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NEOPLASTIC DISEASE

Characterization of Inflammatory Changes Associated with Canine Oligodendroglioma

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Summary

Oligodendroglioma is a common glial tumour in the dog. In human neuropathology, the immune cell microenvironment of gliomas has been investigated; however, the nature of the inflammatory cells within canine gliomas is currently unknown. The aim of this study was to determine the nature of the immune cells and determine an association between the inflammatory cells and tumour grade. Thirty-four (18 of grade II and 16 of grade III) formalin-fixed and paraffin wax-embedded samples of canine oligodendroglioma were evaluated by light microscopy and immunohistochemistry for expression of CD3, PAX5, Iba-1, HLA-DR, Mac387 and CD31. Variations in immune cell recruitment and activation were evident in all cases. Infiltrating $CD3^+$ T lymphocytes were common in most cases. $PAX5^+$ B lymphocytes were less common and restricted to perivascular cuffs within or around the tumour. Iba-1^+ cells were common within the tumour and formed a dense infiltrate around the tumour in a subset of cases. HLA-DR^+ cells were common within the tumour and in a subset of cases formed perivascular cuffs. Iba-1^+ cells typically had prominent ramified processes suggestive of activated microglia, while the HLA-DR^+ cells had a more rounded morphology typical of amoeboid microglia. Rare $Mac387^+$ macrophages were found in the tumour parenchyma, while increased numbers of $Mac387^+$ monocytes were noted within the vasculature. No association or significance was established between the immune cell infiltrate and the grade of the tumour (all $P \geq 0.16$). This study establishes that there is a robust population of immune cells within canine oligodendrogliomas and indicates that further study is needed to determine the role of these cells in tumour pathogenesis and progression.

Keywords: dog; immune cells; microglia; oligodendroglioma

Introduction

Oligodendroglioma is one of the most common primary brain tumours in the dog with proportional prevalence of 20% of all intracranial neoplasia (Vandevelde *et al.*, 2012; Song *et al.*, 2013). There are clear breed predilections for oligodendroglioma, most notably amongst the brachycephalic breeds including boxers and bulldogs (Vandevelde *et al.*, 2012; Song *et al.*, 2013). In addition, anatomical sites of increased incidence include the frontal lobes, especially in the vicinity of the lateral ventricles, and the pyriform lobe (Vandevelde *et al.*, 2012; Song *et al.*, 2013). Although metastatic spread within the ventricular system or meninges can occur, most tumours form solitary, but locally infiltrative, masses. Generally, the tumours are composed of sheets of round to polygonal cells with hyperchromatic nuclei and sparse cytoplasmic processes. Increased malignancy is associated with intratumoural necrosis, glomeruloid vascular proliferation and marked atypia (Koestner *et al.*, 1999; Vandevelde *et al.*, 2012). In human neuropathology, oligodendrogliomas are graded based on their histological features as either grade II or grade III (Reifenberger *et al.*, 2007).

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The inflammatory microenvironment associated with neoplasia has an important role in the stages of tumour development including initiation, progression and invasion (Sowers *et al.*, 2014). Chronic mediators of inflammation, especially lymphocytes and macrophages, are often found widely throughout a variety of tumours; however, their significance is either unknown or varies depending on the specific tumour. In primary central nervous system (CNS) neoplasia, the role of infiltrating and resident immune cells in initiating and promoting tumourigenesis remains poorly understood; however, it is clear that in human glioblastoma multiforme (GBM), immune cells have the ability to kill cancer cells and increased infiltrates of $CD3^+$ T cells have been associated with prolonged survival (Gomez and Kruse, 2006; Kmiecik *et al.*, 2013). In cancers, including GBM, tumour cells produce inhibitory molecules and immunosuppressive comp ounds that effectively abrogate this immune response, suggesting an important role for inflammatory cells in the control of tumour progression (Gomez and Kruse, 2006). Little is known regarding the immune cell microenvironment in human oligodendroglioma, as the majority of the research has been focused on GBM.

The immune cell microenvironment includes infiltrating leucocytes as well as resident cells. In the CNS, the most important resident cells are the microglia. The number of microglia is approximately equivalent to the number of neurons within the CNS. These cells are derived from circulating haemopoietic cells that populate the brain during development *in utero* and transform into microglia (Yang *et al.*, 2010). They are typically classified, based on their morphology, as ramified, activated or amoeboid—phagocytic. Due to their shared origin and similar function to circulating monocytes and macrophages, these cells play a vital role in the CNS immune response. Microglia traditionally produce and respond to a variety of chemokines and cytokines including interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 (Yang *et al.*, 2010). Although microglia were originally thought to have antiglioma activity, recent evidence supports the notion that microglia (with and without the support of infiltrating macrophages) play an important role in glioma progression (Graeber *et al.*, 2002; Schartner *et al.*, 2005). This notion is further supported by data indicating that microglia are an important source of matrix metalloproteinases, vascular endothelial growth factor and epidermal growth factor, all of which can play a role in development, invasion and angiogenesis within gliomas (Yang *et al.*, 2010). Downstream targets of inflammation (including transcription factors and other signalling molecules) have recently become an important avenue of research as new therapies are developed to aid in immunomodulation as a possible therapy for gliomas (Sen, 2011).

Microglia can be recognized in tissue sections by numerous immunohistochemical markers including ionized calcium adapter protein (Iba)-1 and human leucocyte antigen (HLA)-DR. While these markers recognize all macrophages, including microglia, newly infiltrated macrophages can be defined by expression of intracellular myeloid-related proteins (MRPs) (Soulas *et al.*, 2011). One such MRP, MRP14, is expressed by recently infiltrated monocytes and macrophages and can be recognized by the antibody Mac387 (Soulas *et al.*, 2011; Raposo *et al.*, 2013). Mac387 does not react with microglia and therefore serves as a useful marker for the identification of circulating monocytes and recently emigrated macrophages (Sasaki *et al.*, 1996).

The presence and role of immune cells within intracranial tumours of the dog are poorly understood. In canine meningiomas, macrophages and a variety of lymphocyte subtypes infiltrate the tumours; however, it is not known whether infiltrating cells are related to progression or overall survival in this tumour (Boozer *et al.*, 2012). Similar characterization of the immune cell infiltrate of canine gliomas is lacking. The aim of the present study was to perform a retrospective analysis of 34 cases of canine oligodendroglioma to determine the presence and distribution of infiltrating B and T lymphocytes, infiltrating macrophages and resident microglia and their relationship to features of malignancy.

Materials and Methods

The pathology database at Cornell University, College of Veterinary Medicine, was searched for cases of canine oligodendroglioma between 1990 and 2012. All necropsy examinations were performed within 12—24 h of death. Tissues were collected, fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (5 μm) were stained with haematoxylin and eosin (HE). Histological grading of the tumours was performed based on the human World Health Organization (WHO) classification of tumours of the CNS as follows: grade II, diffuse infiltration, moderate cellularity, monomorphic cells and occasional mitoses; grade III, marked mitotic activity, abundant microvascular (glomeruloid) proliferation, large regions of necrosis and marked cellular atypia (Reifenberger *et al.*, 2007). This human grading scheme correlates to the currently accepted, albeit more dated, WHO classification of tumours of the nervous system of domestic animals with respect to two recognized grades of

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oligodendroglioma (Koestner et al., 1999). Confirmation of a predominant oligodendrocyte component was confirmed by immunohistochemistry (IHC) for Olig2 (see below).

To characterize the immune cell infiltrate, primary antibodies specific for the following markers were used: CD3 (directed at a cell surface protein expressed on T lymphocytes; Supplementary Fig. 1), PAX5 (directed at a transcription factor for B-lymphocyte development), Iba-1 (directed at a calcium binding protein expressed in macrophages; Supplementary Fig. 2), HLA-DR (directed at a class II molecule of the major histocompatibility complex expressed on tissue macrophages and microglia; Supplementary Fig. 3), MAC387 (directed at the cytoplasmic antigen calprotectin found in circulating monocytes and macrophages matured from blood monocytes) and CD31 (directed at a cell adhesion molecular found on endothelial cells). All labelling procedures used 5 μm tissue sections that were dewaxed in xylene, hydrated in graded ethanols and subsequently blocked with 3% H_2O_2. Pretreatment for MAC387, HLA-DR, Iba-1 and Olig2 involved microwaving for 20 min in a 0.01 citrate buffer followed by 20 min of cooling. All steps were followed by washing in Tris-buffered saline (TBS). Prior to the application of the primary antibodies, all slides were treated with an avidin–biotin protein block (Invitrogen; Carlsbad, California, USA) and a serum-free protein block (Dako; Carpinteria, California, USA) for 10 min at room temperature. The incubation times, dilutions, clones and manufacturers for each antibody are listed in Supplementary Table 1. Secondary antibodies were either biotinylated horse anti-mouse IgG (for CD3, PAX5, HLA-DR, MAC387 and CD31) (Vector Laboratories, Burlingame, California, USA) or biotinylated goat anti-rabbit IgG (for Iba-1 and Olig2) (Vector Laboratories), diluted at 1 in 200 and applied for 30 min at room temperature. Tertiary antibody was Vectastain ABC Elite reagent (Vector Laboratories) incubated for 30 min at room temperature. Iba-1, HLA-DR and MAC387 reactions were 'developed' with 3, 3′ diaminobenzidine (DAB) chromogen (Dako) and counterstained with Mayer's haematoxylin (Dako). CD3 and PAX5 reactions were performed using the Leica BOND MAX IHC and ISH staining system with standardized protocols (Leica; Buffalo Grove, Illinois, USA). For these two antibodies, antigen retrieval was performed with Novocastra Bond Epitope Retrieval Solution 2 (Novocastra Laboratories, Newcastle upon Tyne, UK). CD3 labelling was 'visualized' using DAB chromogen (Leica) and PAX5 labelling was 'visualized' using mixed refined RED (Leica).

Co-localization of monocytes within blood vessels was achieved via double labelling IHC for MAC387 and CD31. All sections were treated with MAC387 as described above, except that the microwave antigen retrieval was replaced with proteinase K in order to maintain continuity with the CD31 antigen retrieval. Following treatment with MAC387 and development in DAB, sections were washed in TBS and received a universal protein block (KPL Incorporated; Gaithersburg, Maryland, USA) for 30 min at room temperature. Sections were then incubated with the CD31 antibody. Secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories) diluted at 1 in 200 and incubated for 30 min at room temperature. Tertiary antibody was Vectastain ABC–AP Kit (Vector Laboratories) incubated for 30 min at room temperature. The slides were developed with Vector Red (Vector Laboratories) and counterstained with Mayer's haematoxylin (Dako). Negative controls consisted of adjacent sections of tissue with irrelevant species and isotype-matched antibodies. Positive control tissue consisted of canine lymph node for all antibodies. Additionally, all antibodies were applied to adult canine brain sections to determine control immunoreactivity and normal distribution within the CNS.

Expression Analysis and Statistics

The immunohistochemically labelled sections were analyzed by one of the authors (ADM) by light microscopy. Cell counts for CD3, PAX5, MAC387 and HLA-DR were performed sequentially in 10 random ×400 fields per case. A mean value was then determined based on the cell counts for each marker. Because Iba-1 immunoreactivity was difficult to determine by counting cells, expression was estimated based on increased or decreased expression within and adjacent to the tumour on light microscopical examination of 10 ×400 fields. The Shapiro–Wilk test indicated that most (10 of 14 grade-specific) IHC results were non-Gaussian ($P < 0.0012$ in Shapiro–Wilk tests); given also that the number of dogs in each WHO grade was <18, we used non-parametric methods for description and testing. Numerical data were described as minimum, median and maximum. Comparisons between WHO grades were made using the Wilcoxon rank-sum test, 2-tailed. The questions of interest were whether the WHO grade related to the various IHC results. The primary question concerned CD3 and was tested using alpha = 0.05. The secondary questions concerned MAC387 in tumours or blood and used alpha = 0.025 (a Bonferroni correction to adjust for multiplicity), and all other tests used the even lower alpha of 0.01. Fisher's exact test

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was used to determine if there was an association between the WHO grade and whether the dog was brachycephalic. Finally, Spearman's rank correlations were used to describe the associations among the IHC results; this was done on the subset of 24 dogs without missing data for these seven assays. No significant associations between the tested variables were determined. All statistical analyses were performed using Statistix® 10 (2013; Analytical Software, Tallahassee, Florida, USA).

Results

Clinicopathological Features

Thirty-four dogs were included in the study. All cases were subjected to necropsy examination. Ages ranged from 1 to 13 years with a median age of 8.0 years. Boxers and mixed-breed dogs were the most commonly represented breeds (each represented by seven dogs out of 34), followed by bulldogs (4/34), Boston terriers (3/34), Staffordshire bull terriers (2/34), Labrador retrievers (2/34) and one each of bull mastiff, cocker spaniel, German shorthair pointer, Jack Russell terrier, poodle, pitbull, Shar pei, wire-haired fox terrier and Yorkshire terrier. One of the mixed-breed dogs was a Staffordshire bull terrier/boxer cross. Sex was not recorded for one of the cases; the others included 16 neutered females, three entire females, eight neutered males and six entire males.

Gross, Microscopical and Immunohistochemical Features

Grossly, tumours were most prevalent as solitary masses in the frontal region of the brain, where they were in close association with the lateral ventricle (16/34), and in the pyriform lobe (7/34). Less common locations included the prefrontal cortex (2/34), cerebellum (1/34), meninges as a diffuse oligodendrogliomatosis (1/34) and temporal lobe (1/34). Six cases were recorded in the spinal cord (3/6 cervical, 2/6 thoracic and 1/6 undefined). Grossly identifiable haemorrhage was present in 11/34 cases, otherwise tumours had the typical gross appearance of an oligodendroglioma, namely relatively well-defined, gelatinous masses with peritumoural oedema and a midline shift if an intracerebral mass.

Histologically, all tumours had features consistent with an oligodendroglioma. Briefly, tumour cells were round to polygonal with hyperchromatic nuclei and inapparent to moderate amounts of cytoplasm. The degree of atypia and mitotic activity, as well as presence of necrosis and glomeruloid blood vessels, were used to assign a grade according to the WHO CNS tumour classification. Sixteen of 34 cases were grade II and 18/34 cases were grade III, with the latter cases having more pronounced atypia, abundant glomeruloid blood vessels and large regions of necrosis (Fig. 1). Additionally, 10/34 (7/10 were grade III) cases had regions of microcalcification scattered throughout the tumours. In addition, all tumours displayed diffuse, strong intranuclear immunoreactivity for Olig2 (data not shown).

The results of the immunohistochemical analysis are included in Supplementary Table 2. $CD3^+$ T lymphocytes were found in 30/32 (94%) cases and conformed to two distinct patterns, which were always equally evident in the cases. In one pattern, the $CD3^+$ cells were evenly and widely distributed throughout the tumour (Fig. 2), while the less common pattern was increased numbers of perivascular $CD3^+$ cells (Fig. 2, inset). An increase in peritumoural $CD3^+$ cells was not observed in any of the cases; however, scattered $CD3^+$ cells were noted in the meninges in all cases. Two cases showed no immunoreactivity to CD3, including in the meninges, which was likely due to prolonged fixation and not indicative of a complete lack of $CD3^+$ cells. $PAX5^+$ B lymphocytes were noted in 10/34 (29%) cases and in all cases cells were restricted to perivascular cuffs within the tumour (Fig. 3). No parenchymal $PAX5^+$ cells were noted in any of the cases and no $PAX5^+$ cells were noted in the adjacent, non-neoplastic brain parenchyma. Similarly, no $PAX5^+$ cells were present in the meninges.

Iba-1 immunoreactivity was assessed both within the neoplasm as well as in the adjacent non-neoplastic neuroparenchyma. In 26/34 (76%) cases there was a marked increase in intratumoral Iba-1 immunoreactivity. In these cases, $Iba\text{-}1^+$ cells had

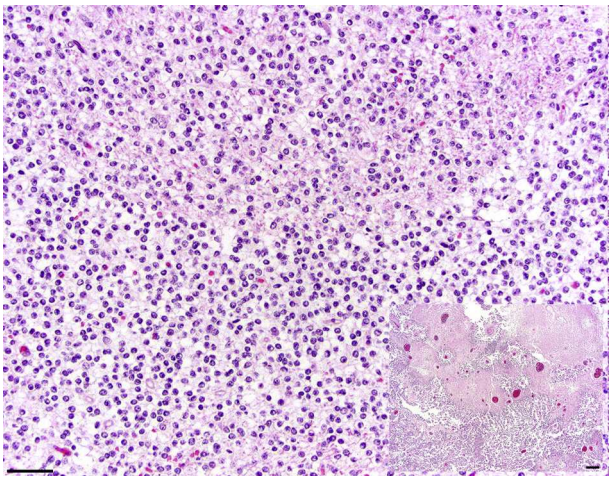

Fig. 1. Canine oligodendroglioma, case 15, grade II. Monomorphic population of neoplastic oligodendrocytes with absence of necrosis, atypia and vascular proliferation. Inset: Canine oligodendroglioma, case 26, grade III. Large tracts of necrosis dissect the tumour. HE. Bar, 50 μm.

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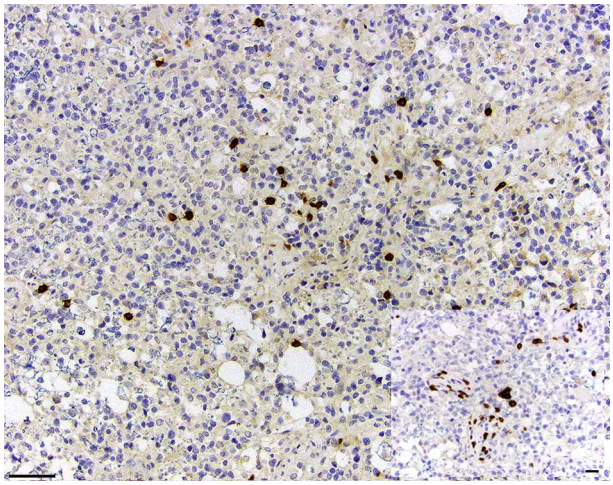

Fig. 2. Canine oligodendroglioma, case 14, grade II. Multiple $CD3^+$ T lymphocytes are scattered throughout the tumour. Inset: $CD3^+$ cells also form perivascular cuffs. IHC. Bar, 50 μm; inset bar, 20 μm.

prominent, branching, interdigitating cell processes that were often uniformly distributed throughout the tumour (Fig. 4). A small subset of these intratumoural Iba-1^+ cells had increased cytoplasmic immunoreactivity and a more rounded phenotype with loss of cytoplasmic branching. In addition, clusters of Iba-1^+ cells were commonly scattered throughout the tumours, while no clusters of positive cells were noted in the unaffected parenchyma. Eight of 34 (23.5%) cases did not have appreciable differences in Iba-1 immunoreactivity and cellular morphology compared with the adjacent non-neoplastic tissue. Finally, in 8/34 (23.5%) cases there was a marked increase in Iba-1^+ microglia/macrophages adjacent to the tumour, often forming a thick rim of cells around the leading edge of the tumour.

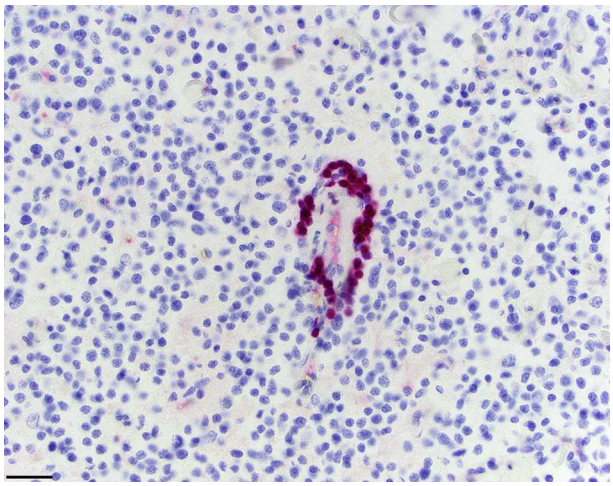

Fig. 3. Canine oligodendroglioma, case 5, grade 2. A single perivascular cuff of $PAX5^+$ B lymphocytes. IHC. Bar, 50 μm.

In general, the distribution of HLA-DR immunoreactive cells parallelled Iba-1 immunoreactivity within the tumour; however, cells labelled with HLA-DR were typically more rounded and lacked the ramified, branching processes evident in the Iba-1^+ cells (Fig. 5). Increased numbers of HLA-DR immunoreactive cells also formed cuffs around blood vessels both within tumours and at their periphery (Fig. 6). While the immunoreactivity of HLA-DR within the tumour samples was similar to Iba-1, in the normal, non-neoplastic parenchyma, HLA-DR did not uniformly label all microglia/macrophages, but rather displayed regional immunoreactivity. Five cases lacked sufficient tissue to perform HLA-DR IHC.

In both the normal brain parenchyma and within the tumour samples, rare $Mac387^+$ macrophages were present in a majority of cases. Significant infiltration of the tumour parenchyma with $Mac387^+$ cells was not a feature; however, in cases in which there was substantial haemorrhage, numerous $MAC387^+$ cells were present in these regions (interpreted as extravasated blood monocytes and neutrophils). An increase in intravascular $Mac387^+$ monocytes was found in both grade II and grade III tumours. This increase in positive cells included vessels within and around the tumour and was especially prominent in the grade III tumours. In cases where there were increased numbers of $Mac387^+$ monocytes within the reactive vasculature, an increase in $Mac387^+$ macrophages was not observed in the adjacent tumour parenchyma (Fig. 7). Containment within the vasculature was confirmed with double labelling immunohistochemical studies for CD31 and Mac387 (Fig. 7, inset).

Statistical Analysis

There were no associations between WHO grade and IHC results (all $P \geq 0.16$; Table 1). The considerable overlaps in the IHC results between WHO grades (Table 1) suggest that these findings are not just the result of low power arising from the limited number of cases available. Brachycephalism and WHO grade also were unrelated ($P > 0.74$). None of the IHC results were related to any of the others; all rank correlations had absolute value ≤ 0.48 (all $P \geq 0.02$; all but one $P \geq 0.10$).

Discussion

In the dog, oligodendroglioma is one of the two most common intraparenchymal CNS tumours. These tumours often have characteristic histological findings with small, round to ovoid cells, inapparent to

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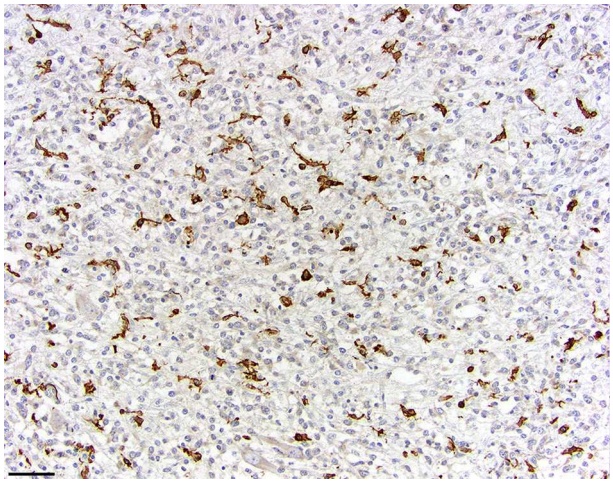

Fig. 4. Canine oligodendroglioma, case 31, grade II. Macrophages within the tumour have diffuse immunoreactivity for Iba-1, with prominent ramified, interdigitating processes typical of microglia. IHC. Bar, 50 μm.

vacuolated cytoplasm and scattered lakes of extracellular mucin. These tumours are subdivided into grade II and grade III, with the latter having marked atypia, large regions of necrosis and abundant reactive (glomeruloid) vasculature (Vandevelde *et al.*, 2012). Despite the known histological features of canine oligodendroglioma, there is no literature defining the associated immune cell infiltrate or whether any of the infiltrating cells correlate with tumour grade. Therefore, we hypothesized that a robust immune cell population could be found in canine oligodendroglioma and we further hypothesized that these cells were, at least partially, correlated with the grade of the tumour.

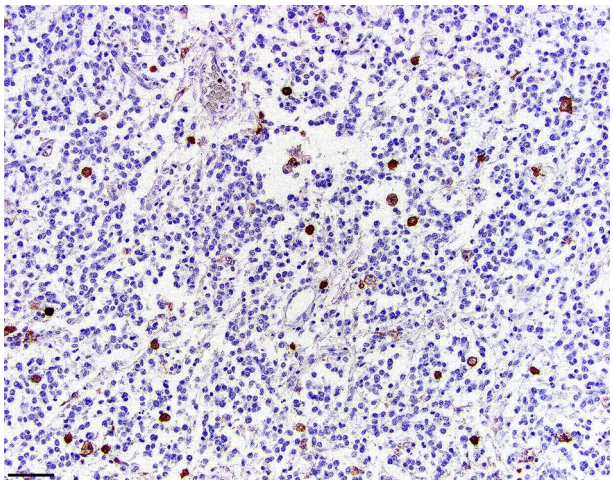

Fig. 5. Canine oligodendroglioma, case 31, grade II. HLA-DR$^+$ cells are scattered within the tumour. These cells lack the prominent ramified processes seen in HLA-DR$^+$ microglia from normal canine brain. IHC. Bar, 50 μm.

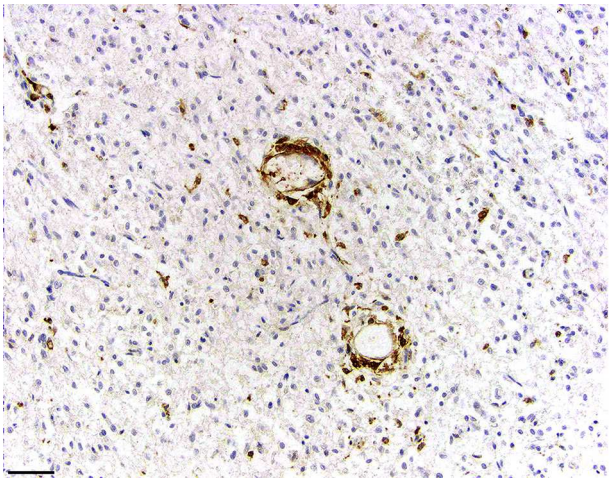

Fig. 6. Canine oligodendroglioma, case 7, grade III. Prominent perivascular cuffs of HLA-DR$^+$ cells. IHC. Bar, 50 μm.

The immune cell microenvironment of tumours can be influenced by a variety of parameters including infiltrating immune cells, the local cytokine milieu, local immunomodulatory cells (i.e. microglia in the CNS, Kupffer cells in the liver) as well as regional necrosis and hypoxia that draws in inflammatory cells (Sowers *et al.*, 2014). Indeed, differences in the immune cell infiltrate may also correlate with the breed of the dog (Villaescusa *et al.*, 2012). In human gliomas, infiltrating immune cells and their concomitant mediator release affects many facets of tumour development, including proliferation and angiogenesis, and in some studies is correlated with

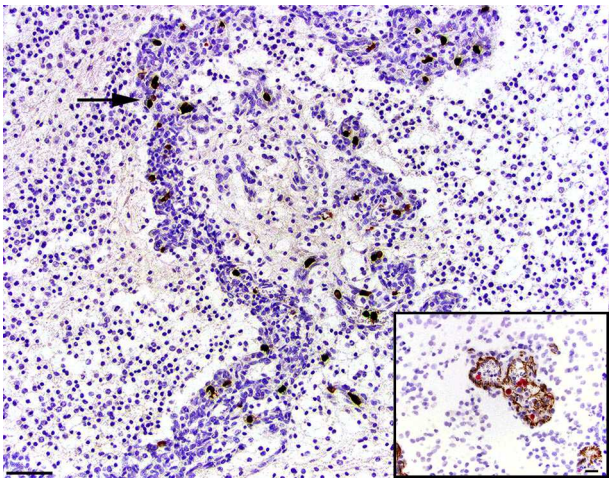

Fig. 7. Canine oligodendroglioma, case 32, grade III. Within the proliferative vasculature are numerous Mac387$^+$ monocytes (arrow). Inset: canine oligodendroglioma, case 33, grade III. Blood vessels indicated via CD31 immunoreactivity (brown) contain large numbers of intravascular Mac387$^+$ monocytes (red). IHC. Bar, 50 μm; inset bar, 20 μm.

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Table 1
Immunohistochemistry and statistical relationship to WHO grade

Immunohistochemical target	n=	*Grade II*			n=	*Grade III*			P *value (between WHO grades)*
		Min	*Med*	*Max*		*Min*	*Med*	*Max*	
CD3*	16	0.2	1.8	10.5	16	0.0	2.2	8.6	0.76
PAX5*	18	0.0	0.0	0.6	16	0.0	0.0	0.3	0.19
Iba-1 (within tumour)†	18	1.0	2.0	2.0	16	1.0	2.0	2.0	0.56
Iba-1 (near tumour)†	18	1.0	1.0	2.0	16	1.0	1.0	2.0	0.87
HLA-DR*	14	1.8	8.9	18.6	15	0.6	7.7	20.5	0.60
Mac387 (within the tumour)*	16	0.2	0.9	7.2	15	0.0	0.9	7.8	0.66
Mac387 (within the blood vessels)*	16	0.0	3.8	15.8	15	0.0	1.5	18.1	0.16

Min, minimum; Med, median; Max, maximum.
*Cell counts of CD3, PAX5, HLA-DR and Mac387 are expressed as the mean of twenty ×400 fields.
†Iba-1 cell counts are expressed as 1 (no difference in immunoreactivity) or 2 (increased immunoreactivity).

tumour grade (Sowers *et al.*, 2014). In human GBM, where infiltrating immune cells have been best defined, both neutrophil and eosinophil infiltration is a common phenomenon that presumably promotes the formation of reactive oxygen species and drives subsequent epigenetic changes within the tumour cells (Atukeren *et al.*, 2010; Sowers *et al.*, 2014).

Large numbers and a wide variety of lymphocytes infiltrate many different tumours. In canine meningiomas, variable numbers of T lymphocytes, including regulatory T cells and B lymphocytes, are reported within and adjacent to the tumours (Boozer *et al.*, 2012). In human astrocytomas, increased numbers of infiltrating $CD3^+$ lymphocytes correlated with increased survival time in patients with glioblastoma in one study (Kmiecik *et al.*, 2013). In the present study, $CD3^+$ T lymphocytes were common within the tumour, regardless of grade, and could be found in one of two patterns: either single cells evenly dispersed throughout the tumour or as perivascular aggregates. Although the cells could still play a role in tumour progression, based on no differences between tumour grades, it is impossible to correlate the tumour grade with the number of infiltrating T cells. B lymphocytes, as defined by PAX5 immunoreactivity in the current study, were only found rarely, which is consistent with findings in canine meningiomas and human gliomas where infiltrating B lymphocytes appear to play no appreciable role in tumour pathogenesis. Pax5 has been shown to be an effective marker for B cells in the dog and in many cases provides more consistent B-cell immunoreactivity than other B cell markers, including CD79a (Willmann *et al.*, 2009).

Microglia are mesoderm-derived, inherent macrophages of the CNS that can be identified immunohistochemically by a variety of markers including Iba-1, HLA-DR and CD163 (Yang *et al.*, 2010; Ide *et al.*, 2011). These cells have an important role in antigen presentation and are key players in the immune system of the CNS. Although Iba-1 is a pan-phagocyte marker, the Iba-1-positive cells in the current study were most consistent with microglia based on morphology. In the current study, phenotypic changes in microglia were found consistently within the tumour parenchyma. This is not entirely surprising because large numbers of macrophage-lineage cells have long been observed in cases of human glioma. Presumably, the local proliferation of microglia/macrophages is promoted by the production of chemoattractants from both the neoplastic cells and the inflammatory cells. In human astrocytoma, the production of MCP-1 by the neoplastic cells has been used as evidence for the recruitment of macrophages (Prat *et al.*, 2000).

In the brain, HLA-DR immunoreactivity is found in microglia; however, the immunoreactivity can be varied across different regions of the brain (Gehrmann *et al.*, 1993). In the current study, $HLA\text{-}DR^+$ microglia displayed fewer ramified processes and increased amoeboid morphology when compared with the $Iba\text{-}1^+$ microglia/macrophages. Although HLA-DR and Iba-1 are both microglial markers, the differential labelling patterns in the present study suggests that, similar to the variations seen in human microglial labelling patterns with HLA-DR, there are potentially different subsets of microglia present in the canine brain (Gehrmann *et al.*, 1993). Alternatively, in switching to a more amoeboid phenotype, HLA-DR is upregulated and Iba-1 is downregulated. The amoeboid microglial morphology is expected in tumours that have abundant necrosis due to the phagocytic capabilities of the cells; however, this morphology was present in the tumours regardless of the presence or absence of necrosis. Although early research suggested that microglia had a role in tumour rejection, or at least in slowing its growth pattern, more recent research

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indicates that microglia have a tumour promoting effect (Yang *et al.*, 2010). This has been further corroborated by data indicating that IL-10, derived from microglia has the ability to promote glioma growth and infiltration (Wagner *et al.*, 1999). The role that microglia/macrophages have in tumour progression and malignancy remains poorly characterized and continued research is needed.

Mac387 is a monocyte/macrophage marker widely used in experimental settings. In blood monocytes, its immunoreactivity is thought to indicate recent release from the bone marrow and it is therefore an indicator of newly emigrated cells (Soulas *et al.*, 2011). In the present study, small numbers of Mac387$^+$ cells were found in the tumour parenchyma, similar to the unaffected tumour parenchyma where only sporadic positive cells could be found. In contrast, Mac387$^+$ cells were often found within blood vessels in and around the tumours. However, even in cases where there were large numbers of Mac387$^+$ cells within the vasculature, there was no evidence of emigration into the adjacent tumour tissue. Double labelling studies in these cases confirmed that the Mac387$^+$ cells were predominately confined to the blood vessels. The significance of the increased numbers of immunoreactive intravascular monocytes is not clear. It is possible that local production of cytokines and chemokines drew the monocytes to the vasculature and that once there they contributed to the local tumour microenvironment through local inflammation and release of angiogenic factors. Further research into the role that newly emigrated monocytes and macrophages have in glioma pathogenesis and progression is needed.

In conclusion, we present data to indicate that alterations in recruited and tissue-specific immune cells are common in canine oligodendroglioma. Since all the samples used were obtained at necropsy examination, we were not able to study the dynamic changes that occur with respect to immune cell infiltration over the time of the tumour; however, correlating dynamic changes in immune cell infiltrates with tumour grade based on biopsy samples is a future goal. Based on statistical analysis, the grade of the tumour did not have a role in the pattern of immune cell infiltrate at the time of necropsy examination. CD3$^+$ lymphocytes were found in both grades of tumours in a similar pattern, while B lymphocytes were only rarely found within the tumours and do not appear to play a significant role in immune cell microenvironment. Differential labelling of Iba-1 and HLA-DR suggests different subsets of microglia/macrophages infiltrating the tumour and possibly indicate distinct roles in the pathogenesis and propagation of the tumour. Finally, new emigrating Mac387$^+$ macrophages were found in very low numbers in the tumour parenchyma; however, increased numbers of these cells were found sequestered within tumoural and peritumoural vasculature. The role that these macrophages have with respect to tumour progression or whether they are just drawn by a supportive cytokine environment will require further research. Inflammation can regulate various stages of tumour initiation, progression and invasion and the data presented herein reveal a varied immune cell infiltrate in canine oligodendroglioma.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Supplementary data

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