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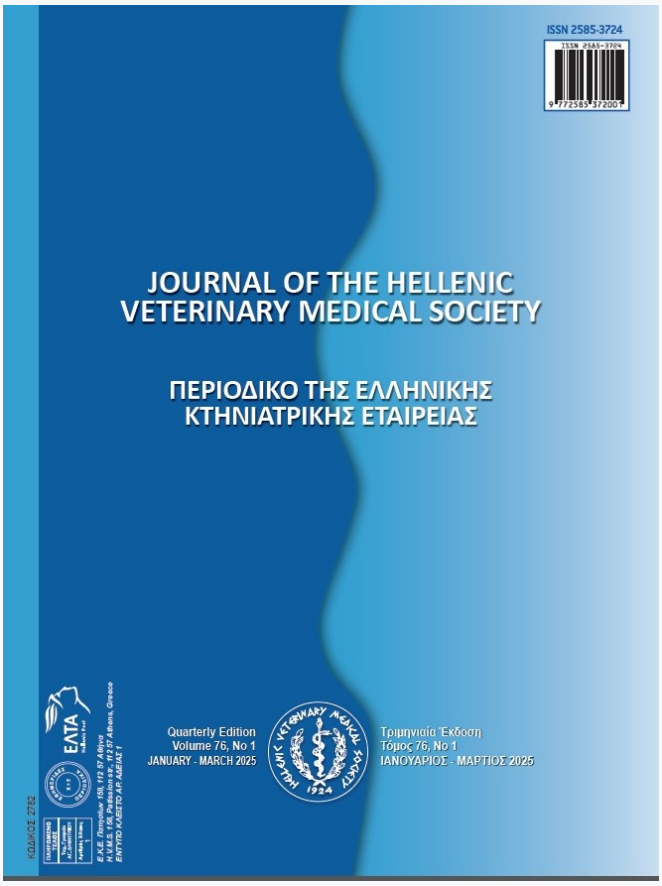

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Exploring the FoxP3+ Lymphocytes and Their Correlation with VEGF in Canine Histiocytic Tumors

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ABSTRACT: Histiocytic proliferative diseases in dogs include reactive diseases such as cutaneous histiocytosis and systemic histiocytosis and neoplastic diseases such as cutaneous histiocytoma, histiocytic sarcoma, and disseminated histiocytic sarcoma. The aim of our study was to investigate the intensity of VEGF staining with FoxP3-positive T lymphocytes in various histiocytic diseases in dogs. The study evaluated 23 cases diagnosed with histiocytic tumors. Immunostaining with Iba-1, E-cadherin, and CD3 antibodies was performed for differential diagnosis. Because of strong CD3 positivity, lymphoma was diagnosed in 2 cases. The diagnosis of histiocytic tumor was confirmed in 12 cases with intense Iba-1 positivity. In the other 9 cases, although Iba-1-positive histiocytic cells were detected in the tumor tissue, these cells were scattered and did not indicate a histiocytic origin. Histopathologic examination of the 12 Iba-1-positive cases revealed that 4 cases were cutaneous histiocytomas, 3 cases were cutaneous histiocytosis, and 5 cases were histiocytic sarcomas (1 hemophagocytic histiocytic sarcoma). Mild E-cadherin positivity was detected in only one cutaneous histiocytoma and one histiocytic sarcoma. CD3- and FoxP3-positive T lymphocytes were counted and evaluated in 12 Iba-1-positive cases. In addition, VEGF staining revealed different degrees of positivity. Statistical analyses revealed a significant increase in CD3 positivity in histiocytic sarcoma and cutaneous histiocytosis cases compared to cutaneous histiocytoma cases in the group comparison. According to the correlation results for CD3, FoxP3, and VEGF levels, a moderate negative correlation was found only between FoxP3 and VEGF. These findings suggest that an increase in the number of FoxP3-positive Tregs in canine histiocytic tumors may negatively correlate with VEGF-mediated angiogenesis.

Keywords: Dog; FoxP3; histiocytic tumor; T-cell;VEGF

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INTRODUCTION

Histiocytic malignant tumors are aggressive tumors that are fatal in dogs of different breeds worldwide (Janeway et al., 1999; Shortman and Liu, 2002). Histiocytic disorders have been reported in humans, dogs, rats, and rarely in cats (Affolter and Moore, 2002; Azakami et al., 2006; Gross et al., 2005; Moore, 2017). These disorders are more common in dogs than cats. Histiocytic diseases in dogs include cutaneous histiocytoma (CCH), cutaneous Langerhans cell histiocytosis, reactive histiocytosis (cutaneous histiocytosis [CH] and systemic histiocytosis), histiocytic sarcoma (HS) and hemophagocytic histiocytic sarcoma (HpHS). It is known that CCH and LCH are derived from Langerhans cells, reactive histiocytosis from activated interstitial dendritic cells, and histiocytic sarcoma (HS) from interstitial dendritic cells, whereas HpHS, which is classified as a distinct subtype, is derived from macrophages (Moore, 2017).

It is known that the activity of regulatory T cells (Tregs) is closely related to the expression of the transcription factor Forkhead Box P3 (FoxP3). FoxP3-regulatory T cells (FoxP3 Treg) are a special group of T lymphocytes that play an important role in immune homeostasis and modulation of the immune response (Campbell and Koch, 2011). Tregs serve as key cells in the development and maintenance of peripheral immune tolerance (Elkord et al., 2010). Normally, these cells can prevent harmful autoimmune responses (Nishikawa and Sakaguchi, 2014), but they can also impair beneficial immune responses such as anti-tumour immunity (Piersma et al., 2008; Whiteside, 2014).

In general, immune regulation may be mediated by regulatory cytokines (e.g., tissue growth factor (TGF)-β, interleukin (IL)-10) or cellular interactions (Lan et al., 2007). It is now known that specialized T helper cells with regulatory properties (Tregs) play an important role in immune regulation (Sakaguchi et al., 2008). Deletion of these cells or mutations of related transcription factors lead to severe cases of autoimmune diseases and chronic inflammatory disorders in rats, as they have also been described in mice and humans (Brunkow et al., 2001; Moes et al., 2010). In addition to their regulatory capacity, Tregs are characterized by the highly conserved transcription factor FoxP3, which plays a critical role in stabilizing CD4 and CD25 expression and regulatory properties (Fontenot et al., 2003;; Sakaguchi et al., 1995).

Angiogenesis, defined by the formation of new vessels and predicted by microvessel density, is a crucial event for the growth, development, and metastasis of various tumors in mammals (Potente et al., 2011). Normal angiogenesis or tumor angiogenesis is controlled by growth factors and their respective receptors (Terme et al., 2012). Hypoxia in the intratumoral compartment induces the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), leading to proliferation, migration and formation of new blood vessels of endothelial cells (De Brot et al., 2015; Yu and Rak, 2003).

Induction of angiogenesis is mediated by many cells, including Treg cells that produce proinflammatory cytokines and VEGF, which is upregulated by hypoxia (Berk and Muthuswamy, 2011; Facciabene et al., 2011). Tregs may contribute to tumor angiogenesis through both indirect and direct mechanisms and may indirectly promote angiogenesis by suppressing the activities of Th1 effector T cells that release angiostatic cytokines such as TNFα and IFNγ (Casares et al., 2003; Yan et al., 2011). However, a negative correlation between Tregs and angiogenesis has also been reported (Li et al., 2018).

The aim of our study was to investigate the intensity of VEGF staining with FoxP3-positive lymphocytes and the correlation between them in canine histiocytic tumors.

MATERIAL AND METHODS

Histopathology and immunohistochemistry

For this study, 23 blocks from the department's archives were used that were suspected of having a histiocytic tumor in the dog by histopathologic examination. Using a microtome (Leica RM 2155), 5 μm-thick sections were taken from these blocks on a microscope slide (Menzel Gläser) for histopathological staining. The sections were deparaffinized in xylene (Xylene - Merck Millipore:108661) and dehydrated in an alcohol series. Sections were stained with hematoxylin and eosin (H&E) for histopathological analysis (Luna, 1968).

For immunohistochemical confirmation of histiocytic tumors, staining with ionized calcium binding adapter protein (Iba-1), CD-3, and E-cadherin antibodies were performed. CD3 positivity was detected in almost all tumor cells in two cases initially diagnosed as histiocytic tumors, leading to a diagnosis of lymphoma. Iba-1 staining in the remaining 21 cases

revealed strong positivity in 12 tumors, whereas only histiocytes distributed in the tumor tissue were positive in 9 tumors. Therefore, only 12 tumors diagnosed as histiocytic were included in the study. These tumors were also immunohistochemically stained with FoxP3 and VEGF.

*I*mmPRESS® Excel Amplified Polymer Staining Kit (Vector Lab, anti-mouse IgG, peroxidase, MP -7602) was used for immunohistochemical staining of CD3, E-cadherin, and VEGF. For this purpose, sections were taken from paraffin blocks on 5 µm-thick lysine slides using a microtome (Leica RM 2155) and subjected to routine follow-up procedures. For antigen retrieval, slides placed in citrate buffer solution (pH: 6) were incubated in an autoclave at 120 degrees for 15 minutes. Staining was then performed according to the protocol of the mentioned kit. A dilution ratio of 1:25 was used for CD3 antibody (Dako, M7254, clone F7.2.38), a dilution ratio of 1:25 was used for E-cadherin (Dako, M3612, clone NCH-38), and a dilution ratio of 1:20 was used for VEGF (Thermo, MA5-13182, clone JH121). DAB was used as chromogen. Sections counterstained with Mayer's hematoxylin (Merck, 109249) for 20 seconds were dried, coverslipped with Entellan, and examined under the microscope. Dog spleen for CD3, dog skin for E-cadherin, and vascular staining in tumors for VEGF were considered positive controls.

ImmPRESS® HRP Goat Anti-Rat IgG, Mouse adsorbed Polymer Detection Kit, Peroxidase (MP -7444-15) was used for FoxP3 staining. Although similar to other antibody staining protocols, this protocol applied the primary antibody (Invitrogen, 14-5773-82, clone FJK-16s) diluted 1/100, followed directly by ImmPRESS polymer reagent without an enhancer antibody step, using DAB (ImmPACT EqV Reagent) as the chromogen. Dog spleen was used as a positive control. In contrast to the above protocols, a 1/100 diluted primary antibody (Wako, 019-19741, polyclonal) was used to stain Iba-1, followed by a secondary anti-rabbit antibody (Vector Lab ImmPRESS® Excel Amplified Polymer Staining Kit, peroxidase, MP -7601). In addition, AEC (Thermo Fisher Scientific, 001122) was used as a chromogen. The antibody information is shown in Table 1.

When immunohistochemical staining was evaluated, tumor tissue was scored as positive or negative without scoring for Iba-1 and E-cadherin. However, when CD3 and FoxP3 staining were evaluated, cells with positivity were counted in four randomly selected fields at 400× magnification in the tumor tissue. The intensity of VEGF staining was determined semi-quantitatively with a score of 0-4 (Maae et al., 2011). Considering the distribution and severity of staining, the scoring was as follows: 0: no positivity, 1: mildly positive, 2: moderately positive, 3: strongly positive, and 4: very strongly positive.

Statistical analysis

The analysis of all the results obtained was performed using appropriate statistical methods and the program Minitab® 16.1.1. The Ryan-Joiner normality test was used to determine whether the data obtained had a normal distribution. Tukey analysis (ANOVA) was used to determine the differences in CD3, FoxP3, and VEGF immune levels between CCHs, cutaneous histiocytoses, and HSs. In addition, a Pearson correlation test was performed to determine the relationship between CD3, FoxP3, and VEGF levels. The correlation ranges of r value were evaluated as follows: insignificant correlation between 0-0.3, low correlation between 0.3-0.5, moderate correlation between 0.5-

Table-1. Information on antibodies used in the study

Antibody	Manufacturer	Catalog Number	Origin	Clone	Dilution	Chromogen	Duration
CD3	Dako (Agilent Technologies)	M7254	Mouse	F7.2.38	1:25	DAB	ON
E-cadherin	Dako (Agilent Technologies)	M3612	Mouse	NCH-38	1:25	DAB	ON
FoxP3	Invitrogen (Thermo Fisher Scientific)	14-5773-82	Rat	FJK-16s	1:100	DAB	ON
Iba-1	Wako (Fujifilm Wako Pure Chemical Corporation)	019-19741	Rabbit	Poly	1:100	AEC	ON
VEGF	Invitrogen (Thermo Fisher Scientific)	MA5-13182	Mouse	JH121	1:20	DAB	ON

ON: Overnight

0.7, high correlation between 0.7-0.9, and 0.9-1 very high correlation (Hinkle et al., 2003; Mukaka, 2012).

RESULTS

Histopathological and Immunohistochemical Findings

The diagnosis was made as lymphoma because of intense positivity on staining with CD3 antibody in 2 of 23 cases. Intense Iba-1 positivity was detected in 12 of the remaining 21 cases, and the diagnosis of histiocytic tumor was confirmed. In the other 9 cases, varying degrees of Iba-1-positive histiocytic cell infiltrates were found only within the tumor tissue. Therefore, these 9 cases were not evaluated in the study. It was noted that only 2 cases with Iba-1 positivity were found to be positive for E-cadherin when evaluated. These cases were one CCH and one HS. It was concluded that the cases with E-cadherin negativity and without epitheliotropism were CH derived from dendritic cells. While mild to severe CD3-positive lymphocyte infiltrates were observed in cases with CCH, CD3-positive lymphocyte infiltrates were intense in cases with CH and HS.

Of the 12 cases whose histiocytic origin was confirmed by immunohistochemistry, 4 were diagnosed as CCH, 3 as CH, 4 as HSs, and 1 as HpHS. In benign histiocytic tumors, infiltrates were observed to spread from the superficial dermis to the depths and in some cases reached the subcutis. The neoplastic cells were found to have round-oval nuclei and prominent nucleoli and generally to have an eosinophilic cytoplasm. Few mitotic figures and mild anisocytosis and anisokaryosis were noted. In 4 cases with epidermal tropism, the diagnosis was made as CCH. In these cases, varying degrees of lymphocyte infiltrates were noted. In 3 cases without epithelial tropism, the diagnosis was made as CH. In these cases, very severe lymphocyte infiltration was observed. Patients diagnosed with HS generally had marked anisocytosis and anisokaryosis and the presence of multinucleated giant cells. A large number of mitotic figures were conspicuous. Intense lymphocyte infiltrates were also observed in these cases.

When CD3 was evaluated, the score was 4 in four of the HS cases and 3 in one. One of the CCHs had a score of 1, two had a score of 2, and one had a score of 4. In all cases of CH, the score was 4. When scoring for FoxP3, the score was 4 in two of the cases with HS, a score of 2 in one, and a score of 1 in two. For CCHs, the score was 3 in one, a score of 2 in one, and a score of 1 in two. For CH, the score was 2 in two and a score of 1 in one. Finally, in the evaluation of VEGF positivity, the value was 1 in one case of HS, 3 in three cases, and 4 in one case; in one case of CCH, the value was 1, 2 in two cases, and 4 in one case; and in all cases of CH, the value was 2. Immunostaining positivity and scores are shown in Table 2. Also, histopathological and immunohistochemical characteristics of the histiocytic tumors are demonstrated in Figure 1.

Statistical Findings

When CD3 levels were statistically compared in CCHs, CH, and HS, CD3 density was significantly higher in CH and HS than in CCHs ($P < 0.05$). When FoxP3 and VEGF levels were compared, no significant difference was found between these tumors. The results are shown in Table 3.

According to Pearson correlation analysis, there was no significant correlation between CD3 and FoxP3 and CD3 and VEGF, whereas a moderately significant negative correlation was found between FoxP3 and VEGF with an r value of -0.595 ($P < 0.05$). The results are shown in Table 4.

DISCUSSION

There are several well-defined histiocytic proliferative disorders in dogs. CCH has features of Langerhans cells and is a benign tumor that can regress spontaneously. In addition, cutaneous Langerhans cell histiocytosis represents another group of diseases that can spread from multiple skin lesions to lymph nodes and internal organs. However, individual skin lesions have similarities to CCH. Apart from these tumors, reactive histiocytosis (cutaneous and systemic) is a proliferative disorder resulting from activation of interstitial dendritic cells. Reactive histiocytosis has extensive lymphocyte-rich infiltrates. HS is a complex and malignant tumor that originates from interstitial dendritic cells and can form in the spleen, lymph nodes, lung, bone marrow, skin, subcutaneous tissue, brain, and joint regions. Although HS arises from interstitial dendritic cells, another variant, HpHS, originates from macrophages in the red pulp of the spleen. In our study, three histiocytomas, four CH, four HS, and one HpHS were diagnosed. Histopathologically, histiocytomas are epidermotropic and usually epidermal invasive (Moore, 2017). In one case study, histiocytes in CCHs were observed to invade the epidermis individually or in clusters at a rate exceeding sixty percent (Moore et al., 1996). Epidermal invasion is

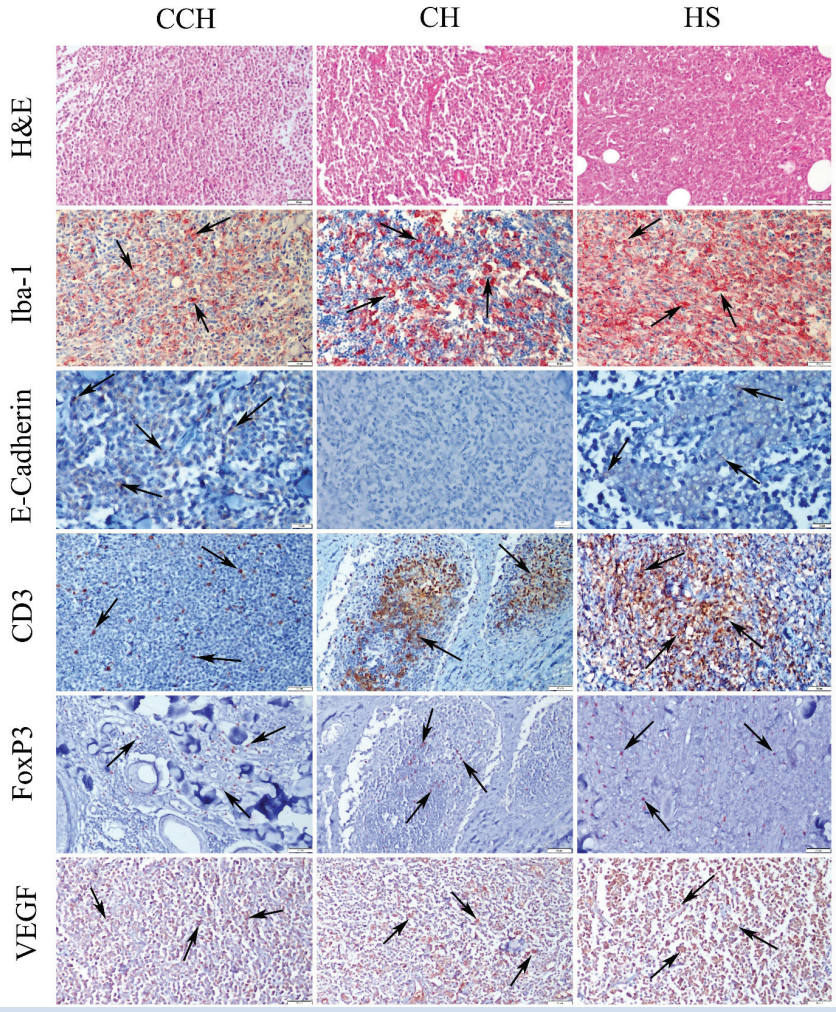

Figure-1: Histopathological and immunohistochemical characteristics of some histiocytic tumors in the study. Strong positivity for Iba-1 in all tumors. Mild to moderate positivity for E-cadherin in CCH and HS, but negativity in CH. Mild CD3 positive cell infiltration in CCH, but numerous positive cells in CH and HS. Mild to moderate FoxP3 positivity in all tumors. Moderate positivity of VEGF in all tumors (arrows indicate the positive stainings).

Table-2. Information and immunohistochemical staining results of canine histiocytic tumors.

No	Race	Gender	Age	Location	Diagnosis	Iba-1	E-Cadherin	CD3 Score	FoxP3 Score	VEGF Score
1.	-	Female	1 year	Left anterior extremity skin	CCH	+	+	1	3	1
1.	Cross	Male	3 year	Ocular	CCH	+	-	2	1	4
2.	-	Male	4 year	Ocular	CCH	+	-	2	1	2
3.	French Bulldog	Male	6 year	Hip area skin	CCH	+	-	4	2	2
4.	Kangal	Male	6 year	Hind limb skin	CH	+	-	4	2	2
5.	-	Male	4 month	Lip	CH	+	-	4	2	2
6.	-	Female	11 year	Vagina	CH	+	-	4	1	4
7.	Cross	Female	1 year	Conjunctiva	HS	+	-	4	1	4
8.	-	-	-	Neck region skin	HS	+	-	4	4	3
9.	Terrier	Female	14 year	Cheek	HS	+	+	3	1	3
10.	-	-	-	Dermal	HS	+	-	4	2	3
11.	-	-	10 year	Spleen	HpHS	+	-	4	4	1

CCH: Canine cutaneous histiocytoma, **CH**: Cutaneous histiocytosis **HS:** Histiocytic sarcoma, **HpHS:** Hemophagocytic histiocytic sarcoma

Table-3. Statistical data of CD3, FOXP3 and VEGF scores.

Tumor / Antibody	CD3	FoxP3	VEGF
CCH	2.250±0.629 [b]	1.750±0.479 [a]	2.250±0.629 [a]
CH	4.000±0.000 [a]	1.667±0.333 [a]	2.667±0.667 [a]
HS	3.800±0.200 [a]	2.400±0.678 [a]	2.800±0.490 [a]
P value	0.024*	0.788	0.709

* P < 0.05 is considered significant.
a,b: Data with different letters in the same column is significant

Table 4. Pearson correlation test comparisons of CD3, FoxP3 and VEGF scores.

		CD3	FoxP3
FoxP3	Pearson correlation	0.150	
	Sig. (2-tailed)	0.641	
VEGF	Pearson correlation	0.208	**-0.595**
	Sig. (2-tailed)	0.516	**0.041**

not observed in CH; instead, perivascular histiocyte infiltration in the deep dermis and subcutis is more prominent in these tumors (Moore, 2017). In our study, we evaluated the presence of epidermal invasion in histiocytic disease that was histopathologically benign and diagnosed CCH in cases in which this condition was observed. In contrast, in cases in which epidermal invasion and perivascular infiltrates were not observed, the diagnosis was CH.

Langerhans cells, dendritic cells, and macrophages can all give rise to histiocytic tumors. First and foremost, confirmation of the histiocytic origin is required in the differential diagnosis of these tumors. Iba-1 and E-cadherin were used in our study for this purpose. Iba-1 is expressed specifically in cells of the monocyte/macrophage lineage, including microglia (Köhler, 2007; Schulze et al., 2008). Iba-1 has been found to be positive in all canine histiocytic tumors (Moore, 2017). Iba-1 has been found to be highly positive in CCH, HS, and reactive histiocytosis, but negative in other round cell tumors (melanoma, lymphoma, mastocytoma, and plasmacytoma) (Pierezan et al., 2014). Only 12 of the tumoral cells in our study showed intense Iba-1 positivity.

Among leukocytes, E-cadherin expression is specific for Langerhans cells and is used specifically for the diagnosis of histiocytomas (Moore, 2004). Although E-cadherin expression is a valuable indicator of Langerhans cell differentiation in CCH, the incidence of E-cadherin expression in these tumors is unknown, and CCHs without E-cadherin expression are also known to exist (Moore, 2014). Ramos-Vara and Miller (2011) investigated the presence of E-cadherin in 116 canine round cell tumors and observed E-cadherin positivity in 95 cases (13 CCHs, 54 plasmacytomas, 14 mastocytomas, 10 epitheliotropic lymphomas, and 5 HSs). Hirako et al. (2015) reported a Langerhans cell-derived primary cutaneous HS in a Pembroke Welsh Corgi dog and reported that this tumor was positive for E-cadherin with Iba-1. Pazdzior-Czapula *et al.* (Paździor-Czapula et al., 2015) reported E-cadherin positivity in two HSs. In a previous study (Ipek et al., 2021), we also observed E-cadherin positivity in 3 HS, but we found E-cadherin negativity in three of 4 CCH and one malignant histiocytosis. Similarly, E-cadherin positivity was observed in only one of the four histiocytoma cases in our current study. While E-cadherin was negative in cases with CH, E-cadherin was positive in only one of 5 HS.

FoxP3, an X-chromosome-encoded intracellular forkhead transcription factor, has been identified as a key factor in natural Treg suppressive phenotype and is a highly specific marker of mouse Tregs. FoxP3 is also found to be stably upregulated in human Tregs (Fontenot et al., 2003; R.Walker et al., 2003). The involvement of Tregs in human and animal cancer is of great interest (Beyer and Schultze, 2006; Biller et al., 2010). In human cancer patients, an increase in CD4 + CD25 + FoxP3 + Treg numbers has been observed in peripheral blood, lymph nodes, and various neoplasms, and this increase has been linked to a poor prognosis (Bates et al., 2006; Biller et al., 2010; Curiel, 2007; O'Neill et al., 2009; Tominaga et al., 2010; Wolf et al., 2003). However, although some studies failed to discover a predictive significance (Grabenbauer et al., 2006), others found that the presence of FoxP3+ Treg in tumors is linked with a better progno-

sis (Carreras et al., 2006). Tregs have also been found in both healthy and cancer-bearing dogs. Tregs were found in higher numbers in peripheral blood of dogs with melanoma, osteosarcoma, mammary gland cancer, and lymphoma (Biller et al., 2010; Miller et al., 2006; O'Neill et al., 2009; Tominaga et al., 2010). The presence of neoplastic cells is expected to drive local proliferation or selective migration of Tregs to tumor infiltrating regions, therefore the number of Tregs in canine neoplasms was much greater than in the peripheral blood of dogs with cancer (Oh et al., 2014). Tregs have been demonstrated to boost the production of immunosuppressive IL-10 and TGF-beta, although research on their prognostic impact is limited (Abrams et al., 2010). There was no significant difference in FoxP3 positive lymphocyte density between histiocytoma, CH, and HS in our study. As a result, a link between FoxP3 positive Tregs and cancer could not be established. According to Belluco et al. (2020), there is no correlation between CD3 and FoxP3 positive lymphocyte infiltrations in canine CCHs. In our study, the correlation between CD3 and FoxP3 positive lymphocyte infiltrations was also determined to be negligible.

In recent years, research in human oncology has focused on the role of Treg in cancer patients. Immune cells commonly penetrate the tumor microenvironment, according to histopathological analyses (Carvalho et al., 2014; Sakai et al., 2018). According to reports of poor prognosis and significant Treg infiltrations in human malignancies, CD4+ CD25+ T cells impair anti-tumoral activity. However, the underlying mechanisms are not completely understood (Liu et al., 2016; Sakaguchi et al., 2008; Sakai et al., 2018; Wang et al., 2012). These cells' influence on the immune system not only avoids the production of powerful immune responses, which can be damaging, as previously mentioned, but also encourages neoplastic growth and dissemination (Biller et al., 2007). Multiple infiltrating Tregs have been reported in a wide range of tumors, including seminoma, intestinal lymphoma, breast carcinoma, and HS in dogs, as in people (Carvalho et al., 2016; Kim et al., 2013; Maeda et al., 2016; Marcinowska et al., 2017). Furthermore, increased Treg infiltration in breast cancer has been linked to poor prognosis characteristics such greater histological grade, lymphatic invasion, and tissue necrosis (Kim et al., 2012; Sakai et al., 2018). Another research found CD4 + FoxP3 + T lymphocytes in healthy dogs' peripheral blood. In that study, 4.3 percent of T cells were found to be CD4+ FoxP3+, and this figure increased to 7.5 percent in cancer-affected dogs. A comparable rise was seen in lymph nodes, where the percentage of normal Treg was 9.8 percent and 17.1 percent in lymph nodes with malignancies (Biller et al., 2007). Garden et al. (2011) found that the prevalence of metastases in all tumor types was related with a significant infiltration of CD4+ FoxP3+ cells. In this investigation, we found low to moderate FoxP3 positive lymphocyte infiltrates in histiocytoma and CH cases, but significant infiltration in two HS cases. Even while there is a difference between tumors, it is not statistically significant. As a result, it was assumed that FoxP3 positive lymphocyte infiltrates were unrelated to cancer. However, as compared to histiocytoma patients, CD3 positive T cells were shown to be considerably greater in CH and HS. This might imply that the total T lymphocyte density is linked to cancer. In fact, Marcinowska et al. (2017) examined CD3 and FoxP3 infiltrations in 40 dogs with HS and found that FoxP3 infiltrates were detected in a larger percentage of soft tissue HS, which had a better prognosis than disseminated and more malignant splenic HS. Despite the fact that such a comparison was not possible due to the small sample size in our investigation, the FoxP3 score was found to be high in the HpHS case identified in the spleen. However, in a soft tissue HS, the score was also found to be high.

FoxP3 reduces angiogenesis in breast cancers via downregulating VEGF, according to Li et al. (2018). However, several studies have found that FoxP3 is linked to enhanced angiogenesis in a variety of malignancies (Giatromanolaki et al., 2008; Zhan et al., 2012). According to Luznik et al. (2020), the involvement of Tregs in angiogenesis differ depending on tissue and disease. Previous research has linked Tregs to enhanced angiogenesis in malignancies and reproductive diseases, but also to tissue ischemia, chronic inflammation, and reduced angiogenesis in ocular tissues. However, it was also noted that such research often remained at the correlational level, and the link between FoxP3 and angiogenesis has yet to be thoroughly defined. In our study, FoxP3 infiltrates ranging from low to high were seen in all canine histiocytic disorders. The correlation analysis of FoxP3 and VEGF scores revealed a statistically significant and moderate negative connection. This data might imply that FoxP3+ Tregs inhibit VEGF-mediated angiogenesis in canine histiocytic disorders, which is line with the observations of Li et al. (2018). Muir et al. (2017) discovered that the prevalence of FoxP3+ cells in canine B lymphomas is unrelated to Ki67, a

neoplastic cell proliferation marker. Carreras et al. (2006) also discovered that FoxP3+ Tregs are linked to improved survival in follicular lymphomas. Based on these data, it is clear that FoxP3+ Tregs are linked with a poor prognosis in some cancer types, whereas they may be associated with an unclear or improved prognosis in others. Unfortunately, case follow-up in terms of prognosis was not possible in our investigation, but an interpretation was established based on malignancy and VEGF levels of the cases.

Some current cancer therapies have been proven to influence the presence and function of Tregs via reducing their effects. Low-dose cyclophosphamide, an immunosuppressive medication, fludarabine and gemcitabine, which impede cellular DNA synthesis, paclitaxel, a mitotic inhibitor, and tyrosine-kinase inhibitors are examples of these (Hatziioannou et al., 2017). Specific therapeutics targeting Treg-expressed molecules have also been reported in animal models, and human clinical trials are currently ongoing (Bulliard et al., 2014; Curti et al., 2013). Although Tregs have been linked to enhanced angiogenesis in many cancers, as observed in some previous research, FoxP3+ cell infiltrations may be linked to lower VEGF and hence decreased angiogenesis in both cancer and other disease states. As a result, the therapeutic choices for FoxP3+ Tregs should be tailored to the tissue and condition. Although our findings suggest a negative association between FoxP3 and VEGF in canine histiocytic tumors, further molecular research is needed to fully confirm the relationship between FoxP3+ cell infiltrations and angiogenesis and prognosis in a larger number of tumor tissues.

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CONFLICT OF INTEREST

None declared.

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