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RESEARCH ARTICLE

Combination Chemotherapy with Firocoxib Induces Reduction of Regulatory T Cells in Dogs with Multicentric B Cell Lymphoma

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ABSTRACT

The aim of this study was to quantify T cells (CD4+, CD8+ and Treg), in dogs with multicentric lymphoma treated with a conventional CHOP protocol or with one in which firocoxib was used instead of prednisone. Twenty-one dogs with lymphoma were divided into two treatment groups (G1=10 and G2=11): those that received the conventional CHOP protocol for 19 weeks (CHOP group) and those that received CHOP protocol with firocoxib instead of prednisone (CHOF group). Twelve clinically healthy dogs were used as control. The percentage of Treg cells were three times greater in animals with lymphoma than in the control group ($P<0.05$). During treatment, the reduction in Treg cells was slower in animals treated with firocoxib than in those that received prednisone. No significant difference ($P>0.05$) was observed in survival time between the two treatment protocols. It can be concluded that the use of firocoxib instead of prednisone in CHOP protocol can induce Treg cell remission in dogs with multicentric lymphoma without interfering with the survival time of these animals.

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INTRODUCTION

Multicentric lymphoma is one of the most common neoplasias in dogs and corresponds to 7-24% of all canine neoplasias (Richards and Suter, 2015). Although its aetiology remains unclear, it is believed that lymphomas have a multifactorial origin through which immuno-suppression can significantly influence the process of tumorigenesis (Grivennikov *et al*., 2011). Chemotherapy protocols that promote longer remission and survival times often take 19 weeks and consist on the combination of vincristine, prednisone, cyclophosphamide and doxorubicin(CHOP) (Vail *et al*., 2013).

Regulatory T cells (Treg) modulate immune responses in physiological and several infectious, allergic, autoimmune, and neoplastic conditions (Cools *et al*., 2007). In dogs, Treg cells have been identified through the use of specific canine anti-CD4 and murine Foxp3 antibodies (Biller *et al*., 2007, Horiuchi *et al*., 2009, O'Neill *et al*., 2009). Recent studies suggest that the increase in Treg cells can influence the development of different types of malign tumours in dogs (Sakai *et al*., 2017; Lisiecka *et al*., 2019) and favour metastasis by supressing type 1 immunity, providing immunotolerance for tumoral cells (Horiuchi *et al*., 2009).

It is believed that the increase in COX-2 expression by tumoral cells stimulates the development of Treg cells (Yaqub *et al*., 2008). In human patients with lung cancer, the infiltration of Treg cells into the tumour is associated with COX-2 expression (Shimizu *et al*., 2010). From a therapeutic perspective, studies in mice with Lewis pulmonary carcinoma have shown that celecoxib can reduce Treg cells (Lee *et al*., 2009).

The use of coxibs in cancer therapy is rowing and its anti-tumoral effects are well documented in several canine neoplasias (Knapp *et al*., 1994.; Saito *et al*., 2014). However, few studies have been done on its benefits in lymphoma treatment or on its effect on Treg cells. Thus, the aim of this study was to quantify the T cells (CD4+, CD8+ and Treg) in dogs with multicentric lymphoma

treated with conventional CHOP protocol or with one in which prednisone was replaced with firocoxib.

MATERIALS AND METHODS

The protocol used in this study was reviewed and approved by the Ethics Committee on the Use of Animals (CEUA) of the UNESP – Universidade Estadual Paulista, Campus Jaboticabal, Brazil (protocol number 027665/10).

Animals: Twenty-one dogs diagnosed with lymphoma were divided into two treatment groups (G1=10 and G2=11): the CHOP group, which received the conventional 19-weeks CHOP protocol (Chun, 2009); and the CHOF group, which received the same protocol but had firocoxib instead of prednisone (Table 1 and 2). Histopathology of tumours was performed according to the method by Valli *et al*. (2011) (Fig. 1).

Twelve clinically healthy dogs were used as controls, six of which were males and six females aged between 2 and 14 years. This group consisted of nine crossbreed dogs, one Doberman, one Beagle and one Labrador.

Subpopulations of T lymphocytes: The percentage of $CD4^+$, $CD8^+$ and Treg cells present in peripheral blood was determined by flow cytometry at the time of diagnosis (M0), after the first round of chemotherapy (M5) and after the last week of chemotherapy (M20) (Table 2). Animals from the control group were subjected to the same evaluation, at a single time. Blood samples (15mL) were collected in heparinized tubes and the mononuclear cells isolated using a modified version of the technique described by Biller *et al*. (2007), employing the antibodies described in Table 3.

The blood samples were diluted (v:v) in sterile and separated by Ficoll-Paque PLUS gradient (GE®). After centrifugation at 1500 rpm for 30 minutes, the mononuclear cell ring was isolated, resuspended in PBS, and centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the leucocyte ring resuspended in 2mL ACK erythrocyte lysing buffer for 5 minutes before being centrifuged. The cells were washed and resuspended in 1mL PBS prior to countingin a Neubauer chamber. Autologous serum (5µL) was added to each tube and incubated for 40 min to block unspecific reactions. The tubes were subsequently incubated with superficial and intracellular antibodies for 30 minutes.

The suspended mononuclear cells were identified and quantified according to their size, granulation and fluorescence intensity by flow cytometry using antigens for PanT, CD4+ and CD8+ cells, and intracellular Foxp3+. Cells were washed in PBS and 100µL PBS-formalin solution added. A total of $1x10^6$ cells were used for cell-surface marking and $2x10^6$ cells for intracellular marking.

Six tubes of mononuclear cells were obtained per animal: Tube 1 – non-marked cells; Tube 2 – cells marked with control isotypes; Tube 3 – cells marked with CD4 FITC, CD8 PE, and PanT APC antibodies; Tube 4 –non-marked cells; Tube 5 – cells marked with control isotypes; Tube 6 – cells marked with CD4 FITC, PanT APC, and Foxp3 PE antibodies. The cells from Tubes 4, 5, and 6 were subjected to membrane permeabilization with permeabilization/fixation and permeabilization solutions (eBioscience® cat 00-5223 and cat 00-8333, respectively).

Cytoflourimetric analysis was performed within 24 hours of blood sampling. Cellular preparation was carried out using FACSCanto II (BD®). At least fifty thousand events on the lymphocyte gate were collected per tube and analysed using the FlowJo programme (Fig. 2). Mouse anti-IgG2a antibody was used as the control isotype antibody for each fluorochrome.

Statistical analysis: T test was used to compare two variables that were normally distributed; however, the Mann Whitney test was used when these were not normally distributed. The F-test of the ANOVA was used hen three normally distributed variables were compared. The Tukey test was used to compare pairs of means. Non-parametric ANOVA (Kruskal-Wallis) was used when variables were not normally distributed and the Dunn test used to compare pairs. Survival curves were estimated and compared between groups by the Kaplan-Meier method and the log-rank test. Significance was considered at 5% ($P<0.05$). Analysis was performed using the statistical programme GraphPad Prism 5 (*GraphPad Prim5 software package*, GraphPad Software, San Diego, CA, USA).

Table 1: Age, sex, breed, histological type, immunophenotype, and staging of dogs with multicentric lymphoma treated with CHOP or CHOF protocols

ID	Age (years)	Sex	Breed	Histological Type	Immunophenotype	Clinical Stage
CHOP						
1	5	M	Rottweiler	Immunoblastic	B	IIIb
2	9	F	Poodle	Burkitt Like	B	Vb
3	5	F	Rottweiler	DLC	B	IIIb
4	6	M	Rottweiler	Lymphoblastic	B	IVb
5	5	M	Mixed	DLC	B	Iva
6	8	F	Pinscher	DLC	B	Vb
7	11	F	Rottweiler	DLC	B	IVb
8	5	F	Rottweiler	DLC	B	Va
9	11	F	Poodle	DLC	B	IVb
10	7	M	Mixed	DLC	B	Iva
CHOF						
11	8	F	Rottweiler	DLC	B	Iva
12	12	F	Mixed	DLC	B	IVb
13	13	F	A. Pitbull	DLC	B	IVa
14	6	F	Basset H.	DLC	B	IVb
15	9	M	Mixed	Follicular grade III	B	IVa
16	4	M	Maltese	DLC	B	IIIa
17	13	F	Boxer	DLC	B	IVa
18	12	M	Mixed	DLC	B	IVa
19	7	F	Fox Terrier	DLC	B	IVb
20	9	F	A. Pitbull	DLC	B	IVa
21	4	M	Maltese	DLC	B	IVa

M: male; F: female; DLC: diffuse large cell; not otherwise specified.

RESULTS

Data are expressed as mean ± standard error of the mean (SE). Dogs with lymphoma showed significantly (P<0.0001) greater percentage of Treg cells (17.08±1.62) than the control group (5.67±1.62) (Fig. 3).

There was a significant (P<0.0001) difference in the percentage of Treg cells between dogs treated with prednisone and controls. During treatment, the percentage of Treg cells reduced significantly at M5 (P<0.0001), reaching levels equivalent to the control group (P>0.05) and remaining stable up to M20 (P>0.05). In dogs treated with firocoxib, the percentage of Treg cells showed similar behaviour to those treated with prednisone; however, a significant (P<0.0001) reduction in those levels was only observed later, at M20. Before the start of treatment (M0), the mean percentage of Treg cells of the CHOP group was significantly (P<0.0114) higher than that of the CHOF group; however, no significant difference (P>0.05) was observed between the groups at any other moment (Table 4).

No significant difference (P>0.05) was observed in the mean percentage of $CD4^+$ and $CD8^+$cells between the treatment groups throughout chemotherapy, nor between the treatment and control groups (Table 5). Even though there was a reduction in the percentage of Treg cells, no significant (P=0.4201) difference was observed between survival times (Fig. 4).

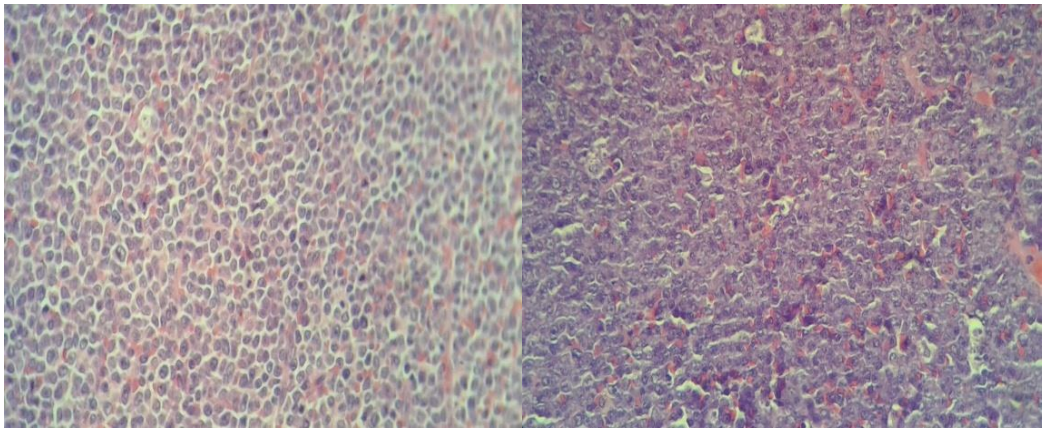

Fig. 1: Diffuse large cell lymphoma B: Notice multiple, peripherally located nucleoli (H&E, ×40).

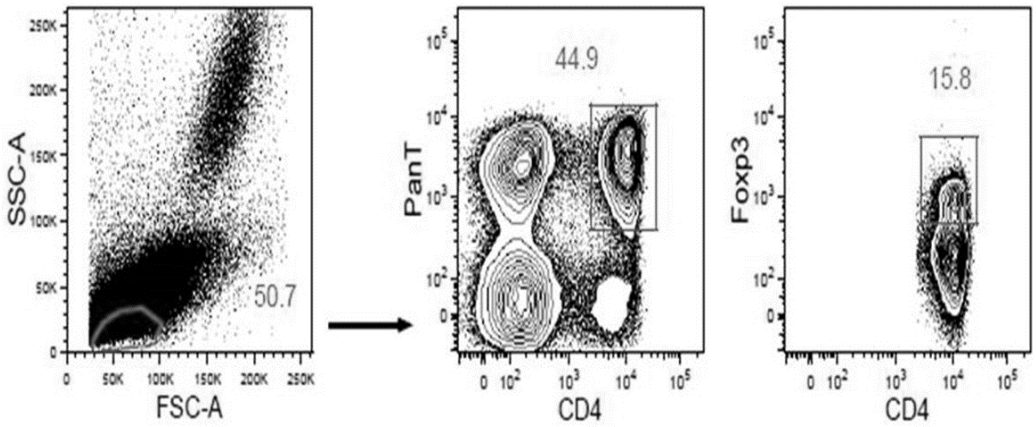

Fig. 2: Representative fluxogram of the lymphocyte gate of a dog with multicentric lymphoma from which a gate of $CD4^+$ T lymphocytes was selected followed by the identification of a gate of $CD4^+Foxp3^+$ lymphocytes (Treg).

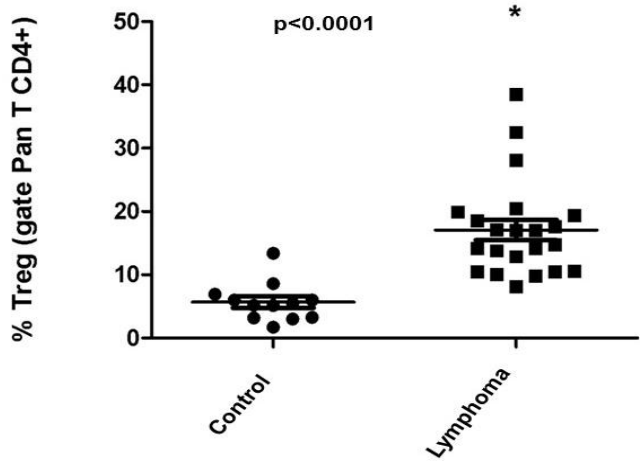

Fig. 3: Percentage of regulatory T cells (Treg) in the peripheral blood of healthy dogs (control) and dogs with multicentric lymphoma.

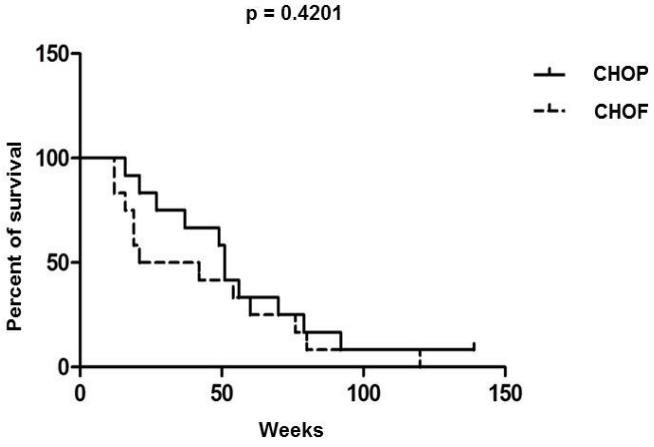

Fig. 4: Survival rate and times of dogs with multicentric lymphoma treated with CHOP or CHOF.

Table 2: Chemotherapy protocol used in dogs with multicentric lymphoma

Weeks	Vincristine 0.7 mg/m² IV	Cyclophosphamide 250mg/m² PO	Doxorubicin 30mg/m² IV	Prednisone or firocoxib** PO
1*	X			x
2		X		x
3	X			x
4			X	x
5*				
6	X			
7		X		
8	X			
9			X	
10				
11	X			
12		X		
13	X			
14			X	
15				
16	X			
17		X		
18	X			
19			X	
20*				

*Blood sampling times for quantification of T lymphocytes; **Prednisone: 2 mg/Kg every 24h, in the first week; 1.5 mg/Kg in the second week; 1 mg/Kg in the third week; and 0.5 mg/Kg in the fourth week. Firocoxib: 5 mg/Kg every 24h.

Table 3: Control isotypes and antibodies used in flow cytometry and their respective volume and manufacture

Control Isotypes	Manufacturer		Volume
Mouse IgG1-Negative Control FITC	AbDSerotec		1µL
Rat IgG2a Iso Control PE	EBioscience		1µL
APC Mouse IgG1k Isotype Control	BD Pharmingen™		1µL
Antibodies	**Manufacturer**	**Clone**	**Volume**
Rat anti dog CD4: FITC	AbDSerotec	YKIX302.9	1µL
Rat anti dog CD8: RPE	AbDSerotec	YCATE55.9	1µL
APC mouse anti dog PanT cell marker	BD Pharmingen™	FJK-16s	1µL
Anti mouse/rat Foxp3 PE	EBioscience	LSM 8.358	2µL

Table 4: Mean ± SE of the percentage of regulatory T cells in the control group and in the treatment groups CHOP and CHOF at times (M) 0, 5, and 20.

	Control	M0	M5	M20
	N=12	N=10	N=10	N=7
CHOP	5.67 ± 0.89^{A}	20.66 ± 2.49^{Ba}	8.69 ± 1.30^{Aa}	8.64 ± 1.74^{Aa}
	N=12	N=9	N=11	N=5
CHOF	5.67 ± 0.89^{A}	12.79 ± 0.77^{Bb}	8.14 ± 0.74^{ABa}	5.90 ± 1.13^{Aa}

Different capital letters in the same row and different lower case letters in the same column represent significant difference between means by the Tukey test. Significance was considered at 5%. SE: Standard error of the mean.

Table 5: Mean ± SE of the percentage of $CD4^+$ and $CD8^+$ cells in the control group and in the treatment groups CHOP and CHOF at times (M) 0, 5, and 20.

	Control	M0	M5	M20
		$CD4^+$		
	N=12	N=10	N=10	N=7
CHOP	51.01±4.92	45.52±4.66	51.61±3.45	59.50±4.48
	N=12	N=9	N=11	N=5
CHOF	51.01±4.92	53.27±2.34	52.96±2.98	52.35±1.34
		$CD8^+$		
	N=12	N=10	N=10	N=7
CHOP	25.87±4.39	25.87±4.39	20.37±3.87	20.73±2.16
	N=12	N=9	N=11	N=5
CHOF	25.87±4.39	23.91±2.91	23.51±2.43	23.98±3.04

DISCUSSION

The percentage of Treg cells in animals with lymphoma was three times higher than in those from the control group, as similarly reported in dogs with different neoplasias (Biller *et al*., 2007; Horiuchi *et al*., 2009; O'Neill *et al*., 2009, Mitchell *et al*., 2012; Mucha *et al*., 2016). O'Neill *et al*. (2009) observed a significant increase in Treg cells of dogs with tumours in comparison to controls, with the greatest percentages being recorded in dogs with carcinoma, followed by those with sarcoma, lymphoma, and mastocytoma. Tominaga *et al*. (2010) also observed that dogs with oral cavity melanoma showed greater percentage of Treg cells in peripheral blood than control animals. More recently, Munhoz *et al*. (2016) described an increase in Treg cells of dogs with multicentric lymphomas when compared to controls.

The immunosuppression role of Treg cellsin stimulating cancer development has been well documented. These cells are known to reduce the anti-tumoral immunity established by the $CD4^+$ and $CD8^+$cells through the production of immunosuppressant cytokines such as TGF-β and IL-10, which in turn promote the conversion of conventional T cells ($CD4^+$) into Treg(Strauss *et al*., 2007). In dogs with cancer, this relationship is supported by the findings of Biller *et al*. (2007), who have demonstrated that Foxp3 mRNA expression in activated lymphocytes cultured with IL-2 and TGF- β.

In the present study, a reduction in circulating Treg cells was observed in both treatment groups. In addition to the classic anti-tumoral effects of anti-neoplastic agents, it is known that doxorubicin and cyclophosphamide can induce a reduction in Treg cells in dogs with different neoplasias, including lymphomas (Mitchell *et al*., 2012; Munhoz *et al*., 2016). During treatment, the reduction in the percentage of Treg cells observed in dogs treated with firocoxib was slower than in those treated with prednisone, suggesting that the later promotes a more immediate response while the former a more delayed one.

Prednisone is present in the majority of chemotherapy protocols used in the treatment of lymphoma, due to its ability to induce cytolysis of neoplastic lymphocytes (Vail *et al*., 2013). Although there are no reports on the benefits of using firocoxib as part of chemotherapy in the treatment of dogs with lymphoma, the relationship between COX-2 and Treg cells in dogs with melanoma has been previously described. Tominaga *et al*. (2010) demonstrated that the increase in the expression of TGF-β, COX-2, and PGE2 by the tumour induces an *in situ* conversion of $CD4^+$into Treg cells and promotes their proliferation in the tumoral microenvironment.

The $CD4^+$ and $CD8^+$cells have a cytotoxic effect on tumoral cells and are present in several types of neoplasias in humans and murine models (Gerdemann *et al*., 2011). The infiltration of Treg cellsinto the tumours directly inhibits the anti-tumoral functions of $CD4^+$ and $CD8^+$cells through the secretion of immunosuppressant cytokines by the Treg cells, such as IL-10 and TGF-β (Curiel, 2007). However, in the present study, $CD4^+$ and $CD8^+$numbers did not vary at any given time during chemotherapy or between treatments. These results are in agreement with other studies in dogs with cancer (Walter *et al*., 2006,

Mitchell *et al*., 2012). In fact, Walter *et al*. (2006), when studying $CD4^+$ and $CD8^+$cells in dogs with lymphoma and osteosarcoma, observed that the function of these cells remained unchanged following chemotherapy. These authors have demonstrated that dogs are able to develop an IgG response specific to the KLH antigen (*keyhole limpet hemocyanin*) responsible for this effect.

The increase in the number of immunosuppressant Treg cells has been associated with a worse prognosis, as demonstrated in dogs with malignant mammary tumors (Carvalho *et al*., 2016) and dogs with diffuse large B cell lymphoma, in which an increase in the number of Treg cells was observed in animals with shorter survival time (Pinheiro *et al*., 2014). In the present study, no difference was observed in the survival time between the treatment groups, suggesting that treatment with firocoxib could promote survival times similar to those of traditional CHOP and, thus, be an alternative option of treatment to patients with lymphoma that have comorbidities that contraindicate the use of prednisone, such as diabetes and hyperadrenocorticism.

It can be concluded that the use of firocoxib instead of prednisone in CHOP protocol can induce Treg cell remission in dogs with multicentric lymphoma without compromising the survival time of these animals. However, further studies on the interaction between COX-2 and Treg cellsin dogs with lymphoma are needed for innovative therapeutic strategies directed at these targets to be developed.

Authors contribution: LAA, MTC and AES, were responsible for the design and development of the project. LAA, TDM, LSS, PCJ and SGC, responsible for the collection of material and treatments of patients. LAA, TDM and DMF, for performing flow cytometry. FRS responsible for the classification of lymphomas. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version**.**

REFERENCES

Biller BJ, Elmslie RE, Burnett RC, *et al*., 2007. Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer. Vet Immunol Immunopathol 116:69-78.

Carvalho MI, Pires I, Prada J, *et al*., 2016. Intratumoral FoxP3 is associated with angiogenesis and prognosis in malignant canine mamary tumors. Vet Immunol Immunopathol 178:1-9.

Chun R, 2009. Lymphoma: which chemotherapy protocol and why? Top. Companion Anim Med 24:157-62.

Cools N, Ponsaerts P, Van Tendeloo VFI, *et al*., 2007. Regulatory T cells and human disease. Clin Dev Immunol 2007:891-95.

Curiel TJ, 2007. Regulatory T-cell development: is Foxp3 the decider? Nat Med 13: 250-3.

Gerdemann U, Katari U, Christin AS, *et al*., 2011. Cytotoxic T lymphocytes simultaneously targeting multiple tumor-associated antigens to treat EBV negative lymphoma. Mol Ther 19:2258-68.

Grivennikov SI, Greten FR and Karin M, 2011. Immunity, Inflammation, and Cancer. Cell 140:883-99.

Horiuchi Y, Tominaga M, Ichikawa M, *et al*., 2009. Increase of regulatory T cells in the peripheral blood of dogs with metastatic tumors. Microbiol Immunol 53:468-74.

Knapp DW, Richardson RC, Chan TC, *et al*., 1994. Piroxicam therapy in 34 dogs with transitional cell carcinoma of the urinary bladder. J Vet Intern Med 8:273-8.

Lee SY, Choi HK, Lee KJ, *et al*., 2009. The immune tolerance of cancer is mediated by IDO that is inhibited by COX-2 inhibitors through regulatory T cells. J Immunother 32:22-8.

Lisiecka U, Kostro K, Dudek K, *et al*., 2019. Evaluation of T regulatory lymphocytes and serum concentration of selected cytokines in dogs with perianal tumors. Vet Immunol Immunopathol 207:10-7.

Mitchell L, Dow SW, Slansky JE, *et al*., 2012. Induction of remission results in spontaneous enhancement of anti-tumor cytotoxic T-lymphocyte activity in dogs with B cell lymphoma. Vet Immunol Immunopathol 145:597-603.

Mucha J, Rybicka A, Dolka I, *et al*., 2016. Immunosuppression in dogs during mamary cancer development. Vet Pathol 53:1147-53.

Munhoz TD, Anai LA, Fonseca DM, *et al*., 2016. Regulatory T cells in dogs with multicentric lymphoma: peripheral blood quantification at diagnosis and after initial stage chemotherapy. Arq Bras Med Veterinária e Zootec 68:1-9.

O'Neill K, Guth A, Biller B, *et al*., 2009. Changes in Regulatory T Cells in Dogs with Cancer and Associations with Tumor Type. J Vet Intern Med 23:875-81.

Pinheiro D, Chang YM, Bryant H, *et al*., 2014. Dissecting the regulatory microenvironment of a large animal model of non-Hodgkin lymphoma: evidence of a negative prognostic impact of FOXP3+ T cells in canine B cell lymphoma. PLoS One:e105027.

Richards KL and Suter SE, 2015. Man's best friend: what can pet dogs teach us about non-Hodgkin's lymphoma? Immunol Rev 263:173-91.

Saito T, Tamura Dand Asano R, 2014. Usefulness of selective COX-2 inhibitors as therapeutic agents against canine mammary tumors. Oncol Rep 31:1637-44.

Sakai K, Maeda S, Yamada Y, *et al*., 2018. Association of tumour-infiltrating regulatory T cells with adverse outcomes in dogs with malignant tumours. Vet Comp Oncol 16:330-6.

Shimizu K, Nakata M, Hirami Y, *et al*., 2010. Tumor-infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. J Thorac Oncol 5:585-90.

Strauss L, Bergmann C, Szczepanski M, *et al*., 2007. A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. Clin Cancer Res 13:4345-54.

Tominaga M, Horiuchi Y, Ichikawa M, *et al*., 2010. Flow cytometric analysis of peripheral blood and tumor-infiltrating regulatory T cells in dogs with oral malignant melanoma. J Vet Diagn Invest 22:438-41.

Vail DM, Pinkerton ME and Young KM, 2013. Canine lymphoma and lymphoid leukemias. In: Small Animal Clinical Oncology. 5th Ed, Elsevier, Missouri, USA pp:608-27.

Valli VE, San Myint M, Barthel A, *et al*., 2011. Classification of canine malignant lymphomas according to the World Health Organization criteria. Vet Pathol 48:198-211.

Walter CU, Biller BJ, Lana SE, *et al*., 2006. Effects of chemotherapy on immune responses in dogs with cancer. J Vet Intern Med 20:342-7.

Yaqub S, Henjum K, Mahic M, *et al*., 2008. Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner. Cancer Immunol. Immunother 57:813-21.