Veterinary Immunology and Immunopathology 239 (2021) 110305

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Isolation and immunophenotyping by flow cytometry of canine peripheral blood and intraepithelial and lamina propria duodenal T lymphocytes

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ARTICLE INFO

Keywords:
Flow cytometry
Intraepithelial lymphocytes
Lamina propria lymphocytes
T lymphocytes
Dog
Peripheral blood lymphocytes

ABSTRACT

The gut associated lymphoid tissue (GALT) effector sites play a crucial role on the pathogenesis of many immune-mediated gastrointestinal diseases. The lymphocytes at these effector sites are principally T cells which present important morphological, phenotypical and functional differences. Flow cytometry (FC) is one of the most commonly used techniques to characterize intestinal lymphocytes in human and animal models. Published studies with a focus on dogs for intraepithelial lymphocytes (IEL) immunophenotyping exist in very limited numbers. Moreover, no lamina propria lymphocytes (LPL) isolation protocols in the canine species have been described for FC evaluation. In addition to immune intestinal dysregulation, imbalances in the peripheral blood immune system have been described in both human and animal gastrointestinal disorders. The aim of this study was to provide a protocol for canine IEL and LPL isolation for FC immunophenotyping of T cells subsets. Specifically, T helper, T cytotoxic, activated Th and Tc lymphocytes, regulatory, double negative, double positive, IFN-γ and IL-4 producing T cells, and to compare their respective populations between these effector sites and with the blood stream compartment in healthy dogs. The potential relationship of these cells distributions with age, sex and breed was also evaluated. This study included sixteen healthy dogs of different sexes and breeds with a mean age of 4.55 ± 2.93 years old. The selected protocols for the three immune compartments showed proper cell yield, purity, viability, and the absence of phenotypic and functional disturbances. Histologically, an adequate separation of the duodenal epithelium from the lamina propria was also observed. All the proposed T cells subsets were identified in the three immune compartments studied, showing some statistically significant differences in their distributions at these locations that highlight the importance of their individual evaluation. This study provides an adequate method for canine small intestine IEL and LPL isolation for FC immunophenotyping and is key for future studies on the gastrointestinal immune system associated with different canine diseases.

1. Introduction

Gastrointestinal disease frequently results in pets being brought in to veterinary practices. Notably, inflammatory bowel disease (IBD) is one of the most relevant chronic problems in dogs with an immune-mediated etiology and is the principal cause of chronic diarrhea and vomiting (Allenspach, 2011; Jergens and Simpson, 2012). In the last decades, the study of the gastrointestinal immunology in human medicine has provided valuable information on the pathogenesis of chronic immune-mediated gastrointestinal diseases and has allowed the development of new biological drugs (Argollo et al., 2017; Park et al., 2017).

The gastrointestinal tract has its own complex immune system denominated gut associated lymphoid tissue (GALT). GALT is an important part of the mucosal immune system which constitutes approximately 70 % of body lymphoid tissue and could be divided into inductive and effector sites (Brandtzaeg, 2009; Garden, 2013). At the

Abbreviations: CD, clusters of differentiation; DTT, 1,4-dithiothreitol; FC, flow cytometry; GALT, gut associated lymphoid tissue; HBSS, Hank's Buffered Salt Solution; IBD, inflammatory bowel disease; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; PE, phycoerythrin; PECy7, phycoerythrin-cyanine dye; RT, room temperature; WSAVA, World Small Animal Veterinary Association.

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https://doi.org/10.1016/j.vetimm.2021.110305
Received 2 September 2020; Received in revised form 22 July 2021; Accepted 27 July 2021
Available online 28 July 2021

inductive site, composed of Peyer patches, isolated lymphoid follicles and mesenteric lymph nodes, APCs initiate the immune response by presenting the antigen to the naïve B and T lymphocytes. Nevertheless, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) are the effector sites, which carry out elimination, tolerance, and inflammation responses (Brandtzaeg, 2009; Garden, 2013; van de Pavert and Mebius, 2010). IEL and LPL are primarily T lymphocytes and they both present important morphological, phenotypical and functional differences while playing a crucial role for the immune response (Maynard and Weaver, 2009).

To date, immunohistochemistry or PCR have been the most widely employed techniques for canine IEL and LPL studies (German et al., 2001; Wagner et al., 2018). Flow cytometry (FC) allows for the simultaneous analysis of a larger number of cells from different areas and using multiple antigens (Adan et al., 2017). The description of isolation and proper separation methods of IEL and LPL in humans and other species such as cats, has allowed the use of FC for the intestinal lymphocyte characterization from endoscopic biopsies (Carrasco et al., 2013; Goodyear et al., 2014; Howard et al., 2005). However, there is a scarcity in studies about canine IEL immunophenotyping using FC (Haas et al., 2014; Luckschander et al., 2009; Sonea et al., 2000) and, to our knowledge, canine LPL isolation techniques have not been previously described.

After naïve lymphocytes activation at the inductive sites, they start a migration through the mesenteric lymph nodes and the thoracic duct to circulate in the bloodstream. These activated lymphocytes selectively re-enter the tissues where they were printed thanks to the expression of homing adhesion molecules such as $\alpha_4\beta_7$ and CCR9, being in these cases, the effector sites of the GALT (Agace, 2010). In addition, lymphocyte movements from the lamina propria to the epithelial compartment have been described, and vice versa. Activated lymphocytes from the GALT are able to recirculate into other mucosal tissues due to the expression of the adhesion molecule MAdCAM-1 by all the mucosae vasculatures (Agace, 2008, 2010; Maynard and Weaver, 2009). This fact has been described in human medicine as the possible cause of extra intestinal IBD manifestations (Greuter and Vavricka, 2019). It should be noted that imbalances in the peripheral blood immune system have been described in both human and veterinary IBD patients (Dong et al., 2013; Galler et al., 2017). To our knowledge, the IEL and the LPL populations have not been characterized by FC neither compared with the PBL subpopulations in dogs.

Therefore, the objectives of this study were: (1) to describe the technique to isolate and stain canine IEL and LPL for FC study; (2) to characterize the different IEL and LPL T cells populations by FC in healthy dogs; (3) to compare IEL, LPL and PBL subpopulations in order to establish similarities and differences between these three immune compartments in healthy dogs. Finally, (4) to determine correlations between age, sex, breed and the PBL, IEL and LPL immunophenotypes.

2. Materials and methods

2.1. Animals

Sixteen healthy dogs of different breeds, age and sex were included in the study. All of them were presented to the Veterinary Medicine Teaching Hospital of the Complutense University of Madrid for elective or routine consultations (e.g., orchiectomy or ovariohysterectomy) and without history of gastrointestinal signs (Supplementary Table S1).

To qualify for the study, all the animals had to meet the following inclusion criteria: older than one year, normal physical examination and absence of abnormalities in the complete blood count and basic biochemistry, negative serology to ehrlichiosis/anaplasmosis (*Ehrlichia* spp./*Anaplasma* spp.) and leishmaniosis (*Leishmania infantum*), common infectious diseases in our geographical environment, absence of diarrhea and/or vomiting in the last six months and absence of treatment with antibiotics or immunosuppressive drugs in the last month prior to the inclusion in the study.

Their participation in the study was always carried out through informed consent of the owners. All procedures and protocols were 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).

2.2. Samples collection and study design

Upper digestive endoscopy was performed during the anesthesia of dogs for other purposes such as neutering/spaying. Video Endoscopes of variable lengths and diameters were used according to the size of the dog (Fujinon [Europe] Gmblt, Willich, Germany). In order to perform a proper macroscopic evaluation of the gastrointestinal tract, food and water were removed from dogs 24 and 12 h prior to the endoscopy, respectively. To confirm the absence of macroscopic gastrointestinal lesions during endoscopic explorations, the World Small Animal Veterinary Association (WSAVA) endoscopic guidelines for canine IBD activity index (Washabau et al., 2010) and the Slovak et al. (2015) endoscopic activity score (Slovak et al., 2015) were used. The forms were always filled out by the same two experienced endoscopists.

Seven duodenal biopsy specimens (average weight of 35 mg) were taken from each dog using pinch biopsy forceps with smooth-edged oval cups. The samples were collected in complete media- RPMI-1640 supplemented with 2 mM L-glutamine, Amphotericin B, Streptomycin, Penicillin, and 2 % FBS (all from Merck, St. Louis, Missouri, USA), placed in ice and processed within 1 h of collection for immunophenotyping study. An additional six to seven biopsy specimens were taken from gastric and duodenal mucosa and preserved in 10 % buffered formaldehyde for histologic evaluation and scoring, using the WSAVA guidelines for histopathologic evaluation of gastrointestinal inflammation (Washabau et al., 2010) and the simplified histopathologic scoring system for gastrointestinal inflammation (Allenspach et al., 2019).

Furthermore, six milliliters of peripheral blood were obtained from all dogs by jugular venipuncture during the upper endoscopy procedure and collected in tubes with EDTA for PBLs FC analysis. Blood samples were also processed within 1 h of collection.

The complete recovery of the animals was guaranteed after all the procedures. None of the dogs developed any complications after the endoscopy.

2.3. IEL isolation

The protocol used for removing the epithelial layer from the duodenal biopsies and IEL isolation was a modification of a previously described method for human colonic biopsies (Carrasco et al., 2013). Briefly, the duodenal biopsies were placed in 13 mL of a calcium- and magnesium-free Hank's Buffered Salt Solution (HBSS, Biowest, Nuaillé, France) with 1 mM 1,4-dithiothreitol (DTT, Merck, St. Louis, Missouri, USA) and 1 mM EDTA (Merck, St. Louis, Missouri, USA) and incubated for 90 min in a shaking water bath at 120 rpm and 37 °C. Then, the supernatant containing IEL was filtered through a 40 μm nylon cell strainer (Biowest, Nuaillé, France), centrifuged (10 min, 400 g), washed in PBS (Merck, St. Louis, Missouri, USA) with 2 % FBS and resuspended in complete media.

2.4. LPL isolation

After epithelial layer removal (Fig. 1), the duodenal biopsies were processed for LPL isolation. As previously reported (Carrasco et al., 2013), tissue specimens were introduced in 5.5 mL of washing solution containing HBSS with 5 % FBS and 0.36 % of glucose (Biowest, Nuaillé, France) and then, incubated 10 min at 37 °C with constant stirring to remove the remaining DTT and EDTA.

Subsequently, in order to release the lymphocytes from the lamina propria, biopsies were placed in 5.5 mL of PBS solution supplemented

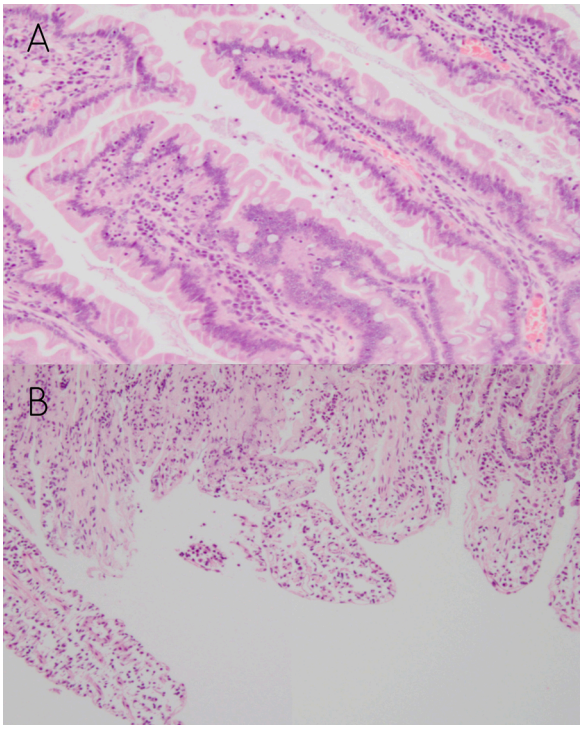

Fig. 1. Effect of IEL isolation protocol in duodenal biopsy. Histological appearance of the duodenal biopsies before ILE isolation protocol (A) and after that (B). H&E (magnification: x20).

with 2 mM Amphotericin B, Streptomycin, Penicillin and 0.5 % of BSA (Merck, St. Louis, MO, USA) and were re-incubated for 16.5 h in a shaking water bath at 120 rpm and 37 °C. Finally, the supernatant with LPL was filtered, centrifuged and resuspended in complete media as previously detailed.

2.5. PBL isolation

Firstly, 6 mL of peripheral blood was centrifuged by density gradient to obtain PBMCs (Histopaque 1077®, Merck, St. Louis, MO). PBMCs were washed using a washing solution with PBS and 2 % of FBS and then adding 4.1 mM bicarbonate to eliminate erythrocyte debris. For lymphocyte purification, PBMCs were resuspended in complete media and were cultured for 90 min at 37 °C in a humidified atmosphere containing 5 % of CO_2. The lymphocyte-enriched supernatant was centrifuged and resuspended in complete media as described earlier for the FC assay.

2.6. Flow cytometry

Phenotypic characterization of freshly isolated PBLs, LIEs and LPLs was performed using two-, three- or four-color flow cytometry. Anti-canine specific, anti-human and anti-bovine cross-reactive mAb against different lymphocyte clusters of differentiation (CD) and other lymphocyte's molecules and conjugated with FITC, phycoerythrin (PE), Alexa Fluor 647, Allophycocyanin, and phycoerythrin-cyanine dye (PECy7) were employed (Table 1). All the mAb were obtained from Bio-Rad (Oxford, UK) except rat anti-human FoxP3 which was supplied by Thermo Fisher (Massachusetts, USA).

Isolated lymphocytes were exposed to various combinations of these mAb for detection of specific surface and intracellular molecules resulting in the characterization of different lymphocytes populations: T lymphocytes (CD45+CD3+ lymphocytes), Th (CD3+CD4+ lymphocytes), T cytotoxic or 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 or Treg (CD3+CD4 + CD25+FoxP3+ lymphocytes), IFN-γ producing T lymphocytes (CD3+ IFN-γ+ lymphocytes) and IL-4 producing T lymphocytes (CD3+IL4+ lymphocytes) lymphocytes. Furthermore, the CD4/CD8 ratio was calculated. The tubes and the amount of each antibody employed are listed in Table 2. Antibody titration was previously developed to determine the optimal working concentration.

Table 1
List of canine-specific and cross-reactive mAb used in flow cytometric evaluation of canine peripheral blood lymphocytes, IEL and LPL.

Antigen	Conjugation	Clone	Specificity	Isotype
CD45	Allophycocyanin	YKIX716.13	Rat anti-dog	IgG2b
CD3	FITC	CA17.2A12	Mouse anti-dog	IgG1
CD4	PECy7	YKIX302.9	Rat anti-dog	IgG2a
CD8	Alexa Fluor 647	YCATE55.9	Rat anti-dog	IgG1
CD25	PE	P4A10	Mouse anti-dog	IgG1
FoxP3	Allophycocyanin	FJK-16s	Rat/mouse anti-human	IgG2a
IL-4	PE	CC303	Mouse anti-bovine	IgG2a
IFN-γ	Alexa Flour 647	CC302	Mouse anti-bovine	IgG1

FC staining protocol was used as previously described by our research group (Villaescusa et al., 2012b) with modifications. Briefly, freshly isolated cells from each immunological compartment were incubated for 10 min at room temperature (RT) with normal serum derived from rats and mice (2 μL of each one, Bio-Rad, Oxford, UK) for Fc receptor blocking. Subsequently, mAbs combinations indicated in Table 2 were added to the lymphocytes and incubated for 30 min at RT. After each mAb incubation, cells were washed in 2 mL of PBS/1 % BSA solution, centrifuged (400 *g*, 10 min) and resuspended in 200 μL of commercially available cell fix-solution (BD CellFIX, Becton-Dickinson, San Jose, CA, USA). For FoxP3 intracellular detection, a fixation/permeation protocol was conducted with the cells after the mAbs surface staining using Leucoperm® kit (Bio-Rad, Oxford, UK). Briefly, once cells were washed from the first staining, the pellet was resuspended in 100 μL of fixation reagent, mixed gently and incubated for 30 min at RT in darkness. Then, cells were washed as indicated previously, re-suspended again with permeation reagent and incubated with rat/-mouse anti-human FoxP3 for 30 min at RT. Subsequently, cells were washed and prepared for FC analysis by being resuspended in cell fix-solution.

Finally, for IFN-γ and IL-4 producing T lymphocytes characterization, freshly isolated PBLs, LIEs and LPLs were stimulated and incubated PMA (10 ng/mL, Enzo®, New York, USA) and ionomycin (0.4 μM, StressMarq BioscienceINC, Victoria, Canada) for 24 h at 37 °C in

Table 2
mAb combinations and antigens detected. The amount of each antibody is given in parenthesis.

Tube	1st incubation mAb added	2nd incubation mAb added	Surface antigens detected	Intracellular antigens detected
1	Rat anti-dog CD45 (3 μL), mouse anti-dog CD3 (3 μL)		CD45, CD3	–
2	Mouse anti-dog CD3 (3 μL), CD25 (5 μL), rat anti-dog CD4 (3 μL), CD8 (3 μL)	–	CD3, CD25, CD4, CD8	–
3	Mouse anti-dog CD3 (3 μL), CD25 (5 μL), rat anti-dog CD4 (3 μL)	Rat/mouse anti-human FoxP3 (5 μL)	CD3, CD25, CD4	FoxP3
4	Mouse anti-dog CD3 (3 μL)	Mouse anti-bovine IL-4 (5 μL), IFN- γ (5 μL)	CD3	IL4, IFN- γ

humidified atmosphere containing 5 % CO_2. Brefeldin A (1 μg/mL, StressMarq Bioscience INC, Victoria, Canada) was added in the last 10 h of incubation to inhibit cytokine secretion by breaking the cell cytoskeleton. Then, the staining for surface and intracellular antigens was performed as described above.

Samples were analyzed using FACSCalibur flow cytometer (Becton Dickinson) and Cell-Quest and FlowJo V10 software. Lymphocytes' gate was selected initially by forward- and side-scattered properties and the studied lymphocytes subsets were detected by gating the different positive or negative CDs (Supplementary Fig. S1). When a two-color FC analysis was necessary for the lymphocytes subset characterization, a simple dot plot was performed (for T cells, IFN-γ, and IL-4 producing T lymphocytes). For the three-color Th, Tc, DN and DP lymphocytes analysis, firstly CD3 positive cells were gated. After that, this CD3+ population was displayed into a CD4/CD8 dot plot to select these lymphocyte subsets based on the positive or negative expression of each mAbs. Activated Th and Tc subsets analyses were achieved displaying the previously gated CD3+CD4+ or CD3+CD8+ cells into a CD25 histogram and selecting the CD25 positive lymphocytes. Finally, the same strategy was used for the four-color Treg analysis: a first selection of CD3+ cells followed by a CD4 + CD25+ dot plot gating, and a final FoxP3+ selection within these CD3+ and CD4 + CD25+lymphocytes using a histogram (Fig. 2). Gates were set using the fluorescence minus one and the appropriate isotope controls (Supplementary Fig. S2). At least, 10.000 events were acquired from lymphocytes' gate.

2.7. Statistical analysis

Statistical analysis was carried out by the "Departamento de Apoyo a la Investigación de Servicios Informáticos" of the Complutense University of Madrid, using the commercially available statistical software SAS, version 9.4 (SAS Institute, Cary, NC, USA). Normal distribution of data was determined by the Kolmogorov-Smirnov test. Differences between the immunological compartments studied, age, sex and breed were evaluated by T Student's test in the case of a normal data distribution or by Wilcoxon's signed rank test in the absence of a normal data distribution. For correlations, Spearman correlation coefficients were employed with the significance level set at $P < 0.05$. The Bonferroni correction was applied in multiple comparison analysis.

3. Results

3.1. Animals

The group of animals included in the study consisted of seven females and nine males. The mean age was 4.55 ± 2.93 years old and mean body weight was 18.92 ± 15.91 kg. Seven dogs were crossbreeds and nine dogs were purebred: American Staffordshire (n = 1), Beagle (n = 1), English Setter (n = 1), Great Dane (n = 1), Greyhound (n = 2), Jack Russell Terrier (n = 1), Maltese (n = 1) and West Highland White Terrier (n = 1). Mean WSAVA endoscopic score was 0.38 ± 0.89 (range 0–27) for the esophagus, 6.86 ± 4.74 (range 0–33) for the stomach and 4.69 ± 2.91 (range 0–33) for the duodenum. Mean quantitative endoscopic value according to Slovak et al. index was 0.5 ± 0.65 (range 0–6) for the stomach and 2.19 ± 1.11 (range 0–8) for the duodenum and qualitative mucosal assessment was 0.5 ± 0.65 (range 0–3) for the stomach and 1.38 ± 0.89 (range 0–4) for the duodenum. Histopathological score using WSAVA guidelines was 3.55 ± 1.67 (range 0–27) for the stomach and 4.67 ± 2.16 (range 0–27) for the duodenum. According to the simplified histopathologic scoring system developed by Allenspach et al., the mean for the stomach was 2.73 ± 1.1 (range 0–15) and for the duodenum, 3.73 ± 1.71 (range 0–18).

3.2. Isolation of PBL, IEL and LPL for FC analysis

The cell yield obtained after the isolation protocols was 1.6×10^6 cells/mL for PBL, 3.8×10^6 cells/mL for IEL and 1.1×10^6 cells/mL for LPL. Regarding purity, most of the cells gated in the lymphocyte regions of the three immunological compartments studied were CD45+ (PBL: mean 96.43 ± 3.18 %, IEL: mean 84.46 ± 9.16 % and LPL: mean 89.55 ± 8.86 %).

Cell viability after the isolation protocols was 92 % for PBL and 98 % for IEL. When considering all the LPL isolated, a viability of 46 % was achieved, but further studies employing 7AAD allowed to confirm that viable and dead cells could be accurately distinguished based on light scatter characteristics. When only viable LPL were gated, the viability increased to 98 % (Fig. 3). Only these cells were used for the lymphocyte subsets analysis.

3.3. Immunophenotyping of PBL, IEL and LPL by FC

Summarized data including mean, standard deviation, median, as well as minimum and maximum of all lymphocyte subpopulations studied in each immunological compartment are shown in Table 3. The majority of the lymphocytes from peripheral blood and from the effector GALT sites were T cells (CD3+). The percentage of T lymphocyte population was lower within the duodenal epithelium compared to the lamina propria ($P = 0.012$). The percentage of Th lymphocytes localized in the duodenal epithelium was significantly lower in comparison with the lymphocyte population from the bloodstream ($P = 0.001$) and from the intestinal lamina propria ($P = 0.001$). When comparing Tc lymphocytes in the immunity sites studied, this subset was higher within the duodenal epithelium compared to the peripheral blood ($P = 0.014$). Furthermore, the CD4/CD8 ratio within the intestinal epithelium was significantly lower than in the bloodstream ($P = 0.001$) and in the duodenal lamina propria ($P = 0.001$).

The double positive T cells population was minimal within the three compartments (1.06 ± 0.55 % of PBL, 1.00 ± 1.17 % of IEL and 1.53 ± 0.88 % of LPL). Nevertheless, the percentage of double positive T cells from the duodenal lamina propria was higher in comparison with the epithelium ($P = 0.014$). On the other hand, a non-depreciable percentage of double negative T cells were detected both in the bloodstream and in the duodenal epithelium and lamina propria, with 12.70±3.33 %, 25.03±12.13 % and 24.13±9.62 %, respectively. In addition, the percentage of peripheral double negative T cells was statistically lower compared to the intestinal epithelium ($P = 0.003$) and lamina propria ($P = 0.001$).

Regarding the different activation status of Th and Tc lymphocytes among compartments, the percentage of duodenal lamina propria activated Th cells was higher than the percentage of this lymphocyte subset identified in the peripheral compartment ($P = 0.002$) and in the duodenal epithelium ($P = 0.001$). However, peripheral blood activated Th lymphocytes were higher in comparison with the intestinal epithelium ($P = 0.001$). Activated Tc lymphocytes were higher within the intestinal epithelium compared to the lamina propria ($P = 0.001$).

The relative count of Treg cells in the three immune compartments was low (2.75 ± 2.12 % of PBL, 1.73 ± 1.13 % of IEL and 4.24 ± 3.06 % of LPL), being this lymphocytes subset statistically lower in the intestinal epithelium than in the lamina propria ($P = 0.001$).

Finally, IFN-γ producing T lymphocytes were more abundant than IL-4 producing T lymphocytes in the three compartments. The expression of IFN-γ and IL-4 by peripheral T lymphocytes was higher than by these epithelial ($P = 0.001$ and $P = 0.002$, respectively) and lamina propria ($P = 0.004$ and $P = 0.012$, respectively) subsets.

3.4. Influence of age, sex and breed in the PBL, IEL and LPL immunophenotype

Relationship between age and the immunophenotype from the three immunological compartments showed that Tc PBL increased significantly with age (R = 0.56; $P = 0.025$) while the ratio CD4/CD8 decreased (R = −0.745; $P = 0.001$).

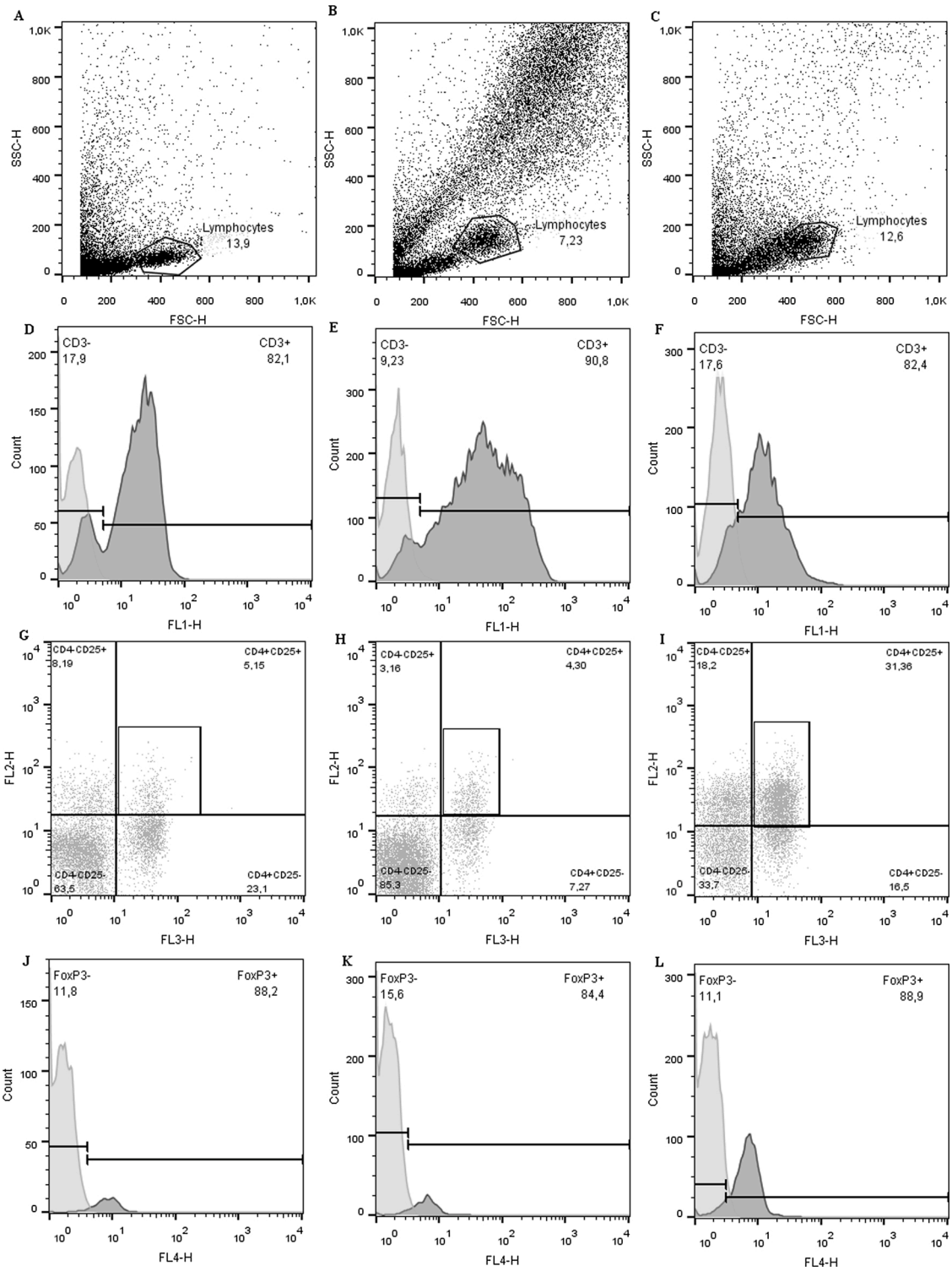

Fig. 2. Flow cytometry for Treg cells characterization in peripheral blood, intestinal epithelium and lamina propria. A, B and C: representative dot plot showing the forward/sideward scatter (FCS/SSC) properties of the analyzed PBL (A), IEL (B) and LPL (C) gating the lymphocyte population. D, E and F: representative histograms of CD3 expression in PBL (D), IEL (E) and LPL (F) with negative cells on the left and positive cells on the right side of the graphs. Numbers in the right upper corners of the graphs indicates the percentages of the respective positive populations. G, H and I: representative dot plots of CD3+ gated cells labeled with mAbs against CD25 and CD4 in PBL (G), IEL (H) and LPL (I). CD25 positive cells are displayed in the upper left corner while CD4 cells are displayed in the lower right corner of the graphs. CD25+CD4+ cells are displayed in the upper right corner of the graphs. The adjacent numbers indicate the percentage of the respective positive populations. J, K, L: representative histograms of FoxP3 expression in PBL (J), IEL (K) and LPL (L) with negative cells on the left and positive cells on the right side of the graphs. The percentages of Treg cells were calculated for the overall T cells from each immune compartment.

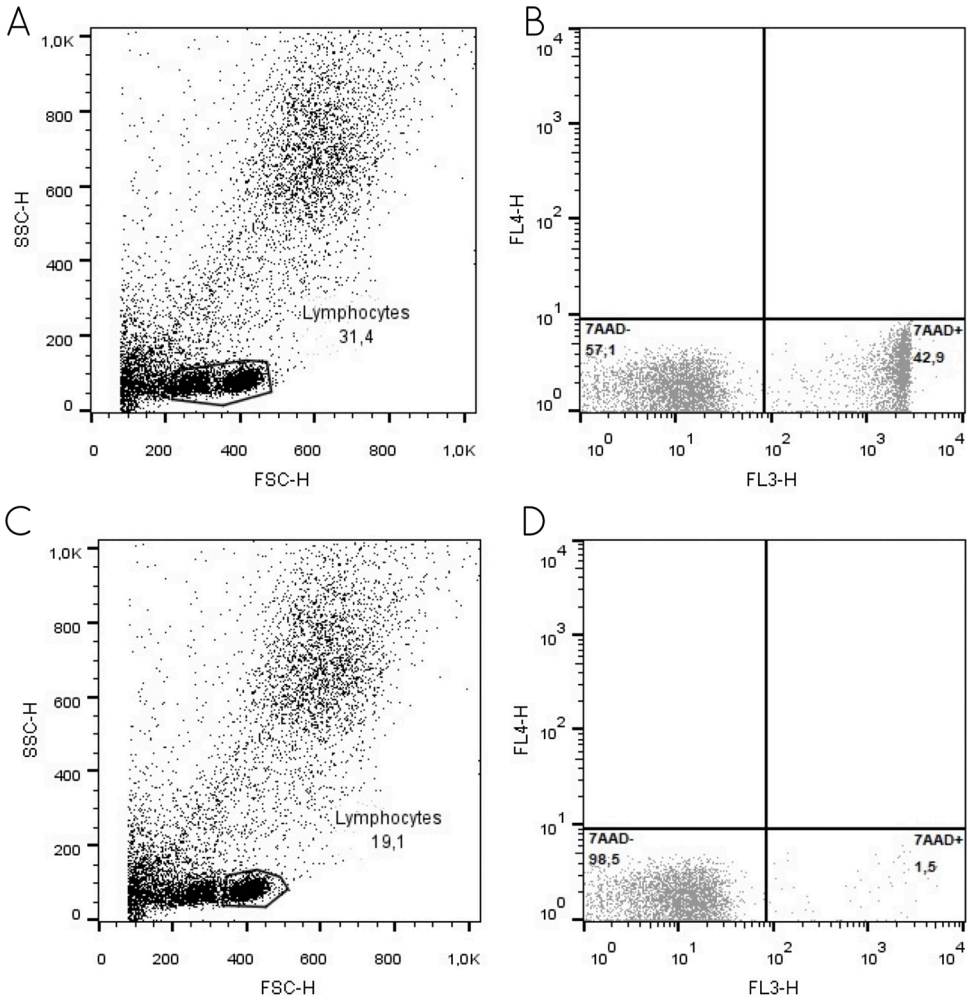

Fig. 3. Differences in LPL viability gating the entire lymphocytes region in forward and side scatter dot plot (A and B) or gating the assumed viable cells based on light scatter characteristics in forward and side scatter dot plot (C and D). Viability was measured using 7AAD.

No differences were found when comparing PBL, IEL and LPL subsets between males and females.

No statistically significant association was observed between breed (crossbreed *versus* purebred) and the immunophenotype studied.

4. Discussion

Peripheral immunophenotype imbalances accompany some gastrointestinal immunomediated diseases such as IBD in dogs and humans (Dong et al., 2013; Galler et al., 2017). These alterations may reflect the immune cell trafficking and the significance of a depth lymphocyte characterization in the three main immune compartments involved in the gastrointestinal immune response (Agace, 2010; Greuter and Vavricka, 2019). In this study, we describe and compare for the first time, the main T lymphocyte populations from the bloodstream and the duodenum in healthy dogs. Our results show important differences between the peripheral immune compartment and the duodenal epithelium, and between the duodenal IEL and LPL. PBL and LPL seem to be the most coincidental immune compartments.

Based on our results and on the fact that the lymphocytes placed in the intestinal epithelium and lamina propria are constituted by different cell types with their own properties and functions (German et al., 1999; Maynard and Weaver, 2009), the separated study of these populations becomes crucial for a correct understanding of gastrointestinal immunity. The first objective of this study was to describe a method for IEL and LPL isolation from intestinal biopsies of dogs for FC analysis. While these methods have been previously described and standardized in other species such as humans, mice or cats (Carrasco et al., 2013; Howard et al., 2005; Weigmann et al., 2007), to the best of the authors' knowledge, there is only one preliminary study of colonic LPL isolation in the dog for a FC characterization (Luiz et al., 2011). In our study, a previously described method for human IEL isolation was modified (Carrasco et al., 2013) and a non-enzymatic LPL isolation method was used after testing many others (data not shown) (Carrasco et al., 2013). These protocols have provided us proper cell yield and purity and were able to separate practically the entire intestinal epithelium without damaging the lamina propria (Fig. 1). After an in depth bibliographic review, this is the first description of the isolation of IEL and LPL from canine duodenal biopsies for FC analysis in the same study. However, it should be considered that the high time-consuming protocols required in this study led to the use of fixed cells. Cell viability was 92 % for PBL and 98 % for IEL. When analyzing LPL, two different cell subsets could be identified based on light scatter properties. Further studies (data not shown) allowed verification that one of the subsets included dead cells, while the cell viability of the other subset was 98 %. Therefore, cells could be gated to ensure that only live LPL were analyzed (Fig. 3). However, it is strongly recommended the inclusion of a viability marker and to work with alive cells in future studies.

In addition, the use of a flow cytometer with a higher number of lasers would led to a better characterization of the lymphocyte populations, adding important markers for leucocyte characterization, such as anti-CD45, in all the tubes of the study, and avoiding hard

Table 3
FC analyses of PBL, IEL and LPL subpopulations. *Activated Th and Tc subsets are expressed as the percentage of Th or Tc cells positive to CD25. Paired superscript letters indicate statistical differences ($P < 0.05$) between lymphocytes subsets from the three immunological compartments. *Abbreviations:* Mean (Mn), Standard Derivation (SD), Median (Med), Minimum (Min), Maximum (Max).

	PBL					IEL					LPL				
	Mn	±SD	Med	Min	Max	Mn	±SD	Med	Min	Max	Mn	±SD	Med	Min	Max
T lymphocytes (CD3+)	87.19	5.33	88.53	74.52	95.59	76.34[l]	11.94	80.03	54.24	89.88	83.41[l]	9.04	85.38	69.58	97.61
Th lymphocytes (CD3+CD4+)	43.06[a]	7.35	42.69	26.39	57.56	9.84[a,m]	5.83	8.77	3.79	25.03	39.89[m]	10.89	40.24	26.40	60.52
Tc lymphocytes (CD3+CD8+)	29.32[b]	8.04	28.00	16.28	42.28	40.39[b]	12.70	40.28	21.77	63.98	29.26	8.64	26.68	18.02	47.24
Double positive T lymphocytes (CD3+CD4+CD8+)	1.06	0.55	1.03	0.21	1.97	1.00[n]	1.17	0.63	0.00	4.75	1.53[n]	0.88	1.48	0.42	3.73
Double negative T lymphocytes (CD3+CD4-CD8-)	12.70[c,h]	3.33	12.36	8.45	18.65	25.03[c]	12.13	27.04	0.00	38.96	24.13[h]	9.62	21.78	12.32	43.34
Activated Th lymphocytes (CD3+CD4 + CD25+)*	3.37[d,i]	1.78	3.09	1.16	8.42	0.31[d,o]	0.17	0.33	0.05	0.57	5.84[i,o]	2.67	5.30	2.71	10.83
Activated Tc lymphocytes (CD3+CD8+CD25+)*	9.87	22.04	3.56	0.95	91.97	13.76[p]	10.31	12.32	1.21	39.22	32.53[p]	9.67	33.50	18.74	49.48
Treg lymphocytes (CD3+CD4 + CD25+Foxp3+)	2.75	2.12	2.28	0.05	5.94	1.73[q]	1.13	1.73	0.26	4.31	4.24[q]	3.06	3.52	0.18	10.52
IFN-γ producing T lymphocytes (CD3+IFN-γ+)	17.42[e,j]	10.30	17.56	2.17	42.87	2.28[e]	3.05	1.44	0.00	10.56	2.26[j]	3.77	0.35	0.00	10.10
IL-4 producing T lymphocytes (CD3+IL4+)	7.78[f,k]	8.86	3.98	0.19	28.10	1.21[f]	1.23	1.13	0.00	3.65	1.37[k]	1.65	0.95	0.00	4.41
Ratio CD4/CD8	1.61[g]	0.62	1.48	0.62	3.17	0.28[g,r]	0.22	0.21	0.07	0.85	1.53[r]	0.75	1.35	0.57	2.94

compensations needed with a four color system. In agreement with previous studies (Byrne et al., 2000; Faldyna et al., 2001; Villaescusa et al., 2012b), our results show a majority of T cells (CD3+) within PBL, as well as a higher population of Th lymphocytes (CD3+CD4+) than Tc lymphocytes (CD3+CD8+). With regard to the duodenal epithelium, a majority of T cells, a low percentage of Th lymphocytes and a high percentage corresponding to the cytotoxic subset have been found in this study. However, we have detected a percentage of CD3+, CD4+ and CD8+ cells slightly lower than other reports (Haas et al., 2014; Luckschander et al., 2009). These differences, which can be considered slight, may be due to the clone or the antibodies source used, to the specific methodology employed or to the animal's characteristics, among others. Our findings in LPL about these subsets show the same phenotypic distribution than other studies performed in humans, mice and cats (Howard et al., 2005; Mennechet et al., 2002; Schieferdecker et al., 1992) being this the first report for canine duodenal LPL employing FC.

Our results show a percentage of DP T-cells in PBL of healthy dogs of 1.06 ± 0.55 %, that are in accordance with the previously described range for this canine lymphocyte population (0.23–2.42 % of PBL) (Bismarck et al., 2012; Rothe et al., 2017; von Buttlar et al., 2015v). For the duodenal epithelium, our results show higher percentages for this lymphocyte population (mean: 1.00 ± 1.17 %) than that described for healthy dogs by Sonea and coworkers but close to two fold less the value that Rabiger et al. reported (Rabiger et al., 2019a; Sonea et al., 2000). LPL DP T cells have not been described in dogs in previous studies up to now, but almost the same percentage as those obtained in our study for this lymphocyte population were found in cats (mean: 1.50 %) (Howard et al., 2005).

On the other hand, peripheral DN T-cells characterized by the expression of CD3 in the absence of CD4 and CD8 expression, may also include a subset of NK cells, called NKT cells, whether no others surface markers are used (Hillhouse and Lesage, 2013). Our results for this peripheral cell subset (PBL DN T cells mean: 12.70 ± 3.33 %) resemble the range previously described in peripheral blood of healthy dogs (~10–15 %) (Alexandre-Pires et al., 2010; Platt et al., 2013; Rabiger et al., 2019b). DN T cells have not been practically studied in the canine intestine. Only one work evaluated the percentage of DN T cells in the IEL compartment, describing 50 % of this cell population (Sonea et al., 2000). However, in human medicine imbalances of this intestinal lymphocyte population have been reported associated to immunomediated diseases such as coeliac disease (Steenholt et al., 2017). Taking into account the high percentages of DN T cells found in the intestine of our healthy dogs, further studies are needed for a deeper characterization of these atypical lymphocytes in this species.

The CD25 antigen can be used for the evaluation of T cells activated phenotype (Brummelman et al., 2018). Our study has detected a low percentage of peripheral blood CD3+CD4 + CD25+ (mean: 3.37 ± 1.78 %) and CD3+CD8+CD25+ (mean 9.87 ± 22.04 %). These outcomes agree with the previously reported for these canine lymphocyte populations (Bismarck et al., 2012), but contrast with the study of Mizuno and coworkers, where the unstimulated PBMCs of four dogs were CD25 negative (Mizuno et al., 2009). Regarding to the epithelial compartment, the percentages of activated Th and Tc lymphocytes (mean: 0.31 ± 0.17 % and mean: 13.76 ± 10.31 %, respectively) detected in our healthy dogs were considerably lower than the described by Rabiger and coworkers for these subsets (~45 % and ~30 %, respectively) (Rabiger et al., 2019a). For this study, the highest percentages of these lymphocyte subpopulations have been found in the duodenal lamina propria (mean: 5.84 ± 2.67 % and mean: 32.53 ± 9.67 %, respectively). Similar results have been reported by Howard et al. in the feline duodenal lamina propria using FC (Howard et al., 2005). The elevated expression of CD25, especially from Tc, in the intestine can be explained by the intense antigen contact that takes place in these effector immune compartments.

Treg cells require IL-2 for survival, which translates into a high expression of CD25 antigen (Brummelman et al., 2018). This fact may suppose that some of the CD25+ Th cells can play regulatory activities. The percentage of peripheral canine Treg cells described in the literature is quite variable (Archer et al., 2018; Palatucci et al., 2018; Pinheiro et al., 2011), being our results (mean: 2.75 ± 2.12 %) within the described ranges (0.05–12 % of PBL). This large range may be due to the

heterogeneity of the dog populations included among studies.

Regarding the FC characterization of small intestinal Treg cells in dogs, to our knowledge, this is the first study that evaluates this population both in IEL and LPL. As previously described in mice (Sujino et al., 2016), the majority of mucosal Treg cells in our study are located in the lamina propria, with a lower percentage placed in the epithelium (IEL mean: 1.73 ± 1.13 %, LPL mean: 4.24 ± 3.06 %).

Because of the high IFN-γ and IL-4 expression by Th1 and Th2 subsets, these cytokines are the most used for the detection and characterization of these lymphocyte populations (Pedersen et al., 2002). Nevertheless, these cytokines are also produced by other T lymphocyte subpopulations such as Tc, double positive and double negative T cells (including the NKT cells) showing a quite important role in the proinflammatory immune response (Ho and Miaw, 2016; Palatucci et al., 2018; Xie et al., 2009). In concordance with Cortese and coworkers our results show a higher percentage of IFN-γ-producing T PBLs than IL-4-producing T PBLs (mean: 17.42 ± 10.30 % and mean: 7.78 ± 8.86 %, respectively) in healthy dogs (Cortese et al., 2015). Canine intestinal cytokine profile has been previously evaluated using mRNA quantification by real-time qPCR (Peters et al., 2005; Schmitz et al., 2012), but FC has never been used. This study shows lower percentages of IFN-γ producing T cells in IEL and LPL (mean: 2.28 ± 3.05 % and mean: 2.26 ± 3.77 %, respectively) than previously described for these human immune compartments in healthy patients (Van Damme et al., 2001). However, the percentages of IL-4 producing T cells observed in our dog population in both intestinal locations (IEL mean: 1.21 ± 1.23 % and LPL mean: 1.37 ± 1.65 %) are in concordance with the proportion described by van Damme and coworkers (IEL mean: 1.1 % range 0.2–2 % and LPL mean: 1.6 % range 0.6–3.8 %) (Van Damme et al., 2001).These differences could be due to different activation protocols or by species differences, but further studies are needed to understand these results and to establish a better protocol for the identification of these cells in the dog intestine.

Influence of age, sex and breed in the peripheral immunophenotype have been previously described in dogs (Faldyna et al., 2001; Villaescusa et al., 2012a, b), but their effects in canine IEL and LPL have never been reported. We did not find significant differences based on sex and breed in our study group. However, the small number of animals of single breeds included in our study prevents us from drawing a definitive conclusion about the influence of breed on the intestinal immunophenotype. Furthermore, the large statistical deviations found for the majority of the lymphocyte's subpopulations described highlights the important individual immunophenotype variations. We have found an increase with age in the percentage of Tc lymphocytes in PBL and a decrease of CD4/CD8 ratio in the dogs that were part of this study. The same has been previously described in dogs (Faldyna et al., 2001; Reis et al., 2005; Villaescusa et al., 2012a). Some authors have associated this phenomenon with an oligoclonal and altered expansion of Tc cells, which, in humans, results in an increase of morbidity and mortality risk in the elderly (Provinciali et al., 2009). With regard to the intestinal lymphocytes, in agreement with our results, these cells do not appear to show these aging changes in other studies (Dock et al., 2017). Nevertheless, when analyzing our results it should be considered that all the animals included in this study were prime adult dogs, with the inclusion of other range ages being necessary to prove these findings.

In summary, this study provides an accurate method for canine small intestine IEL and LPL isolation for FC immunophenotyping. A characterization and comparison of the principal T lymphocyte subsets in PBL, IEL and LPL establishing the differences between each immune compartment has been achieved. This work lays the foundations for future studies on the gastrointestinal immune system associated with different diseases.

Founding sources

This study was supported by Proyecto Santander of Complutense University of Madrid, Spain (reference PR41/17-21023).

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors thank to "Departamento de Apoyo a la Investigación de Servicios Informáticos", "Centro de Ayuda a la Investigación de Citometría de Flujo y Microscopía Confocal" of the Complutense University of Madrid and to Jacinto Delgado from "Hospital Veterinario Vetersalud Indra".

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110305.

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