Research paper

Canine multicentric lymphoma exhibits systemic and intratumoral cytokine dysregulation

Joana N.R. Dias[a], Mariana Lopes[a], Conceição Peleteiro[a], Gonçalo Vicente[b], Telmo Nunes[a], Luísa Mateus[a], Frederico Aires-da-Silva[a], Luís Tavares[a], Solange Gil[a,*]

[a] CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, 1300-477 Lisbon, Portugal
[b] Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Universidade de Lisboa, Av. da Universidade Técnica, 1300-477, Lisboa, Portugal

ARTICLE INFO

ABSTRACT

Non-Hodgkin Lymphoma (NHL) is among the most common neoplasias in dogs and humans. Owing to remarkable similarities with its human counterpart, the canine lymphoma (cNHL) model has been proposed as a powerful framework for rapid and clinically relevant translation of novel immunotherapies. However, the establishment of cNHL as a predictive preclinical model has been hampered by the limited characterization of the canine immune system. Cytokines are key players of the interaction between tumor and its microenvironment. In human NHL, multiple cytokines have been linked to the development of lymphoma and are relevant biomarkers for treatment response and prognosis. In contrast, few studies have investigated cytokines in cNHL. Within this context, this study aimed to investigate cytokine regulation in cNHL.

A multicentric cNHL biobank was successfully constructed. Cytokine mRNA profiles in tumor tissue and circulating PBMC were analyzed by qRT-PCR and compared to a healthy control group. Specific primers were used to evaluate Th1, Th2 and Th17 responses. Systemic cytokine concentrations were measured using a commercial canine multiplex assay which included IL-2, IL6, IL-10 and TNF-α, and compared to a healthy control group. Our results demonstrated a dysregulation of cytokine mRNA expression, representative of the tumor microenvironment and systemic response in cNHL. Intratumoral cytokine response revealed a significant downregulation of humoral and Th1 responses. The systemic response demonstrated a distinct mRNA pattern, however immunosuppression also prevailed. Cytokine serum quantification showed a significant increase of IL-10 concentration in cNHL. Significant differences in hematological parameters were described and a correlation between IL-6 protein serum levels and neutrophil count was shown. Finally, data analysis demonstrated that baseline pretreatment IFN-γ tissue mRNA levels were correlated to survival outcome, predicting a favorable response to chemotherapy.

Altogether, these results revealed that cNHL presents a local and systemic dysregulation in cytokine response. By confirming and extending previous research, our work contributed for the evaluation of potential cytokine candidates for diagnostic, prognostic purposes and therapeutic intervention, therefore adding value to comparative oncology.

1. Introduction

Non-Hodgkin Lymphoma (NHL) is among the most common neoplasias in dogs and humans. Owing to remarkable similarities with its human counterpart, the canine lymphoma (cNHL) model has been proposed as a powerful framework for rapid and clinically relevant translation of novel immunotherapies (Park et al., 2016). However, the establishment of cNHL as a predictive preclinical model has been hampered by the limited characterization of the canine immune system.

Several efforts have been made to develop immunotherapeutic approaches for dogs. (Ito et al., 2015; Jain et al., 2016; Marconato et al., 2015; O'Connor and Wilson-Robles, 2014; Weiskopf et al., 2016). To date, two mAbs - CD20-positive B cell (Blontress®) and CD52 positive T cell lymphoma (Tactress®) - have been approved by the US Department of Agriculture and are commercially available in the USA and Canada (Regan and Dow, 2015). Nevertheless, the reported therapeutic efficacy

of these mAbs is suboptimal and substantially inferior to results reported in human patients. These results show that, apart from the importance of developing specific and sophisticated immunotherapies for veterinary settings in order to surpass the lack of cross-species reactivity to human immunotherapies, the limited characterization of the immune system of these animals may also hinder recent efforts to develop novel therapeutic tools, such as mAbs and CAR-T cells (chimeric antigen receptor T cells) (Hartley et al., 2018; Maekawa et al., 2017; Panjwani et al., 2016). Above all, the canine immune system has not been deeply studied and characterized compared to the human immune system. For example, relatively little is known about lymphocyte subtypes and expression and regulation of their receptors, or about canine cytokines that support their function. This also implies that there are no predictive tests to assess which patient may benefit from what type of immunotherapy (Klingemann, 2018).

NHL are an heterogeneous group of lymphoproliferative malignancies with variable patterns of behavior and responses to therapy. NHL development and invasion depend on the dynamic and complex interplay between tumor cells and non-neoplastic cells and on their interaction with the surrounding stroma/matrix environment. Such interactions are usually modulated by several cytokines (Grant and Bollard, 2017; Malaponte et al., 2016). Immune dysregulation of the host cytokine environment has been associated with lymphoma pathogenesis in humans. In light of this, the evaluation of cytokine and immune stimulatory molecules prior to lymphoma diagnosis has provided insights into the etiology of these cancers and has been exploited as predictable biomarkers of increased risk of developing lymphoma in patients with primary and acquired immune deficiencies (Chiu et al., 2017; Purdue et al., 2013). Furthermore, changes in cytokine signaling and regulation have also been correlated with disease presentation, tumor progression, response to therapy and prognosis (Vaidya and Witzig, 2014). By reflecting cytokine expression of nascent tumors cells and/or reactive tumor-associated immune cells, these molecules have also been regarded as potential early biomarkers of human lymphoma (Vendrame and Martínez-Maza, 2011).

An impaired cellular immunity in dogs with lymphoma has been evidenced by *in vitro* lymphocyte blastogenesis, survival of allogeneic skin grafts, and response to tuberculin challenge exposure after sensitization with Bacille Calmette Guérin (Calvert et al., 1982; Dutta et al., 1978; Weiden et al., 1974). Furthermore, a dysfunction in humoral response of dogs with lymphoma was also identified with suppressed antibody responses to sheep red blood cells and to primary and secondary immunization with bacteriophage, being reported (Medleau et al., 1983; Weiden et al., 1974). In fact, serum IgG concentration of dogs diagnosed with lymphoma were significantly lower when compared with healthy control dogs (Weiden et al., 1974). Moreover, autoimmune diseases, such as immune-mediated thrombocytopenia, have been also associated with a higher risk of developing lymphoma in dogs, compared to a normal population (Keller, 1992). Nevertheless, the role of the immune system in the pathogenesis of canine lymphoma and its interactions with the surrounding microenvironment have not yet been deeply investigated and few studies have addressed cytokine dysfunction (Bryan, 2016; Calvalido et al., 2016).

Within this context, the present study aimed to gain better understanding of the systemic and intratumoral cytokine dysregulation/dysfunction in canine lymphoma. Considering that high quality basic, clinical and translational cancer research requires prompt access to well-preserved biological samples, multiple samples were collected from dogs diagnosed with lymphoma to establish a biobank. These samples were used for the relative quantification of Th1/Th2/Th17 cytokine mRNA expression in paired tissue samples of affected lymph nodes and circulating peripheral blood mononuclear cells (PBMC). Its comparison with healthy control samples allowed assessing for the first time cytokine mRNA profiles associated with multicentric canine lymphoma. Moreover, the comparison of putative cytokine serum quantification of dogs diagnosed with lymphoma with those of healthy control

dogs complemented mRNA expression studies. Systemic immune status evaluation included blood counts analysis and determined significant differences compared to healthy controls. These results were further correlated with serum cytokine levels. Finally, we evaluated whether intratumoral and systemic cytokine levels might predict a favorable survival outcome following CHOP protocol. Results demonstrated that there was a positive correlation between intratumoral immune response and prognosis.

2. Material and methods

2.1. Biological samples

2.1.1. Animals

Patients with canine multicentric lymphoma were followed at the oncology unit of the Veterinary Medicine Faculty – University of Lisbon (FMV/UL)'s -Teaching Hospital where clinical evaluations were conducted. On a preliminary phase, with diagnostic and staging purposes, a complete history, clinical signs and physical examination were assessed. Complete blood count and biochemistry profile were performed, as well as abdominal and thoracic imaging exams. Histopathological evaluation of lymph nodes was performed after node biopsy. This histopathological evaluation included a morphologic examination, classification of lymphoma into grade subcategories and immunophenotyping to determine the immunophenotype present – B or T. Immunohistochemistry markers included CD3, CD20, CD79αcy and PAX-5. This clinical and laboratory examination allowed staging the dogs using the World Health Organization (WHO) system (Owen, 1980).

Inclusion criteria comprised dogs recently diagnosed with multicentric lymphoma by clinical examination and cytological examination of lymph node fine-needle aspirate that have not yet begun therapy. Exclusion criteria included dogs who have begun chemotherapy and who have received steroids or other immunotherapeutic agent within the last eight weeks of study enrollment or dogs who have become severely ill.

2.1.2. Establishment of a canine multicentric lymphoma biobank

All sample collection was conducted with written pet owner consent in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and approved by the Animal Care and Use Committee of FMV/UL. Blood samples allowed the isolation of plasma and serum, as well as the extraction of DNA (DNeasy Blood & Tissue, Qiagen, Hilden, Germany) and mRNA (RNeasy Protect Animal Blood System, Qiagen), that were stored at -80 ˚C. Additionally, PBMC were isolated by Ficoll gradient method (Biocoll Separating Solution, BioChrom®, Fisher Scientific, New Hampshire, USA) and following cell viability assessment, aliquots of 5×10^6 cells were suspended in 90% Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Paisley, UK) and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) and kept in liquid nitrogen. Sterile biopsy lymph nodes samples were divided, 1/3 was fined cut and stored at -80 ˚C in RNAlater® (Invitrogen, Life Technologies, Paisley, UK), 1/3 was formalin-fixed and 1/3 stored in liquid nitrogen after lymphoma cell isolation. Briefly, solid tissue was cut, passed through a cell strainer (Cell Strainer, BD Falcon®), suspended in Roswell Park Memorial Institute–1640 (RPMI-1640) medium (Gibco) supplemented with 20% FBS and penicillin 100 U/ml plus streptomycin 0.1 mg/ml (Gibco), and isolated through Ficoll gradient method (Biocoll Separating Solution, BioChrom®). Cell viability were assessed and for storage, aliquots of 5×10^6 cells were suspended in 90% FBS and 10% DMSO and kept in liquid nitrogen. Clinical follow-up information about all cases was gathered from electronic medical records. All dogs that participated in this study were client-owned animals which joined the study during their diagnostic assessment. All sampled animals stayed with their owners after sample collection.

2.1.3. Control groups

Whole blood samples were collected from 9 canine healthy donors and processed for serum, plasma and PBMC isolation and storage, as previously mentioned. All animals were submitted to clinical examination at FMV/UL's-Teaching Hospital and their health status was screened through the execution of hematological and biochemistry profile and blood-borne parasites serology. Inclusion criteria included dogs aged between two and seven years, with a normal clinical examination and normal blood parameters. Exclusion criteria comprised dogs diagnosed with chronic diseases, such as heart disease, chronic kidney disease, endocrine disease or cancer, or whom have fall acutely ill and/or subjected to medications within the last 60 days. All sample collection was conducted with written pet owner consent in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and approved by the Animal Care and Use Committee of FMV/UL. All dogs that participated in this study were client-owned animals which joined the study voluntarily. All sampled animals stayed with their owners after sample collection. Due to ethical concerns raised by the collection of lymph nodes from healthy animals without medical indication, sterile biopsy lymph nodes samples were collected from a healthy control group of 8 dogs housed at the animal facility of Faculty of Veterinary Medicine - Universiteit Utrecht. All sample collection was performed after donors were sacrificed by intravenous administration of 65 mg/kg sodium pentobarbital. Lymph node samples were fined cut and stored at -80 °C in RNAlater®.

2.2. Relative quantification of cytokine expression by real-time quantitative polymerase chain reaction

For total RNA extraction, lymph node samples stored in RNAlater® and PBMC samples were thawed and processed using RNeasy Mini Kit (Qiagen), according to the manufacturer's instruction. To eliminate possible contaminant DNA, total RNA samples were subjected to DNAse treatment, using RNase-free DNase Set (Promega; Wood Hollow road, Madison, USA), following the manufacturer's instructions. Thereafter, cDNA was synthesized using Transcriptor High Fidelity (Roche, Basel, Switzerland) following the manufacturer's instructions and used as a template for Real-Time quantitative Polymerase Chain Reaction (qRT-PCR). Total RNA and cDNA purity were assessed by 1% agarose gel electrophoresis. The primers used for each gene were published in the literature and the respective authors and sequences are presented in Table 1. Despite the DNAse treatment, and to preclude genomic DNA amplification, primers covered putative exon–exon junctions. Optimization experiments for each amplification system were previously performed (data not shown). All qPCR amplification efficiencies exceeded 90%. Primers were obtained from a commercial manufacturer (Metabion International AG, Germany). The mRNA transcription of the Ribosomal protein L27 gene (RPL27) had no significant statistical differences (p > 0.05) regarding cNHL and control groups, therefore this gene was considered a suitable housekeeping gene (Table 1). qRT-PCR was performed in duplicate wells on StepOne Plus realtime analyzer (Applied Biosystems, Foster City, CA, USA). The PCR assays comprised, in each reaction, 2 µl of each primer (final concentration of 100 nM), 2 µl of cDNA (1 ng), 4 µl of sterile water and 10 µl of SYBr (Applied Biosystems, Warrington, UK) in a total volume of 20 µl per reaction. Thermocycling conditions consisted of an initial denaturation of 10 min at 95 °C, followed by 40 cycles of amplification (95 °C for 15 s and annealing at 60 °C for 1 min). A final melting curve stage consisted of 95 °C for 15 s, 60 °C for 1 min followed by a ramp rate and heating of samples until 95 °C with a 0.3 °C/s ramp rate. The melting curves obtained were used to verify the specificity of each amplicon and finally PCR products were sequenced. Expression of target genes was normalized against that of the housekeeping gene. The $2^{-\Delta\Delta CT}$ method was used as described by Perkin-Elmer Applied Biosystems to assess relative mRNA expression quantification between lymphoma group and control group experiments (Livak and Schmittgen, 2001).

2.3. Multiplex cytokine immunoassay

Milliplex® MAP magnetic bead panel based on Luminex® xMAP® technology (CCYTOMAG-90 K, Millipore GmbH, Am Kronberger Hang 5, 65824 Schwalbach/Ts., Germany) was used to measure the final product from four different cytokines in canine serum: IL-2, IL-6, IL-10 and TNF-α. The cytokine panel selection was based on previous works (Axiak-Bechtel et al., 2014; Calvalido et al., 2016) and on our cytokine mRNA panel assortment. The method was performed according to the manufacturer's instructions. All samples were analyzed in duplicates. The data were analyzed using the Bio-Plex Manager 4.0 software (Bio-Rad, Hercules, CA). The observed concentration of each analyte for each sample was calculated using a standard curve generated from the seven standards and a blank provided by the manufacturer. If a sample concentration was extrapolated outside the standard curve and designated as "Value extrapolated beyond standard range" by the software, that sample concentration was accepted as the calculated value (Levin et al., 2014). The minimum detectable concentrations for the four cytokines according to the manufacturer was 12.2 pg/ml. Significance of differences of the mean value of each serum cytokine concentration between cNHL group and control group was analyzed.

2.4. Statistical analysis

All the statistical analyses were carried out using R-software. Normality test was performed using Shapiro-Wilk test. The distribution of all cytokine concentrations and most clinical variables did not pass the normality test and groups were therefore compared using the Mann–Whitney U-test. Parameters that demonstrated normal distribution within both the lymphoma group and the control group were analyzed with unpaired t-tests. Correlations of variables were evaluated using Spearman rank correlation. To avoid treatment related bias, survival correlation included animals submitted to CHOP protocol, excluding three non-treated animals and one animal that is currently alive. The significance level was set at 5%.

3. Results

3.1. Establishment of a canine multicentric lymphoma biobank

High quality clinical and basic cancer research, as well as the development of novel prevention, early diagnostics and treatment approaches, relies on prompt access to biological samples. Tumor samples are usually restricted to formalin-fixed paraffin-embedded tissues, which cannot meet the requirement of current research. In fact, most RNA and protein studies require samples to be cryopreserved. Thus, we have been successfully constructing a canine multicentric lymphoma biobank (Table 2). Canine lymphoma is an heterogeneous malignancy with several presentation forms, however multicentric lymphoma is the most diagnosed form (Ettinger, 2003), accounting for ± 75% of the cases. Considering its high prevalence, we focused on constructing a naïve multicentric canine lymphoma. Twenty-two dogs were included in the multicentric lymphoma biobank consisting on thirteen males and nine females with the median age of 9.1 ± 3.4 years (range 3–17). The breeds represented were mixed-breed, Labrador retriever and other breeds. Eight male dogs were enrolled in the tissue sample control group and nine dogs (four males and five females) were included in the PBMC control group. The samples collected will be used for current studies and are an important source for proposed and future research works. There was no significant difference between the lymphoma group and control group regarding age, sex, and weight (p > 0.05). Based on staging evaluation, 45% (10/22) of the animals presented clinical stage V and 55% (12/22) presented clinical stage IV. All cases were classified into B and T cell subtypes as designated in the WHO classification system. Results are presented in Table 2.

Table 1
Detailed primers and conditions used for real-time PCR assays.

Gene	Accession number	Sequence (5′–3′)	Amplicon size (bp)	Source
IL-1β	NM_001037971	FW-GAAGAAGCCCTGCCCACA RV-AATTATCCGCATCTGTTTTGCAG	104	Henriques et al. (2016) [a]
IL-2	NM_001003305	FW-TTGTCGCAAACAGTGCACCTA RV-CCTGGAGAGTTGGGGGTTCT	131	Henriques et al. (2016) [a]
IL-4	NM_001003159	FW- CTCACCAGCACCTTTGTCCA RV- GTCAGCTCCATGCACGAGTC	107	Henriques et al. (2016) [a]
IL-6	NM_001003301	FW-CTGGCAGGAGATTCCAAGGAT RV-TCTGCCAGTGCCTCTTTGC	167	Henriques et al. (2016) [a]
IL-8	NM_001003200	FW-TTGCTCTCTTGGCAGCTTTTG RV-TTTGGGATGGAAAGGTGTGG	122	Henriques et al. (2016) [a]
IL-10	NM_001003077	FW-ACATCAAGAACCACGTGAACTCC RV-ACTCACTCATGGCTTTGTAGACACC	177	Henriques et al. (2016) [a]
IL-12p40	AF091134.1	FW-CAGCAGAGAGGGTCAGAGTGG RV-ACGACCTCGATGGGTAGGC	109	Peters et al. (2005) [b]
IL-17A	NM 001165878.1	FW-CACTCCTTCCGGCTAGAGAA RV-CACATGGCGAACAATAGGG	72	Schmitz et al. (2012) [c]
TNF-α	NM_001003244	FW-CTGCCTCAGCCTCTTCTCCTT RV-CTGGGCAAGAGGGCTGATTA	133	Henriques et al. (2016) [a]
TGF-β1	NM_001003309	FW-AGCCCGAGGCGGACTACTAC RV-CGGAGCTCTGATGTGTTGAAGA	130	Henriques et al. (2016) [a]
IFN-γ	NM_001003174	FW-GCTGTAACTGTCAGGCCATGTT RV-TGTTTTGTCACTCTCCTCTCTCCA	140	Henriques et al. (2016) [a]
RPL27	NM_001003102	FW-TCGTCAACAAGGATGTCTTCAGAG RV-TCTTGCCAGTCTTGTACCTCTCCT	96	Henriques et al. (2016) [a]

[a] (Henriques et al., 2016).
[b] (Peters et al., 2005).
[c] (Schmitz et al., 2012).

3.2. Blood cell counts analysis

To assess the immune status of lymphoma diagnosed dogs and to better understand the relationship between cytokine expression and hematological parameters, comparison of red and white cell counts between cNHL and control group was conducted. This analysis demonstrated that cNHL patients presented a significant lower hemoglobin and hematocrit ($p < 0.01$) compared to the control group. All anemias were classified as non-regenerative, normocytic and normochromic, compatible with anemia related to inflammatory/chronic disease. Furthermore, cNHL group demonstrated a significant higher count of total leucocytes ($p < 0.05$) and monocytes ($p < 0.05$); and a significant lower count of eosinophils ($p < 0.01$). There was no significant difference in lymphocytes, neutrophils and platelets counts between groups (Fig. 1).

3.3. Transcriptional profile of cytokines expression in lymph nodes of cNHL patients

To assess the immune changes of affected lymph nodes of cNHL patients, relative quantification of cytokine expression by qRT-PCR was performed. The analysis demonstrated a clear immune imbalance of

Table 2
cNHL patient clinical characteristics and corresponding tissue and blood samples.

Patient (n = 22)	Age (Years)	Sex*	Breed	WHO Classification	Tumor sample[a]	PBMC sample[b]*	Serum sample[c]	CHOP protocol[d]
1	12	M	Schnauzer	DLBCL	+	+	+	–
2	15	M	Mixed-breed	PTCL	+	–	+	–
3	8	M	Labrador	DLBCL	+	+	+	+
4	12	M	Golden Retriever	Blastic NK Lymphoma	+	+	+	+
5	6	M	Rottweiller	DLBCL	+	+	+	+
6	6	F	Basset Hound	DLBCL	+	+	+	+
7	12	F	Pitbull	Follicular	+	+	+	+
8	12	F	Husky	DLBCL	+	–	+	–
9	8	M	Mixed-breed	DLBCL	+	+	+	+
10	11	F	Mixed-breed	DLBCL	+	+	+	+
11	9	F	Boxer	DLBCL	+	+	+	+
12	7	M	Pointer	DLBCL	+	+	+	+
13	17	F	Mixed-breed	DLBCL	+	+	+	+
14	3	F	German Shepherd	DLBCL	+	+	+	+
15	8	M	Mixed-breed	DLBCL	+	+	+	+
16	9	M	Mixed-breed	T Zone Lymphoma	+	+	+	+
17	4	M	Golden Retriever	DLBCL	+	+	+	+
18	8	M	Beagle	PTCL	+	+	+	+
19	5	M	Labrador	DLBCL	+	–	+	+
20	9	F	Toy poodle	DLBCL	+	–	+	+
21	11	F	Dobermann	DLBCL	+	+	+	+
22	9	M	Afghan hound	DLBCL	+	+	+	+

*Sex M – Male, F – Female; PBMC – peripheral blood mononuclear cells; DLBCL – Diffuse Large B Cell Lymphoma; PTCL – Peripheral T Cell Lymphoma; NK – Natural Killer; [a] + tumor sample, - no tumor sample; [b] + PBMC sample, no PBMC sample; [c] + Serum sample, - no serum sample; [d] + submitted to CHOP protocol, - no CHOP protocol.
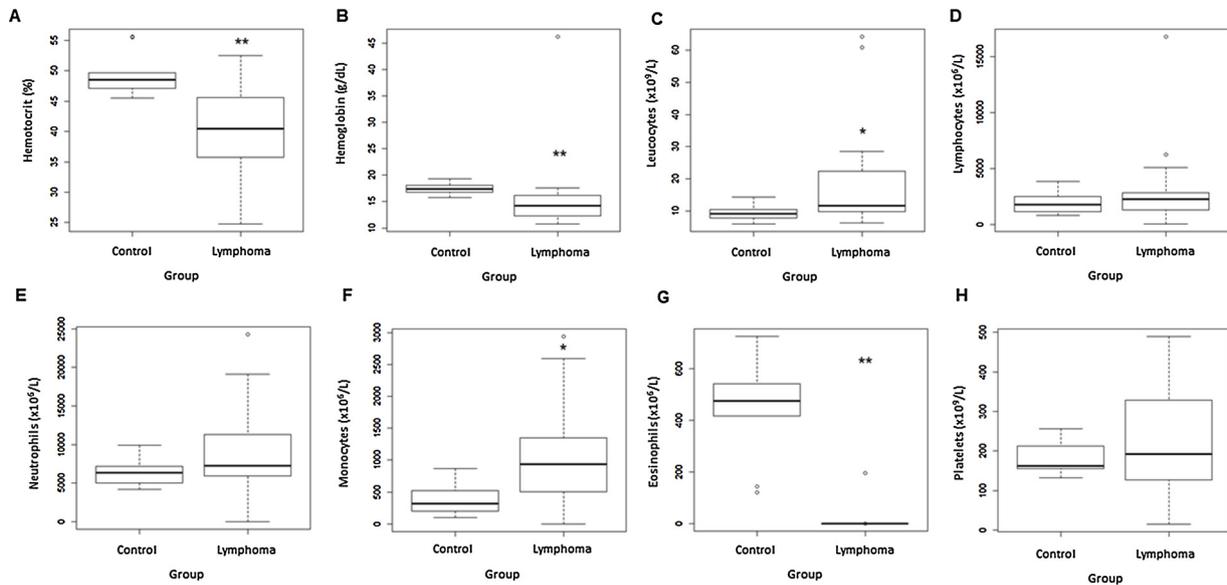
Fig. 1. Differences in blood cell counts of cNHL group compared to the control group. Data of hematological parameters, including hemoglobin, hematocrit, leucocytes, lymphocytes, neutrophils, monocytes, eosinophils and platelets are presented as box and whiskers (A-H). * $p < 0.05$; ** $p < 0.001$.
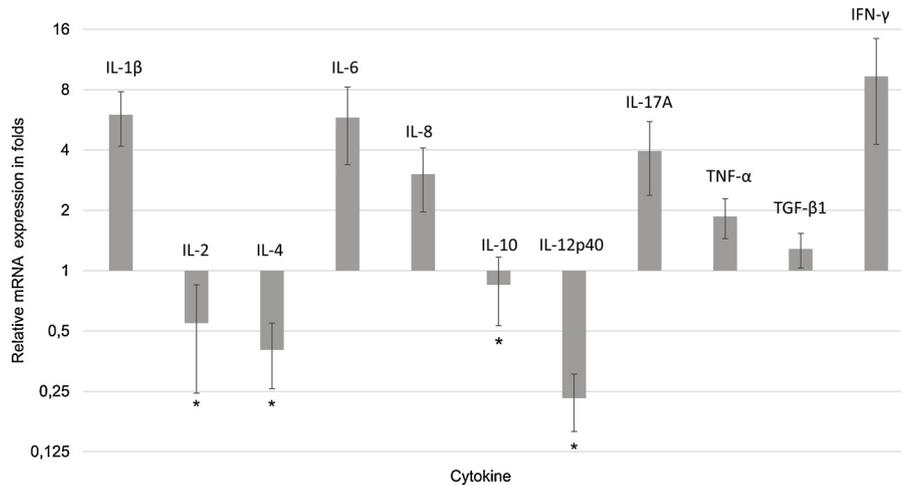

Fig. 2. Relative cytokine mRNA expression in cNHL tumor tissue. Results are expressed as a fold difference between mean ± SEM of mRNA expression level normalized to housekeeping gene RPL27 in cNHL affected lymph nodes samples and a control group with healthy donors. * $p < 0.05$.

cytokine mRNA expression in naïve cNHL patients, compared to healthy donor lymph nodes samples. As shown in Fig. 2, cNHL presented a tendency of upregulation of IL1-β, IL-6, IL-8, IL-17A, TNF-α, transforming growth factor-β (TGF-β) and IFN-γ and a trend of downregulation of IL-2, IL-4, IL-10 and interleukin-12 subunit p40 (IL-12p40). The downregulation of the mRNA expression of IL-2, IL-4, IL-10 and IL-12p40 was statistically significant ($p < 0.05$). There were no significant correlations between cytokine mRNA expression in the cNHL patients lymph nodes with clinical characteristics and blood counts.

3.4. Transcriptional profile of cytokines expression in circulating PBMC of cNHL patients

In order to evaluate possible differences in the transcriptomic profile of cytokine expression in circulating PBMC of cNHL patients and healthy donors, a relative quantification of cytokine mRNA expression by qRT-PCR was carried out. As shown in Fig. 3, this experiment demonstrated a distinct profile of cytokine expression compared with the former lymph node analysis, revealing a trend of upregulation of IL1-β, IL-8, IL-10 and IL-12p40 and a trend of downregulation of IL-17A, TNF-α and TGF-β. Importantly, the downregulation of IL-17A, TNF-α and

TGF-β was statistically significant ($p < 0.05$). Again, a low cytokine expression profile was detected. Moreover, the residual expression levels of IL-6, IL-4 and IFN-γ did not allow the subsequent analysis and comparison between circulating PBMC mRNA expression between cNHL and control/healthy groups. No significant correlation between cytokine mRNA expression in the circulating PBMC of cNHL patients with clinical characteristics and blood counts were found. Finally, no significant correlation between cytokine mRNA expression between cNHL lymph node and circulating PBMC was displayed.

3.5. Serum cytokine levels in cNHL patients

Cytokines protein concentrations in sera collected from cNHL biobank dogs were analyzed by the selected canine cytokine multiplex panel and compared to those from the healthy donor group. The cytokine panel selection was based on previous works (Axiak-Bechtel et al., 2014; Calvalido et al., 2016) and on our cytokine mRNA panel assortment. All samples presented low detectable values of IL-2, IL-6, IL-10 and TNF-α. The comparison of cytokine levels demonstrated that IL-10 concentrations in cNHL dogs were significantly higher compared to healthy donor samples ($p < 0.05$). No significant differences
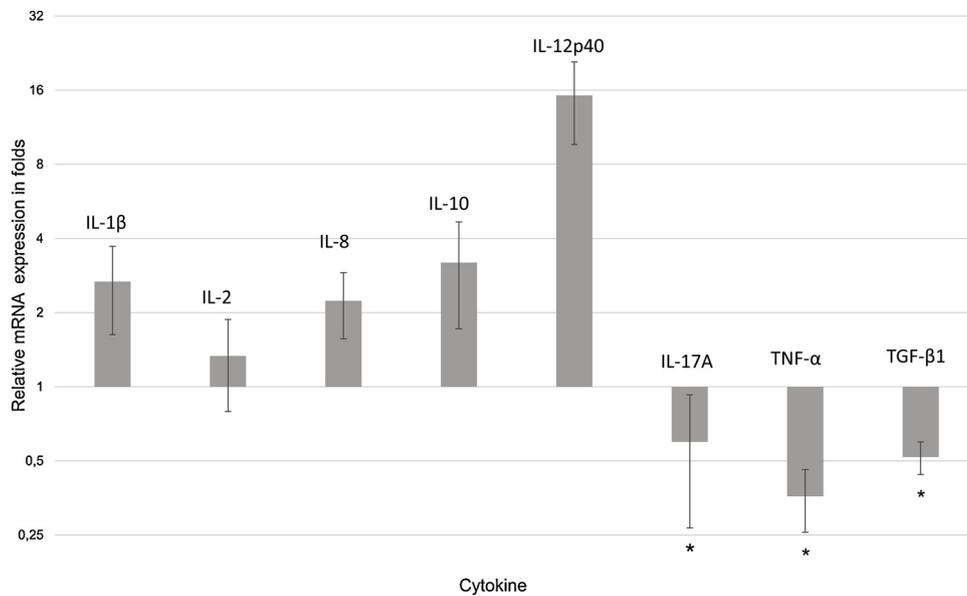
Fig. 3. Relative cytokine mRNA expression in cNHL circulating PBMC. Results are expressed as a fold difference between mean ± SEM of mRNA expression level normalized to housekeeping gene RPL27 in cNHL PBMC and a control group with healthy donors. * p < 0.05.
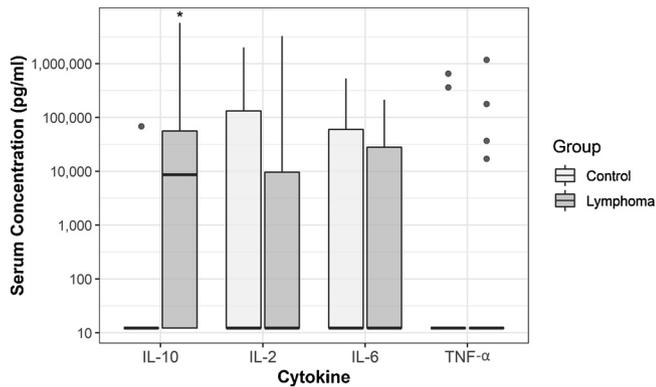
Fig. 4. Differences in serum cytokine concentrations of cNHL group compared to control group. Data are presented as box and whiskers and a log10 scale was used for the y axis for an optimal visualization of results. * p < 0.05.

between sera concentration of IL-2, IL-6 and TNF-α of cNHL and control groups were found (Fig. 4). No significant correlation between cytokine mRNA expression in the lymph node or the circulating PBMC of cNHL patients with cytokine sera concentration were detected. Regarding correlation of cytokine sera concentration with hematological parameters and clinical characteristics, a positive correlation of IL-6 sera levels with neutrophil counts (p = 0.013; rho = 0.546) was found.

3.6. Correlation of cytokine expression and patient survival

To investigate whether pretreatment cytokine expression in intratumoral and systemic samples could predict whether patients would respond favorably to CHOP protocol, correlation analysis with survival clinical outcome was performed. This study demonstrated that IFN-γ level was positively correlated with patient survival following chemotherapy (p = 0.044). This correlation was considered moderate (rho = 0.481) (Fig. 5).

4. Discussion

To the best of our knowledge, this is the first study addressing intratumoral cytokine dysregulation in lymphoma-bearing dogs, along with systemic cytokine dysfunction. To date, a limited number of other
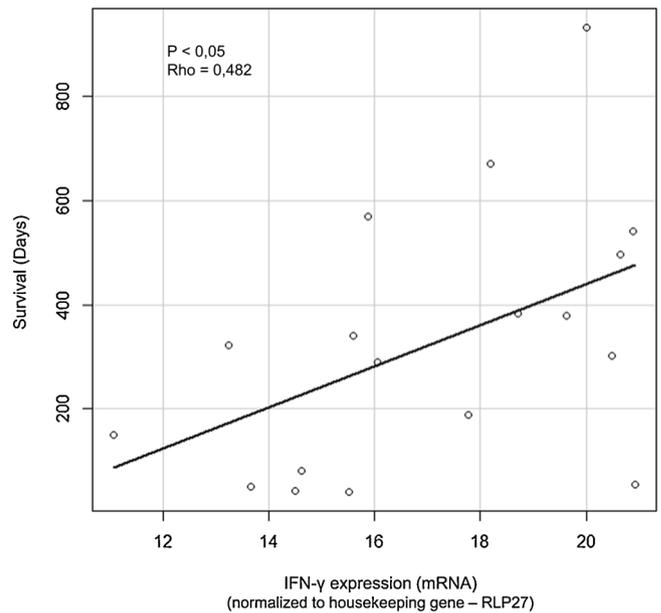
Fig. 5. Correlation of IFN-γ mRNA expression and survival clinical outcome in cNHL tissue samples. Results are presented as Spearman rank test correlating survival days and CT values normalized to housekeeping gene RPL27.

studies have explored the immune status of dogs diagnosed with lymphoma, particularly cytokine expression (Axiak-Bechtel et al., 2014; Calvalido et al., 2016). On one hand, Axiak-Bechtel and collaborators recently reported for the first time an imbalance in both pro- and anti-inflammatory cytokine production, however their work focused mainly on the chemotherapy induced remission effects on the immune response of dogs diagnosed with lymphoma as a risk factor for sepsis (Axiak-Bechtel et al., 2014). On the other hand, Calvalido and collaborators investigated circulating cytokines in lymphoma-bearing dogs as potential disease biomarkers (Calvalido et al., 2016). Even though these studies represented groundbreaking research about canine lymphoma immunity, many challenges remain. This is particularly true at a time when canine lymphoma model is being proposed as a powerful platform for rapid and clinically relevant translation of novel high impact immune therapies and immune combination therapies.

Within this context, in the present study we aimed to investigate cytokine regulation in canine lymphoma. For this purpose, cytokine gene expression was evaluated in tumor samples and circulating PBMCs, and serum concentration of putative cytokines was measured using multiplex assay.

Cytokines are molecular messengers that mediate immune intercellular communication to generate a coordinated, robust and rapid immune response to a target antigen (Lee and Margolin, 2011). In tumorigenesis, theoretically these cytokines directly promote immune effector and stromal cells at the tumor site and stimulate tumor cell recognition by cytotoxic effector cells (Anestakis et al., 2015). However, there is growing evidence that cytokine signaling in many tumors reveal an immune suppressive or anti-inflammatory function, being involved in tumor immunosurveillance evasion (Esquivel-Velázquez et al., 2015; Israelsson et al., 2017; Mager et al., 2016). As a result, the investigation of cytokine role in tumor-driven molecular mechanisms might provide opportunities to explore novel therapeutic approaches.

Cytokine research can rely on measuring serum concentration of biologically active cytokines or by assessing their expression in the affected organs or in circulating leukocytes (Maissen-Villiger et al., 2016). In this study, we chose to focus on the measurement of cytokine mRNA expression levels using qRT-PCR. The primary reason for that was the limited availability of validated canine assays that hampers the range of cytokines that can be evaluated (Maissen-Villiger et al., 2016). In addition, the high specificity, sensitivity and stability of quantitative evaluation of cytokine expression by real-time PCR method enables the analysis of samples with very low mRNA expression and can be a valuable tool to establish disease-related cytokine profiles (Giulietti et al., 2001). The expression of mRNA does not necessarily reflect the real cytokine concentration but, being the result of the upstream path of activation leading to gene expression, it offers valuable insights into cytokine regulation pathways (Gygi et al., 1999). Furthermore, although the protein quantification provides a more predictive insight of its effect, it does neither evaluate its biological activity at the tissue level nor the effect of potential inhibitors (Cavaillon et al., 1992). As a result, our work aimed to fill in the gap in lymphoma cytokine mRNA profile research considering that previous studies focused only on measuring serum cytokine concentrations. Our study also included a protein-based assay to complement cytokine gene expression studies and to allow correlation with previously published work.

In this study, we assessed the mRNA profile of 11 cytokines in tissue samples of canine lymphoma affected lymph nodes compared to the mRNA profile of normal lymph nodes from healthy control dogs. Our results demonstrated a dysregulation of cytokine expression, representative of the tumor microenvironment in canine lymphoma. Notably, key mediators of Th2 response, including IL-4 and IL-10 expression were significantly downregulated in the lymph node of dogs with canine lymphoma. Furthermore, IL-12 - required for Th1-cell differentiation and IL-2 - principal growth factor for T cells, were also significantly downregulated, indicating a component of immunosuppression in cancer microenvironment (Setrerrahmane and Xu, 2017).

Although not statistically significant, there was a prominent tendency to the upregulation of IL1-β, IL-6, IL-8, and TNF-α, cytokines that mediate inflammatory response. Moreover, we found a trend of upregulation of IL-17A expression, another proinflamatory cytokine, associated with tumor cell proliferation and neoangiogenesis in hNHL (Ferretti et al., 2015). Finally, IFN-γ expression, the main cytokine implicated in cell-mediated immunity primarily through its effects on the monocyte/macrophage population, also shown a tendency of upregulated expression in canine lymphoma. These results suggest that some dogs diagnosed with cNHL may present a higher inflammatory response, however this response was found to be heterogeneous and therefore not statistically significant.

The downregulation of both Th2 and Th1 cytokine responses in cNHL is in line with the postulated hypothesis that the immunosuppressive nature of the tumor microenvironment is proving to be a major barrier for the treatment of B cell malignancies in human medicine (Nicholas et al., 2016).

Nevertheless, in the case of human NHL, the contribution of the tumor microenvironment to the pathogenesis and tumor survival remains poorly understood (Fowler et al., 2016). Moreover, cytokine expression was considered scarce and consequently it was never fully investigated, existing a lack of studies that evaluated mRNA cytokine expression in human NHL (Herreros et al., 2008; Hsu et al., 1993; Jones et al., 2002).

Herein, we described for the first time a distinct systemic cytokine mRNA profile expressed by circulating PBMC. However, similarly to the immune response observed in tumor microenvironment, in the circulating PBMC a major immunosuppressive component of the immune response prevailed, with a significant downregulation of IL-17A, TNF-α and TGF-β. The expression of IL-6, IL-4 and IFN-γ were undetectable in both groups, making the results for this cytokine negligible by this method. The significant downregulation of IL-17A documented herein is consistent with previous studies that report a significant decrease of Th17 cells in the PBMC of patients with NHL and B-cell NHL and a remarkably decreased IL-17AF plasma levels in B-NHL patients (Lu et al., 2016). Furthermore, although not statistically significant, IL-12p40 and IL-10 expression demonstrated a tendency to upregulation.

In addition, we reported a significant increase of IL-10 serum levels in canine lymphoma patients. IL-10 is an immunosuppressive cytokine produced by many different cells of the immune system, including T and B lymphocytes, macrophages, monocytes, dendritic cells, and NK cells (Purdue et al., 2007) but can also be produced by neoplastic B lymphocytes (Mocellin et al., 2005; Purdue et al., 2007). By suppressing antigen presenting cells, IL-10 enables tumor cells to escape immune system (Xiu et al., 2015). Moreover, IL-10 may promote the upregulation of bcl-2 expression, contributing for the protection of malignant cells from apoptosis (Blay et al., 1993). Elevated IL-10 levels have been found in patients with NHL and were associated with poor prognosis (Blay et al., 1993; Lech-Maranda et al., n.d.). These results are in line with previous studies that also demonstrated an elevated IL-10 serum level and an increased IL-10 production by circulating PMBC following peptidoglycan stimulation in canine lymphoma patients (Axiak-Bechtel et al., 2014; Calvalido et al., 2016). There were no significant differences between IL-6, TNF-α and IL-2 serum levels of canine lymphoma and control group. Calvalido and collaborators reported a IL-6 serum elevation only in T-cell lymphoma group (Calvalido et al., 2016). Considering that our studied lymphoma group is composed mainly by B-cell lymphoma cases, these findings are consistent with previously published data. In addition, the hypothesis of a systemic immunosuppression in canine lymphoma patients is supported by previous work that had observed a significant depression of CD4+ and CD8+ T-cell numbers before initiation of chemotherapy (Walter et al., 2006).

Remarkably, a moderate positive correlation between IFN-γ mRNA expression in lymphoma affected lymph node and survival following CHOP protocol treatment, was found. These results indicate that the promotion of an effective Th1 response may benefit cNHL patients, supporting the rationale for the use of immunotherapies that stimulate an effector T cell response.

Considering the close relationship between cytokines and peripheral blood cells, we carried out a comparison between red and white blood cell counts of cNHL patient and control group. Similar to previous studies, our results demonstrated a significant higher count of leucocytes and monocytes in cNHL patients (Calvalido et al., 2016; Perry et al., 2011). Furthermore, our data revealed a significant lower eosinophil count, as well as a lower hemoglobin and hematocrit, compatible with a non-regenerative normochromic anemia. In fact, anemia is a well-known hematological finding in cNHL, being associated with poor prognosis (Calvalido et al., 2016; Madewell and Feldman, 1980). Moreover, a positive correlation between neutrophil count and IL-6

serum level was found in our cNHL patients. IL-6 regulates neutrophil trafficking during the inflammatory response by orchestrating chemokine production and leukocyte apoptosis. As such, this finding demonstrates that IL-6 might be involved in the inflammatory response in cNHL (Fielding et al., 2008). In fact, in humans IL-6 has been associated with paraneoplastic inflammatory syndrome symptoms, such as weight loss, night sweats and fever (Burger, 2013).

A major strength of this investigation was the careful criteria selection that allowed to include well-characterized naïve canine multicentric lymphoma cases in the lymphoma group and healthy donors in the control group. Indeed, the successful construction of multicentric cNHL biobank was a major output of this work. Maintaining the construction of this biobank will ensure the availability of multiple, properly preserved samples for future research studies. Furthermore, simultaneous analyses were done with paired tissue and corresponding PBMC samples thus opening a possibility to compare the cytokine mRNA expression at the local and systemic level. Furthermore, our own long-term experience and access to a well-established qRT-PCR assay allowed a parallel measurement of mRNA expression for a broad panel of cytokines that cover Th cytokine profiles of major interest. However, this study also presented several limitations such as the limited number of patients included and the heterogeneity of the lymphoma diagnosed cases. In the future, it will be important to include larger study groups categorized accordingly to cNHL subtypes. This is particularly true in the case of the inflammatory cytokines expression, considering that the high individual heterogenicity may have prevented to attain statistical significance and a larger number of patients might have contributed for the better understanding of these cytokines mRNA profile in cNHL. Lastly, a major drawback of this work was the inability to perform multiplex immunoassay on tumors extract due to kit specifications.

5. Conclusions

Taken together, these results suggest that similarly to its human counterpart, cNHL reveals a local and systemic dysregulation in cytokine response. In fact, an immunosuppressive cytokine dysfunction appears to be a constitutive component of the immune status of cNHL. By confirming and extending previous investigations, our work contributed for the evaluation of potential cytokine candidates for diagnostic and prognostic purposes and therapeutic intervention by cytokine inhibition and/or immunomodulatory strategies. Nevertheless, future studies using larger cohorts are needed to elucidate in detail the role of cytokines in the establishment and disease progression of cNHL. Importantly, Axiak-Bechtel and collaborators reported that chemotherapy protocols appeared to fail to restore immune response in cNHL (Axiak-Bechtel et al., 2014). Although these results must be confirmed, it could indicate that harnessing the immune system with immunotherapeutic approaches might be the long-waited solution to improve the prognosis of cNHL.

In conclusion, this work contributes for the better understanding of cNHL immune status, essential for the establishment of this animal model as a predictive preclinical surrogate for human NHL, mutually benefiting these uniquely co-dependent species and opening up perspectives in comparative oncology.

Authors' contribution

JD established the cNHL biobank, performed and analyzed the majority of the experiments and wrote the manuscript; ML assisted in the RT-qPCR experiments and statistical analysis; CP performed the histopathological evaluation; GV assisted in the biological sample collection; TN designed and assisted the statistical analysis; LM supervised the RT-qPCR experiments; FAS assisted the experimental design and reviewed the manuscript; LT supervised the work and reviewed the manuscript and SG was responsible for the research concept, experimental work supervision and manuscript revision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All sample collection was conducted with pet owner written consent in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Animals and approved by the Animal Care and Use Committee of FMV/UL.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Authors' information

JNRD – DVM, Msc, PhD, Researcher at FMV. ML – DVM, Msc. CP - DVM, PhD, Full Professor at FMV. GV – DVM. TN – DVM, Invited Professor. LM – DVM, PhD, Associate Professor at FMV. FAS – PhD, Researcher at FMV. LT – DVM, PhD, Full Professor at FMV. SG – DVM, PhD, Assistant Professor at FMV.

References

Anestakis, D., Petanidis, S., Kalyvas, S., Nday, C.M., Tsave, O., Kioseoglou, E., Salifoglou, A., 2015. Mechanisms and applications of interleukins in cancer immunotherapy. Int. J. Mol. Sci. 16, 1691–1710. https://doi.org/10.3390/ijms16011691.

Axiak-Bechtel, S., Fowler, B., Yu, D.H., Amorim, J., Tsuruta, K., DeClue, A., 2014. Chemotherapy and remission status do not alter pre-existing innate immune dysfunction in dogs with lymphoma. Res. Vet. Sci. 97, 230–237. https://doi.org/10.1016/j.rvsc.2014.07.009.

Blay, J.Y., Burdin, N., Rousset, F., Lenoir, G., Biron, P., Philip, T., Banchereau, J., Favrot, M.C., 1993. Serum interleukin-10 in non-Hodgkin's lymphoma: a prognostic factor. Blood 82, 2169–2174.

Bryan, J.N., 2016. The current state of clinical application of serum biomarkers for canine lymphoma. Front. Vet. Sci. 3. https://doi.org/10.3389/fvets.2016.00087.

Burger, R., 2013. Impact of Interleukin-6 in hematological malignancies. Transfus. Med. Hemother. 40, 336–343. https://doi.org/10.1159/000354194.

Calvalido, J., Wood, G.A., Mutsaers, A.J., Wood, D., Sears, W., Woods, J.P., 2016. Comparison of serum cytokine levels between dogs with multicentric lymphoma and healthy dogs. Vet. Immunol. Immunopathol. 182, 106–114. https://doi.org/10.1016/j.vetimm.2016.10.009.

Calvert, C.A., Dawe, D., Leifer, C.E., Brown, J., 1982. Lymphocyte blastogenesis in dogs with lymphosarcoma. Am. J. Vet. Res. 43, 94–101.

Cavaillon, J.M., Munoz, C., Fitting, C., Misset, B., Carlet, J., 1992. Circulating cytokines: the tip of the iceberg? Circ. Shock 38, 145–152.

Chiu, Y.-H., Chung, C.-H., Lin, K.-T., Lin, C.-S., Chen, J.-H., Chen, H.-C., Huang, R.-Y., Wu, C.-T., Liu, F.-C., Chien, W.-C., Chiu, Y.-H., Chung, C.-H., Lin, K.-T., Lin, C.-S., Chen, J.-H., Chen, H.-C., Huang, R.-Y., Wu, C.-T., Liu, F.-C., Chien, W.-C., 2017. Predictable biomarkers of developing lymphoma in patients with Sjögren syndrome: a nationwide population-based cohort study. Oncotarget 8, 50098–50108. https://doi.org/10.18632/oncotarget.15100.

Dutta, S., Novilla, M., Bumgardner, M., 1978. Lymphocyte responsiveness to mitogens and quantitation of T and B lymphocytes in canine malignant lymphoma. Am. J. Vet.

Res. 455–458.

Esquivel-Velázquez, M., Ostoa-Saloma, P., Palacios-Arreola, M.I., Nava-Castro, K.E., Castro, J.I., Morales-Montor, J., 2015. The role of cytokines in breast Cancer development and progression. J. Interferon Cytokine Res. 35, 1–16. https://doi.org/10.1089/jir.2014.0026.

Ettinger, S.N., 2003. Principles of treatment for canine lymphoma. Clin. Tech. Small Anim. Pract. Med. Oncol. 18, 92–97. https://doi.org/10.1053/svms.2003.36622.

Ferretti, E., Di Carlo, E., Ognio, E., Guarnotta, C., Bertoni, F., Corcione, A., Prigione, I., Fraternali-Orcioni, G., Ribatti, D., Ravetti, J.L., Ponzoni, M., Tripodo, C., Pistoia, V., 2015. Interleukin-17A promotes the growth of human germinal center derived non-Hodgkin B cell lymphoma. Oncoimmunology 4. https://doi.org/10.1080/2162402X.2015.1030560.

Fielding, C.A., McLoughlin, R.M., McLeod, L., Colmont, C.S., Najdovska, M., Grail, D., Ernst, M., Jones, S.A., Topley, N., Jenkins, B.J., 2008. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. J. Immunol. Baltim. Md 1950 (181), 2189–2195.

Fowler, N.H., Cheah, C.Y., Gascoyne, R.D., Gribben, J., Neelapu, S.S., Ghia, P., Bollard, C., Ansell, S., Curran, M., Wilson, W.H., O'Brien, S., Grant, C., Little, R., Zenz, T., Nastoupil, L.J., Dunleavy, K., 2016. Role of the tumor microenvironment in mature B-cell lymphoid malignancies. Haematologica 101, 531–540. https://doi.org/10.3324/haematol.2015.139493.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25, 386–401. https://doi.org/10.1006/meth.2001.1261.

Grant, C., Bollard, C.M., 2017. Developing T-cell therapies for lymphoma without receptor engineering. Blood Adv. 1, 2579–2590. https://doi.org/10.1182/bloodadvances.2017009886.

Gygi, S.P., Rochon, Y., Franza, B.R., Aebersold, R., 1999. Correlation between protein and mRNA abundance in yeast. Mol. Cell. Biol. 19, 1720–1730. https://doi.org/10.1128/MCB.19.3.1720.

Hartley, G., Elmslie, R., Dow, S., Guth, A., 2018. Checkpoint molecule expression by B and T cell lymphomas in dogs. Vet. Comp. Oncol. https://doi.org/10.1111/vco.12386.

Henriques, S., Silva, E., Silva, M.F., Carvalho, S., Diniz, P., Lopes-da-Costa, L., Mateus, L., 2016. Immunomodulation in the canine endometrium by uteropathogenic Escherichia coli. Vet. Res. 47. https://doi.org/10.1186/s13567-016-0396-z.

Herreros, B., Sanchez-Aguilera, A., Piris, M.A., 2008. Lymphoma microenvironment: culprit or innocent? Leukemia 22, 49–58. https://doi.org/10.1038/sj.leu.2404970.

Hsu, S.-M., Waldron, J.W., Hsu, P.-L., Hough, A.J., 1993. Cytokines in malignant lymphomas: review and prospective evaluation. Hum. Pathol. 24, 1040–1057. https://doi.org/10.1016/0046-8177(93)90183-H.

Israelsson, P., Labani-Motlagh, A., Nagaev, I., Dehlin, E., Nagaeva, O., Lundin, E., Ott, U., er Mincheva-Nilsson, L., 2017. Assessment of cytokine mRNA expression profiles in tumor microenvironment and peripheral blood mononuclear cells of patients with high-grade serous carcinoma of the ovary. J. Cancer Sci. Ther. 9, 422–429. https://doi.org/10.4172/1948-5956.1000453.

Ito, D., Brewer, S., Modiano, J.F., Beall, M.J., 2015. Development of a novel anti-canine CD20 monoclonal antibody with diagnostic and therapeutic potential. Leuk. Lymphoma 56, 219–225. https://doi.org/10.3109/10428194.2014.914193.

Jain, S., Aresu, L., Comazzi, S., Shi, J., Worrall, E., Clayton, J., Humphries, W., Hemmington, S., Davis, P., Murray, E., Limeneh, A.A., Ball, K., Ruckova, E., Muller, P., Vojtesek, B., Fahraeus, R., Argyle, D., Hupp, T.R., 2016. The development of a recombinant scFv monoclonal antibody targeting canine CD20 for use in comparative medicine. PLoS One 11, e0148366. https://doi.org/10.1371/journal.pone.0148366.

Jones, E.A., Pringle, J.H., Angel, C.A., Rees, R.C., 2002. Th1/Th2 cytokine expression and its relationship with tumor growth in B cell non-Hodgkin's lymphoma (NHL). Leuk. Lymphoma 43, 1313–1321. https://doi.org/10.1080/10428190290026385.

Keller, E.T., 1992. Immune-mediated disease as a risk factor for canine lymphoma. Cancer 70, 2334–2337.

Klingemann, H., 2018. Immunotherapy for dogs: running behind humans. Front. Immunol. 9. https://doi.org/10.3389/fimmu.2018.00133.

Lech-Maranda, E., Bienvenu, J., Michallet, A.-S., Houot, R., Robak, T., Coiffier, B., Salles, G., 2019. n.d. Elevated IL-10 Plasma Levels Correlate With Poor Prognosis in Diffuse Large B-cell Lymphoma. pp. 7.

Lee, S., Margolin, K., 2011. Cytokines in Cancer immunotherapy. Cancers 3, 3856–3893. https://doi.org/10.3390/cancers3043856.

Levin, M., Romano, T., Matassa, K., De Guise, S., 2014. Validation of a commercial canine assay kit to measure pinniped cytokines. Vet. Immunol. Immunopathol. 160, 90–96. https://doi.org/10.1016/j.vetimm.2014.04.001.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods San Diego Calif. 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Lu, T., Yu, S., Liu, Y., Yin, C., Ye, J., Liu, Z., Ma, D., Ji, C., 2016. Aberrant circulating Th17 cells in patients with B-Cell Non-Hodgkin's lymphoma. PLoS One 11. https://doi.org/10.1371/journal.pone.0148044.

Madewell, B.R., Feldman, B.F., 1980. Characterization of anemias associated with neoplasia in small animals. J. Am. Vet. Med. Assoc. 176, 419–425.

Maekawa, N., Konnai, S., Takagi, S., Kagawa, Y., Okagawa, T., Nishimori, A., Ikebuchi, R., Izumi, Y., Deguchi, T., Nakajima, C., Kato, Y., Yamamoto, K., Uemura, H., Suzuki, Y., Murata, S., Ohashi, K., 2017. A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. Sci. Rep. 7, 8951. https://doi.org/10.1038/s41598-017-09444-2.

Mager, L.F., Wasmer, M.-H., Rau, T.T., Krebs, P., 2016. Cytokine-induced modulation of colorectal Cancer. Front. Oncol. 6. https://doi.org/10.3389/fonc.2016.00096.

Maissen-Villiger, C.A., Schweighauser, A., Dorland, H.A., van Morel, C., Bruckmaier, R.M., Zurbriggen, A., Francey, T., 2016. Expression profile of cytokines and enzymes mRNA in blood leukocytes of dogs with leptospirosis and its associated pulmonary hemorrhage syndrome. PLoS One 11, e0148029. https://doi.org/10.1371/journal.pone.0148029.

Malaponte, G., Hafsi, S., Polesel, J., Castellano, G., Spessotto, P., Guarneri, C., Canevari, S., Signorelli, S.S., McCubrey, J.A., Libra, M., 2016. Tumor microenvironment in diffuse large B-cell lymphoma: matrixmetalloproteinases activation is mediated by osteopontin overexpression. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1863, 483–489. https://doi.org/10.1016/j.bbamcr.2015.09.018. Tumor Microenvironment Regulation of Cancer Cell Survival, Metastasis, Inflammation and Immune Surveillance.

Marconato, L., Stefanello, D., Sabattini, S., Comazzi, S., Riondato, F., Laganga, P., Frayssinet, P., Pizzoni, S., Rouquet, N., Aresu, L., 2015. Enhanced therapeutic effect of APAVAC immunotherapy in combination with dose-intense chemotherapy in dogs with advanced indolent B-cell lymphoma. Vaccine 33, 5080–5086. https://doi.org/10.1016/j.vaccine.2015.08.017.

Medleau, L., Dawe, D.L., Calvert, C.A., 1983. Immunosuppressive effects of cyclophosphamide, vincristine, and L-asparaginase in dogs. Am. J. Vet. Res. 44, 176–180.

Mocellin, S., Marincola, F.M., Young, H.A., 2005. Interleukin-10 and the immune response against cancer: a counterpoint. J. Leukoc. Biol. 78, 1043–1051. https://doi.org/10.1189/jlb.0705358.

Nicholas, N.S., Apollonio, B., Ramsay, A.G., 2016. Tumor microenvironment (TME)-driven immune suppression in B cell malignancy. Biochim. Biophys. Acta BBA Mol. Cell Res. 1863, 471–482. https://doi.org/10.1016/j.bbamcr.2015.11.003. Tumor Microenvironment Regulation of Cancer Cell Survival, Metastasis, Inflammation and Immune Surveillance.

O'Connor, C.M., Wilson-Robles, H., 2014. Developing T cell cancer immunotherapy in the dog with lymphoma. ILAR J. Natl. Res. Counc. Inst. Lab. Anim. Resour. 55, 169–181. https://doi.org/10.1093/ilar/ilu020.

Owen, L.N., 1980. TNM Classification of Tumours in Domestic Animal.

Panjwani, M.K., Smith, J.B., Schutsky, K., Gnanandarajah, J., O'Connor, C.M., Powell, D.J., Mason, N.J., 2016. Feasibility and safety of RNA-transfected CD20-specific chimeric antigen receptor t cells in dogs with spontaneous B cell lymphoma. Mol. Ther. 24, 1602–1614. https://doi.org/10.1038/mt.2016.146.

Park, J.S., Withers, S.S., Modiano, J.F., Kent, M.S., Chen, M., Luna, J.I., Culp, W.T.N., Sparger, E.E., Rebhun, R.B., Monjazeb, A.M., Murphy, W.J., Canter, R.J., 2016. Canine cancer immunotherapy studies: linking mouse and human. J. Immunother. Cancer 4. https://doi.org/10.1186/s40425-016-0200-7.

Perry, J.A., Thamm, D.H., Eickhoff, J., Avery, A.C., Dow, S.W., 2011. Increased monocyte chemotactic protein-1 concentration and monocyte count independently associate with a poor prognosis in dogs with lymphoma. Vet. Comp. Oncol. 9, 55–64. https://doi.org/10.1111/j.1476-5829.2010.00235.x.

Peters, I.R., Helps, C.R., Calvert, E.L., Hall, E.J., Day, M.J., 2005. Cytokine mRNA quantification in histologically normal canine duodenal mucosa by real-time RT-PCR. Vet. Immunol. Immunopathol. 103, 101–111. https://doi.org/10.1016/j.vetimm.2004.08.020.

Purdue, M.P., Hofmann, J.N., Kemp, T.J., Chaturvedi, A.K., Lan, Q., Park, J.-H., Pfeiffer, R.M., Hildesheim, A., Pinto, L.A., Rothman, N., 2013. A prospective study of 67 serum immune and inflammation markers and risk of non-Hodgkin lymphoma. Blood 122, 951–957. https://doi.org/10.1182/blood-2013-01-481077.

Purdue, M.P., Lan, Q., Kricker, A., Grulich, A.E., Vajdic, C.M., Turner, J., Whitby, D., Chanock, S., Rothman, N., Armstrong, B.K., 2007. Polymorphisms in immune function genes and risk of non-Hodgkin lymphoma: findings from the New South Wales non-Hodgkin Lymphoma Study. Carcinogenesis 28, 704–712. https://doi.org/10.1093/carcin/bgl200.

Regan, D., Dow, S., 2015. Manipulation of innate immunity for Cancer therapy in dogs. Vet. Sci. 2, 423–439. https://doi.org/10.3390/vetsci2040423.

Schmitz, S., Garden, O.A., Werling, D., Allenspach, K., 2012. Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease. Vet. Immunol. Immunopathol. 146, 87–91. https://doi.org/10.1016/j.vetimm.2012.01.013.

Setrerrahmane, S., Xu, H., 2017. Tumor-related interleukins: old validated targets for new anti-cancer drug development. Mol. Cancer 16. https://doi.org/10.1186/s12943-017-0721-9.

Vaidya, R., Witzig, T.E., 2014. Prognostic factors for diffuse large B-cell lymphoma in the R(X)CHOP era. Ann. Oncol. 25, 2124–2133. https://doi.org/10.1093/annonc/mdu109.

Vendrame, E., Martínez-Maza, O., 2011. Assessment of pre-diagnosis biomarkers of immune activation and inflammation – insights on the etiology of lymphoma. J. Proteome Res. 10, 113–119. https://doi.org/10.1021/pr100729z.

Walter, C.U., Biller, B.J., Lana, S.E., Bachand, A.M., Dow, S., 2006. Effects of chemotherapy on immune responses in dogs with cancer. J. Vet. Intern. Med. 20, 342–347.

Weiden, P.L., Storb, R., Kolb, H.J., Ochs, H.D., Graham, T.C., Tsoi, M.S., Schroeder, M.L., Thomas, E.D., 1974. Immune reactivity in dogs with spontaneous malignancy. J. Natl. Cancer Inst. 53, 1049–1056.

Weiskopf, K., Anderson, K.L., Ito, D., Schnorr, P.J., Tomiyasu, H., Ring, A.M., Bloink, K., Efe, J., Rue, S., Lowery, D., Barkal, A., Prohaska, S., McKenna, K.M., Cornax, I., O'Brien, T.D., O'Sullivan, M.G., Weissman, I.L., Modiano, J.F., 2016. Eradication of canine diffuse large B-Cell lymphoma in a murine xenograft model with CD47 blockade and Anti-CD20. Cancer Immunol. Res. 4, 1072–1087. https://doi.org/10.1158/2326-6066.CIR-16-0105.

Xiu, B., Lin, Y., Grote, D.M., Ziesmer, S.C., Gustafson, M.P., Maas, M.L., Zhang, Z., Dietz, A.B., Porrata, L.F., Novak, A.J., Liang, A.-B., Yang, Z.-Z., Ansell, S.M., 2015. IL-10 induces the development of immunosuppressive CD14+HLA-DRlow/− monocytes in B-cell non-Hodgkin lymphoma. Blood Cancer J. 5, e328. https://doi.org/10.1038/bcj.2015.56.