Journal of Comparative Pathology 212 (2024) 20–26

Contents lists available at ScienceDirect

Journal of Comparative Pathology

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

Neoplastic disease

Immunophenotype investigation in feline intestinal non-B-cell lymphoma

Birgitt Wolfesberger [a,*], Gabriele Gradner [a], Barbara C. Rütgen [b], Katharina M. Hittmair [a], Ingrid Walter [b], Taryn A. Donovan [c], Miriam Kleiter [a], Alexander Krischak [a], Iwan A. Burgener [a], Andrea Fuchs-Baumgartinger [b]

[a] Department for Companion Animals and Horses, University of Veterinary Medicine Vienna, 1210 Vienna, Austria
[b] Department of Pathobiology, University of Veterinary Medicine Vienna, 1210 Vienna, Austria
[c] Department of Anatomic Pathology, The Schwarzman Animal Medical Center, 510 East 62nd Street, New York 10065, USA

ARTICLE INFO

Article history:
Received 31 January 2024
Accepted 24 May 2024

Keywords:
cats
flow cytometry
granzyme B
immunohistochemistry
LGL lymphoma
non-B-cell lymphoma
WHO classification

ABSTRACT

Lymphoma is the most common tumour of domestic cats, developing most frequently in the small intestine. Feline small intestinal lymphoma predominantly demonstrates a T-cell immunophenotype identified by standard immunopositivity for T cells with CD3 or immunopositivity for B cells with CD20. In contrast, a wide spectrum of immunohistochemical antibodies are applied in humans to diagnose the various specific lymphoma subtypes according to the WHO classification. Our aim was to augment our knowledge of immunophenotypes in feline non-B-cell lymphomas forming macroscopic masses in the intestinal tract. We evaluated the combined immunohistochemistry and flow cytometry findings from 15 cases. Neoplastic lymphoid cells were immunopositive for CD3 in 93% (14/15), granzyme B in 87% (13/15), CD5 in 20% (3/15), CD8 in 13% (2/15), CD4 in 7% (1/15) and CD56 in 7% (1/15) of cases. Cytotoxic granules indicating a cytotoxic origin of the neoplastic cells were identified by histopathology only in 13% (2/15) and by cytology in 47% (7/15) of the cases. Without immunohistochemical labelling of the cytotoxic protein granzyme B, the cytotoxic status would have been missed in 46% (6/13) of the cytological and in 85% (11/13) of the histopathological slides. These findings suggest that more complex immunophenotyping may advance our understanding and help prognosticate small intestinal T-cell lymphoma in cats.
© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

Lymphoma represents the most frequently diagnosed and clinically most important malignant tumour in cats [1]. It develops primarily in the gastrointestinal tract with the small intestine representing the most affected site [2,3]. Neoplasms of lymphoid cells have been described morphologically as large or small cell lymphoma and immunophenotypically as B- or T-cell lymphoma. In cats, B-cell lymphomas predominate in the stomach and T-cell lymphomas in the small intestine [4–6]. Attempts to classify lymphomas with more detail in veterinary medicine have included stratification of tumours in classifications adopted from human medicine, such as the National Cancer Institute Working Formulation, the updated Kiel classification and, most recently, the state-of-the-art World Health Organization (WHO) classification system [4,7–9].

In human medicine, besides using clinical, morphological and genetic features, advanced immunophenotyping allows identification of different lymphoma subtypes according to the WHO classification. For example, intestinal T-cell or natural killer (NK)-cell lymphomas are defined by using a broad group of antibodies against various antigens [10]. In contrast, for routine feline lymphoma evaluation, T-cell lymphoma immunophenotyping is often limited to CD3 expression, precluding a more detailed diagnosis. In routine diagnostic practice, no specific antibodies are used to identify neoplastic feline NK cells; their presence is typically assumed when neither CD3 nor CD20 are expressed.

The aim of this study was to investigate more complex immunophenotyping in 15 cats with small intestinal, mass-forming non-B-cell lymphoma using a combination of immunohistochemistry (IHC) and flow cytometry (FCM).

* Corresponding author.
E-mail address: birgitt.wolfesberger@vetmeduni.ac.at (B. Wolfesberger).

https://doi.org/10.1016/j.jcpa.2024.05.004
0021-9975/© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

2. Materials and methods

2.1. Patients and tissue specimens

Inclusion criteria for this study encompassed confirmation of a non-B-cell lymphoma with immunophenotyping by histopathology and FCM plus stratifying lymphoma subtypes according to the WHO classification [9]. Only lymphomas affecting the small intestine and/or mesenteric lymph nodes, with macroscopic masses verified by abdominal ultrasound at presentation, were included. Initial diagnostics were performed via ultrasound-guided fine needle aspiration of the abdominal masses followed by Romanowsky staining (Diff-Quik) and cytological evaluation of the slides.

Specimens for histopathological examination and FCM were all obtained by laparoscopic resection of the tumour mass. Data were collected using the digital information system of the University of Veterinary Medicine Vienna from 2016 to 2021.

2.2. Histopathology and immunohistochemistry

Resected tumours were fixed in 10% neutral buffered formalin. Representative areas of the tumorous tissue were routinely processed and embedded in paraffin wax and 3–4 μm sections were stained with haematoxylin and eosin (HE).

For identification of B-cell and T-cell lineages, IHC was performed using a LabVision Autostainer (Thermo Fisher Scientific, www.thermofisher.com). Slides were pre-treated with heat in citrate buffer (pH 6.0) for 15 min for antigen unmasking. To decrease background staining, the slides were incubated in H_2O_2 block (Thermo Fisher Scientific) for 5 min and in Ultra V Block (Thermo Fisher Scientific) for another 10 min. A polyclonal rabbit anti-human antibody specific for CD3 (A452, 1:1000; Dako, www.agilent.com) and a monoclonal rabbit anti-human antibody specific for CD20 (ab27093, 1:1000; Abcam, www.abcam.com) were used as pan-T-cell and pan-B-cell markers, respectively. The samples were incubated with the primary antibodies for 30 min and with the secondary antibody for 30 min (BrightVision Poly HRP anti-rabbit IgG; Immunologic, www.immunologic.nl). For visualization diaminobenzidine (DAB; Large Volume DAB Plus Substrate System for 5 min; Thermo Fisher Scientific) was used as chromogen. Slides were counterstained with Mayer's haematoxylin, dehydrated, placed into Neo Clear and mounted in Neo-Mount (both Merck, www.merckgroup.com).

The reactivity of antibodies against granzyme B, CD56 and CD57 in feline lymphoid cells has been validated [5,11,12]. For immunohistochemical labelling of the proteins CD56, CD57 and granzyme B, slides were pre-treated with heat in citrate buffer (pH 6.0) for 30 min. A monoclonal mouse anti-human antibody specific for CD56 (LS-B12970-50, 1:150; LSBio, www.lsbio.com), a monoclonal mouse anti-human antibody specific for CD57 (C6680-100TST, 1:500; Sigma-Aldrich, www.sigmaaldrich.com) and polyclonal rabbit anti-human antibody specific for granzyme B (ab4059, 1:400; Abcam) were used. The slides were incubated with the primary antibodies overnight at 4°C and with the secondary antibodies for 30 min (BrightVision Poly-HRP anti-mouse or anti-rabbit; Immunologic), followed by incubation with DAB solution and nuclear staining with haemalaun.

The primary antibody was omitted from negative controls and isotype controls were also used. A feline B-cell lymphoma from the ileocecal region was used as a negative control. Normal feline spleen, lymph node and small intestine served as positive controls.

Samples were categorized according to the WHO classification described by Valli *et al* [9]. All samples were evaluated by two independent experienced veterinary anatomical pathologists (AFB and TAD).

2.3. Flow cytometry

Immediately after surgery the tissue was transferred into a 15 ml vial (Greiner Bio-One, www.gbo.com) with 10 ml cell culture medium (RPMI; PAA Laboratories, www.gehealthcare.com) supplemented with 10% inactivated fetal calf serum (FCS; PAA Laboratories) and 100 U/ml penicillin/0.1 mg/ml streptomycin (PAA Laboratories). The samples were stored at 4°C for a maximum of 12 h until further processing depending on the time of surgery. A single cell suspension was prepared by mincing the tissue of the intestinal tract through a sieve (mesh size 40 μm). The single cell suspension was put in phosphate buffered saline (1 × PBS) without Ca^{2+} and Mg^{2+} (PAA Laboratories) and centrifuged at 1,300 U/min (353 *g*) for 6 min (Thermos Heraeus Multifuge 1S–R; Thermo Fisher Scientific) and resuspended in 1 ml PBS. For immunophenotyping aliquots of the single cell suspensions were labelled with monoclonal antibodies against CD3, CD4, CD5, CD8 and CD21 (respectively clones CD3-12, vpg34, FE1.1B11, vpg9, CA2.1D6; Bio-Rad, www.bio-rad.com). The viability dye eBioscience Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific) was used for live/dead discrimination. Cells only and corresponding isotype controls to all corresponding antibodies were used as controls. Most of the monoclonal antibodies (mABs) were directly conjugated to fluorochromes. For each analysis, 5×10^5 to 1×10^6 cells were incubated with the mAbs or the corresponding isotype controls for 20 min on ice. After a washing step (PBS without Ca^{2+} and Mg^{2+}) cells labelled with unconjugated mAbs or unconjugated isotype controls were incubated with anti-mouse secondary antibodies (Bio-Rad) and incubated for an additional 20 min on ice. After a final washing step all labelled cells were immediately analysed in a FACSCanto II Flow Cytometer (BD Biosciences, www.bdbiosciences.com). For fixation and permeabilization prior to labelling intracellular antigen anti-CD3, the IntraStain-Kit (Dako) was used according to the manufacturer's instructions.

Gating was performed for all samples using the forward scatter/side scatter (FSC/SSC) dot plot, representing the size and the granularity of the cells/events. The target lymphoid population was gated and the dead cells excluded by viability stain. The remaining living cells within the gate were used for analysis of their antigen expression. All samples were evaluated by the same experienced veterinary clinical pathologist (BCR).

3. Results

3.1. Patients

Fifteen cats fulfilled the inclusion criteria with presence of non-B-cell lymphoma solely in the small intestine and/or mesenteric lymph nodes, with macroscopic masses identified with abdominal ultrasound and additional FCM results. All animals were diagnosed at the University of Veterinary Medicine Vienna.

3.2. Immunophenotyping and cytotoxic granules

Eighty-seven percent (13/15) of the study cases were granzyme B positive, demonstrating a cytotoxic phenotype (Figs. 1 and 2). Cytotoxic granules were demonstrated by histopathology in 13% (2/15) and cytologically in 47% (7/15) of the slides. Visualization of cytotoxic granules corresponded with positive IHC results.

Antibodies against CD20 (IHC) and CD21 (FCM) were applied to assess B-cell immunophenotype; the respective results correlated with each other as all samples were devoid of labelling, thereby confirming the T-cell origin of lymphomas in 93% of cats. Only one cat was negative for CD3 (in IHC and FCM), CD20 and CD21. This

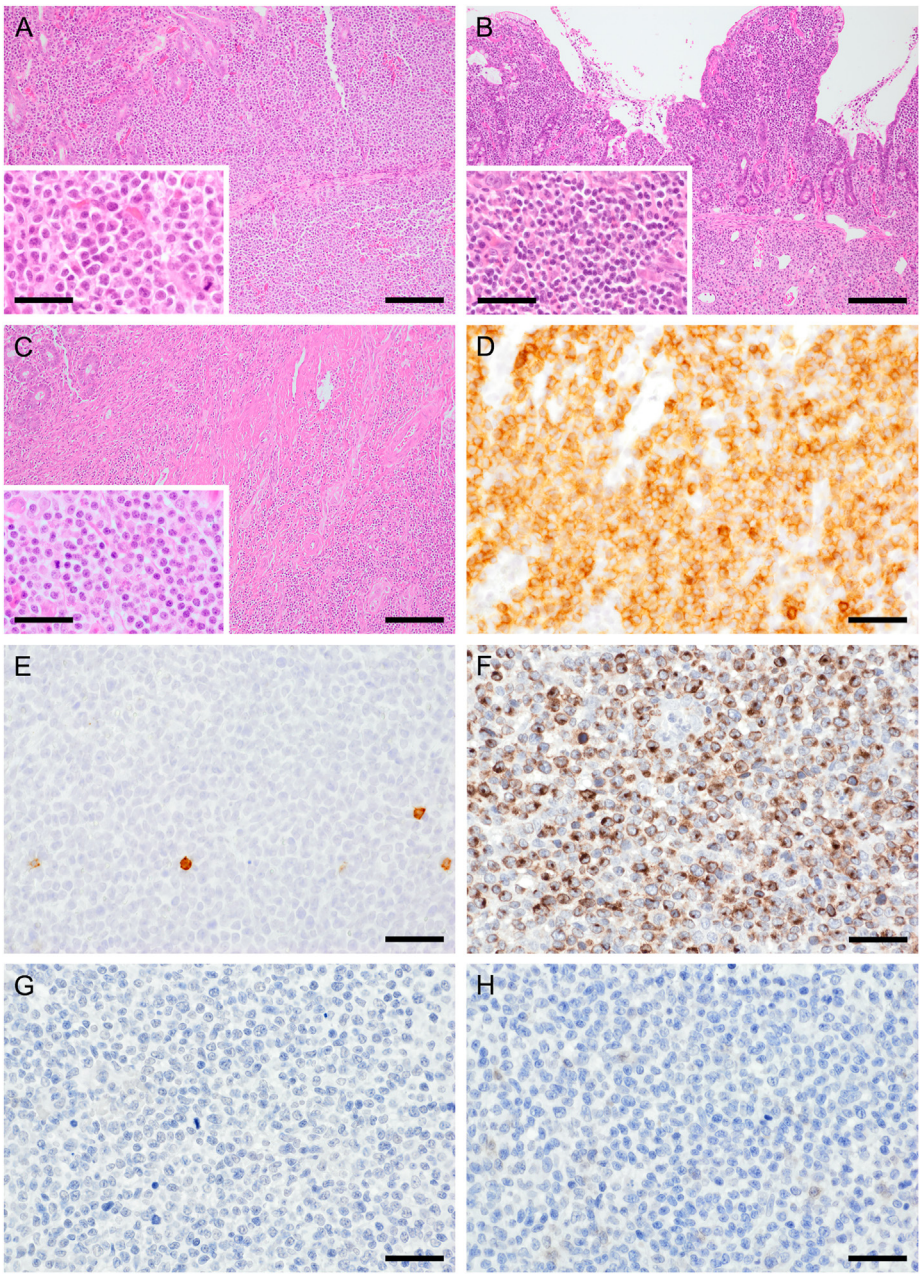

Fig. 1. T-cell lymphoma, small intestine, cat. (**A**) EATL/EATL type 1. Transmural infiltration of intestine with lymphoid cells that have large (>2 × diameter of an erythrocyte) round or irregularly folded nucleus with dispersed chromatin. HE. Bar, 160 μm. Inset: detail of neoplastic cells. HE. Bar, 40 μm. (**B**) iTLPD-GI/EATL type 2. Transmural infiltration of intestine with small lymphoid cells (<2 × diameter of an erythrocyte) that have chromatin-dense nuclei. HE. Bar, 160 μm. Inset: detail of neoplastic cells. HE. Bar, 40 μm. (**C**) ITCL (NOS)/PTCL, unspecified. Infiltration of intestinal submucosa and muscularis with intermediate-sized lymphoid cells (1.5–2 × diameter of an erythrocyte). HE. Bar, 160 μm. Inset: detail of neoplastic cells. HE. Bar, 40 μm. (**D**) Intense surface and membranous cytoplasmic immunopositivity for CD3. IHC. Bar, 40 μm. (**E**) Proliferating lymphoid cells immunonegative for CD20. IHC. Bar, 40 μm. (**F**) Intracytoplasmic immunopositivity for granzyme B. IHC. Bar, 40 μm. (**G**) Neoplastic cells immunonegative for CD56. IHC. Bar, 40 μm. (**H**) Neoplastic cells immunonegative for CD57. IHC. Bar, 40 μm.

sample expressed CD56, indicating a NK-cell lymphoma (Fig. 2). Immunolabelling results are presented in Table 1.

3.3. World Health Organization classification

In the classification of lymphoid neoplasms adopted by the WHO, as applied for use in animals [9], intestinal T-cell lymphomas of intraepithelial lymphocytes are classified as enteropathy-associated T-cell lymphoma (EATL). EATL type 1 (large cell neoplasm) is described as a commonly transmural neoplasm of lymphoid cells with a round or irregularly folded large nucleus (>2 × diameter of an erythrocyte). EATL type 2 (small to intermediate cell neoplasm) is most commonly a mucosal neoplasm of chromatin-dense, mature (nucleus <2 × diameter of an erythrocyte) lymphoid cells. The heterogeneous group of all non-specific T-cell lymphomas is encompassed under the basket term 'unspecified peripheral T-cell lymphoma' (PTCL) and NK-cell lymphomas are not further described in the adopted WHO classification.

According to this classification, 60% (9/15) of the cases in this study matched EATL type 1 and 13% (2/15) EATL type 2. In 20%

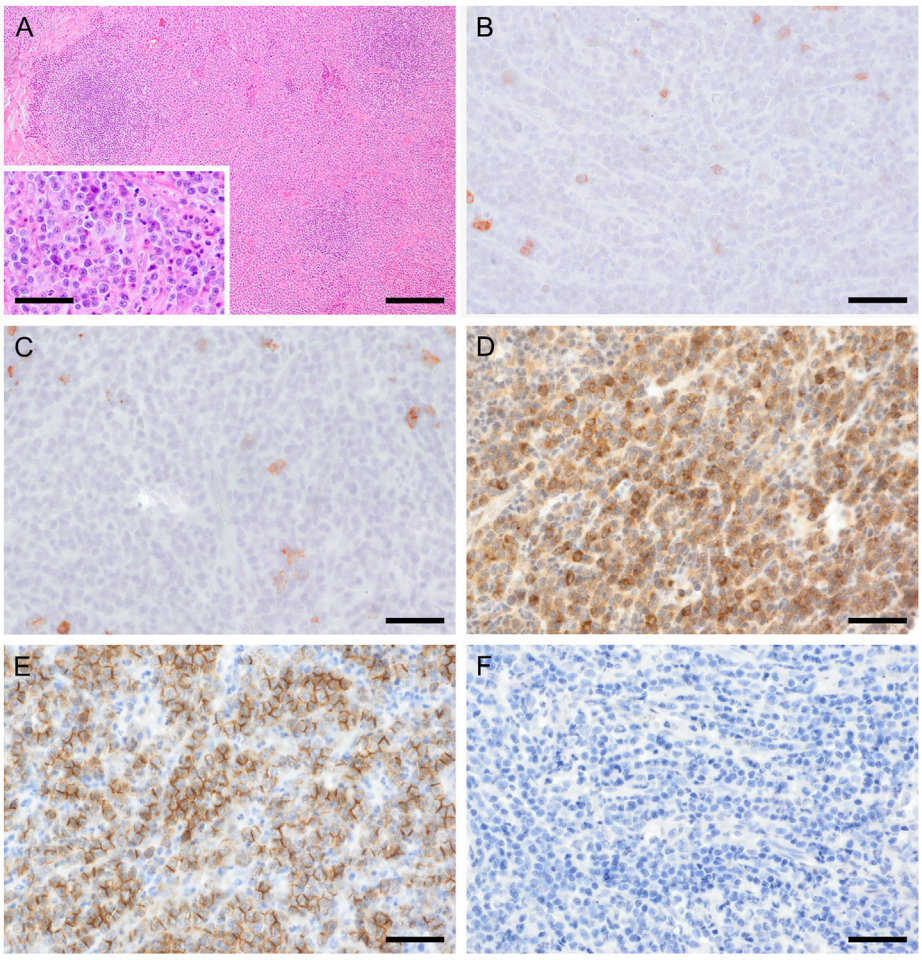

Fig. 2. NK-cell lymphoma, mesenteric lymph node, cat. (**A**) Diffuse infiltration of node with intermediate-sized lymphoid cells (1.5–2 × diameter of an erythrocyte) with round to indented nucleus, accompanied by eosinophils. Residual follicles present. HE. Bar, 160 μm. Inset: detail of neoplastic cells. HE. Bar, 40 μm. (**B**) Proliferating lymphoid cells immunonegative for CD3. IHC. Bar, 40 μm. (**C**) Proliferating lymphoid cells immunonegative for CD20. IHC. Bar, 40 μm. (**D**) Intracytoplasmic immunolabelling of neoplastic cells for granzyme B. IHC. Bar 40 μm. (**E**) Intense membranous immunopositivity for CD56. IHC. Bar, 40 μm. (**F**) Neoplastic cells immunonegative for CD57. IHC. Bar, 40 μm.

Table 1
Immunophenotypic distribution of lymphoma in cats

Immunophenotype	Technique	Number of cats	Percentage
CD3+	IHC	14	93
CD3+	FCM	14	93
Granzyme B+	IHC	13	87
CD4− CD8−	FCM	12	80
CD5+	FCM	3	20
CD8+	FCM	2	13
CD4+	FCM	1	7
CD56+	IHC	1	7
CD57+	IHC	0	0
CD4+ CD8+	FCM	0	0
CD21+	FCM	0	0
CD20+	IHC	0	0

CD, cluster of differentiation; IHC, immunohistochemistry; FCM, flow cytometry.

(3/15) of the cases the lymphoma did not seem to have arisen in the lamina propria or intraepithelial compartment (infiltration almost without involvement of the mucosa or only the mesenteric node was affected). In these cases, a diagnosis of a PTCL was made (Fig. 1). The CD56 immunopositive case (1/15) indicating a NK lymphoma had a severe diffuse infiltration of the mesenteric lymph node and, to a lesser degree, infiltration of the small intestinal lamina propria by lymphoid cells with an intermediate size (× 1.5–2 the diameter of an erythrocyte) round to indented nucleus accompanied by eosinophils and fibrosis (Fig. 2).

Epitheliotropism was observed in 11% (1/9) of EATL type 1, in 50% (1/2) of EATL type 2 but not in PTCL or NK-cell lymphoma. The number of mitotic figures in 10 high-power fields (2.37 mm^2, field number 22 mm) ranged from 25 to 117 in EATL type 1, 2 to 21 in EATL type 2, 30 to 87 in PTCL and was 19 in the NK-cell lymphoma.

The former [13] and more recent nomenclature [10] of the WHO classification used in human medicine and the current nomenclature of the WHO classification in veterinary medicine [9] is shown in Table 2. Proposed veterinary terminology adapted from human medicine and immunophenotypes of the individual patients are provided in Table 3.

4. Discussion

Lymphomas developing from cytotoxic T lymphocytes or NK cells can develop cytotoxic proteins such as granzymes. In feline neoplasms, granzyme B has been documented in a T-cell large granular lymphocytic (LGL) lymphoma in the brain [14] and in alimentary lymphomas [5,15]. Granzyme B immunopositivity was found in 87% of our feline intestinal lymphoma cases, contrasting with the findings of Ii *et al* [15], who reported 16% positivity for this marker. This discrepancy may be explained by study design, as Ii

Table 2
WHO classification of subtypes of the most important T-cell lymphoma entities in the gastrointestinal tract in human medicine and veterinary medicine

Current WHO in HM [10]	Former WHO in HM [13]	Current WHO in VM [9]
EATL CD3+, cytotoxic+, CD5−, CD4− CD8−, CD56−	**EATL** CD3+, cytotoxic+, CD5−, CD4− CD8−, CD56−	**EATL type 1** CD3+
MEITL CD3+, cytotoxic+, CD5−, CD4−, CD8+, CD56+	**Type II EATL** CD3+, cytotoxic+, CD5− CD4−, CD8+, CD56+	**EATL type 2** CD3+
iTLPD-GI CD3+, cytotoxic−/+, CD5+, CD4+ or CD8+, CD56−	not described	not described
ITCL (NOS) T-cell lymphomas that do not fit into a specific category	**PTCL (NOS)** T-cell lymphomas that do not fit into a specific category	**PTCL (Unspecified)** T-cell lymphomas that do not fit into a specific category

WHO, World Health Organization; HM, human medicine; VM, veterinary medicine; +, immunopositive; −, immunonegative.
EATL, enteropathy-associated T-cell lymphoma; MEITL, monomorphic epitheliotropic intestinal T-cell lymphoma; ITCL, intestinal T-cell lymphoma; iTLPD-GI, indolent T-cell lymphoproliferative disorder of the gastrointestinal tract; PTCL, peripheral T-cell lymphoma; NOS, not otherwise specified; cytotoxic, cytotoxic granule-associated proteins.

Table 3
Immunophenotype of feline lymphoma samples using IHC and FCM and their resulting diagnosis according to the current and a proposed WHO classification in veterinary medicine

No.	Immunophenotype	Current WHO classification in VM [9]	Proposed WHO classification in VM
1	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
2	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
3	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
4	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
5	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
6	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
7	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
8	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 1	EATL
9	CD3+ GB− CD5− CD4− CD8− CD56−	EATL type 1	EATL
10	CD3+ GB+ CD5− CD4− CD8− CD56−	EATL type 2	iTLPD-GI
11	CD3+ GB+ CD5+ CD4− CD8+ CD56−	EATL type 2	iTLPD-GI
12	CD3+ GB+ CD5− CD4− CD8− CD56−	PTCL unspecified	ITCL (NOS)
13	CD3+ GB+ CD5+ CD4− CD8− CD56−	PTCL unspecified	ITCL (NOS)
14	CD3+ GB− CD5+ CD4+ CD8− CD56−	PTCL unspecified	ITCL (NOS)
15	CD3− GB+ CD5− CD4− CD8+ CD56+		NK-cell lymphoma

WHO, World Health Organization; VM, veterinary medicine; +, immunopositive; −, immunonegative; EATL, enteropathy-associated T-cell lymphoma; ITCL, intestinal T-cell lymphoma; iTLPD-GI, indolent T-cell lymphoproliferative disorder of the gastrointestinal tract; PTCL, peripheral T-cell lymphoma.
NOS, not otherwise specified; NK, natural killer.

et al included larger numbers of small cell lymphomas (6% with granzyme B expression) as compared with large cell lymphomas (43% immunopositive for granzyme B) [15].

In a comprehensive series of 120 cases of feline gastrointestinal lymphoma, nine transmural T-cell lymphomas had morphological features of LGL differentiation, characterized by eosinophilic cytoplasmic granules. All nine LGL cases were immunopositive for granzyme B. However, as the other 94 T-cell lymphomas in the study were not stained with granzyme B, the percentage of positive cases may have been underestimated [5]. The difficulty in detecting cytoplasmic granules by histopathology on HE-stained slides has been described [16]. In the present study we found eosinophilic cytoplasmic granules in only 13% of the cases by histopathology. In contrast, cytoplasmic evaluation of cells obtained with fine needle aspiration revealed eosinophilic cytoplasmic granules in 47% of the slides. Nevertheless, the cytotoxic differentiation of a large portion of these feline intestinal T-cell lymphomas would have been overlooked without IHC, which revealed the presence of the cytotoxic protein granzyme B in 87% of the cohort.

According to the WHO classification for animals, 60% of the feline samples had characteristics of EATL type 1. Large lymphoid cells with a large round or irregularly folded nucleus transmurally infiltrated the intestinal wall. All EATL type 1 cases were immunonegative for CD4, CD8, CD5 and CD56. However, they were immunopositive for CD3, confirming T-cell immunophenotype, and all but one cat was granzyme B positive. In this way not only the morphological but also the immunophenotypic similarities to EATL in human medicine were striking. The human counterpart is characterized by the presence of CD3, CD7, CD103 and cytotoxic markers such as granzyme B, perforin or TIA1 and the absence of CD4, CD5, CD8 and CD56. EATLs are postulated to originate from small intestinal intraepithelial lymphocytes [10]. γδ T lymphocytes, which are much less common in adults than αβ T lymphocytes, have a predilection for epithelial-rich tissues such as the gastrointestinal tract [17]. Most intraepithelial γδ T lymphocytes in the human gut epithelium were described as CD4 and CD8 double negative [18]. This finding was confirmed in intraepithelial lymphocytes of juvenile and adult specific pathogen free cats, substantiating the hypothesis that EATL type 1 also originates from these cells in felines [19]. In veterinary medicine, the division of lymphoma subtypes into EATL types 1 and 2, based on the human medical classification, goes back to 2012 [5] and since 2016 has been included in the classification of lymphoid neoplasms adopted by the WHO, as applied for use in animals [9]. In human medicine, EATL was originally subdivided into EATL and type II EATL [13]. These terms have been changed to EATL and monomorphic epitheliotropic intestinal T-cell lymphoma (MEITL) [10]. An additional entity named indolent T-cell proliferative disorder of the gastrointestinal tract (iTLPD-GI) was also included in 2017 [10]. iTLPD-GI is a clonal T-cell disorder originating in the mucosa, mostly in the small intestine and the colon in humans. This subtype of lymphoma comprises monomorphic, small, CD3-positive lymphoid cells with round nuclei, inconspicuous nucleoli and scant pale staining cytoplasm, resembling small mature lymphocytes. In cats, similarities between EATL type 2 and iTLPD-GI have been observed, including morphology, cellular growth pattern, proliferation activity and

clinical outcome [6]. However, making a definitive diagnosis of this indolent low-grade lymphoproliferative disorder can be difficult in both humans and animals, given the overlapping histopathological aspects of inflammation and neoplasia [20,21]. In veterinary medicine, EATL type 2 is sometimes also named feline low-grade intestinal T-cell lymphoma (LGITL), although this name is not represented as a subtype in the WHO classification for animals. Recently, immunohistochemical characterization of 11 feline LGITL samples revealed consistent expression of CD3, CD56 negativity in 95% of the cases and variable CD4/CD8 expression, emphasizing similarities to the human subtype iTLPD-GI [12]. In humans the cells can also express CD4 or CD8 with rare double negativity or double positivity. CD8-positive cells usually are TIA1 positive but granzyme B negative, although exceptions exist. CD56 expression is typically negative with some exceptions. Additionally, other pan T-cell markers such as CD2, CD5 and CD7 are also often positive [22].

In the current study small lymphoid cells of two intestinal samples were CD3 positive, granzyme B positive and CD56 negative, thus showing more morphological and immunophenotypic similarities to iTLPD-GI than to MEITL (previous name type II EATL in human medicine). The typical immunophenotype of MEITL consists of positive labelling for CD3, CD8 and CD56. The cytotoxic marker TIA1 is usually expressed, but granzyme B or perforin staining is less common [10]. None of our lymphoma samples had such a staining pattern. Therefore, it would be less confusing to consider eliminating the term EATL type 2 in veterinary medicine and substituting it with another term (eg, iTLPD-GI) if the above criteria are met.

In human medicine the lymphoid cells of iTLPD-GI are described as being present in the lamina propria; however, dissemination into the muscularis mucosae and submucosa can occur, with absent full-thickness expansion and formation of tumour masses [20]. In veterinary medicine, Moore *et al* [5] described EATL type 2 as mucosal T-cell lymphoma with an infiltration above the muscularis mucosae and Valli *et al* [9] stated that this lymphoma type presents commonly as a mucosal tumour. Nevertheless, in a study with 22 cats with low-grade lymphoma sharing clinical, histopathological and immunophenotypic aspects with iTLPD-GI, Freiche *et al* [12] observed 68% infiltration of neoplastic lymphocytes into the submucosa, 48% into the muscularis and 24% into the serosa. Additionally, transmural infiltration occurred in four cases of small-cell type lymphoma in the study of Moore *et al* [5]. In the two cases of small cell intestinal lymphoma found in the current study, transmural infiltration of the tumour cells was also present. A limitation of this study was that due to the small number of patients, a statistical analysis comparing tumour behaviour and clinical outcome for the different lymphoma subtypes was not feasible. In the large study of Moore *et al* [5] the median survival time of cats with large-cell T-cell lymphoma was significantly shorter, with 1.5 months compared to 28 months in the small-cell T-cell lymphoma group. It has also been observed [15] that cats diagnosed with granzyme B-positive intestinal T-cell lymphoma had a shorter median survival time (169 days) than negative cases (965 days).

If a T-cell lymphoma of the alimentary tract in humans did not match a specific entity, the tumour was referred to as peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS) and more recently as intestinal T-cell lymphoma, not otherwise specified (ITCL, NOS) [10,13]. ITCL, NOS is not considered a distinct entity. Unreliable diagnoses due to suboptimal biopsies or fragmentary immunophenotypic results and borderline cases are confined to this group, keeping well-defined subtypes such as EATL or MEITL homogeneous [10]. In veterinary medicine, the term peripheral T-cell lymphoma, unspecified is currently used in the WHO classification [9]. In the present study PTCL, unspecified was diagnosed in 20% of cases with infiltration of the intestinal wall without involvement of the mucosa or if the mesenteric lymph node was the only site affected. Morphology and immunophenotype in this subset were relatively heterogeneous, which also has been described in human cases. Therefore, adoption of the term 'intestinal T-cell lymphoma, not otherwise specified' is a possible consideration for veterinary pathologists.

All the feline samples in the present cohort except one were immunopositive for CD3. Immunolabelling using ICH and FCM correlated fully with each other. CD3 is expressed on all T cells and, therefore, is the most reliable T-cell marker. The one exception that labelled negative for CD3 was immunopositive for granzyme B, CD8 and CD56. NK cells contain cytoplasmic granule molecules such as granzyme linked with cytotoxicity. They are also characterized by the absence of surface CD3 and by the expression of CD56 in humans and cats [23,24]. CD56 is considered a highly useful marker for identifying NK cells [10]. CD8 expression was detected on 30–40% of human [25] and on 10–20% of feline NK cells [24]. Thus, the one case described above could be assigned as NK-cell lymphoma, which is not designated as a specific subtype in the current WHO classification for animals. It seems that NK-cell lymphomas are rare in dogs and cats. We found only one CD56-positive case as did another study from 2021 [12], whereas all intestinal lymphomas were CD56 negative in a large study with 50 cats [15]. One case report described a lymphoma immunopositive for CD56 and immunonegative for CD3 and CD57, interpreted as a blastic NK-cell lymphoma [11].

The CD57 antigen is a marker for senescent T lymphocytes and mature NK cells [26]. In humans, T-cell large granular lymphocyte lymphocytic leukemia routinely expresses CD57 on mature cytotoxic T cells [10]. Little is known about the expression of CD57 in animals. In one case series, three of six cats with LGL lymphoma in the small bowel and lymph nodes were immunoreactive with an anti-CD57-like antibody [27]. One report of hepatosplenic lymphoma in a calf identified CD57 expression [28]. All our feline intestinal lymphoma cases were immunonegative for CD57.

Antibodies against CD57, CD56 and granzyme B work well on formalin-fixed, paraffin-embedded (FFPE) tissues of cats [5,11,12]. The assessment of CD4 and CD8 expression is much more difficult. Mouse anti-feline antibodies for evaluating CD4 and CD8 expression on frozen biopsies of cats can be used [12,16], although obtaining frozen samples is not routinely practised in most clinics and the quality of frozen sections is much lower than in FFPE tissues. Alternatively, FCM, performed in the current study, can be an option for assessing the CD4 and CD8 status in feline lymphoid tissues [29,30], although the equipment needed to perform FCM is not available to all veterinary clinicians and pathologists. Recently, a commercially unavailable rat antibody against CD8 was used in FFPE tissues of Japanese cats with intestinal lymphoma [15]. Intriguingly, 93% (13/14) of large T-cell lymphomas in the small intestine were CD8 positive. In contrast, none of the large T-cell lymphomas from our Austrian cat cohort expressed CD8. The discrepancy between these findings could be attributable to regional or case-related differences, therefore investigations in larger groups of cats are warranted.

5. Conclusion

We have demonstrated that most of the mass-forming lymphomas in the small intestinal tract of the investigated cats were CD4-/CD8-/CD56-/CD3+/granzyme B+, suggesting cytotoxic T-cell origin. Routine cytological and histological evaluation of these neoplastic populations would have overlooked the cytotoxic differentiation, suggesting that a larger immunohistochemical panel could improve the diagnostic workup of intestinal T-cell lymphomas in cats. Recommended panels include antibodies against

CD4 and CD8 for FCM and against CD20, CD3, CD56 and granzyme B for IHC.

Funding

Funding for this study was provided by the Department for Companion Animals and Horses and the Department for Pathobiology located at the University of Veterinary Medicine Vienna, Austria.

Acknowledgments

The authors thank Claudia Höchsmann, Madeleine Lunardi and Klaus Bittermann for their expert technical support.

Declaration of competing interests

The authors declared no conflicts of interest in relation to the research, authorship or publication of this article.

References

[1] MacVean DW, Monlux AW, Anderson Jr PS, Silberg SL, Roszel JF. Frequency of canine and feline tumors in a defined population. Vet Pathol 1978;15:700–15.
[2] Collette SA, Allstadt SD, Chon EM, Vernau W, Smith AN, Garrett LD, et al. Treatment of feline intermediate- to high-grade lymphoma with a modified university of Wisconsin-Madison protocol: 119 cases (2004–2012). Vet Comp Oncol 2016;14:136–46.
[3] Vail DM, Moore AS, Ogilvie GK, Volk LM. Feline lymphoma (145 cases): proliferation indices, cluster of differentiation 3 immunoreactivity, and their association with prognosis in 90 cats. J Vet Intern Med 1998;12:349–54.
[4] Pohlman LM, Higginbotham ML, Welles EG, Johnson CM. Immunophenotypic and histologic classification of 50 cases of feline gastrointestinal lymphoma. Vet Pathol 2009;46:259–68.
[5] Moore PF, Rodriguez-Bertos A, Kass PH. Feline gastrointestinal lymphoma: mucosal architecture, immunophenotype, and molecular clonality. Vet Pathol 2012;49:658–68.
[6] Wolfesberger B, Fuchs-Baumgartinger A, Greß V, Hammer SE, Gradner G, Knödl K, et al. World Health Organisation classification of lymphoid tumours in veterinary and human medicine: a comparative evaluation of gastrointestinal lymphomas in 61 cats. J Comp Pathol 2018;159:1–10.
[7] Valli VE, Jakobs RM, Norris A, Cuoto CG, Morrison WB, McCaw D, et al. The histologic classification of 602 cases of feline lymphoproliferative disease using the National Cancer Institute working formulation. J Vet Diagn Invest 2000;12:295–306.
[8] Chino J, Fujino Y, Kobayashi T, Kariya K, Goto-Koshino Y, Ohno K, et al. Cytomorphological and immunological classification of feline lymphomas: clinicopathological features of 76 cases. J Vet Med Sci 2013;75:701–7.
[9] Valli VEO, Kiupel M, Bienzle D. Hematopoietic system. In: Maxie MG, editor. Pathology of domestic animals. St. Louis: Elsevier Inc; 2016. p. 213–35.
[10] Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. Revised 4th ed., vol 2. Lyon: International Agency for Research on Cancer; 2017.
[11] Hirabayashi M, Chambers JK, Sugawara M, Ohmi A, Tsujimoto H, Nakayama H, et al. Blastic natural killer cell lymphoma/leukaemia in a cat. FMS Open Rep 2019;5:2055116919863080.
[12] Freiche V, Cordonnier N, Paulin MV, Huet H, Turba ME, Macintyre E, et al. Feline low-grade intestinal T cell lymphoma: a unique natural model of human indolent T cell lymphoproliferative disorder of the gastrointestinal tract. Lab Invest 2021;101:794–804.
[13] Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed., vol 2. Lyon: International Agency for Research on Cancer; 2008.
[14] Tsuboi M, Uchida K, Park ES, Kotera Y, Seki T, Takahashi M, et al. Systemic T cell large granular lymphocyte lymphoma with multifocal white matter degeneration in the brain of a Japanese domestic cat. J Vet Med Sci 2010;72:795–9.
[15] Ii T, Chambers JK, Nakashima K, Goto-Koshino Y, Mizuno T, Uchida K. Intraepithelial cytotoxic lymphocytes are associated with a poor prognosis in feline T-cell lymphoma. Vet Pathol 2022;59:931–9.
[16] Roccabianca P, Vernau W, Caniatti M, Moore PF. Feline large granular lymphocyte (LGL) lymphoma with secondary leukemia: primary intestinal origin with predominance of a CD3/CD8(alpha)(alpha) phenotype. Vet Pathol 2006;43:15–28.
[17] Shiromizu CM, Jancic CC. γδ T lymphocytes: an effector cell in autoimmunity and infection. Front Immunol 2018;9:2389.
[18] Lundqvist C, Baranov V, Hammarström S, Athlin L, Hammarström ML. Intraepithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 1995;7:1473–87.
[19] Roccabianca P, Woo JC, Moore PF. Characterization of the diffuse mucosal associated lymphoid tissue of feline small intestine. Vet Immunol Immunopathol 2000;75:27–42.
[20] Sanguedolce F, Zanelli M, Zizzo M, Luminari S, Martino G, Soriano A, et al. Indolent T-cell lymphoproliferative disorders of the gastrointestinal tract (iTLPD-GI): a review. Cancers 2021;13:2790.
[21] Marsilio S, Freiche V, Johnson E, Leo C, Langerak AW, Peters I, et al. ACVIM consensus statement guidelines on diagnosing and distinguishing low-grade neoplastic from inflammatory lymphocytic chronic enteropathies in cats. Vet Intern Med 2023;37:794–816.
[22] Soderquist CR, Patel N, Murty VV, Betman S, Aggarwal N, Young KH, et al. Genetic and phenotypic characterization of indolent T-cell lymphoproliferative disorders of the gastrointestinal tract. Haematologica 2020;105:1895–906.
[23] Greer JP, Mosse CA. Natural killer-cell neoplasms. Curr Hematol Malig Rep 2009;4:245–52.
[24] Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Grauwet KL, et al. Natural killer cells: frequency, phenotype and function in healthy cats. Vet Immunol Immunopathol 2012;150:69–78.
[25] Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood 1990;76:2421–38.
[26] Kared H, Martelli S, Ng TP, Pender SL, Larbi A. CD57 in human natural killer cells and T-lymphocytes. Cancer Immunol Immunother 2016;65:441–52.
[27] Darbès J, Majzoub M, Breuer W, Hermanns W. Large granular lymphocyte leukemia/lymphoma in six cats. Vet Pathol 1998;35:370–9.
[28] Nozaki S, Sasaki K, Ando M, Kadota K. Natural killer-like T-cell lymphoma in a calf. J Comp Pathol 2006;135:47–51.
[29] Bernardi S, Martini V, Perfetto S, Cozzi M, Comazzi S. Flow cytometric analysis of mediastinal masses in cats: a retrospective study. Front Vet Sci 2020;7:444.
[30] Rütgen BC, Baszler E, Weingand N, Wolfesberger B, Baumgartner D, Hammer SE, et al. Composition of lymphocyte subpopulations in normal and mildly reactive peripheral lymph nodes in cats. J Feline Med Surg 2022;24:77–90.