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Peripheral and intestinal T lymphocyte subsets in dogs with chronic inflammatory enteropathy

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Abstract

Background: Dysregulated T lymphocyte response is thought to play a key role in chronic intestinal inflammation (CIE).

Objectives: To evaluate the presence of changes in peripheral and intestinal T lymphocyte subsets and to describe potential immune and inflammatory biomarkers in dogs with CIE.

Animals: Sixteen healthy dogs and 26 dogs were diagnosed with CIE.

Methods: Prospective case-control study evaluating peripheral and intestinal T lymphocytes using flow cytometry and inflammatory markers obtained from complete blood cell counts.

Results: Dogs with CIE had higher peripheral activated T helper (Th) lymphocytes (87/μL [18-273] CIE, 44/μL [16-162] healthy control (HC, $P = .013$) and regulatory T cells (Treg; 108/μL [2-257] CIE, 34/μL [1-114] HC, $P = .004$). In the intestinal epithelium, CIE dogs presented lower percentages of Th (4.55% [1.75-18.67] CIE, 8.77% [3.79-25.03] HC, $P = .002$), activated Th cells (0.16% [0.02-0.83] CIE, 0.33% [0.05-0.57] HC, $P = .03$) and CD4/CD8 ratio (0.08 [0.02-0.39] CIE, 0.21 [0.07-0.85] HC, $P = .003$). Conversely, higher percentage of activated T cytotoxic cells (20.24% [3.12-77.12] CIE, 12.32% [1.21-39.22] HC, $P = .04$) and interferon-gamma (IFN-γ) producing T lymphocytes (7.36% [0.63-55.83] CIE, 1.44% [0.00-10.56] HC, $P = .01$) within the epithelium was observed. In the lamina propria the percentage of Treg lymphocytes was higher (6.02% [1.00-21.48] CIE, 3.52% [0.18-10.52] HC, $P = .02$).

Conclusions and Clinical Importance: Systemic and intestinal immune alterations occur in dogs with CIE suggesting that blood IFN-γ producing T lymphocytes and the

Abbreviations: BCS, body condition score; BSA, bovine serum albumin; CBC, complete blood count; CCECAI, clinical canine chronic enteropathy activity index; CD, clusters of differentiation; CIBDAI, clinical IBD activity index; CIE, chronic inflammatory enteropathy; CVMTH, Complutense Veterinary Medicine Teaching Hospital; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FC, flow cytometry; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; HBSS, Hank's Buffered Salt Solution; HC, healthy control; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocyte; IFN-γ, interferon-gamma; IL, interleukin; IRE, immunosuppressant-responsive enteropathy; LPL, lamina propria lymphocyte; mAbs, monoclonal antibodies; NLR, neutrophil-to-lymphocyte ratio; PBL, peripheral blood lymphocyte; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PLR, platelet-to-lymphocyte ratio; SII, systemic immune-inflammation index; SRE, steroid-responsive enteropathy; Tc, T cytotoxic; Th, T helper; TLI, trypsin-like immunoreactivity; Treg, regulatory T cells; WSAVA, World Small Animal Veterinary Association.

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systemic immune-inflamation index (SII) could potentially serve as biomarkers for the disease.

KEYWORDS
flow cytometry, IBD, intraepithelial lymphocytes, lamina propria lymphocytes, peripheral blood lymphocytes, T lymphocytes

1 | INTRODUCTION

Chronic inflammatory enteropathy (CIE) comprises a group of disorders of unknown etiology characterized by the persistence and recurrence of signs of gastrointestinal (GI) disease.[1-3] It is the main cause of chronic vomiting and diarrhea in dogs.[1,4] Interrelated immunologic, dietary, genetic, and environmental factors appear to play a role in its pathogenesis.[1,2,4] However, it is considered an immune-mediated disease because of an exacerbated and abnormal immune response to antigens, responsible for the clinical signs.[1,4,5]

Most immune components play a role in CIE.[5,6] In human inflammatory bowel disease (IBD), T cells and their products at the intestinal epithelium and lamina propria, the effector sites of the gut-associated lymphoid tissue (GALT), appear to have a greater impact on the immune response developed.[7-10] IBD is considered a systemic disease because of extraintestinal manifestations in a high percentage of the patients.[11] Some authors suggest that the cause of these extraintestinal manifestations is the migration of lymphocytes from GALT effector sites because of the expression of adhesion molecules such as MAdCAM-1.[11,12] In dogs, extraintestinal manifestations are not common[13-15] although changes in the peripheral immunophenotype in dogs with CIE have been described.[16-18]

Chronic inflammatory enteropathy in dogs is diagnosed after an exclusion protocol of all underlying causes of intestinal inflammation, accompanied by histologic evidence of an inflammatory mucosal infiltrate.[1,4] Immunosuppression or immunomodulation is considered the treatment of choice, therefore this entity is often referred to as steroid-responsive enteropathy (SRE) or immunosuppressant-responsive enteropathy (IRE).[1]

Advances in the understanding of the immunopathogenesis of IBD in humans have led to the development of successful immunotherapies[19,20] and the description of useful immune and complete blood count-derived inflammatory biomarkers for the prediction of treatment response and disease progression.[21-24] However, knowledge of immune changes during CIE in dogs is still limited.[5]

The aim of this study was to evaluate changes in the blood and intestinal immunophenotype in dogs with CIE. The following specific objectives were considered: (1) to describe the major peripheral blood (PBL), intestinal intraepithelial (IEL), and lamina propria (LPL) T lymphocyte populations by flow cytometry (FC) in dogs with CIE; (2) to compare these lymphocyte subpopulations between dogs with CIE and healthy dogs; (3) to evaluate the possible association of PBL subpopulations and complete blood count-derived inflammatory parameters with clinical, endoscopic, and histopathologic characterization in dogs with CIE in search of immunologic and inflammatory biomarkers of the disease.

2 | MATERIALS AND METHODS

All procedures and protocols of this study were approved by the Animal Research Committee of the Complutense Veterinary Medicine Teaching Hospital (CVMTH), the Complutense University of Madrid, and the Community of Madrid under reference number: PROEX 175/18. Informed consent of the owners was always required for the dogs to participate in the study.

2.1 | Study groups

2.1.1 | Healthy control dogs

Dogs of different breeds, age, and sex that were presented to the CVMTH for elective or routine consultations (eg, orchiectomy or ovariohysterectomy) were included in the healthy control (HC) group after meeting these inclusion criteria: normal physical examination, absence of abnormalities in the complete blood count (CBC) and basic biochemistry, negative serology for the most common vector-borne diseases in our geographical area (*Ehrlichia* spp./*Anaplasma* spp. and *Leishmania infantum*), absence of signs of GI disease in the last 6 months and absence of treatment with antibiotics or immunosuppressive drugs in the last month before sample collection.

2.1.2 | CIE dogs

This group consisted of dogs presented to the Gastroenterology and Endoscopy Service of the CVMTH with sings of chronic GI disease (including vomiting, diarrhea, and weight loss among other less frequent) and were diagnosed with CIE. The diagnosis was made via a thorough physical examination, CBC, serum biochemistry panel, immunofluorescence antibody test for *Leishmania infantum* and *Ehrlichia* spp./*Anaplasma* spp., serial fecal analysis, diagnostic imaging (abdominal ultrasound and/or radiographs), resting cortisol/ACTH test and TLI (trypsin-like immunoreactivity). Dogs underwent an upper GI endoscopy and biopsy sampling to confirm the inflammatory process after having completed a minimum of 2 dietary trials of at least 3 weeks on a hydrolyzed protein-based or novel protein

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diets to rule out food-responsive enteropathy.[25,26] Clinical severity was assessed for each dog using the clinical IBD activity index (CIBDAI) and the clinical canine chronic enteropathy activity index (CCECAI).[25,27] The following CBC-derived inflammatory markers were also evaluated: neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII, calculated as [neutrophils × platelets]/lymphocytes).

2.2 | Sample collection and endoscopic and histopathologic scoring

All dogs underwent upper GI endoscopy for sample collection. HC dogs were anesthetized for other purposes such as neutering/spaying and CIE dogs for diagnostic purposes. Food and water were removed from all the dogs for 24 and 12 hours, respectively. Video endoscopes of various lengths and diameters were used depending on the size of the dog (Fujinon [Europe] GmbH, Willich, Germany). Macroscopic GI changes were scored by 3 experienced endoscopists (A.S., M.G-S., and F.R-F.) according to the World Small Animal Veterinary Association (WSAVA) endoscopic guidelines for dogs with CIE activity index[28] and the Slovak et al endoscopic activity score.[29]

At least 6 adequate biopsy specimens were collected from the gastric and duodenal mucosa of each dog and were preserved in 10% buffered formaldehyde for histologic evaluation and scoring by an experienced pathologist using the WSAVA guidelines for histopathologic evaluation of GI inflammation[28] and the simplified histopathologic scoring system for GI inflammation proposed by Allenspach et al.[30]

For the immunophenotyping study, additional 7 adequate duodenal biopsies were collected in complete media RPMI-1640 supplemented with 2 mM L-glutamine, amphotericin B, streptomycin, penicillin, and 2% fetal bovine serum (FBS; Merck, St. Louis, Missouri), placed on ice, and processed within 1 hour of collection. For PBL FC analysis, 6 mL of peripheral blood were also collected from all dogs by jugular venipuncture, collected in ethylenediaminetetraacetic acid (EDTA) tubes, and processed within 1 hour of collection.

2.3 | PBL, IEL, and LPL isolation

The protocols proposed by Agulla et al[31] for isolation of lymphocytes from the 3 immune compartments of interest, PBL, IEL, and LPL, were used. Briefly, duodenal biopsies were placed in Hank's Buffered Salt Solution (HBSS, Biowest, Nuaillé, France) containing 1 mM 1,4-dithiothreitol (DTT) and 1 mM EDTA (both from Merck, St. Louis, Missouri) and incubated in a shaking bath to achieve release of IEL, that were resuspended in complete medium.

Tissue samples were then placed in phosphate buffered saline (PBS) supplemented with 2 mM amphotericin B, streptomycin, penicillin, and 0.5% bovine serum albumin (BSA; Merck, St. Louis, Missouri) and re-incubated in a shaking bath to detach LPL, that were resuspended in complete media.

For PBL isolation, 6 mL of peripheral blood were centrifuged through a density gradient to obtain the peripheral blood mononuclear cells (PBMCs; Histopaque 1077, Merck, St. Louis, Missouri), that were resuspended in complete media and cultured for 90 minutes at 37°C in a humidified atmosphere containing 5% of CO_2. The lymphocyte-enriched supernatant was centrifuged and resuspended in complete media for FC analysis.

2.4 | Flow cytometry

Freshly isolated PBLs, LIEs, and LPLs were analyzed for characterization of the T lymphocyte populations of interest using various combinations of anti-canine, anti-human, and anti-bovine cross-reactive monoclonal antibodies (mAb) against different lymphocyte molecules (Table 1). The analysis was performed using 2-, 3-, or 5-color flow cytometry. T lymphocytes (CD45+ CD3+ lymphocytes), T helper (Th, CD3+ CD4+ lymphocytes), T cytotoxic (Tc, CD3+ CD8+ lymphocytes), activated Th and Tc subsets (CD3+ CD4+ CD25+ and CD3+ CD8+ CD25+ lymphocytes, respectively), double positive T lymphocytes (CD3+ CD4+ CD8+ lymphocytes), double negative T lymphocytes (CD3+ CD4− CD8− lymphocytes), regulatory T cells (Treg, CD3+ CD4+ CD25+ FoxP3+ lymphocytes), T lymphocytes producing interferon-gamma (IFN-γ; CD3+ IFN-γ+ lymphocytes), and T lymphocytes producing interleukin 4 (IL-4; CD3+ IL4+ lymphocytes) were analyzed. Additionally, the CD4/CD8 ratio was calculated.

For FC staining, the protocols previously described by Agulla et al[31] were also followed. For characterization of IFN-γ and IL-4-producing T lymphocytes, freshly isolated PBLs, IELs, and LPLs were stimulated and incubated with phorbol myristate acetate (10 ng/mL, Enzo, New York) and ionomycin (0.4 μM, StressMarq Bioscience INC, Victoria, Canada) for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2, and brefeldin A (1 μg/mL, StressMarq Bioscience INC, Victoria, Canada) was added during the last 10 hours of incubation to inhibit cytokine secretion before staining.

Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), and Cell-Quest and FlowJo V10 software. The lymphocyte gate was initially selected based on forward and side scatter properties, and the lymphocyte subsets studied were detected by gating the different positive or negative CDs using dot plots or histograms as appropriate (Figure 1). Antibody titration was previously developed to determine the optimal working concentration. Gates were set using fluorescence minus 1 and the appropriate isotype controls. A minimum of 10 000 events were acquired from the lymphocyte gate.

2.5 | Statistical analysis

Statistical analysis was performed with commercially available statistical software SAS, version 9.4 (SAS Institute, Cary, North Carolina). The Kolmogorov-Smirnov test was used to determine the normal distribution of the data. Differences in signalment, clinical, endoscopic,

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TABLE 1 List of canine-specific and cross-reactive mAb used in flow cytometric evaluation of canine PBL, IEL, and LPL.

Antigen	Location	Fluorescence labeling	Clone	Specificity	Isotype
CD45[a]	Surface	APC[b]	YKIX716.13	Rat anti-dog	IgG2b
CD3[a]	Surface	FITC[c]	CA17.2A12	Mouse anti-dog	IgG1
CD4[a]	Surface	PECy7[d]	YKIX302.9	Rat anti-dog	IgG2a
CD8[a]	Surface	Alexa Fluor 647	YCATE55.9	Rat anti-dog	IgG1
CD25[a]	Surface	PE[e]	P4A10	Mouse anti-dog	IgG1
FoxP3[f]	Intracellular	APC[b]	FJK-16s	Rat/mouse anti-human	IgG2a
IL-4[a]	Intracellular	PE[e]	CC303	Mouse anti-bovine	IgG2a
IFN-γ[a]	Intracellular	Alexa Flour 647	CC302	Mouse anti-bovine	IgG1

[a]Obtained from Bio-Rad (Oxford, UK).
[b]APC, allophycocyanin.
[c]FITC, fluorescein isothiocyanate.
[d]PECy7, phycoerythrin-cyanine dye.
[e]PE, phycoerythrin.
[f]Supplied by Thermo Fisher (Massachusetts, USA).

histopathologic, and immunophenotypic data between the 2 groups of dogs were evaluated using Wilcoxon's signed rank test for quantitative and ordinal qualitative variables and contingency tables with Fisher's exact test for non-ordinal qualitative variables. Spearman correlation coefficients were used for correlations. The significance level was set at $P < .05$. The Bonferroni correction was applied in the correlation study to address the issue of multiple comparisons among variables, considering a significance level of $P < .03$ in these cases. The absence of large effect size for nonparametric tests was calculated using the $Z/\sqrt{N}$ formula.

3 | RESULTS

3.1 | Animals

A total of 42 dogs were included in the study: 16 healthy dogs and 26 dogs diagnosed with CIE. The control group consisted of 7 females (2 intact, 5 spayed) and 9 males (5 intact, 4 neutered) with a median age of 4.00 years (range: 1.40-11.00 years) and a median body weight of 13.85 kg (range: 4.50-64.60 kg). The median body condition score (BCS, 1-9) was 5 (range: 4-7). Regarding breed, 7 dogs were crossbred, and 9 dogs were purebred (2 Greyhounds and 1 of each of the following: American Staffordshire, Beagle, English Setter, Great Dane, Jack Russell Terrier, Maltese, and West Highland White Terrier).

The dogs with CIE were 11 females (3 intact, 8 spayed) and 15 males (5 intact, 10 neutered). The median age in this group was 6.00 years (range: 1.80-12.00 years), the median body weight was 12.25 kg (range: 2.20-47.50 kg), and the median BCS was 4 (range: 3-7). The breeds included 8 crossbred dogs and 18 purebred dogs (3 Golden Retrievers and Maltese; 2: Chihuahuas and Labrador Retriever; and 1 of each of the following: Border Collie, Boston Terrier, French Bulldog, German Shepherd, Jack Russell Terrier, Miniature Schnauzer, Pyrenean Mountain, and Yorkshire Terrier).

There were no significant differences in age, sex, reproductive status, breed, body weight, or BCS between the 2 study groups.

3.2 | Clinical, endoscopic, and histopathological evaluation

Chronic inflammatory enteropathy dogs presented a median CIBDAI of 6.5 (range: 3-14) and a median CCECAI of 7.5 (range: 3-17). On the CBC, 7 dogs (27%) presented thrombocytosis, 6 dogs (23%) mild anemia and 3 (12%) hypoproteinemia and hypoalbuminemia. Chronic inflammatory enteropathy dogs showed a significantly higher automated or manual platelet count than HC dogs ($280.50 \times 10^3/\mu L$ [range: $104\text{-}545 \times 10^3/\mu L$] CIE, $188 \times 10^3/\mu L$ [range: $116\text{-}409 \times 10^3/\mu L$] HC, $P = .01$). For all cases, a blood smear was evaluated, manual platelet count was performed when the automated counts were considered to be incorrect, mainly in cases of low automated platelet counts and the presence of platelet aggregation ($n = 5$). In addition, SII was higher in the diseased dogs (632.42×10^3 [range: $304.01\text{-}4564.50 \times 10^3$] CIE, 457.59×10^3 [range: $204.83\text{-}2029.75 \times 10^3$] HC, $P = .01$).

Endoscopic evaluation revealed esophageal lesions in 13/26 CIE dogs (50%) and gastric and duodenal changes in all of them (100%) when the WSAVA endoscopic index[30] was used. The index of Slovak et al[29] identified gastric lesions in 24/26 (92%) of CIE dogs and duodenal changes in 100% of the dogs. Histopathologically, both the WSAVA[28] and the simplified Allenspach et al[30] indexes demonstrated lesions in the stomach and duodenum of all CIE dogs. All diseased animals presented with an infiltrate of lymphocytes and plasma cells in both the lamina propria of the stomach and the duodenum. The endoscopic and histopathologic findings and comparisons between groups are shown in Table 2.

3.3 | Peripheral blood immunophenotype

Summarized data, including mean, SD, median, minimum, and maximum of all lymphocyte subpopulations evaluated in each group, as well as statistical differences between groups, are presented in Table 3. Most of the lymphocytes in peripheral

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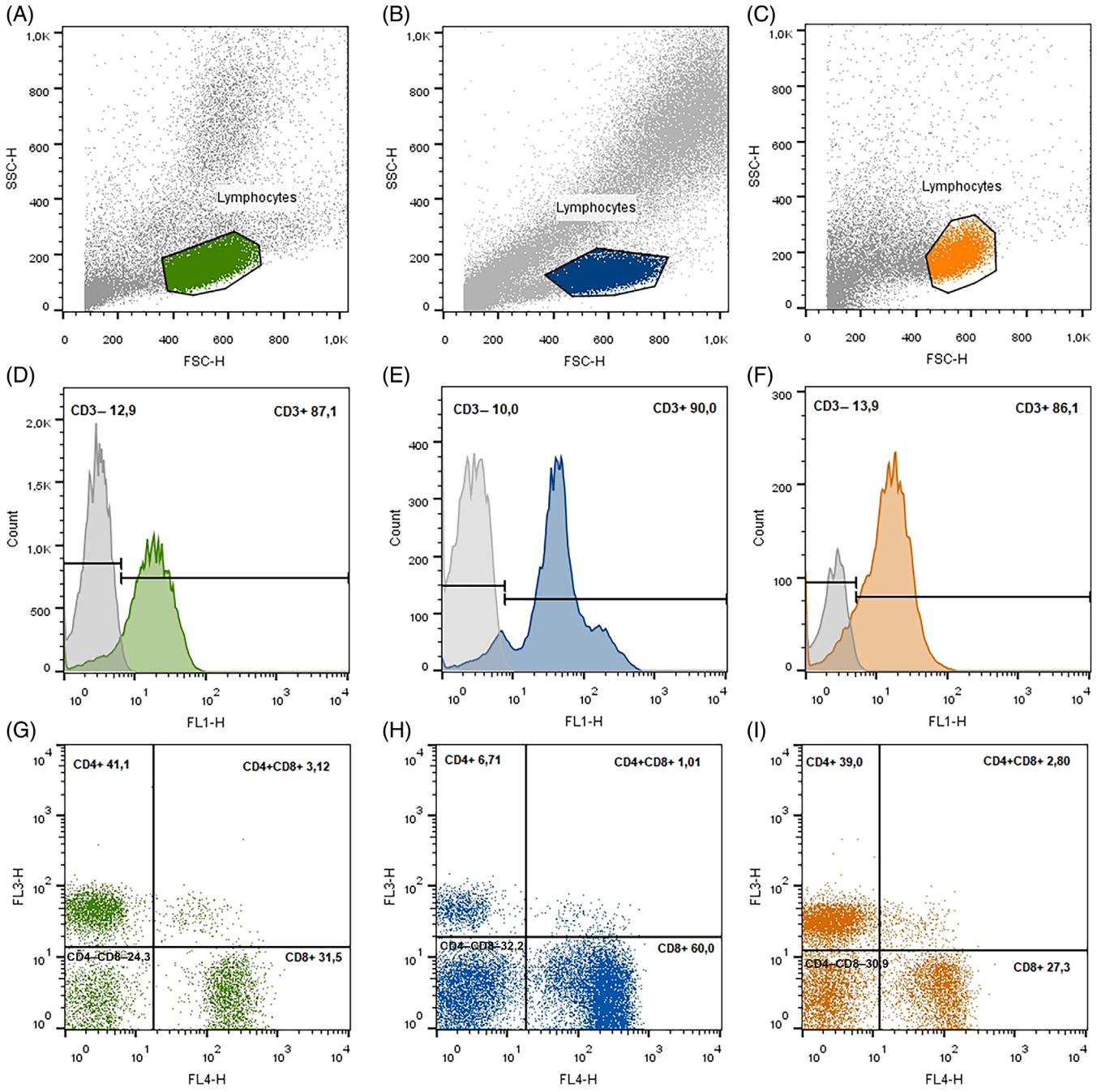

FIGURE 1 Example of flow cytometry characterization. Phenotypes of PBL, IEL, and LPL Th (CD3+ CD4+), Tc (CD3+ CD8+), double positive (CD3+ CD4+ CD8+) and double negative (CD3+ CD4− CD8−) T cells. Data are presented as histograms showing the expression of 1 parameter and as dot plots showing percentages of cells with various properties. (A-C) Representative dot plot showing the forward/side scatter (FCS/SSC) properties of the analyzed PBL (A), IEL (B), and LPL (C) gating the lymphocyte population. (D-F) Representative histograms of CD3 expression in PBL (D), IEL (E), and LPL (F), with negative cells on the left (gray) and positive cells on the right side of the graphs (green, blue, and orange, respectively). All cells on the right of where the negative cells end are considered positive. Numbers in the right upper corners of the graphs indicates the percentages of the respective positive populations. (G-I) Representative dot plots of CD3+ gated cells labeled with mAbs against CD4 and CD8 in PBL (G), IEL (H), and LPL (I). CD4 positive cells are displayed in the upper left corner while CD8 cells are displayed in the lower right corner of the graphs. CD4+ CD8+ cells are displayed in the upper right and CD4− CD8− cells in the lower left corner of the graphs. The adjacent numbers indicate the percentage of the respective positive populations.

blood in both study groups were T cells (87.90% ± 5.33% HC, 86.19% ± 10.39% CIE), and within them the majority were Th cells versus Tc cells. Although the percentage of activated Tc was higher than the percentage of activated Th, CIE dogs had a significantly higher percentage of activated Th than HC dogs (Figure 2A,B).

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Evaluation		Median		
Macroscopic	**∑ values (range)**	**HC (n = 16)**	**CIE (n = 26)**	***P*-value**
WSAVA[28]	Esophagus (0-5)	0	0.5	.05
	Stomach (0-12)	2	6	.001*
	Duodenum (0-15)	5	8	.003*
Slovak et al[29]	Quantitative stomach (0-3)	0	1	.002*
	Quantitative duodenum (0-5)	2	3	.049*
	Qualitative stomach (0-2)	0	1	.001*
	Qualitative duodenum (0-4)	1.5	2	.03*
Histopathologic	**∑ values (range)**	**HC (n = 16)[a]**	**CIE (n = 26)**	***P*-value**
WSAVA[28]	Stomach (1-8)	3	4.5	.48
	Duodenum (2-18)	4	12	.001*
Allenspach et al[30]	Stomach (1-5)	2	3	.34
	Duodenum (2-14)	3	9.5	.001*

Abbreviation: WSAVA, World Small Animal Veterinary Association.
[a]Because of the quality of the biopsies, 5 stomach biopsies and 1 duodenal biopsy of the healthy control dogs could not be evaluated, so the median values are calculated without considering these samples.
**P*-value was significant when <.049.

TABLE 2 Comparison of macroscopic and histological scores between groups using the proposed indexes for endoscopic and histopathological evaluation.

TABLE 3 Results and comparison of the PBL immunophenotype between HC and CIE dogs.

		PBL %						PBL µL					
		Med	**Mn**	**±SD**	**Min**	**Max**	***P*-value**	**Med**	**Mn**	**±SD**	**Min**	**Max**	***P*-value**
T lymphocytes (CD45+ CD3+)	HC	88.53	87.90	5.33	74.52	95.59	.80	1266	1385	407	885	2214	.39
	CIE	86.97	86.19	10.39	46.52	97.67		1471	1491	532	464	2392	
Th lymphocytes (CD3+ CD4+)	HC	42.69	43.06	7.35	26.39	57.56	.95	668	687	233	349	1137	.61
	CIE	43.45	43.21	8.35	26.54	54.94		755	765	347	290	1558	
Tc lymphocytes (CD3+ CD8+)	HC	28.00	29.32	8.04	16.28	42.28	.83	451	446	145	224	768	.73
	CIE	26.36	29.39	9.77	16.52	52.86		478	492	202	205	1021	
Double positive T lymphocytes (CD3+ CD4+ CD8+)	HC	1.03	1.06	0.55	0.21	1.97	.09	16	17	11	3	47	.08
	CIE	1.33	1.67	1.18	0.40	5.82		22	29	27	6	129	
Double negative T lymphocytes (CD3+ CD4− CD8−)	HC	12.36	12.70	3.33	8.45	18.65	.17	174	200	73	116	371	.23
	CIE	14.92	15.10	5.38	7.03	27.39		234	266	148	85	618	
Activated Th lymphocytes (CD3+ CD4+ CD25+)[a]	HC	3.09	3.37	1.78	1.16	8.42	.01*	44	55	37	16	162	.01*
	CIE	4.92	5.74	3.56	1.55	18.25		87	99	64	18	273	
Activated Tc lymphocytes (CD3+ CD8+ CD25+)[a]	HC	3.56	9.87	22.04	0.95	91.97	.97	16	44	99	5	413	.97
	CIE	3.73	6.00	5.64	0.68	22.38		22	29	34	3	158	
Treg lymphocytes (CD3+ CD4+ CD25+ Foxp3+)	HC	2.28	2.75	2.12	0.05	5.94	.003*	34	44	36	1	114	.004*
	CIE	6.03	6.32	3.88	0.20	17.10		108	110	70	2	257	
IFN-γ producing T lymphocytes (CD3+ IFN-γ+)	HC	17.56	17.42	10.30	2.17	42.87	.46	226	267	162	22	707	.45
	CIE	18.91	21.70	16.49	1.13	71.84		389	401	381	24	1588	
IL-4 producing T lymphocytes (CD3+ IL4+)	HC	3.98	7.78	8.86	0.19	28.10	.09	57	132	158	2	503	.10
	CIE	1.73	3.92	5.08	0.00	17.92		28	75	104	0	334	
Ratio CD4/CD8	HC	1.48	1.61	0.62	0.62	3.17	.91						
	CIE	1.46	1.62	0.80	0.50	3.57							

Abbreviations: Max, maximum; Med, median; Min, minimum; Mn, mean; SD, standard deviation.
[a]Activated Th and Tc subsets are expressed as the percentage of Th or Tc cells positive to CD25.
**P*-value <.05, significant differences for lymphocyte populations between HC and CIE dogs.

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Both study groups showed a low percentage and total number of double positive T lymphocytes, Treg lymphocytes, and IL-4 producing T cells, but Treg cells were significantly higher in CIE animals than in the HC group, both in relative and absolute terms (Figure 2A,B).

3.4 | Intestinal immunophenotype

The descriptive results and the comparative study of the IEL and LPL populations between the HC animals and the CIE dogs are detailed in Table 4. In the duodenal epithelium, there was a high percentage of Tc lymphocytes compared with a low percentage of Th cells. Furthermore, the percentage of Tc lymphocytes was higher in CIE dogs than in the HC group and the percentage of Th lymphocytes was significantly lower, resulting in a significant decrease in the CD4/CD8 ratio at this level in CIE dogs. In addition, the percentage of activated Tc was significantly higher and the percentage of activated Th significantly lower in CIE dogs (Figure 2C-E).

The percentages of intraepithelial double positive T cells, Treg, IL-4, and IFN-γ producing T lymphocytes were low in all study animals, with the percentage of IFN-γ producing T lymphocytes higher in CIE dogs (Figure 2C). On the other hand, the double negative T lymphocyte population was highly represented at the duodenal epithelium.

A substantial percentage of Th, Tc, and double negative T cells were observed in the duodenal lamina propria. In contrast, a low percentage of double positive cells was detected. Activated Tc lymphocytes were higher than the activated Th cells. There were no differences observed between these groups. Cells producing IFN-γ and IL-4 were found in very low percentages in the duodenal lamina propria.

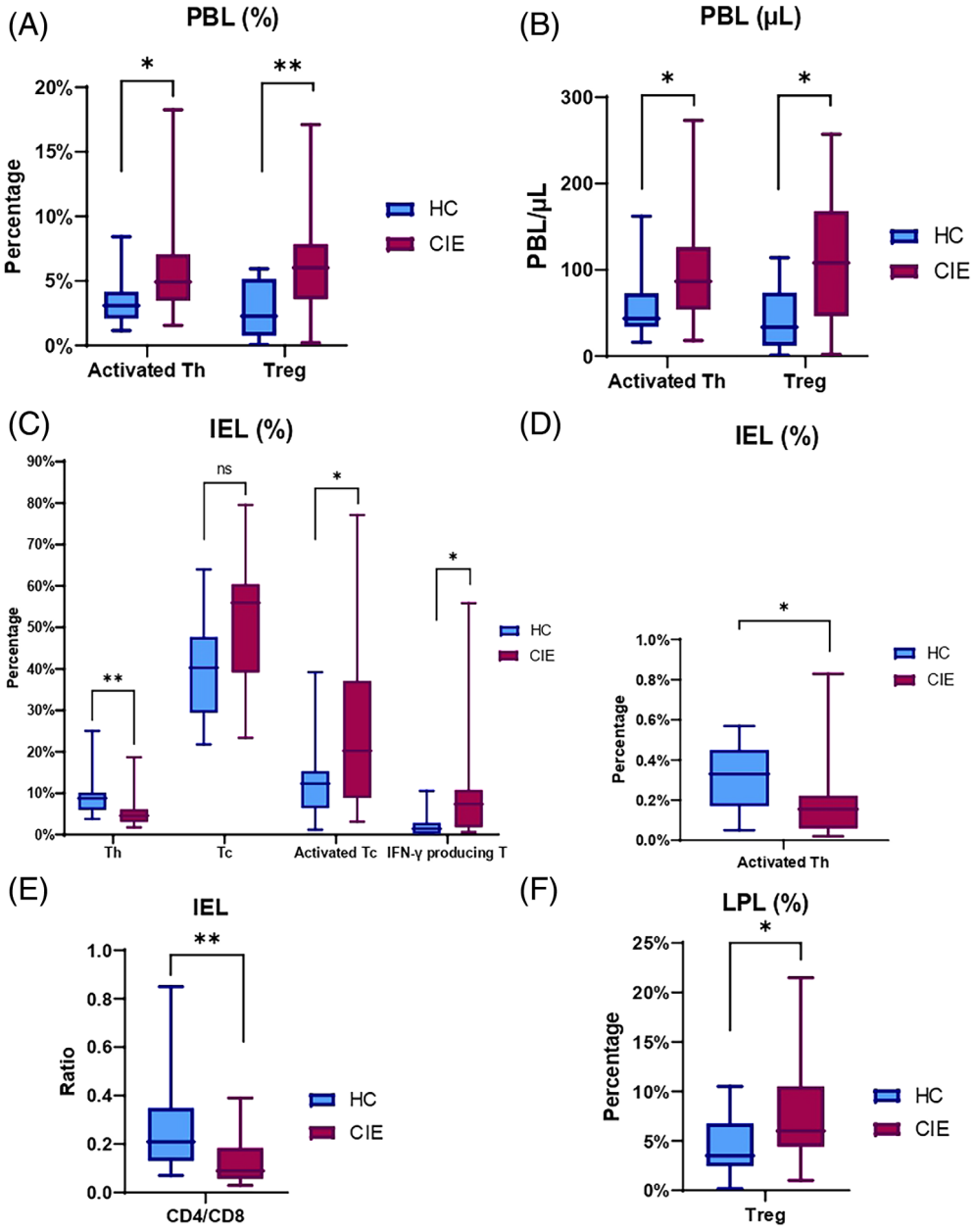

FIGURE 2 Significantly different T lymphocyte subpopulations between the HC group and CIE group in the 3 immune compartments evaluated. (A) Percentages of different T PBL subpopulations. (B) Absolute value of different T PBL subpopulations. (C,D) Percentages of different T IEL subpopulations. (E) Ratio CD4/CD8 from duodenal epithelium. (F) Percentages of different T LPL subpopulations. The lines correspond to the median, the whiskers to the range and the bars to the confidence interval. Significant *P*-values: ns, no significant; <.05*; <.01**.

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TABLE 4 Results and comparison of the IEL and LPL immunophenotype between HC and CIE dogs.

		IEL %						LPL %					
		Med	Mn	±SD	Min	Max	*P*-value	Med	Mn	±SD	Min	Max	*P*-value
T lymphocytes (CD45+ CD3+)	HC	80.03	76.34	11.94	54.24	89.88	.13	85.38	83.41	9.04	69.58	97.61	.52
	CIE	85.01	81.55	11.09	47.04	94.05		86.97	85.44	6.91	71.59	93.70	
Th lymphocytes (CD3+ CD4+)	HC	8.77	9.84	5.83	3.79	25.03	.002*	40.24	39.89	10.89	26.40	60.52	.39
	CIE	4.55	5.33	3.48	1.75	18.67		33.48	36.78	12.96	17.29	67.80	
Tc lymphocytes (CD3+ CD8+)	HC	40.28	40.39	12.70	21.77	63.69	.06	26.68	29.26	8.64	18.02	47.24	.37
	CIE	55.93	50.42	15.00	23.38	79.57		33.60	37.50	17.15	18.56	64.04	
Double positive T lymphocytes (CD3+ CD4+ CD8+)	HC	0.63	1.00	1.17	0.00	4.75	.53	1.48	1.53	0.88	0.42	3.73	.39
	CIE	0.59	0.67	0.59	0.03	2.70		1.59	2.70	2.79	0.00	9.77	
Double negative T lymphocytes (CD3+ CD4− CD8−)	HC	27.04	25.03	12.13	0.00	38.96	.73	21.78	24.13	9.62	12.32	43.34	.11
	CIE	27.57	28.58	9.06	10.97	52.85		16.25	17.37	10.30	0.00	35.77	
Activated Th lymphocytes (CD3+ CD4+ CD25+)[a]	HC	0.33	0.31	0.17	0.05	0.57	.03*	5.30	5.84	2.67	2.71	10.83	.15
	CIE	0.16	0.19	0.18	0.02	0.83		6.34	7.61	5.39	1.63	25.09	
Activated Tc lymphocytes (CD3+ CD8+ CD25+)[a]	HC	12.32	13.76	10.31	1.21	39.22	.04*	33.50	32.53	9.67	18.74	49.48	.53
	CIE	20.24	26.12	21.07	3.12	77.12		33.33	38.09	21.26	2.63	81.32	
Treg lymphocytes (CD3+ CD4+ CD25+ Foxp3+)	HC	1.73	1.73	1.13	0.26	4.31	.96	3.52	4.24	3.06	0.18	10.52	.02*
	CIE	1.50	1.95	1.60	0.12	6.82		6.02	8.01	5.12	1.00	21.48	
IFN-γ producing T lymphocytes (CD3+ IFN-γ+)	HC	1.44	2.28	3.05	0.00	10.56	.01*	0.35	2.26	3.77	0.00	10.10	.37
	CIE	7.36	10.41	13.39	0.63	55.83		1.30	2.21	2.67	0.18	7.31	
IL-4 producing T lymphocytes (CD3+ IL4+)	HC	1.13	1.21	1.23	0.00	3.65	.11	0.95	1.37	1.65	0.00	4.41	.22
	CIE	0.00	1.53	4.01	0.00	17.39		0.22	0.39	0.52	0.00	1.35	
Ratio CD4/CD8	HC	0.21	0.28	0.22	0.07	0.85	.003*	1.35	1.53	0.75	0.57	2.94	.24
	CIE	0.08	0.11	0.08	0.02	0.39		1.27	1.19	0.72	0.29	2.77	

Abbreviations: Max, maximum; Med, median; Min, minimum; Mn, mean; SD, standard deviation.
[a]Activated Th and Tc subsets are expressed as the percentage of Th or Tc cells positive to CD25.
**P*-value <.05, significant differences for lymphocyte populations between HC and CIE dogs.

When comparing the 3 immune compartments studied, the highest percentage of Treg lymphocytes was found in the lamina propria. In addition, the relative number of Treg LPL was also significantly higher in dogs with CIE than in healthy dogs (Figure 2F).

3.5 | Associations between PBL subsets and CBC-derived inflammatory biomarkers and clinical, endoscopic, and histopathological characterization in dogs with CIE

Examination of the associations between PBL and disease characterization showed a moderate negative correlation between the duodenal histopathologic score obtained with the simplified Allenspach et al index and the percentage of IFN-γ-producing T lymphocytes (rho = −0.44, $P = .02$).

Of the CBC-derived inflammatory biomarkers, a moderate positive association was found between the SII and the histopathologic score of the duodenum obtained both with the simplified Allenspach et al index (rho = 0.40, $P = .01$) and the WSAVA index (rho = 0.43, $P = .01$).

4 | DISCUSSION

This study simultaneously evaluates changes in the major T lymphocyte populations in both the peripheral and gut immune compartments in dogs with CIE. In dogs with CIE and human IBD, the interactions between potential etiopathogenic factors lead to a proinflammatory state with an imbalance between pro- and anti-inflammatory cytokines, which is reflected in the intestinal and peripheral lymphocyte immunophenotype.[7,32] Such is the importance of lymphocytes and their products in IBD that these pro-inflammatory molecules are the main targets of most biological treatments used in human medicine, and these treatments also attempt to prevent the return of lymphocytes to the gut.[33,34]

Increased platelet counts are described in both human IBD and in dogs with CIE[35,36] and are associated with disease severity.[35-37] Our results are consistent with these observations, as CIE dogs in the study showed significantly higher platelet counts than HC dogs. The higher platelet count has traditionally been associated with a hypercoagulable state or with the effect of endogenous/exogenous steroids.[38-40] The hypercoagulable state that accompanies the disease in dogs appears to be independent of these cells and is a direct

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consequence of the systemic inflammatory state.[41,42] Consistent with the latter hypothesis, the diseased dogs in our study showed significantly higher SII than the healthy dogs.

Contrary to some authors[30,43] and according to others,[44-46] we found no associations between the clinical score and the macroscopic and histopathologic changes in CIE dogs (data not shown). However, further studies using simplified indexes may be needed to validate our findings.

The effector sites of GALT, the epithelium and lamina propria, carry out elimination, tolerance, and inflammatory responses before antigen interaction at the inductive sites.[47] IEL and LPL are primarily T lymphocytes and show significant phenotypic and functional differences.[48] The similarity between the peripheral and duodenal lamina propria immunophenotypes and the uniqueness of the intestinal epithelial T subpopulations in dogs have been described previously.[31] Therefore, to provide information on the role of acquired immunity in CIE, it was considered necessary to evaluate the 3 immune compartments in this work. However, when interpreting the immunophenotypic results, the high variation found in many of the lymphocyte populations of this study should be considered, as this finding suggests a large individual immune variability, as described by many authors in both healthy dogs and dogs with different diseases.[16,49-52]

Peripheral blood lymphocytes changes have been described in Crohn's disease and ulcerative colitis in humans[53-55] and in dogs with CIE.[16,17] We found a significant increase in the percentage and absolute value of activated peripheral Th lymphocytes in CIE dogs compared with the HC group. We also found an increase in peripheral activated Tc lymphocytes, but not statistically significant. These findings are described in the blood of human IBD patients.[53,56] The proposed hypothesis is that the loss of intestinal permeability would lead to systemic translocation of microbiota products and subsequent activation of the cellular immune response mediated by Th and Tc lymphocytes. Our study supports a state of lymphocytic activation at the peripheral level. Considering that autoantibodies against components of the microbiota have also been identified in the serum of dogs with CIE,[57,58] it would be interesting in future studies to establish their possible relationship. This would provide information on the interplay between 2 important pillars in the etiopathogenesis of the disease in dogs.

In addition, the CIE dogs showed a higher percentage and absolute number of peripheral and a higher percentage of lamina propria Treg lymphocytes than the HC, which is inconsistent with the study that previously evaluated this peripheral lymphocyte subset in this entity in dogs.[18] However, and in agreement with our findings, there is an increase in blood and lamina propria Treg cells in human IBD patients.[59,60] The increased Treg lymphocytes are positive for IL-17 and RORγt markers, suggesting a possible polarization toward the proinflammatory Th17 phenotype because of the inflammatory environment and because they are derived from a common cell precursor or progenitor.[59] Other authors have explained the higher number of peripheral Treg cells as an attempt to restore the loss of immune tolerance that occurs during disease, or because of an alteration in their regulatory functions.[34,60] Furthermore, these findings suggest a greater similarity between these 2 immune compartments during the course of the disease, as previously described in healthy dogs.[31]

We observed a significantly lower percentage of Th lymphocytes and higher population of Tc IEL of CIE dogs compared with HC animals, resulting in a statistically significant lower in the CD4/CD8 ratio. A higher number of Tc lymphocytes at the intestinal epithelium is considered common in patients with Crohn's disease and ulcerative colitis[61-63] and occurs in dogs with CIE.[64] It has been postulated that an alteration in the pathogenic recognition mechanism of epithelial Tc lymphocytes through MHC type I disrupts the balance between immune tolerance and the cytotoxic activity of this cell population, triggering the inflammatory process.[62,65] These Tc lymphocytes would be directly responsible for epithelial barrier damage, loss of immune tolerance, and perpetuation of the inflammatory process.[62,63] It is believed that the epithelial damage resulting from the cytotoxic activity of Tc lymphocytes allows bacterial invasion of the mucosa and increases cellular activation.[66,67] In line with this hypothesis, we observed that CIE dogs had a higher significant percentage of activated Tc lymphocytes than HC. On the contrary, Th lymphocytes of CIE dogs showed a significantly lower activation status, although the low representation of Th lymphocytes in this compartment should be considered.

Crohn's disease, in contrast to ulcerative colitis, is characterized by a Th1 polarization with a predominance of interleukins such as IFN-γ,[7,34] which is consistent with our finding of a significant increase in the percentage of IFN-γ-producing IELs and the absence of differences between both groups for the IL-4-producing T cells at this compartment. This finding suggests the existence of a possible polarization toward a type 1 immune response in the course of chronic inflammatory diseases affecting the small intestine in dogs. However, given the immunological singularity of the epithelial compartment[31] and the lack of differences in the lamina propria, and considering that this is the first study to assess these populations by FC, further studies are needed to confirm these findings.

Potential peripheral immune cell biomarkers have been described in human IBD patients,[67,68] but in veterinary medicine predominantly humoral components such as cytokines, chemokines or antibodies have been evaluated.[58,69] Our results showed a moderate negative correlation between the relative levels of IFN-γ producing T lymphocytes and the severity of duodenal histopathologic lesions. It is important to note that this lymphocyte population was significantly higher in the duodenal epithelium of CIE dogs compared with the HC. Taken together, these data could suggest that a higher demand for these lymphocytes in the duodenal epithelium may lead to a decrease in the bloodstream, although further studies are needed to confirm this hypothesis.

On the other hand, certain CBC-derived inflammatory biomarkers are useful in categorizing the clinical severity of individuals with IBD.[23,70] In dogs with CIE, differing results have been obtained in the evaluation of these indexes.[42,71,72] Our study is consistent with previous observations by some authors,[42] as we did not find a correlation between any of these inflammatory biomarkers and the clinical severity of the disease. Although 1 study did not find a correlation between

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NLR and the severity of histologic lesions in dogs with the disease,[72] we have observed a moderate positive association between SII and the severity of duodenal histopathologic lesions. CIE dogs displayed a notably higher SII than HC. Hence, these findings may suggest that the dogs' systemic inflammatory state reflect in their histopathological lesions, indicating it may serve as a biomarker for prognosis and response to treatment.

These findings propose that peripheral IFN-γ-producing T lymphocytes and SII could serve as immunologic/inflammatory markers in dogs with CIE, although further studies with larger numbers of animals are needed to determine the sensitivity and specificity of these findings and to establish possible cut-points.

Our study describes changes in peripheral and intestinal immunophenotype in CIE dogs. These findings highlight the role of the adaptive immune response, driven by distinct T lymphocyte subpopulations from intestinal and peripheral effector sites. It also emphasizes the advantages of FC for the characterization of specific lymphocyte populations that are inaccessible with other techniques in veterinary medicine, because of the use of multiple antigens simultaneously, and its usefulness in the study of immune compartments other than blood, such as the GALT. This work demonstrates the necessity for further investigation of changes in lymphocytes populations and their metabolites in dogs with CIE.

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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

Approved by the Animal Research Committee of the Veterinary Medicine Teaching Hospital and Complutense University of Madrid, and the Community of Madrid (PROEX 175/18). Written informed consent was obtained from the owners of the animals.

HUMAN ETHICS APPROVAL DECLARATION

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

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