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

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ARTICLE HISTORY (17-255)
Received: July 24, 2017
Revised: March 04, 2019
Accepted: March 06, 2019
Published online: March 22, 2019

Key words:
Canine lymphoma
CHOP
Foxp3
Treg

A B S T R A C T
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 the 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.

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 immunosuppression 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, Horiuichi 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 benign tumours in dogs (Sakai et al., 2017; Lisiecka et al., 2019) and favour metastasis by suppressing 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 counting in 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 superfluous and intracellular antibodies for 30 minutes.

The suspended mononuclear cells were identified and quantified according to their size, granularity 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 1x10⁶ cells were used for cell-surface marking and 2x10⁶ 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).

Cytocentrifugical 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 to compare two groups when variables were normally distributed; however, the Dunn test was 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 |
|----|-------------|-----|-------|-------------------|-----------------|---------------|
| 1  | 5           | M   | Rottweiler | Immunoblastic | B               | IIb           |
| 2  | 9           | F   | Poodle   | Burkitt Like | B               | Vb            |
| 3  | 5           | F   | Rottweiler | DLC           | B               | IVb           |
| 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               | Vb            |
| 10 | 7           | M   | Mixed     | DLC           | B               | Iva           |

CHOF

| ID | Age (years) | Sex | Breed | Histological Type | Immunophenotype | Clinical Stage |
|----|-------------|-----|-------|-------------------|-----------------|---------------|
| 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).
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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 | MD | M0 | M20 |
|---------|----|----|-----|
| 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 |
| 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  |

Table 4: 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 |
|---------|----|----|-----|
| N=12    | N=10| N=10| N=7 |
| CHOP    | 5.67±0.89 20.66±2.49 | 6.69±1.30 | 8.64±1.74  |
| N=12    | N=9 | N=11 | N=5 |
| CHOF    | 5.67±0.89 12.79±0.77 | 8.14±0.74 | 5.90±1.13  |

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

| Control Isotypes | Manufacturer | IsoClone | Volume |
|------------------|--------------|----------|--------|
| Mouse IgG1-Negative | AbDSerotec | 1µL |
| Rat IgG2a IsoControl PE | EBioscience | 1µL |
| APC Mouse IgG1 K Isotype Control | Pharmingen™ | 1µL |
| Antibodies | Manufacturer | Clone | Volume |
| Rat and dog CD4:FITC | AbDSerotec | YKIX02.9 | 1µL |
| Rat and dog CD8: RPE | AbDSerotec | YCATE55.9 | 1µL |
| APC mouse and dog Pan T cell marker | Pharmingen™ | FJK-16s | 1µL |
| Anti mouse/rat Foxp3 PE | EBioscience | LSM 8.358 | 2µL |

Table 2: Chemotherapy protocol used in dogs with multicentric lymphoma

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

*a 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.

DISCUSSION

The percentage of Treg cells in animals with lymphoma was three times higher than those from the control group, as similarly reported in dogs with different neoplasias (Billier et al., 2007; Horiiuchi 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 cells in 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(Straus et al., 2007)). In dogs with cancer, this relationship is supported by the findings of Billier 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 cells into 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, 2009). 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 cells in 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. FR S responsible for the classification of lymphomas. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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