Phenotypic Characterization of Canine Intestinal Intraepithelial Lymphocytes in Dogs with Inflammatory Bowel Disease

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**Background:** Many dogs suffering from inflammatory bowel disease (IBD) are presented to veterinary clinics. These patients are diagnosed based on a history of chronic gastrointestinal signs and biopsy-confirmed histopathologic intestinal inflammation. Intestinal intraepithelial lymphocytes (IEL) are part of the first line of defense in the gastrointestinal immune system. Alterations in IEL subsets may play a role in the pathogenesis of IBD.

**Hypothesis:** The aim of this study was to characterize the phenotypes of IEL in dogs with IBD compared with healthy control dogs.

**Animals:** Intestinal intraepithelial lymphocytes subpopulations of control dogs (n = 5) obtained from endoscopic biopsies (EB) were compared to those obtained from full thickness biopsies (FTB) on the same day. In addition, the phenotypes of IEL from FTB of control dogs (n = 10) were compared with EB of IBD dogs (n = 10). Each participant was scored clinically using the canine inflammatory bowel disease activity index (CIBDAI), and all samples were graded histopathologically. Three-color flow cytometry of isolated IEL was performed using monoclonal antibodies against T- and B-lymphocyte subpopulations.

**Results:** No significant differences in the composition of IEL subpopulations were found in control dogs based on method of biopsy. The IBD dogs had significantly higher CIBDAI and histopathologic scores compared with control dogs and their IEL contained a significantly higher frequency of T cells.

**Conclusions and Clinical Importance:** Endoscopic biopsies provide suitable samples for 3-color flow cytometry when studying canine intestinal IEL and IBD patients show significant changes of major T-cell subsets compared to healthy control dogs.

**Key words:** Dogs; Flow-cytometry; Intestinal immune-cells.

A large number of dogs suffer from chronic or recurrent gastrointestinal signs. Inflammatory bowel disease (IBD) represents a heterogeneous group of disorders characterized by inflammation of the intestinal tract. After excluding infectious, endocrine and neoplastic causes, the diagnosis of canine IBD is established based on histopathologic evidence of intestinal inflammation. In most cases, lymphocytic-plasmacytic cellular infiltration of the mucosa predominates. Furthermore, dogs with IBD have been differentiated clinically with regard to response to therapeutic trials as “diet-responsive,” “antibiotic-responsive,” and “steroid-responsive.”

Dogs may share a similar, multifactorial pathogenesis as do human IBD patients, but little is known about the underlying pathologic mechanisms.

**Abbreviations:**
- Alexa647: Alexa Fluor 647
- WSAVA: World Small Animal Veterinary Association
- APC: cluster of differentiation
- CIBDAI: canine inflammatory bowel disease activity index
- DTT: 1,4-Dithiothreitol
- EB: endoscopic biopsies
- FCS: fetal calf serum
- FITC: fluorescein isothiocyanate
- FSC/SSC: forward scatter/sideward scatter
- FTB: full-thickness biopsies
- HHB: hepes-buffered Hanks’ balanced salt solution
- IBD: inflammatory bowel disease
- IEL: intraepithelial lymphocytes
- IgG: immunoglobulin G
- mAb: monoclonal antibody
- PBS: phosphate buffered saline
- PE: phycoerythrin
- SD: standard deviation
- TCR: T-cell receptor
- WSAVA: World Small Animal Veterinary Association
Factors, disruption of the mucosal barrier, changes in the intestinal microbiome, and dysregulation of the intestinal immune system lead to the breakdown of immunologic tolerance and the onset of IBD. Intestinal intraepithelial lymphocytes (IELs) are an important part of adaptive immunity. Two major subsets can be defined: (1) conventional IEL characterized by T-cell receptor (TCR) αβ expression with co-receptor cluster of differentiation (CD) 4 and CD8αβ and (2) non-conventional IEL expressing TCRαβ or TCRγδ combined with co-receptor CD8αα. Pro- and anti-inflammatory functions are described for both subsets.

Human patients with Crohn’s disease have increased numbers of CD8+ cytotoxic T-cells and TCRγδ+ T-cells in inflamed colonic mucosa. Based on this information, it can be concluded that IEL play an important role in the pathogenesis of canine IBD. Hence, the aim of the present study was to characterize the phenotypes of IEL by flow cytometry. The suitability of intestinal EB for flow cytometric analysis in dogs also was validated. The phenotypic characterization of IEL from control dogs and dogs with IBD was performed to identify differences in their lymphocyte subsets.

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, Tierversuchsge setz [TVG]), and the Federal Ministry for Science and Research [reference number: GZ 68.205/0201-II/3b/2010].

Study Groups

Control Dogs. Ten healthy control dogs of different breeds were included in the study. They were presented to the Clinic for Internal Medicine at the Veterinary University of Vienna for non-gastrointestinal problems. These dogs had not received antibiotic or immunosuppressive treatment in the 10 days before biopsy acquisition and were euthanized for reasons not related to the study. Full thickness biopsies (FTB) and EB from the duodenum were obtained 15–30 minutes post-mortem and were immediately placed in ice-cold phosphate-buffered saline (PBS) and 4% buffered paraformaldehyde solution until processing.

Inflammatory Bowel Disease Dogs. Ten dogs presented to the Clinic for Internal Medicine at the Veterinary University of Vienna with chronic gastrointestinal signs were selected for this prospective study. Inclusion criteria were vomiting, diarrhea, anorexia, weight loss, or some combination of these signs for at least 4 weeks, with no immunosuppressive drugs or antibiotics administered by the owners for at least 10 days before biopsy acquisition. Furthermore, a complete clinical evaluation was performed, including hematology, clinical biochemistry (including canine serum trypsin-like immunoreactivity, vitamin B12, and folate concentrations), urinalysis, fecal flotation, Giardia antigen test, and abdominal ultrasound examination to exclude infectious, endocrine or neoplastic diseases as explanations for the gastrointestinal signs. Owners gave written consent for their dogs to take part in the study. Gastrointestinal endoscopy was performed under general anesthesia, and EB samples from the stomach and descending duodenum were taken with flexible endoscopic biopsy forceps. Endoscopic procedures and sample storage were performed the same as for control dogs. All dogs had intestinal infiltration with inflammatory cells and lesions were graded using the World Small Animal Veterinary Association (WSAVA) guidelines. Based on the chronicity of gastrointestinal signs, the exclusion of underlying infectious, endocrine or neoplastic diseases, and the intestinal histopathologic inflammatory findings, these dogs were diagnosed as suffering from IBD.

Clinical and Histopathologic Scoring

All cases were scored according to the canine inflammatory bowel disease activity index (CIBDAI). All tissue samples from the 3 study groups were graded by a single independent board-certified pathologist according to the WSAVA International Gastrointestinal Standardization Group guidelines. Because a number of samples had suboptimal orientation of mucosal villi, the morphologic criteria of villus stunting were not taken into account. In total, 4 morphologic parameters (epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis) and 4 inflammatory histologic parameters (IEL, lamina propria lymphocytes and plasma cells, lamina propria eosinophils, and lamina propria neutrophils) were scored as 0 = normal, 1 = mild, 2 = moderate, 3 = marked. The sum of the scores from single parameters were totaled and dogs were subdivided into histologic severity groups: WSAVA score of 0 = normal, 1–6 = mild (<25% of the maximal score of 24), 7–12 = moderate (25–50% of the maximal score), 13–18 = severe (50–75% of the maximal score), and >18 = very severe (>75% of the maximal score).

IEL Isolation

Immediately after sample collection, IEL were isolated as previously described. In brief, all duodenal biopsies (FTB and EB) had to contain intact lamina propria mucosa. The FTB samples were cut into pieces approximately 1 cm in length. All specimens were washed 2–6 times in ice-cold PBS or Heps-buffered Hank’s balanced salt solution (HHB) to remove attached feces. Afterward, they were washed twice in HHB with 2% fetal calf serum (FCS), 2 mM 1,4-dithiotreitol, and 0.5 mM EDTA for 20 minutes each time at 37°C with constant stirring. After each wash, cells were passed through a 70 µm nylon cell strainer. Cells then were centrifuged on a discontinuous density gradient with 40% and 70% Percoll (920 × g, 30 minutes, room temperature). The interphase was harvested and then washed twice in HHB containing 5% FCS. Cells were counted in a Neubauer counting chamber and live/dead discrimination was determined using trypan blue exclusion.

Flow Cytometry

After IEL isolation from the biopsy samples, 3-color flow cytometry using anti-canine specific and anti-human cross-reactive monoclonal antibodies (mAb) against CD21, CD79α/μ, CD3-12, TCRαβ, TCRγδ, CD4, CD8α, and CD8β (Table 1) was performed to characterize the cells. For each analysis, 500,000 or 1,000,000 cells were incubated with the listed mAb for 15 minutes at room temperature. Cells were then washed in PBS, without Ca2+ and Mg2+, and supplemented with 3% FCS. Those samples containing mAb without directly conjugated fluorochromes were labeled with anti-mouse secondary antibodies and incubated for an additional 15 minutes at room temperature. For intracellular staining (CD3-12, CD79α/μ), the IntraStain-Kit was used according to manufacturers’ instructions. After the last incubation step, cells were washed again and then analyzed using a FACSCanto II flow cytometer. Data analysis was performed by the FACSDiva software, version 6.1.3.
Statistical Analysis

Age and weight of the 2 dog groups were summarized by descriptive statistics. Data were tested for normal distribution using the Shapiro–Wilk-test. Dog groups were compared by non-parametric tests when the data was not normally distributed. All analyses were performed using IBM SPSS 20.0j software. The level of statistical significance was set at \( P < .05 \).

Results

Descriptive Data—Age, Sex, Weight

The control dogs (n = 10) consisted of several different breeds (4 cross-breeds, 2 Yorkshire Terriers, 1 Boxer, 1 Cocker Spaniel, 1 Maltese, 1 Shi Tzu) and consisted of 5 males (4 intact, 1 neutered), and 5 females (3 intact, 2 neutered). The median (range) age of the dogs was 10.3 years (2.3–15.4 years); age mean \( \pm SD \) was 5.8 \( \pm \) 2.8 years. The median (range) body weight was 21.4 kg (2.3–45.6 kg); body weight mean \( \pm SD \) was 21.6 \( \pm \) 16.6 kg.

The breeds in the IBD group (n = 10) included 2 cross-bred and 1 each of the following breeds: American Staffordshire Terrier, Boxer, Collie, Groenendael, Jack Russell Terrier, Maltese, Pit Bull Terrier and Shar Pei. There were 6 males (2 intact, 4 neutered) and 4 neutered female dogs. IBD dogs had a median (range) age of 6.0 years (1.8–9.6 years), with an age mean \( \pm SD \) of 5.8 \( \pm \) 2.8 years. The median (range) body weight of this group was 24.2 kg (7.2–37 kg), with a body weight mean \( \pm SD \) of 22.3 \( \pm \) 10.4 kg. Control dogs were significantly older compared with IBD dogs (\( P < .05 \)). There was no significant difference between body weights.

Clinical Scoring

All dogs were clinically evaluated by CIBDAI scores. Control dogs had a median (range) CIBDAI score of 1 (0–5), and mean \( \pm SD \) score of 1.7 \( \pm \) 1.7. The IBD dogs were classified as mild, with median (range) CIBDAI score of 4.5 (2–8), and mean \( \pm SD \) score of 4.6 \( \pm \) 2.1. The CIBDAI scores of IBD dogs were significantly higher compared with control dogs (\( P < .01 \); Fig 1).

Histopathologic Examination

Comparison of histopathologic results between IBD dogs and control dogs identified marked differences in inflammatory criteria, but morphologic abnormalities were less diverse. Three control dogs had 1 of the following abnormalities: mild lacteal dilatation, mild mucosal fibrosis, or moderate increase of lamina propria eosinophils. Two control dogs showed no histologic abnormalities. In all remaining control dogs, a mild increase in lamina propria lymphocytes was

Table 1. List of mAb used for flow cytometry.

| mAb   | Clone    | Isotype | Fluorescence Labeling |
|-------|----------|---------|-----------------------|
| CD45  | YKIX716.13\a | IgG2b   | APC                   |
| CD79\b | HM57\b   | IgG1    | PE                    |
| CD21  | B-ly-4\b | IgG1    | APC                   |
| CD3   | CD3-12\b | IgG1    | FITC                  |
| TCR\b | CA15.8G7\a | IgG1    | \( \alpha \)-IgG1-FITC\c\d |
| TCR\b | CA20.8H1\a | IgG2a   | \( \alpha \)-IgG2a-FITC\c\e |
| CD4   | YKIX302.9\a | IgG1    | APC                   |
| CD8\a | YCATE 55.9\a | IgG1    | PE                    |
| CD8\b | CA15.4G2\a | IgG1    | \( \alpha \)-IgG1-APC\c\f |

mAb, monoclonal antibodies; CD, cluster of differentiation; IgG, immunoglobulin G; TCR, T-cell receptor; m, mouse; \( \alpha \)-m, anti-mouse; r, rat; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

\( \alpha \)Anti-canine antibody.
\( \beta \)Anti-human cross-reactive antibody (CD79\b; CD3-12: Serotec, technical datasheet MCA1477; CD21).48
\( \gamma \)Fluorescence labeling was achieved by use of a secondary antibody.
\( \delta \)Goat anti-mouse IgG1-Alexa488: Life Technologies, Carlsbad, CA.
\( \epsilon \)Goat F(ab’\b2 anti-mouse IgG2a-FITC; SouthernBiotech, Birmingham, AL.
\( \zeta \)Goat anti-mouse IgG1-Alexa647; Life Technologies.

Fig 1. Clinical disease scores for individual dogs in each study group. The canine inflammatory bowel disease activity index (CIBDAI) for control dogs (n = 10) and dogs with inflammatory bowel disease (IBD; n = 10) was calculated for individual dogs. Each dot indicates an individual dog score. The horizontal lines show the mean score in each group (**\( P < .01 \)).
reported. The cellular infiltrates in IBD dogs ranged from normal to moderately increased. No abnormalities in morphologic criteria were reported. The WSAVA scores of IBD dogs were median (range) 2 (1–3) and mean ± SD scores of 1.9 ± 0.57. Scores were significantly higher compared with the control dogs that had median (range) 1.9 (0–2) and mean ± SD scores of 0.9 ± 0.57 (P < .01; Fig 2).

**Immunophenotyping by Flow Cytometry**

**Gating of IEL and Analysis of T- and B-cells.** In forward/sideward scatter (FSC/SSC) analysis, isolated IEL were detected as a distinct population (Fig 3A), and represented on average 7.3% of all acquired cells (minimum, 2.4%; maximum, 27.5%). Less than 1% of the gated IEL were B-cells expressing CD21, CD79αcy or both (Fig 3B,C). In contrast, the majority of all cells were CD3+ T-cells (median ± SD in control dogs, 97.6 ± 5.1%; and in IBD dogs, 89.5 ± 6.8% (Fig 3D). Therefore, results were normalized to CD3+ T-cells.

**Immunophenotyping—FTB versus EB.** The cell yield from FTB comprised a mean ± SD of 24,800,000 ± 22,620,000 cells/mL, and median (range) of 15,000,000 cells/mL (9,000,000–64,000,000 cells/mL). The cell yield from EB consisted of mean ± SD of 2,840,000 ± 1,450,000 cells/mL, and median (range) of 2,800,000 cells/mL (1,000,000–5,000,000) cells/mL. In the lymphocyte gate, there were no significant differences in the distribution of lymphocyte subpopulations, comparing IEL from FTB and EB of the same control dog, collected at the same time from the same duodenal localization (data not shown).

**Immunophenotyping—IBD Dogs versus Healthy Control Dogs.** In both healthy and control dogs, the CD8α+ T-cells were predominant (control dogs, 52.9 ± 24.0%; IBD dogs, 53.1 ± 19.3%) compared with CD4+ T-cells (control dogs, 14.2 ± 11.1%; IBD dogs, 7.9 ± 6.3%). (Table 2). In their IEL subsets, IBD dogs had fewer TCRαβ+ T-cells (IBD dogs, 64.4 ± 15.6%; control dogs, 79.7 ± 17%; P = .059;
Table 2. Phenotypes of intestinal IEL from control dogs and dogs with IBD, expressed as percent of CD3+ T-cells.

|                  | CD4⁺ | CD8α⁺ | TCRαβ⁺ | CD8α⁺TCRαβ⁺ | CD8β⁺TCRαβ⁺ | TCRγδ⁺ |
|------------------|------|-------|--------|-------------|-------------|--------|
| Control dogs (n = 10) |      |       |        |             |             |        |
| IEL, mean ±SD    | 14.2 ± 11.1 | 52.9 ± 24 | 79.7 ± 17 | 6.9 ± 3.9   | 44.4 ± 25.4 | 8.4 ± 6.1⁺ |
| IEL, median (range) | 10.5 (4.6–39.1) | 60.7 (15.3–74.8) | 83.4 (44.6–101.4) | 6.2 (2.1–14.8) | 46.1 (11.7–85.9) | 5.8 (0.6–17.7) |
| IBD dogs (n = 10) |      |       |        |             |             |        |
| IEL, mean ±SD    | 7.9 ± 6.3   | 53.1 ± 19.3 | 64.4 ± 15.6 | 6.9 ± 2.4   | 43.7 ± 18.6 | 19.9 ± 8.7⁺ |
| IEL, median (range) | 6.9 (1.1–24.3) | 50.0 (29.2–93.8) | 62.8 (44.6–91.6) | 5.7 (4.6–11.5) | 43.3 (21.0–84.6) | 21.7 (1.5–29.6) |

IEL, intraepithelial lymphocytes; IBD, inflammatory bowel disease.
*Parameters that were not normally distributed were analyzed by the Mann-Whitney test.
*Parameters that were normally distributed were analyzed by Student’s t-test.
*Significant difference between groups (P < .05).

Table 2; Fig 3E) and significantly more TCRγδ⁺ T-cells (IBD dogs, 19.9 ± 8.7%; control dogs 8.4 ± 6.1%; P < .01; Table 2; Fig 4). Immunophenotyping—CD8α⁺ Homodimer versus CD8β⁺ Heterodimer Expression in TCRαβ⁺ and TCRγδ⁺ IEL. Both subpopulations, TCRαβ⁺ and TCRγδ⁺ IEL, can be further described depending on their CD8α⁺ and CD8β⁺ expression pattern. We analyzed these subpopulations by gating on TCRαβ⁺ and TCRγδ⁺ cells in combination with staining for CD8α⁺ and CD8β⁺ expression (Fig 5). Because of the extreme predominance of CD3⁺ T-cells among total IEL (Fig 3d), we concluded that TCRγδ⁺ cells represent TCRαβ⁺ T-cells. In all study groups, the majority of TCRαβ⁺ T-cells identified by this strategy expressed the CD8β⁺ heterodimer (control dogs, 44.4 ± 25.4%; IBD dogs, 43.7 ± 18.6%) and less frequently the CD8α⁺ homodimer (control dogs, 6.9 ± 3.9%; IBD dogs, 6.9 ± 2.4%; Table 2; Fig 5). TCRγδ⁺ T-cells were not analyzed statistically with respect to their CD8 expression pattern because the homodimer CD8α⁺ and the heterodimer CD8β⁺ showed high variability among individuals (Table 2). No significant differences between these subpopulations comparing IBD dogs with control dogs could be detected.

Discussion

Intestinal intraepithelial lymphocytes play a crucial role in the development and maintenance of inflammation in chronic enteropathies in animal models and human IBD patients.17,18 This study was performed to characterize canine intestinal IEL phenotypes in dogs with IBD, because different phenotypes in humans and mice seem to be associated with different disease characteristics18–20,26 and different responses to treatment.27,28

Dogs with IBD had significantly higher WSAVA scores compared with control dogs. These results support the WSAVA scoring system as an appropriate method to verify histopathologic changes in dogs with IBD. Although WSAVA scores in control dogs were lower than in IBD dogs, control dogs were not free of pathological findings. Abnormalities were primarily the result of increased inflammatory cell infiltrates. These findings are similar to previous reports, whereby mild histopathology changes were found in healthy dogs, and characterized predominantly as lymphoplasmacytic infiltration of the lamina propria.21,29 Again, WSAVA scores were lower compared with IBD dogs in those studies.21 An age-dependent increase in intestinal inflammation has been suggested in healthy dogs, but is controversial.21,30,31 The intestinal mucosa is continuously exposed to numerous antigenic stimuli throughout life. The control dogs in this study were older compared with IBD dogs, which makes an age-dependent increase in intestinal inflammation a possible explanation for the mildly increased WSAVA scores in control dogs.

Another goal of the present study was to establish the usefulness of EB for flow cytometry analyses in canine IBD patients, because usually EB are obtained for the diagnosis in IBD dogs.32 However, even for EB diagnosis of IBD there is considerable inter-observer variability in interpretation of histopathology findings,33 and in many cases inadequate sample size or quality of tissue samples is problematic.34 Therefore, we anticipated EB might not be adequate for flow cytometry studies. The IEL from EB and FTB of the same control dogs were isolated, stained and analyzed identically. We found no significant difference with...
respective T-cell subpopulations between EB and FTB, demonstrating that EB are suitable for IEL isolation and evaluation by flow cytometry. Although previous studies have compared FTB with EB in healthy dogs of different breeds,29,35 compared EB to FTB from IBD dogs and healthy Beagle dogs,35 to our knowledge this is the first study comparing flow cytometry data from both biopsy methods. The majority of isolated IEL in the current study were T-cells. Fewer than 1% expressed a common B-cell marker (CD21+, CD79a+). This finding mirrored results of previous studies, whereby IEL were defined predominantly as T-cells either by immunohistochemistry,21,36–38 or flow cytometry. In both control and IBD dogs, CD8α+ T-cells were predominant over CD4+ T-cells, which also is in accordance with previous studies of canine intestinal IEL.25,29,35 Although the percentages of CD4+ cells in both control and IBD dog were comparable to previously published data, percentages of CD8α+ T-cells were not. Previous studies showed 2- to 3-fold higher CD8α+/CD4 T-cell ratios in control and IBD dogs compared to previous canine gastroenterology studies in dogs,29,35 whereas in the present study control dogs had a 5-fold and IBD dogs nearly a 7-fold higher CD8α+/CD4 T-cell ratio. Regional differences may have been caused by altered intestinal bacterial colonization because of different genetic backgrounds or husbandry conditions (e.g., breeding, dietary management). Future studies are necessary to elucidate the reasons for the variation in CD8α+/CD4 T-cell ratio present within IEL.

The IBD dogs had significantly higher percentages of TCRγδ+ T-cell subsets compared to control dogs. TCRγδ+ IEL (CD8αα+ TCRγδ+, CD4+ CD8– TCRγδ–) have important regulatory and protective functions in the healthy gut.39 However, in murine IBD models, a pro-inflammatory role is suspected,40–42 in which IL-17-producing TCRγδ+ T-cells are able to induce colitis.43 Furthermore, direct correlation between numbers of TCRγδ+ T-cells in human intestinal mucosa and disease severity in patients suffering from IBD has been demonstrated.44–46 Similarly, the higher presence of TCRγδ+ lymphocytes in IBD dogs of the present study seems to be linked to greater severity of intestinal inflammation, which was reported in the histopathologic results.

In summary, clinical and histopathologic scores, as well as flow cytometry data for control dogs and dogs with IBD, were compared. The IBD dogs showed significantly higher WSAVA scores as well as CIBDAI scores. An increased percentage of TCRγδ+ T-cells in IBD dogs was a notable finding, indicating that IEL from IBD dogs express a significantly different immunophenotype compared to control dogs. Future studies are needed to further define the functional relevance of this unique T-cell subpopulation.

**Footnotes**

a PAA, Pasching, Austria  
b SAV Liquid Production GmBH, Hochriesstrasse 2, Germany  
c Carl Roth, Karlsruhe, Germany  
d Sigma-Aldrich, Vienna, Austria  
e Merck, Darmstadt, Germany  
f BD Biosciences, San Jose, CA  
g Dako, Glostrup, Denmark  
h AbD Serotec, Raleigh, NC  
i Peter F. Moore, California, CA  
j IBM® Cooperation, Armonk, NY

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