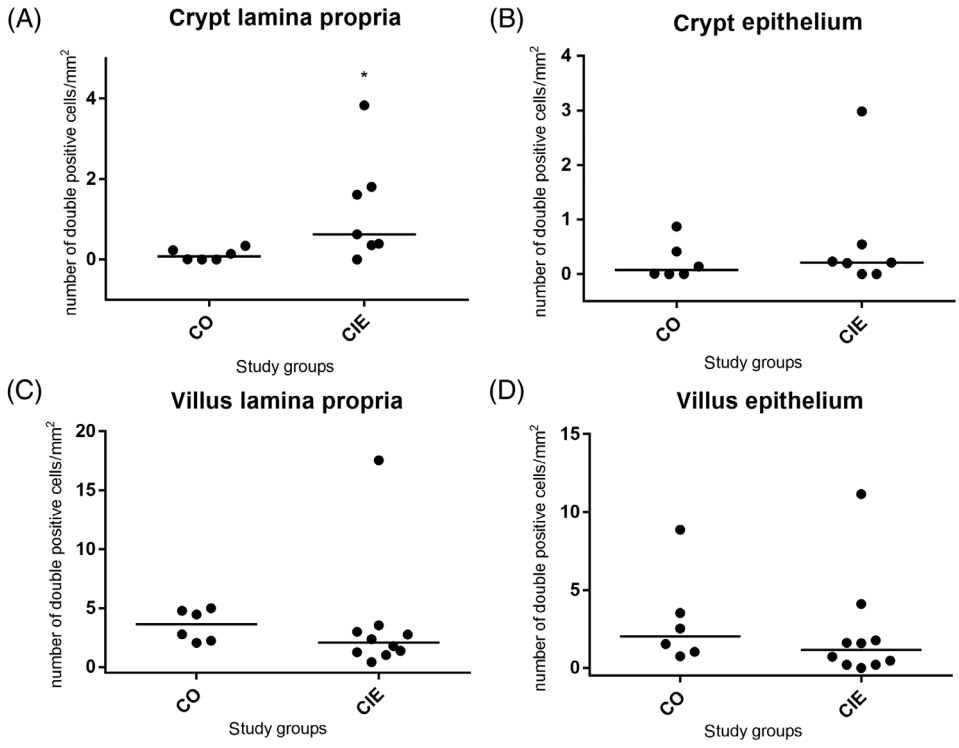

FIGURE 4 Double positive cells in epithelium and lamina propria of villi and crypts. The lamina propria of the crypt area showed a significantly higher expression of Ki-67/cluster of differentiation 3 double positive cells/mm^2 (median: 0.63; interquartile range: 0-0.54; P = .044) compared to crypt epithelium, villus epithelium, and villus lamina propria. *P < .05. Not all areas were always available on each slide of every patient

few, very small endoscopic biopsy samples are available that may or may not be representative of the entire GI tract.[22] Their quality depends on the experience of the operator obtaining the biopsy samples as well as interobserver variations in histopathological evaluation, despite a standardized grading system.[17,23,24] Given the current limitations of the WSAVA scoring system, the demand for supportive diagnostic tools in CIE is increasing. Because hypersensitivity and proliferation of T cells may play a major role in the pathogenesis of CIE,[7] the combination of Ki-67 as a proliferation marker and CD3 as a marker for T cells was used to investigate active T cells in different areas of the intestinal wall. Results of this study show a significant upregulation of Ki-67/CD3 double-positive cells in the crypt area of the LP in dogs with CIE ($P = .044$) compared to CO, indicating that it is a metabolically active region for T cells. However, crypt and villus epithelium as well as villus LP did not show a significant upregulation of double-positive cells. This study represents the first time that proliferation of T cells in the LP of CIE dogs has been evaluated and indicates that this proliferative ratio could be a more useful marker of clinical severity than the currently used WSAVA score and endoscopic grading.[25]

The WSAVA score was not significantly different between the 2 study populations. This finding may be a consequence of the small size of the study population. Although a standardized grading system was used, histopathological interpretation still is subjective and influenced by staining techniques and interobserver variability.[17] Previous studies found no change in the total number of T cells as well as severity of inflammatory infiltrates after treatment, and histological score was not associated with outcome in dogs with CIE.[7,26] Despite the fact that the WSAVA scores comparing CO and CIE were not significantly different, the MD in the CIE group was higher than in the CO. Nevertheless, the CO consisted of beagles, which are not an ideal CO, and most of the CIE dogs were scored mildly. Although the Ki-67/CD3 ratio did not correlate with histopathological scoring, we detected a correlation with CCECAI. Proliferating T cells exhibit increased cytokine production, which may contribute to the clinical signs seen in patients with CIE.[13-16] The CD3 cell infiltrates in the duodenum of dogs with CIE decrease after treatment with cyclosporine, thus decreasing expression of IL-2, crucial for T-cell survival.[8] Therefore, the routine use of immunohistochemical markers such as the Ki-67/CD3 ratio could aid in the interpretation of the histological score together with clinical scoring, because a significant difference was found between the groups in the crypt area in our study.

We found significant upregulation of Ki-67/CD3-positive cells in the LP of the crypt. This finding is in contrast to the normal distribution of T-cells in healthy dogs with an increasing cell density from crypt to villus tip, reflecting exposure to luminal antigens.[27,28] This finding may be a result of higher epithelial destruction because of inflammation in the examined intestinal biopsy samples.[2] Another explanation for this distribution could be that stem cells of the crypt LP show an increased proliferation rate during inflammation and higher amounts of destruction at the villus tip.[29]

Our study had several limitations, mostly because of its retrospective study design. A prospective study and larger population without pretreatment would be desirable for further studies. Because of the retrospective character of our study, no ileal biopsy samples were available from both the CIE group and CO. Therefore, no results are available for the ileum, thus the multifocal character of the lesions in CIE with different degrees of severity in each region may have been missed.[30,31] However, intraepithelial lymphocytes, lymphoplasmacytic infiltrates, and inflammatory changes tend to be more common in the duodenum than in the ileum, and significant differences in the Ki-67/CD3 ratio were identified in our study.[30]

The CIE group included 1 dog with PLE. This fact must be interpreted with caution because PLE is not a single disease but a syndrome that develops in different diseases of different etiologies such as lymphatic obstruction, increased vascular permeability because of cytokines, or mucosal inflammation as in IBD (primarily caused by severe lymphoplasmatic enteritis).[32,33] Another limitation was that not all areas were always available for every patient because of the nature of biopsy samples and cutting techniques.

The patients with CIE received different treatments before endoscopic diagnosis was made, ranging from diet alone, a combination of different diets, antibiotics, prednisolone, and deworming. However, all treatments were discontinued at least 2 weeks before endoscopy. Therefore, pretreatment should not be a confounding factor.

For future prospective studies, it would be preferable to have a CO consisting of dogs of different breeds, sex, and age, avoiding the selection bias of our study. Although no significant difference in age was found between the 2 study populations in our study, dogs with FRD generally are younger than those with SRD.[5] Furthermore, grouping of patients according to their response to treatment for FRD, ARD, and SRD, as well as outcome, would be preferable. Re-evaluation of the status of the disease using endoscopically biopsy samples to determine if the Ki-67/CD3 ratio decreases with improvement in clinical signs also would be needed in future studies to ascertain its value as a prognostic and therapeutic marker.

In conclusion, we report significant upregulation of Ki-67/CD3 double-positive cells in the LP in the crypt area of dogs with CIE as compared to CO as well as a correlation of Ki-67/CD3 with clinical severity as determined by CCECAI score. Given the current limitations of the WSAVA scoring system, the Ki-67/CD3 ratio could be a promising tool to aid in the diagnosis of CIE, but more studies with a larger study population of dogs are needed.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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