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1 **Diagnosis of anaplastic large-cell lymphoma in a dog using CD30**

2 **immunohistochemistry**

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21 Short running title: Diagnosis of canine ALCL using CD30 immunohistochemistry

22

23 **Abstract.** Anaplastic large-cell lymphoma or null-cell lymphoma is a clinical entity reported
24 in people, classified according to the unique appearance of large pleomorphic cells that
25 express CD30. Null-cell lymphoma has also been described in dogs when neither CD3 nor
26 CD79α is expressed by the tumor. We describe a case of lymphoma in the dog in which
27 neoplastic cells did not express routine B- or T-lymphocyte markers on flow cytometry or
28 immunohistochemistry; however, cells immunohistochemically labeled for CD30. The dog in
29 our case died 5 mo after initial presentation, confirming a poor prognosis. Identification of
30 further similar cases in dogs would provide additional prognostic information for this subset
31 of lymphomas. CD30 may also serve as a potential therapeutic target in anaplastic large-cell
32 lymphomas.
33
34 **Key words:** CD30; dogs; immunohistochemistry; lymphoma; null-cell lymphoma.
35

A 10-y-old male neutered Beagle dog was presented to North Downs Specialist Referrals (Bletchingley, Surrey, UK) with unilateral right-sided mandibular lymphadenopathy. Prior medical history included dorsal laminectomy and disc fenestration of an intervertebral disc extrusion (T13/L1) the preceding year. He had also been diagnosed with sciatic neuritis by histologic examination of a sciatic nerve biopsy specimen 9 mo prior to presentation. The dog had received intermittent treatment with prednisolone and cyclosporine. Three months before presentation, the dog developed severe anemia as a result of gastrointestinal hemorrhage, and prednisolone therapy was stopped.

Hematologic examination at the time of presentation indicated no evidence of cytopenias, and cytologic evaluation of a fine-needle aspirate (FNA) of the enlarged mandibular lymph node was carried out by a board-certified veterinary clinical pathologist (B Szladovits). The nucleated cell population was dominated by medium-to-large lymphocytes (Fig. 1). Cells had mostly round, occasionally indented, and rarely lobed, central-to-paracentral nuclei that were typically 1.5–2× and occasionally ≥3× the size of red blood cells. There was moderate anisokaryosis and a finely granular to mildly clumped chromatin pattern. Nuclei had moderately distinct, small-to-medium–sized nucleoli, and occasional cells had a single large nucleolus. Cells had unusually large amounts of mid-blue cytoplasm with low-to-moderate numbers of small vacuoles, and prominent perinuclear clearing (Golgi zone), commonly around the entire nucleus. The mitotic rate was high (≥3 per five 40× fields). Small lymphocytes were rare; occasional neutrophils, rare eosinophils, and rare plasma cells were present. A diagnosis of lymphoma was considered most likely; however, the cellular morphology was considered unusual given the large amount of cytoplasm within the cells. Thoracic radiography and abdominal ultrasonography were unremarkable. Bone marrow evaluation was not performed, and the patient was diagnosed as having stage 1 lymphoma

60 according to the World Health Organization's clinical staging system for lymphoma in
61 domestic animals.[4]

62 Flow cytometric analysis was carried out on the FNA samples of the enlarged lymph
63 node in order to immunophenotype the lymphoma. Samples were stained with canine-specific
64 or cross-reactive fluorochrome-conjugated monoclonal antibodies (mAbs) against both extra-
65 and intracellular antigens (Table 1). Seven-color staining was performed. Cells were stained
66 with mAbs for cell surface antigens for 20 min. Following incubation in buffer (Intracellular
67 fixation and permeabilization buffer, Thermo Fisher Scientific, Paisley, UK) overnight, the
68 cells were stained with mAbs against intracellular antigens for 30 min. All incubations were
69 performed at 4°C in the dark, and cells were washed twice following each of the incubation
70 periods. Flow cytometric data were acquired (FACS Canto II flow cytometer, BD
71 Biosciences, Oxford, UK) and analyzed (Flowjo software, Tree Star, Ashland, OR). All gates
72 were defined by using isotype and "fluorescence minus one" controls. Flow cytometric
73 analysis of viable cells of the FNAs yielded the impression of a predominantly medium-sized
74 population, consistent with the cytologic appearance. The population was negative for all
75 lymphoid antigens, with a high proportional expression of the proliferation marker Ki-67.

76 The enlarged lymph node was surgically excised and, following fixation in 10%
77 neutral-buffered formalin, was processed routinely and embedded in paraffin wax. Sections
78 (4 µm) were stained with hematoxylin and eosin. Histologically, ~90% of the node was
79 effaced by a dense, highly infiltrative, nonencapsulated, poorly differentiated round-cell
80 neoplasm. The neoplastic cells were round-to-oval with distinct cell borders and variable
81 amounts of eosinophilic cytoplasm that usually contained a single, hyperchromatic nucleus
82 that was 2–3× the size of a red blood cell. There was marked anisocytosis and anisokaryosis,
83 including frequent macrokaryosis (Fig. 2). Frequently, some cells exhibited large vesicular
84 nuclei with distinct indentations or binucleation with multiple distinct, magenta, ovoid

85 nucleoli. Fifteen mitotic figures were observed within 10 high-power (400×, 2.37 mm^2)
86 fields, including occasional bizarre mitoses. Numerous individual neoplastic cells were either
87 necrotic or apoptotic. There were many fewer small (mature) lymphocytes within the
88 remaining nodal architecture and large central areas of coagulative necrosis (tumor necrosis
89 and/or the result of prior FNA sampling). The capsule was thickened by fibrous connective
90 tissue that was infiltrated by moderate numbers of small (reactive) lymphocytes and small
91 foci of neutrophils. Subcapsular and medullary sinuses were congested or contained low
92 numbers of hemosiderophages. The neoplastic tissue appeared contained by the thickened
93 capsule within the sections examined. A diagnosis of high-grade large-cell lymphoma was
94 given.

95 Immunohistochemistry (IHC) was performed on serial sections (Bond-Max autostainer,
96 Bond polymer refine detection system, Leica Biosystems, Newcastle-upon-Tyne, UK).
97 Primary antisera were specific for CD3 (polyclonal rabbit anti-human, Dako, Ely, UK; 1 in
98 500 dilution; antigen retrieval in buffer pH 9.0 buffer [ER2, Dako] for 30 min); CD79α
99 (monoclonal mouse anti-human, Dako; 1 in 100 dilution; antigen retrieval in pH 9.0 buffer
100 [ER2] for 10 min); CD18 (mouse anti-canine, clone CA1.4E9, University of California
101 Davis, CA; 1 in 20 dilution; with enzymatic antigen retrieval [Enzyme 1, Dako] for 10 min);
102 and CD30 (monoclonal mouse anti-human, clone Ber-H2, Dako; 1 in 30 dilution; antigen
103 retrieval in pH 9.0 buffer for 20 min). Immunohistochemically, the neoplastic cells diffusely
104 exhibited specific membranous labeling for CD30 (Fig. 3) and did not express CD3, CD79α,
105 or CD18. Negative (Fig. 4) and positive controls were processed with the evaluated slides and
106 were labeled appropriately. A diagnosis of high-grade, poorly differentiated null-cell
107 lymphoma or anaplastic large-cell lymphoma (ALCL) was given.

108 Two months after lymph node removal, lymphadenopathy was noted in the same
109 anatomic site. Cytologic evaluation confirmed a diagnosis of lymphoma. Blood evaluations,

110 thoracic radiography, and abdominal ultrasonography were repeated. Bone marrow aspirate
111 and biopsy of inflamed gingivae were also performed with no evidence of lymphoma at other
112 sites.

113 Treatment was implemented with a modified L-CHOP protocol. No measurable
114 improvement was noted in response to single L-asparaginase (400 IU/kg IM; Medac, Wedel,
115 Germany), vincristine (0.7 mg/m^2 IV; Hospira UK, Warwickshire, UK), or
116 cyclophosphamide (250 mg/m^2 PO; Star Pharmaceuticals, Harrow, UK) treatments, so the
117 protocol was abandoned. Further treatments using doxorubicin (30 mg/m^2 IV; Pharmachemie,
118 Haarlem, The Netherlands), lomustine (70 mg/m^2 PO; Medac), and chlorambucil (5 mg/m^2
119 q48h PO; Aspen Pharma, Dublin, Ireland) also failed to achieve a beneficial effect. The
120 patient underwent a course of palliative radiation therapy (5× once wk 7 Gy fractions) by
121 which time lymphoma was also noted in the left mandibular and the right prescapular lymph
122 nodes. All detectable lymphoid tissues responded favorably to radiotherapy; however,
123 complete remission was not achieved.

124 While receiving treatment, a mass arose on the left upper eyelid and, 2 d after
125 completion of the radiotherapy course, the patient showed markedly reduced alertness. The
126 patient was anesthetized for MRI of the head and neck and cerebrospinal fluid analysis,
127 which gave no indication of lymphoma in the central nervous system; however, there were
128 multiple enlarged lymph nodes of the head and neck. The eyelid mass was removed and was
129 composed of neoplastic cells similar to those described within the mandibular lymph node. A
130 plasma cell origin to this neoplasm was also considered at this stage; however, cells did not
131 exhibit immunohistochemical labeling for MUM-1 (performed at an external commercial
132 laboratory; MUM-1/IRF-4 monoclonal mouse anti-human, Dako; 1 in 100 dilution; antigen
133 retrieval in pH 9.0 buffer [Envision FLEX target retrieval solution, Dako] for 30 min).
134 Neoplastic cells exhibited similar expression of CD30 and did not express CD3 or CD79a.

135 The patient failed to make a satisfactory recovery from anesthesia and died 3 d later. Autopsy
136 was not performed.

137 ALCL was first recognized as a clinical entity in people in 1985 and was classified
138 according to the unique appearance of large pleomorphic cells that express CD30, originally
139 termed Ki-1.[3] ALCL has previously been described in dogs, with 0.8% of 608 cases of canine
140 lymphoma being classified as null-cell type given the lack of expression of either CD3 or
141 CD79α.[6] Further reports of null-cell lymphomas in dogs are rare. The use of CD30 in dogs
142 has been described in a study characterizing testicular neoplasms, in which CD30 was not
143 expressed,[8] and in a case of canine pulmonary lymphomatoid granulomatosis in which a
144 population of cells expressed CD30.[5] CD30 is a transmembrane protein receptor, of the tumor
145 necrosis factor receptor superfamily, which is normally expressed by activated B- or T-
146 lymphocytes.[2,7] Upon activation, CD30 influences cell growth and survival.[1]

147 We suggest that in cases in which lymphoma is strongly suspected histologically,
148 despite neoplastic cells failing to express routine T- or B-lymphocyte markers, CD30 may be
149 used to provide a diagnosis of null-cell lymphoma or ALCL. Given positive CD30 expression
150 and the unique appearance of large, pleomorphic neoplastic cells, a diagnosis of ALCL was
151 made. Identification of further cases of ALCL in dogs would provide additional prognostic
152 information for this subset of lymphomas. Therapeutics that target CD30 in people with
153 Hodgkin lymphoma and ALCL have been developed.[1] The dog in our case had a survival
154 time of 5 mo following initial presentation, despite both chemotherapy and radiotherapy,
155 confirming a poor prognosis.

156 Declaration of conflicting interests

157 The authors declare no potential conflicts of interest with respect to research, authorship, and
158 publication of this article.

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178

179 **Table 1.** Flow cytometry: staining antibodies, isotype controls, and fluorochromes.

Antigen	Source	Clone	Isotype	Fluorochrome
CD3	Bio-Rad	CA17.2A12	Mouse IgG1	FITC
CD4	Thermo Fisher	YKIX302.9	Rat IgG2a	PE-Cyanine7
CD5	Bio-Rad	YKIX322.3	Rat IgG2a	PE
CD8	Thermo Fisher	YCATE55.9	Rat IgG1	PerCP-eFluor 710
CD21	Bio-Rad	CA2.1D6	Mouse IgG1	Alexa Fluor 647
CD34	Bio-Rad	1H6	Mouse IgG1	PE
CD45	Thermo Fisher	YKIX716.13	Rat IgG2b	eFluor 450
CD79b	Bio-Rad	AT107-2	Rat IgG1	FITC
Ki-67	Thermo Fisher	SolA15	Rat IgG2a	eFluor 450

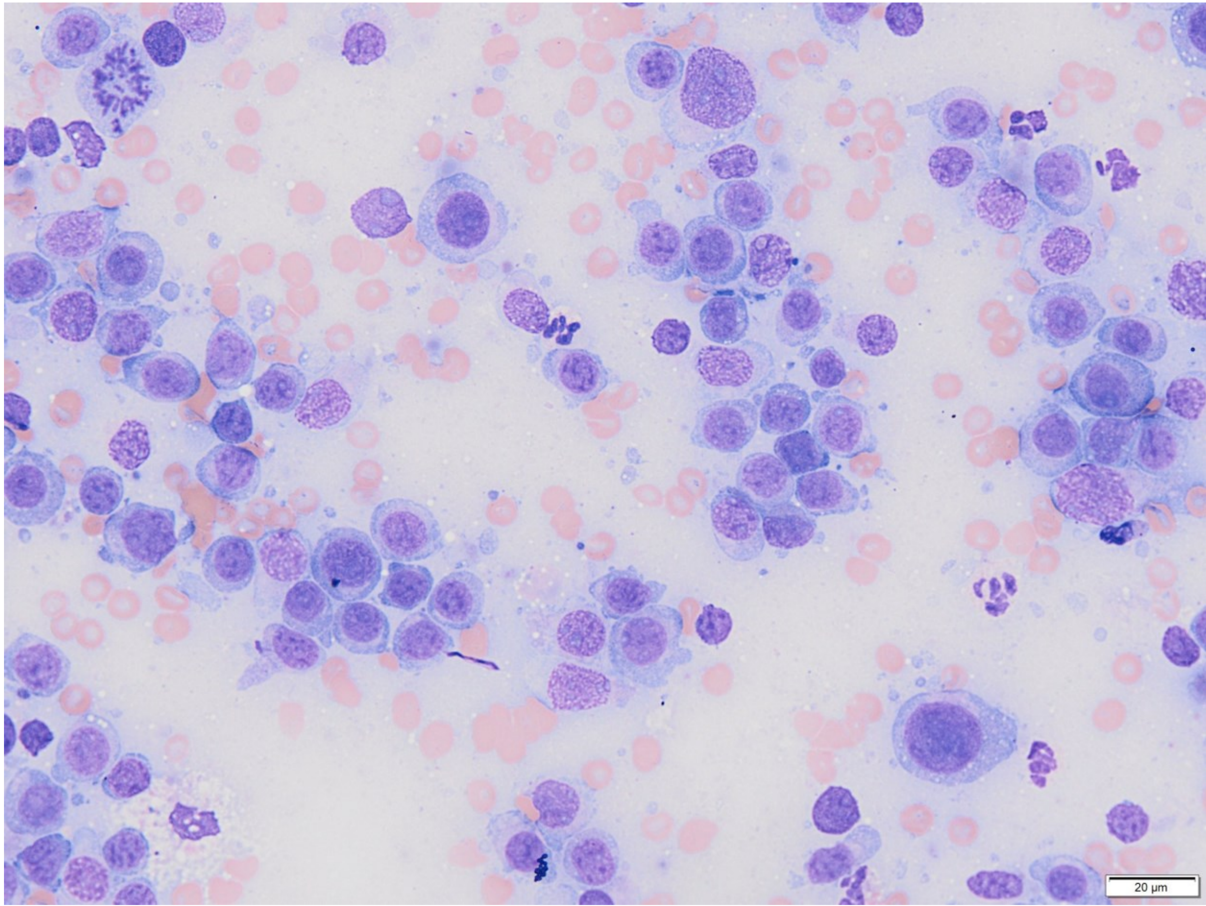
180 FITC = fluorescein isothiocyanate; PE = phycoerythrin. Sources: Bio-Rad, Watford,

181 Hertfordshire, UK; Thermo Fisher Scientific, Paisley, Renfrewshire, UK.

182

183 **Figure 1.** A population of medium-to-large lymphocytes on cytologic evaluation of

184 mandibular lymph node aspirates from a dog. Modified Wright stain. Bar = 20 µm.



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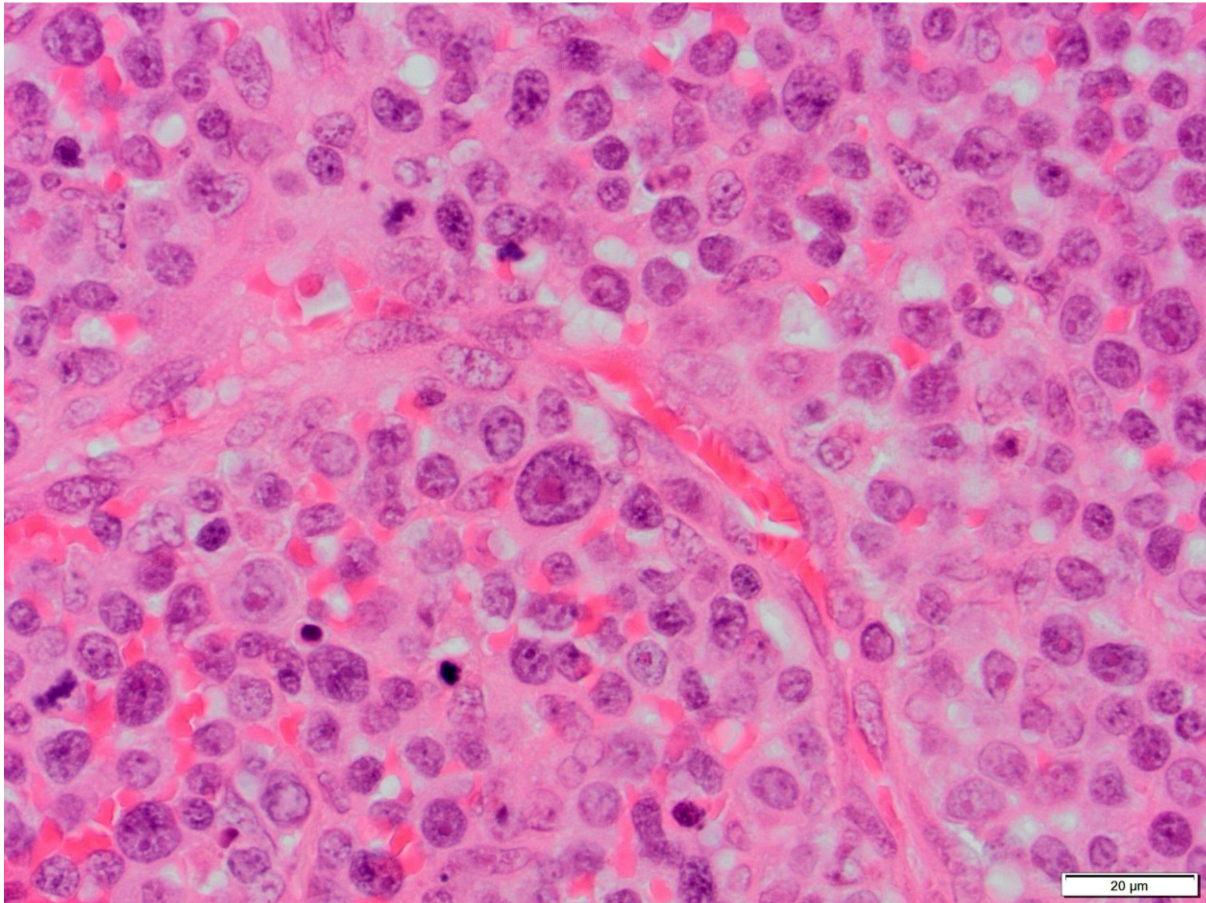
187 **Figure 2.** Neoplastic cells within the mandibular lymph node of a dog exhibit marked

188 anisocytosis and anisokaryosis. Nucleoli are prominent, and mitotic figures are present.

189 H&E. Bar = 20 μm.



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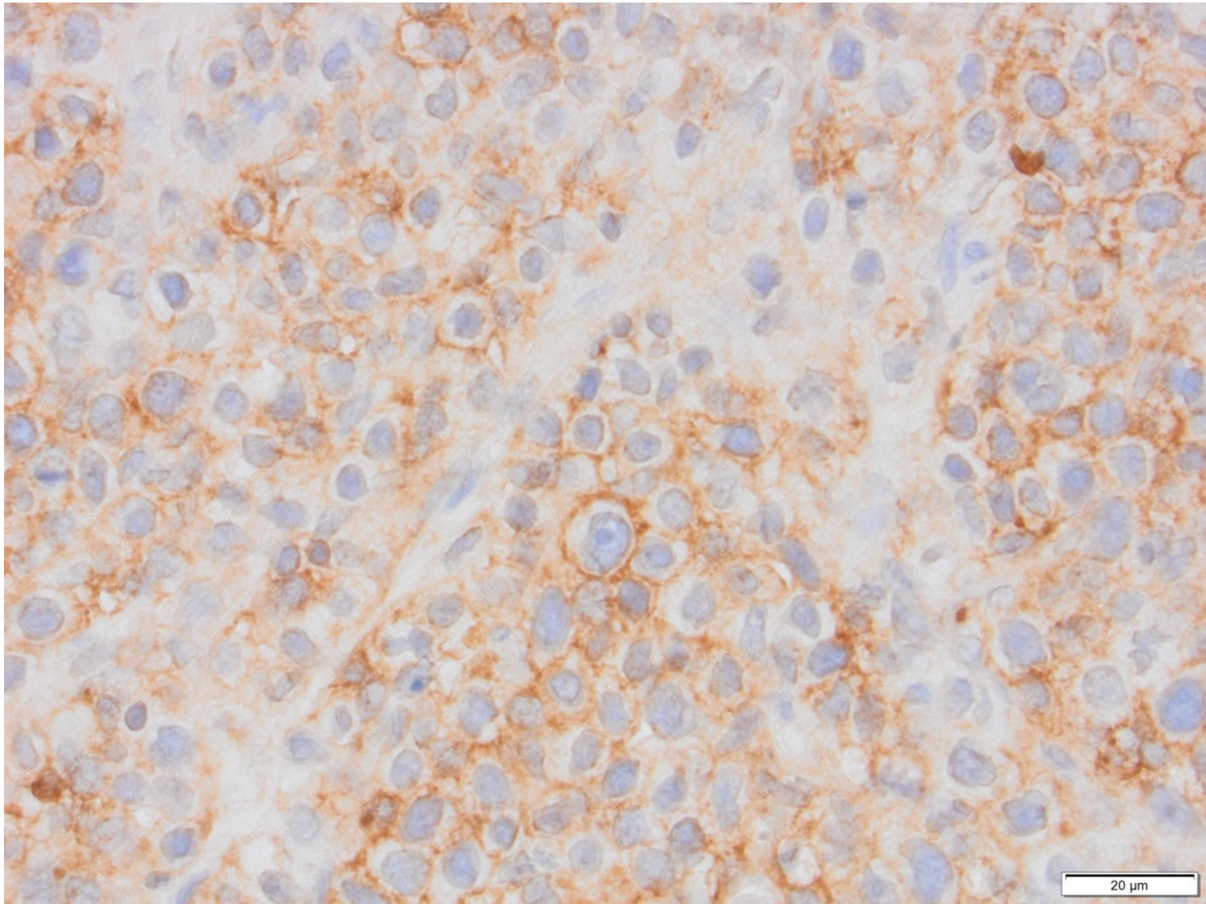
191

192 **Figure 3.** Neoplastic cells within the mandibular lymph node of a dog exhibit specific

193 membranous labeling following CD30 immunohistochemistry. Bar = 20 μm.



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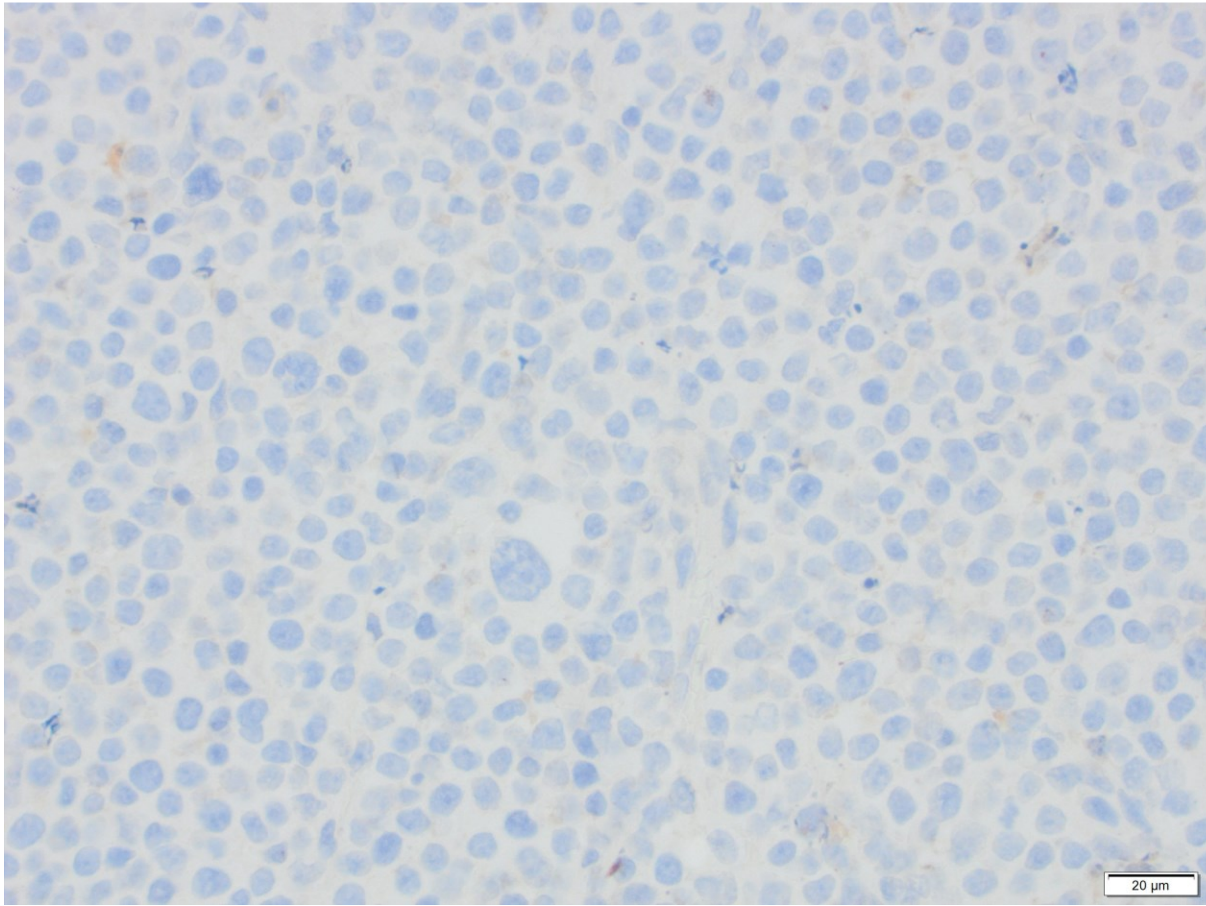
196 **Figure 4.** Neoplastic cells within the mandibular lymph node of a dog do not label for CD30

197 immunohistochemistry in the negative control. Bar = 20 μm.



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