Gamma delta ($\gamma\delta$) T-cell large granular lymphocyte (LGL) lymphoma in a dog

Short Title: $\gamma\delta$ T-cell LGL lymphoma in a dog

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Abstract

A 2-year-6-month-old female neutered Labrador Retriever with Horner syndrome, megaesophagus and a mediastinal mass was referred to the Queen Mother Hospital for Animals of the Royal Veterinary College. A large granular lymphocyte (LGL) lymphoma was diagnosed on cytology; flow cytometry analysis revealed a $\gamma\delta$ -T-cell phenotype (CD3+, CD5+, CD45+, TCR $\gamma\delta$ +, CD4-, CD8-, CD34-, CD21-). Chemotherapy was started with a LOPP protocol (a combination of lomustine, vincristine, procarbazine and prednisolone) followed by bleomycin. Euthanasia was elected by the owners, due to progressive deterioration and lack of quality of life, 28 days after diagnosis. This is the first cytological and immunophenotypic characterization of a canine $\gamma\delta$ T-cell LGL lymphoma with probable mediastinal origin. The role of chemotherapy in delaying the disease progression is still unknown.

Keywords: Flow cytometry, lymphoid tumour, canine

Word count: 2094

Case Presentation

A 2-year 6-months-old female Labrador Retriever presented with a two-week history of retching and coughing. The dog was treated for presumptive kennel cough but failed to improve. Thoracic radiographs revealed soft tissue opacity in the cranioventral thoracic region, with broad effacement of the cardiac silhouette and presence of mild pleural effusion. The patient was subsequently referred to the Queen Mother Hospital for Animals at the Royal Veterinary College for further investigations. On physical examination, vital parameters were normal, but cardiac auscultation revealed bilateral dull heart sounds. On abdominal palpation, cranial organomegaly was suspected. Horner's syndrome was present in the left eye with enophthalmos, miosis, and third eyelid

protrusion. Moderate muscle atrophy of the right temporal muscle was also noted. Neurologic examination localized the lesion in the right trigeminal nerve and sympathetic innervation to the left eye.

Hematology was unremarkable. Biochemical abnormalities included moderate hyperbilirubinemia (10.0 umol/l; reference interval: 0.0-2.4 umol/l) and moderate elevation in alanine aminotransferase (ALT) (255 U/l; reference interval: 13-88 U/l). A free-catch urinalysis revealed poorly concentrated urine (USG: 1.026), haematuria (50 red blood cells per high power field (HPF) (x400), reference interval: 0-5 rbc/hpf), and pyuria (50 white blood cells per HPF (x400), reference interval 0-5 wbc/hpf). A urine culture was performed and no growth was observed.

Thoracic radiographs and computed tomography (CT) scan of the thorax and abdomen revealed a large mass (measuring W9.6 x H7.4 x L10cm) on the ventral aspect of the cranial mediastinum, displacing the trachea and the cranial vena cava dorsally and to the right (Figure 1). Megaesophagus was also present. Cranial mediastinal, tracheobronchial and axillary lymph nodes were mildly enlarged. CT of abdomen revealed no abnormalities. Ultrasound guided fine needle aspirations (FNA) of the mediastinal mass, liver and spleen were obtained.

Cytology of the mediastinal mass revealed the presence of a mixed population of lymphoid cells, the majority being intermediate to large in size (70%) (Figure 2). The nucleus was round, occasionally indented, paracentral to eccentrically placed, with coarse granular chromatin, rarely showing poorly distinct multiple round nucleoli. The cytoplasm was moderate in amounts, basophilic, frequently containing multiple small intracytoplasmic magenta granules. A few atypical mitotic figures were also present. These results were consistent with LGL lymphoma. Cytology from the liver was characterized by very rare clusters of well differentiated hepatocytes, together with an increased number of intermediate size lymphoid cells containing intracytoplasmic magenta granules similar to those described in the mediastinal mass. Occasional macrophages, non-

degenerate neutrophils and rare eosinophils and mast cells were also seen. Fine needle aspiration of the spleen also showed a similar lymphoid population confirming the splenic involvement by the LGL lymphoma. According to these results, and given the normal lymphocytic count in the peripheral blood, the absence of atypical circulating lymphoid cells and the lack of cytopenias in the blood, the patient was classified as stage IVb of the WHO (World Health Organization) staging system for lymphoma in domestic animals ¹.

To further characterize this lymphoproliferative disorder, flow cytometry was performed on samples obtained by fine needle aspiration of the mediastinal mass (Figure 3). This was performed with a three-color analysis system using a BD Accuri C6 flow cytometer and CFlow Plus software (Becton-Dickinson, Franklin Lakes, NJ, USA). Cells were labeled using monoclonal antibodies against extracellular antigens: CD5-FITC (clone YKIX322.3, T-lymphocytes), CD21-PE (clone CA21D6, B-lymphocytes), and CD45-AlexaFluor 647 (clone YKIX716.13, all leukocytes) in one tube; CD3-FITC (clone CA17.2A12, T-lymphocytes), CD8-PE (clone YCATE55.9, T-cytotoxic), and CD4-AlexaFluor 647 (clone YKIX302.9, neutrophils and T-helper lymphocytes) in a second tube; TCR $\gamma\delta$ (clone CA20.8H1, T subpopulation) followed by a labeling step with FITC-conjugated rabbit anti-mouse IgG in a third tube. All antibodies were from Serotec (Oxford, UK) except for TCR $\gamma\delta$ provided by Leukocyte Antigen Biology Laboratory (UC Davis, CA, USA). Additional single color analysis for CD11d (clone CA11.8H2, AbD Serotec, USA) was performed from the University of Cambridge with a FACSCalibur flow cytometer and CellQuest software (Becton-Dickinson, Franklin Lakes, NJ). Gated lymphoid cells expressed CD3, CD5, CD45 and $\gamma\delta$ -TCR, and were negative to the other antibodies tested (CD4, CD8, CD11d, CD21), confirming a $\gamma\delta$ T cell origin.

The patient was started on a modified LOPP protocol (a combination of lomustine, vincristine, procarbazine and prednisone) and clinical management of megaesophagus was instructed. To rule out myasthenia gravis as cause of the megaesophagus, testing for AChRAbs (Anti-Acetylcholine

Receptor Antibody Diagnostic Testing) was performed and was negative. 2M antibody test to rule out masticatory myositis was also performed, and was also negative. Clinical improvement was initially noted, but 4 days later the dog re-presented with increased frequency of cough. Repeated thoracic radiographs were consistent with aspiration pneumonia, likely due to the megaesophagus, and the cranial mediastinal mass appeared reduced in size, although interpretation was difficult due to the presence of consolidated lung lobes. This finding impacted the decision of continuing with the LOPP protocol, due to the potential immunosuppressive adverse effects of it, and lead to the decision of treating the patient with antimicrobials for the pneumonia and bleomycin for the lymphoma control. Bleomycin has not being extensively assessed as therapy for canine lymphoma, although anedoctical reports suggest positive results in relapsed canine lymphoma, as well as lack of immunosuppression ². Two doses were administered (500 IU/kg) subcutaneously two days apart. Unfortunately the patient worsened and the owners elected for euthanasia. A post mortem examination was declined by the owners.

The survival time from diagnosis was 28 days, or 27 days from the start of the chemotherapy treatment.

Discussion

Lymphoma represents one of the most common neoplasms in the dog. It comprises approximately 7 to 24% of all canine neoplasias and 83% of all haematopoietic tumors ³.

In the last twenty years immunophenotyping has become a powerful tool in classifying and approaching canine lymphoma, since T-cell high-grade multicentric forms have been reported to have a worse prognosis with shorter survival times after chemotherapy when compared with B-cell high grade lymphomas ⁴⁻⁸. Further studies have proven the existence of other subpopulations of

lymphoid cells (NK lymphocytes, $\gamma\delta$ T lymphocytes, LGL lymphocytes), which may also undergo monoclonal expansion and generate lymphoproliferative diseases ^{9,10}.

$\gamma\delta$ T-cells represent a small group of post-thymic T-cells that have a distinct T-cell receptor (TCR) on their surface. The majority of T-cells ($\alpha\beta$ T-cells) have a TCR composed of one α chain and one β chain (TCR $\alpha\beta$). In contrast, $\gamma\delta$ T-cells have a TCR characterized by one γ chain and one δ chain (TCR $\gamma\delta$). In humans, $\gamma\delta$ T lymphocytes are mostly localized in the spleen, although low numbers have been found in lymph nodes and some epithelial rich tissues ¹¹. Similar localization of $\gamma\delta$ lymphocytes has been reported in the canine species ¹²⁻¹⁴. The precise function of $\gamma\delta$ lymphocytes is still unclear, although these cells have been proposed to have a role in the first line defense in the epidermal and mucosal epithelial lining ¹⁵. Lymphomas exhibiting a $\gamma\delta$ TCR phenotype include T-cell LGL lymphoma/leukemia. LGL lymphoma/leukemia is a morphological subtype of lymphoma characterized by lymphoid cells containing intracytoplasmic azurophilic granules ⁹. The exact origin of LGLs is unknown, however, studies have shown that large granular lymphocytes may belong at several distinct cell lines, including T lymphocytes (CD3+, mostly CD8+), and expressing either TCR $\alpha\beta$ or TCR $\gamma\delta$, and NK lymphocytes ^{9,16,17}.

In human medicine, $\gamma\delta$ lymphoma is a rare, well-defined entity. WHO classification recognizes two main $\gamma\delta$ lymphoma entities: hepatosplenic T-cell lymphoma (HSTL) and primary cutaneous $\gamma\delta$ T-cell lymphoma (PCGD-TCL). HSTL is characterized by a highly aggressive clinical course and poor prognosis ¹⁸. The presenting clinical features described in the literature are usually limited to constitutional symptoms and hepatosplenomegaly. Cytopenias are frequently observed in the peripheral blood (mainly anemia and thrombocytopenia). Interestingly, the neoplasia is usually confined to the spleen and the liver; peripheral lymphadenomegaly is uncommon, and neoplastic circulating lymphoid cells are usually not observed ¹⁸.

The incidence of $\gamma\delta$ T-cell lymphoma in dogs is reported to be low, with only a few cases reported in veterinary literature^{12,13,19,20}. Only one case report of $\gamma\delta$ T-cell lymphoma with presumed mediastinal involvement exists; nevertheless the mediastinal mass was not analyzed. In this case, immunophenotype was determined by flow cytometry performed on a sample obtained from the left prescapular lymph node (CD45+, CD3+, $\gamma\delta$ TCR +, CD4-, CD8-) and no LGL morphology was reported.¹⁹ To the authors' knowledge, this is the first case report of a $\gamma\delta$ T-cell LGL lymphoma and also the first report with cytologic and flow cytometric/immunophenotypic characterization of a $\gamma\delta$ T-cell LGL lymphoma with probable mediastinal origin.

The real incidence of canine $\gamma\delta$ lymphomas may be underestimated since the availability of the antibody for $\gamma\delta$ TCR is limited and a lymphoma CD3+, CD4-, CD8- may be considered as a T-cell lymphoma with aberrant expression of CD4/CD8 or referred to as an early T lymphoma. The majority of $\gamma\delta$ lymphomas in humans and dogs have a splenic origin while in our patient we suspect a mediastinal or a hepatic origin supported by the fact that the tumor cells were CD11d negative. CD11d is expressed on large granular lymphocytes and on macrophages and T-cells in the splenic red pulp. CD11d positive cells seem to constitute 30% of splenic T-cells and are almost exclusive localized on the red pulp¹³. As in the present case T-cells were CD11d negative, a primary hepatic or a mediastinal origin instead of splenic must be considered. The presence of hyperbilirrubinemia, increased AST and the infiltration of the liver with neoplastic lymphocytes, confirms the involvement of this organ, but due to the widespread of the disease at presentation, the origin of the tumor is uncertain in this case. Splenomegaly and hepatomegaly were not seen, differently from what has been reported in literature^{12,13}, which may reflect a less severe infiltration of these organs. Furthermore, the absence of hepatomegaly could support a mediastinal origin in this case.

Complete blood cell count and blood smear evaluation were unremarkable, while patients of the previously reported cases, showed anemia, thrombocytopenia and leukopenia.^{12,13,19,20} Human

hepatosplenic $\gamma\delta$ T-cell lymphomas also generally present with cytopenias (mostly anemia and thrombocytopenia). The lack of hematologic abnormalities in the current case might reflect a lower stage of the disease compared to the previously reported cases, with a less severe infiltration of the hematopoietic organs such as spleen and bone marrow, or just a different biological behavior.

The patient was classified as stage IVb of the WHO (World Health Organization) staging system for lymphoma in domestic animals. However, it is not possible to completely rule out a stage V of the disease, as bone marrow aspiration was not performed; therefore, despite the lack of circulating cells and of cytopenias, bone marrow infiltration could not be assessed. The WHO staging system is the only available staging system for canine lymphoma and was therefore used to classify the patient's clinical stage in the present case, although there are some controversies as to whether this staging system can only be used for multicentric lymphoma.

The patient was euthanatized due to deterioration (presence of pneumonia, frequent regurgitation) 28 days after diagnosis, despite the fact that the mediastinal mass was seen to be decreasing in size with treatment. Previous reports also show a poor prognosis especially when treatment is not attempted ¹². However, in a previous reported case of $\gamma\delta$ T-cell lymphoma with involvement of peripheral lymph nodes and presence of a mediastinal mass, the patient was alive 24 months after diagnosis, being in remission on week number 10 of a Madison Wisconsin protocol¹⁹. In another study, one dog that received chemotherapy treatment (prednisone, chlorambucil, cyclophosphamide, and CCNU, alone or in combination) survived for 196 days ¹². Given the limited number of cases, any conclusion regarding the efficacy of chemotherapy for the treatment of $\gamma\delta$ lymphoma cannot be made.

Interestingly, all the reported cases had substage b at the presentation, which has being historically considered a strong negative prognostic factor ²²⁻²⁵. This could support the hypothesis that $\gamma\delta$ lymphomas may represent a more aggressive subtype of lymphoma, thus carrying a poor prognosis,

as is already demonstrated in human medicine ¹⁸. Further studies with a series of cases are therefore warranted.

This case report also strengthen the evidence that flow-cytometry is a useful tool for assessing canine lymphomas and that broad panels of antibodies should always be applied in order to precisely characterize different subtypes of what has being historically considered just one single disease. It may be important to investigate a possible $\gamma\delta$ status for all CD3+ lymphomas, especially those resulting CD4-CD8- or CD8+, as a $\gamma\delta$ T -cell lymphoma seems to be related with rapid progression of the disease, and may be associated with a poor prognosis.

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Figures

Figure 1. CT scan image (transversal view) demonstrating a large mass (measuring approximately W9.6 x H7.4 x L10cm) on the ventral aspect of the cranial mediastinum. The mass slightly homogeneously enhances the contrast.

Figure 2. Fine-needle aspirate from a mediastinal mass of a dog with $\gamma\delta$ T-cell LGL lymphoma. (A-B) Monomorphic population of large lymphoid cells containing variable numbers of azurophilic intracytoplasmic granules and atypical mitotic figures. Wright-Giemsa, x50-100 objective.

Figure 3. Results of a flow cytometry analysis from a mediastinal mass of a dog with $\gamma\delta$ T-cell LGL lymphoma. Dot plot on the top left showing cell size on x-axis (FSC: forward scatter) and cell complexity on the y-axis (SSC: side scatter). Further dot plots clockwise indicating expression of several lymphoid markers from the gated cell population (TCR $\gamma\delta$, CD3, CD4, CD5, CD8, CD21, CD45).