UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

CLINICAL AND IMMUNOLOGICAL CHARACTERIZATION OF NATURALLY OCCURRING CANINE LYMPHOMA: DEVELOPMENT AND APPLICATION OF ENGINEERED RECOMBINANT ANTIBODIES FOR DIAGNOSIS AND TREATMENT

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Sanidade Animal

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Abstract

Clinical and immunological characterization of naturally occurring canine lymphoma: development and application of engineered recombinant antibodies for diagnosis and treatment

Non-Hodgkin lymphoma (NHL) is one of the most common causes of cancer-related death worldwide. Although the outcome of NHL patients has improved with current therapies, the rate of mortality is still high. A plethora of new drugs is entering clinical development for NHL treatment; however, the approval of new treatments remains low due in part to the paucity of clinically relevant models for validation. Canine lymphoma (cNHL) shares remarkable similarities with its human counterpart, making the dog an excellent animal model to explore novel therapeutic options. Therefore, driven by the great success achieved by immunotherapies in human NHL, comparative research has focused on the development of similar immunotherapeutic approaches for dogs. However, the successful use of this animal model remains challenging, still lacking the characterization of the canine immune system, of common tumor epitopes, the development of canine-specific/cross-reactive agents and the establishment of preclinical models. Within this context, we aimed to develop novel antibody-based therapies for cNHL, while contributing for the characterization and validation of the cNHL model for translational immune-oncology research. For that purpose, a cNHL biobank was successfully constructed. The clinical cytokine patterns in patients with cNHL were investigated, confirming a local and systemic dysregulation in cytokine response. Furthermore, a positive correlation between intratumoral immune response and a favorable response to chemotherapy indicates that the modulation of the immune response might contribute to improve patient outcomes. With that in mind, a through characterization of canine CD20 expression was conducted and contributed for the validation of this receptor as a potential immunotherapeutic target for cNHL. This motivated the development and identification of a panel of single domain antibodies (sdAbs) with high binding activity and specificity to canine and human CD20. In addition, to develop a novel drug delivery system for cNHL treatment, we described novel methodologies to identify potential targets, while selecting highly specific sdAbs against NHL. This work allowed to select a promising pool of sdAbs that specifically target NHL tumor receptors for the development of a novel antibody drug conjugate (ADC). Furthermore, we conducted a thorough investigation of a novel ADC payload – panobinostat - a potent histone deacetylase (HDAC) inhibitor with strong *in vitro* and *in vivo* antitumor properties in cNHL. Finally, we established a new bioluminescent murine model for monitoring tumor progression and treatment response in preclinical studies. In summary, the work presented herein allowed the establishment of a solid platform for the acceleration of the translational research of novel immunotherapeutic approaches for comparative oncology.

Key-words: canine lymphoma, non-Hodgkin lymphoma, animal model, recombinant antibodies, antibody-drug conjugate, HDAC inhibitors, panobinostat

Resumo

Caracterização clínica e imunológica do linfoma canino como modelo animal: Desenvolvimento de anticorpos recombinantes para tratamento e diagnóstico

O linfoma não-Hodgkin (LNH) é uma das principais causas de morte por neoplasia em todo o mundo, representando 90% de todos os linfomas. O LNH abrange um grupo heterogéneo de tumores, caracterizado pela proliferação de linfócitos malignos, 85-90% dos quais de linfócitos B. Os anticorpos direccionados para o recetor CD20, combinados com quimioterapias convencionais revolucionaram o tratamento do linfoma de células B, melhorando o tempo de remissão e aumentando a taxa de sobrevivência. No entanto, independentemente da terapêutica utilizada, a taxa de mortalidade mantém-se elevada. Uma grande diversidade de novos fármacos encontra-se em fase de desenvolvimento clínico para o tratamento do LNH; no entanto, a aprovação de novos tratamentos permanece baixa devido, em parte, à escassez de modelos clinicamente relevantes para validação. O cLNH e o LNH humano (hLNH) partilham muitas características histopatológicas, moleculares, genéticas e clínicas, desta forma o cão é considerado um excelente modelo animal para explorar novas opções terapêuticas. Consequentemente, a investigação em oncologia comparativa, motivada pelo grande sucesso alcançado pelas imunoterapias no tratamento do hLNH, tem-se focado no desenvolvimento de abordagens imunoterapêuticas semelhantes para cães. Contudo, o sucesso do cLNH como modelo animal tem vindo a revelar-se desafiante, na medida que a validação do seu uso para o desenvolvimento de imunoterapias ainda carece da caracterização do sistema imune canino, das células imunitárias e das moléculas efectoras, da avaliação dos epítopos tumorais comuns, do desenvolvimento de agentes imunoterapêuticos específicos para a espécie canina com potencial reacção cruzada com a espécie humana e do estabelecimento de modelos pré-clínicos para oncologia veterinária.

Neste contexto, o presente trabalho teve como principal objectivo validar o cLNH como modelo animal para o desenvolvimento de novas estratégias imunoterapêuticas para LNH, visando estabelecer uma linha de investigação inovadora para o desenvolvimento de anticorpos recombinantes para o tratamento do cLNH. Neste sentido, desenvolveu-se uma estratégia multidisciplinar constituída por etapas complementares de modo a construir uma sólida plataforma para o desenvolvimento de ensaios clínicos de imunoterapias em oncologia comparativa.

Após uma breve sinopse no capítulo 1 com uma revisão bibliográfica das principais temáticas que contextualizam os objectivos deste trabalho, o capítulo 2 foca-se no papel das citoquinas no cLNH, importantes mediadores da interacção entre as células tumorais e o seu microambiente. No hLNH, diversas citoquinas foram associadas ao desenvolvimento de linfoma e demonstraram ser relevantes biomarcadores da resposta à terapêutica e de prognóstico. Em contraste, poucos estudos investigaram o seu papel no cLNH. Neste

contexto, um dos objectivos deste trabalho foi a avaliação da expressão e regulação das citoquinas no cLNH multicêntrico. Para este fim, foi construído com sucesso um biobanco de cLNH, composto por amostras colhidas de doentes seguidos no Hospital Escolar da FMV-ULisboa. O perfil de ARNm de citoquinas pertencentes à resposta TH1/TH2/TH17 no tecido tumoral e nos PBMC circulantes foi investigado recorrendo à técnica de qRT-PCR e os resultados obtidos comparados com os do grupo de controlo constituído por cães saudáveis. As concentrações de citoquinas sistémicas foram também avaliadas usando um ensaio comercial multiplex canino, incluindo as citoquinas IL-2, IL6, IL-10 e TNF-α, e comparadas com o grupo de animais de controlo. Os resultados demonstraram uma desregulação da expressão de ARNm das citoquinas, representativa do microambiente tumoral e da resposta sistémica presente no cLNH. A regulação intratumoral de citoquinas revelou uma sobreexpressão do ARNm das citoquinas mediadoras da inflamação e uma diminuição significativa da expressão da resposta humoral e TH2 concomitante. Pelo contrário, a resposta sistémica demonstrou um padrão de expressão de ARNm distinto, com predomínio de imunossupressão e incapacidade de montar uma resposta TH1. Por sua vez, a quantificação serológica de citoquinas demonstrou um aumento significativo da concentração da IL-10 no cLNH. Foram descritas diferenças significativas nos parâmetros hematológicos e foi reportada uma correlação entre a concentração serológica da IL-6 e a contagem neutrofílica. De salientar ainda que foi observada uma correlação positiva entre a expressão de ARNm tecidular de IFN-γ pré-tratamento e o aumento do tempo de sobrevida. Ao confirmar e amplificar trabalhos prévios, este trabalho contribuiu para a avaliação de citoquinas com potencial diagnóstico, prognóstico e terapêutico e para a caracterização do sistema imunitário do modelo de cLNH. De destacar ainda a construção bem-sucedida do biobanco de cLNH representou uma importante mais-valia, uma vez que a sua manutenção garantirá a disponibilidade de diversas amostras adequadamente preservadas para trabalhos em curso e futuros projetos.

Atendendo ao sucesso terapêutico do anticorpo monoclonal Rituximab no tratamento do LNH de células B em medicina humana, a comunidade médico-veterinária anseia por uma imunoterapia similar para o tratamento do cLNH. Deste modo, para ultrapassar a limitação da ausência de reactividade cruzada do Rituximab para o epítopo canino, alguns anticorpos monoclonais de primeira geração foram recentemente desenvolvidos, um dos quais obteve já aprovação clínica. No entanto, estes anticorpos têm vindo a apresentar resultados clínicos e científicos decepcionantes, sugerindo que o desenvolvimento de um mAb eficaz no tratamento cLNH poderá requerer uma abordagem mais complexa e inovadora.

Neste contexto, no capítulo 3, procedeu-se ao desenvolvimento de anticorpos recombinantes para o diagnóstico e tratamento de cNHL. Assim, no sub-capítulo 3.1 descreve-se um estudo em que foi investigada a sequência do gene do CD20 canino e a sua expressão génica e proteica no biobanco canino. É reportada ainda uma nova estratégia para o desenvolvimento

de anticorpos anti-CD20 que reconhecem o receptor canino e apresentam reação cruzada com o humano. Os resultados obtidos demonstraram uma sobrexpressão deste receptor em células de linfoma canino, validando assim o CD20 como um potencial alvo de imunoterapia no cão. Para além disso, foi identificada uma nova sequência do CD20 canino, que diverge das sequências anteriormente reportadas. Adicionalmente, através do desenvolvimento de uma biblioteca variada de anticorpos de domínio único (sdAbs) de coelho contra a nova sequência do CD20 canino e da seleção dos melhores sdAbs pela técnica de "phage display" subtractivo em células, foi possível identificar um painel de sdAbs com elevada afinidade e especificidade para o CD20 canino, com reação cruzada com o CD20 humano. Estes sdAbs podem revelar-se promissores para o desenvolvimento de novas alternativas terapêuticas em oncologia comparativa.

Apesar do sucesso do Rituximab, têm vindo a ser descritos vários mecanismos de resistência das células tumorais a este anticorpo, o que justifica a procura contínua por novos mAbs. Entre as terapêuticas baseadas em mAbs, os anticorpos conjugados com fármacos (ADCs) são considerados uma das estratégias mais promissoras, combinando a especificidade de reconhecimento tumoral, propriedades farmacocinéticas e de biodistribuição dos anticorpos com a potência citotóxica das moléculas pequenas. Considerando o potencial que representa o desenvolvimento de um ADC para oncologia comparativa, o subcapítulo 3.2 teve como objectivo explorar uma nova estratégia de selecção de anticorpos altamente específicos para o LNH, com o objectivo de desenvolver um sistema promissor de entrega de fármacos citotóxicos. Para isso, foi construída uma biblioteca de sdAbs de coelho anti-cNHL. A combinação de uma selecção por "phage display" subtrativa em células intactas e de uma análise com uma técnica de alta capacidade, possibilitou a identificação de um painel de sdAbs altamente específicos para o LNH. Este trabalho permitiu assim selecionar um promissor painel de sdAbs que reconhecem receptores de NHL *in vivo*, que no futuro esperamos acoplar a fármacos citotóxicos de modo a desenvolver um novo ADC.

Por sua vez, o capítulo 4 teve como objectivo a identificação e caracterização de um agente citotóxico de elevada potência para o desenvolvimento de ADCs. Considerando o sucesso que os inibidores da histona desacetilase (HDACis) têm vindo a apresentar no tratamento oncológico no Homem, este trabalho visou assim investigar as propriedades antitumorais dos HDACis no LNH canino. Para este efeito, um painel de sete compostos (CI-994, panobinostat, SBHA, SAHA, scriptaid, tricostatina A e tubacina) foi testado quanto à capacidade de inibir o crescimento da linha celular CLBL-1 de linfoma canino difuso de células grandes tipo B. Os resultados obtidos demonstraram que todos os compostos testados apresentaram efeitos inibitórios dependentes da dose sobre a proliferação das células CLBL-1 e promoveram o aumento da acetilação das histonas H3. De todos os HDACis estudados, o panobinostat provou ser o composto mais promissor tendo sido selecionado para posterior avaliação *in vitro* e *in vivo*. A citotoxicidade do panobinostat foi associada à histona H3, à

acetilação da α-tubulina e à indução da apoptose. É importante salientar que o panobinostat inibiu eficazmente o crescimento tumoral num modelo murino de xenotransplante de CLBL-1 e induziu fortemente a acetilação da histona H3 e a apoptose *in vivo*. Em conclusão, estes resultados fornecem novos dados validando os HDACis em geral e especialmente, o panobinostat, como agentes promissores para terapêuticas antitumorais em medicina veterinária.

Por fim, considerando que uma das maiores limitações para a integração do cLNH em estudos comparativos é a falta de modelos murinos adequados para estudos pré-clínicos, o capítulo 5 dedicou-se ao desenvolvimento e caracterização de um novo modelo bioluminescente de cLNH de células B. Para este efeito, foi desenvolvida uma linha celular CLBL-1 que expressa luciferase exibindo fluorescência verde. Um estudo piloto com três densidades celulares diferentes ($0,1 \times 10^6$, $0,5 \times 10^6$ e 1×10^6 células) em ratinhos imunodefiecientes, revelou que todos os ratinhos apresentaram indução tumoral 8 dias após a injecção subcutânea, com 100% de eficiência de enxerto e não foram observadas diferenças significativas no crescimento tumoral entre os grupos. Os tumores eram altamente agressivos e localizados no local de inoculação, reproduzindo características histológicas e imunofenotípicas compatíveis com LNH canino de células B. Além disso, os tumores foram detectados e quantificados por imagiologia bioluminescente. Consequentemente, foi realizado um ensaio terapêutico com o panobinostat, tendo-se observado que o tratamento com 20 mg/kg inibiu significativamente o crescimento tumoral, tendo a imagiologia bioluminescente permitido a monitorização e quantificação da resposta tumoral à terapêutica. Este estudo permitiu assim validar um modelo de cLNH de células B que oferece uma elevada eficiência de indução tumoral, preservação das características tumorais e monitorização da progressão tumoral através da utilização de um método não invasivo.

Em suma, os trabalhos apresentados nesta tese constituem importantes pilares para a construção de uma sólida plataforma de aceleração da investigação translacional de inovadoras estratégias imunoterapêuticas em oncologia comparativa.

Palavras-chave: linfoma canino, linfoma non-Hodgkin, modelo animal, anticorpos recombinantes, conjugado anticorpo-fármaco, inibidores HDAC, panobinostat

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List of Abbreviations and Symbols

%	Percentage
®	Registered trademark
ACT	Adoptive cell therapy
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cell-mediated cytotoxicity
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BLI	Bioluminescence imaging
B-NHL	B-cell non-Hodgkin lymphoma
BSA	Bovine serum albumin
CAR	Chimeric antigen receptor
CCNU	Lomustine®
CD	Cluster Designation
CDC	Complement dependent cytotoxicity
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity Determinating Region
CH	Região constante da cadeia pesada da imunogloblina
CHOP	Cyclophosphamide, doxorubicin, vincristine and prednisone
CL	Região constante da cadeia leve da imunogloblina
CML	Chronic myeloid leukemia
cNHL	Canine non-Hodgkin Lymphoma
COP	Cyclophosphamide, vincristine and prednisone
CR	Complete response
CTCL	Cutaneous T-cell lymphoma
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DAR	Drug to antibody ratio
DLBCL	Diffuse large B-cell lymphoma
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
E. coli	*Escherichia coli*

ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
ε	Epsilon
Fab	Antigen-binding fragment
FBS	Fetal bovine serum
Fc	Crystallizable fragment
FcRn	Neonatal Fc receptor binding
FCS	Fetal calf serum
FcγR	Fc gamma receptor
FDA	US Food and Drug Administration
FL	Follicular lymphoma
FNA	Fine needle aspiration
Fv	Variable fragment
g	Gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
H&E	Hematoxylin and eosin stain
H_2O	Water molecule
HBI	Half body irradiation
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitors
HER2	Human Epidermal growth factor Receptor 2
HL	Hodgkin lymphoma
HLA-DR	Human Leukocyte Antigen – DR isotype
hNHL	Human non-Hodgkin Lymphoma
HSP	Heat shock protein
HSPPC	Immunogenic tumor specific peptides
Ig	Immunoglobulin
IL-10	Interleukin-10
IL-12p40	Interleukin-12 subunit p40
IL-17A	Interleukin-17
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6

IL-8	Interleukin-8
ILSG	International Lymphoma Study Group
INF-γ	Interferon-γ
L-CHOP	L-asparginase - Cyclophosphamide, doxorubicin, vincristine and prednisone
LMI	Large multivalent immunogen
M	Molar
mAbs	Monoclonal Antibodies
MDR	Multidrug resistance
MHC	Major histocompatibility complex
min	Minute
MOPP	Methchlorathamine, vincristine, procarbazine, and prednisone
NHL	Non-Hodgkin lymphoma
NK	Natural killer
º	Degree
ºC	Degrees celcius
PAAR	PCR for Antigen Receptor Rearrangements
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Progressive response
PD-1	Programmed death 1
PDL-1	Programmed death ligand 1
PE-A	Pseudomonas exotoxin A
pH	Negative decimal logarithm of the hydrogen ion activity in a solution
Phage	Bacteriophage
PK/PD	Pharmacokinetic/pharmacodynamic
PR	Partial response
qRT-PCR	Real-Time quantitative Polymerase Chain Reaction
R-CHOP	Rituximab - Cyclophosphamide, doxorubicin, vincristine and prednisone
REAL	Revised European American Lymphoma Classification
RLP27	Ribosomal Protein L27
RNA	RiboNucleic Acid
RPMI-1640	Roswell Park Memorial Institute–1640
SB	Super broth medium
scFv	Single chain antibody fragment

SD	Stable disease
sdAbs	Single domain antibody fragment
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis;
SEM	Stantard error of the mean
TAA	Tumor associated antigens
TBI	Total body irradiation
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
TGI	Tumor growth inhibition
TH1	Helper T cells type 1
TH2	Helper T cells type 2
TIL	Tumor infiltrating lymphocytes
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
USDA	U.S. Department of Agriculture
VEGFR2	Vascular endothelial growth factor receptor 2
VH	Heavy-chain variable region
VL	Light-chain variable region
WHO	World Health Organization
α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
λ	Lambda
μ	Mu
μg	Microgram

Chapter 1

Bibliographic review and objectives

1.1. Cancer immunotherapies – challenges and prospects

In 2015 alone, cancer was responsible for 8.8 million deaths worldwide in countries of all income levels, ranking second place in the leading causes of death, behind cardiovascular diseases (Fitzmaurice et al., 2017). Owing to population growth, aging, and adoption of lifestyle behaviors associated with cancer risk, this number is expected to rise by about 70% over the next 20 years (Fitzmaurice et al., 2017; Torre, Siegel, Ward, & Jemal, 2016). Still, even though these impressive numbers demonstrate that cancer burden remains a major challenge worldwide, recent developments in personalized medicine and novel treatment approaches, such as immunotherapy, have raised hope of significantly improving cancer survival (Global Burden of Disease Cancer Collaboration, 2017).

The concept of harnessing the host's immune system to treat cancer can be traced back decades, however only in recent years immunotherapies have emerged as a clinically validated and effective treatment strategy (Yang, 2015). Nowadays, cancer immunotherapy positions as a fast-growing field and rapidly became the fourth pillar of cancer care, along with surgery, cytotoxic therapy and radiotherapy (Emens et al., 2017). More recently the successes of clinical breakthroughs, such as checkpoint inhibitors and engineered T cells, revitalized the field and highlighted the opportunities that immunotherapeutic approaches can offer, which culminated in the nomination of "cancer immunotherapy" as 2013's Breakthrough of the Year by *Science* (Farkona, Diamandis, & Blasutig, 2016; Marks, 2017).

However, by transforming the cancer therapeutic landscape, this complex modality brought unique challenges to the drug discovery and development industry. In fact, as more cancer patients have received immunotherapies, some of the major drawbacks of these treatments have become clear. One of the major issues is to determine the sub-populations of patients who will respond and who will experience at times significant toxicities (Ventola, 2017). As a

matter of fact, the challenge now is to extend the range of patients that benefit from immunotherapy while minimizing treatment-related adverse events. To address this, it is crucial to identify factors predictive of response that may help to properly select patients for treatment, identify rational combination therapies, and define progression and resistance (Klevorn & Teague, 2016). This is particularly critical when developing cancer immunotherapies, considering that the patient's immune system is expected to be as significant as tumor-related aspects when determining response and toxicity (Park et al., 2016).

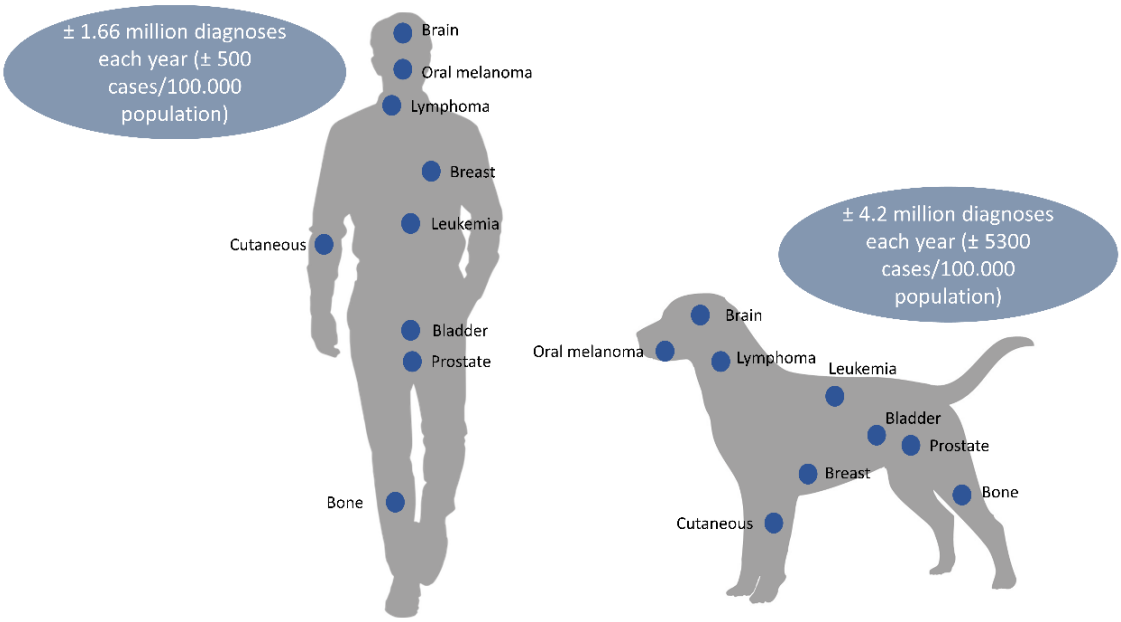
Clinical translation of cancer immunotherapy depends on preclinical investigation and rodent models have been the foundation of preliminary basic investigation and safety assays (Malaney, Nicosia, & Davé, 2014). However, these models underrepresent the heterogeneity and complex interaction between the human immune cells and cancers. As a matter of fact, laboratory mice rarely develop spontaneous tumors, are housed under specific-pathogen free conditions that markedly influences immune development, and incompletely model key characteristics of the tumor/immune microenvironment, creating challenges for clinical translation. As a result, these murine models have failed to correlate with clinical success rates, demonstrating an urgent need for innovative pre-clinical models (Biemar & Foti, 2013; Kohnken, Porcu, & Mishra, 2017; Kola & Landis, 2004). Thereby, the use of alternative animal models is pivotal to bridge the translational gap between murine models and human clinical studies. In particular, preclinical models with intact immune systems that closely mimic the human immune system, display comparable, spontaneous oncogenesis and immune interactions to humans, and that can model key immunotherapeutic outcomes such as efficacy, dose response, and toxicity, will be critical for progress in translational cancer immunotherapy research (Park et al., 2016).

Thus, comparative medicine offers an important platform with innovative complex cross-species models that allow the research of novel therapeutic strategies and agents for diseases that are common to animals and humans (Figure 1) (Henry & Bryan, 2013; Porrello, Cardelli, & Spugnini, 2006). In particular, the canine model represents a powerful resource of models for cancer immunotherapy research. Dogs are an appealing outbred combination of companion animals that experience spontaneous cancer development in the setting of an intact immune system (Park et al., 2016). Besides, naturally occurring tumors in dogs present many clinical, pathological, immunologic, molecular, diagnostic and therapeutic similarities to those observed in humans, that are difficult to reproduce in other models (Gardner, Fenger, & London, 2016; Pinho, Carvalho, Cabral, Reis, & Gärtner, 2012; Ranieri et al., 2013; Rowell, McCarthy, & Alvarez, 2011). This allows studying the complex immune interactions during

the course of treatment while also addressing long-term efficacy and toxicity of cancer immunotherapies (Park et al., 2016).

Nevertheless, the integration of the canine model in immunotherapeutic approaches research requires diagnosis, staging and treatment response assessment optimization and standardization, to perform large and organized clinical trials and to achieve conformity when analyzing data (Marconato et al., 2017).

Figure 1 - The remarkable similarity in cancers shared by human and dog



The cancers shown are found in both human and canine populations, and several ongoing studies emphasize the similarities at the genomic level. The incidence of cancers in both species is shown, highlighting the more than 2.5×number of cancers diagnoses in pet dogs each year. Comparative oncology is a growing trans-disciplinary field that harnesses these data, adding evidence to support a shared pathogenesis. Adapted from (Schiffman & Breen, 2015).

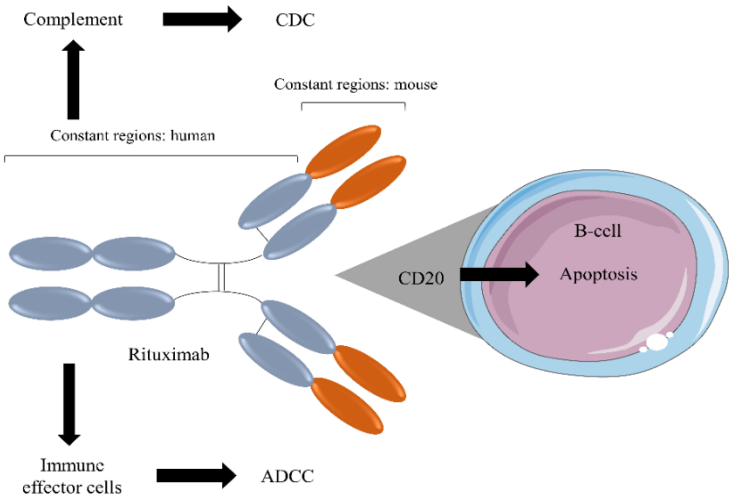
1.2. Non-Hodgkin Lymphoma

Lymphomas are solid tumors of lymphoid cells and are divided into Hodgkin's lymphoma and Non-Hodgkin lymphoma (NHL) (Hennessy, Hanrahan, & Daly, 2004). NHL is the fourth most common cancer and the sixth most common cause of cancer-related death in the United States and its incidence has nearly doubled since the early 70s (Kong, Barisone, Sidhu, O'Donnell, & Tuscano, 2015; Siegel, Miller, & Jemal, 2015). NHL represents 90% of all lymphomas and encompasses an heterogeneous group of cancers characterized by the proliferation of malignant lymphocytes, 85–90% of which arise from B lymphocytes, whereas the remaining derive from T cells or NK cells. This diverse group of malignancies usually

develops in the lymph nodes, but can occur in almost any tissue, ranging from the more indolent follicular lymphoma to the more aggressive diffuse large B-cell and Burkitt's lymphoma (Shankland, Armitage, & Hancock, 2012). NHL patients typically present with persistent painless lymphadenopathy, but some patients may present with constitutional symptoms or with involvement of organs other than the lymphoid and hematopoietic system (Ansell, 2015).

The basis of treatment selection requires an accurate diagnosis, a careful staging of the disease, and the identification of adverse prognostic factors. Regardless, NHL patients most commonly receive chemoimmunotherapy as initial treatment. Radiation therapy may be performed if patients have early-stage disease (Ansell, 2015). Response rates to conventional chemotherapy are generally greater than 50%; however, most patients eventually relapse. Moreover, the toxicity of conventional chemotherapy often limits its efficacy (Kong et al., 2015). In the last decades, the scientific community has been reporting cases of therapeutic success using monoclonal antibodies (mAbs) in the treatment of NHL in humans. One of the most successful examples has been the application of mAbs targeting the surface antigen CD20 (Rituximab®) (Figure 2) in combination with chemotherapy regimen CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone), which has revolutionized the treatment of B-cell lymphoma by significantly improving disease-free interval and overall survival, with minimal toxicity (Ito, Brewer, Modiano, & Beall, 2015; Motta et al., 2010). Even though current therapy strategies have significantly improved prognosis of patients diagnosed with NHL, a significant fraction of patients relapse or are refractory to these treatments. Several treatment shortcomings have been identified as research priorities, however rituximab resistance and refractory/relapsed disease represent major current and emerging challenges (Chao, 2013; Molina, 2008; Zappasodi, de Braud, & Di Nicola, 2015).

Figure 2 –Schematic illustration of the mechanism of action of rituximab.



The antibody labels B lymphocytes, which have the CD20 cell marker. These cells are then killed by one of three mechanisms: antibody-dependent cytotoxicity, complement-dependent cytotoxicity, or stimulation of apoptosis.

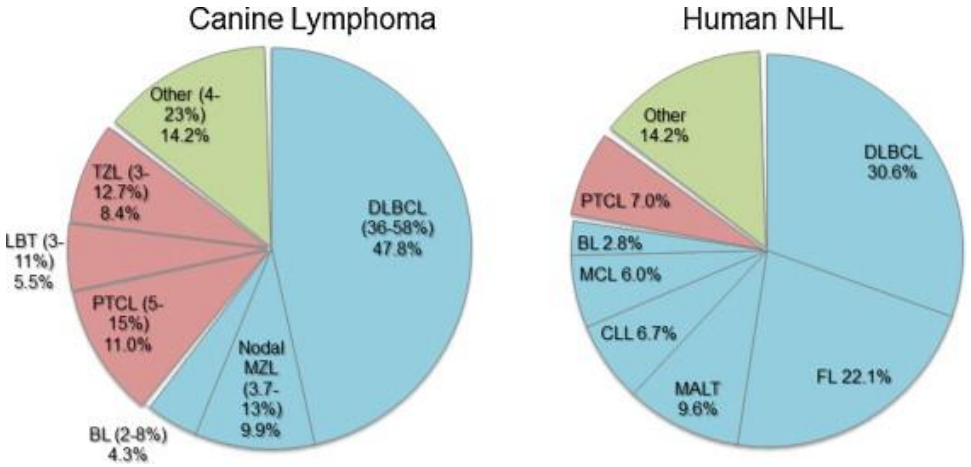
1.3. Rationale for a canine model of lymphoma

For a long time, research in lymphoma has benefited from traditional mouse models, however the paucity of truly representative models has hindered complete understanding of disease biology and drug development. With the introduction of genomics technology, nontraditional animal models have been more accessible and the leverage of these opportunities may represent a novel strategy to accelerate disease research and new drug discovery (Richards & Suter, 2015). Furthermore, there is an increasing number of studies demonstrating that spontaneously arising lymphoma in dogs could be an invaluable resource to study the biology and treatment of this disease (Marconato, Gelain, & Comazzi, 2013). As such, the canine lymphoma (cNHL) model may help to bypass many of the limitations associated with the use of murine models while presenting other additional advantages (Ito, Frantz, & Modiano, 2014; Khanna et al., 2006).

The canine lymphoma shares many remarkable similarities with its human counterpart (Ito et al., 2014; Kisseberth et al., 2007; Ponce et al., 2010; Rütgen et al., 2010, 2012). The incidence of cNHL of 15–30/100 000 is similar to human incidence (Hahn, Bravo, Adams, & Frazier, 1994; Vail & MacEwen, 2000), though recent studies indicate that the incidence of cNHL may be higher (Hansen & Khanna, 2004). Classification and grading schemes of cNHL were designed to reflect the equivalent in people and facilitate comparison. In fact, the 2008 revised

World Health Organization (WHO) classification based on the Revised European American Lymphoma (REAL) classification system, which attempts to group lymphomas by cell type, phenotypic, genetic and molecular aspects, is the current standard for the diagnosis and classification of human lymphoma, also serves as the basis for the current canine recommendations (Seelig, Avery, Ehrhart, & Linden, 2016; Swerdlow, 2008). The use of these current WHO guidelines as a template, allowed to describe 20 cNHL entities, among nearly 50 discrete subtypes of human NHL (hNHL) (Figure 3). Moreover, B-cell lymphoma is more prevalent than T-cell lymphoma in both species and the most common type of NHL is the same in both humans and dogs is diffuse large B-cell lymphoma (Seelig et al., 2016). Finally, treatment modalities for cNHL are similar to those used for human lymphoma (radiation, corticosteroids, chemotherapy) and CHOP-based chemotherapy agents are typically used to treat it. Response to treatment and resistance also present clinical patterns similar to hNHL (Richards & Suter, 2015).

Figure 3 - Relative distribution of common subtypes of cNHL and hNHL.

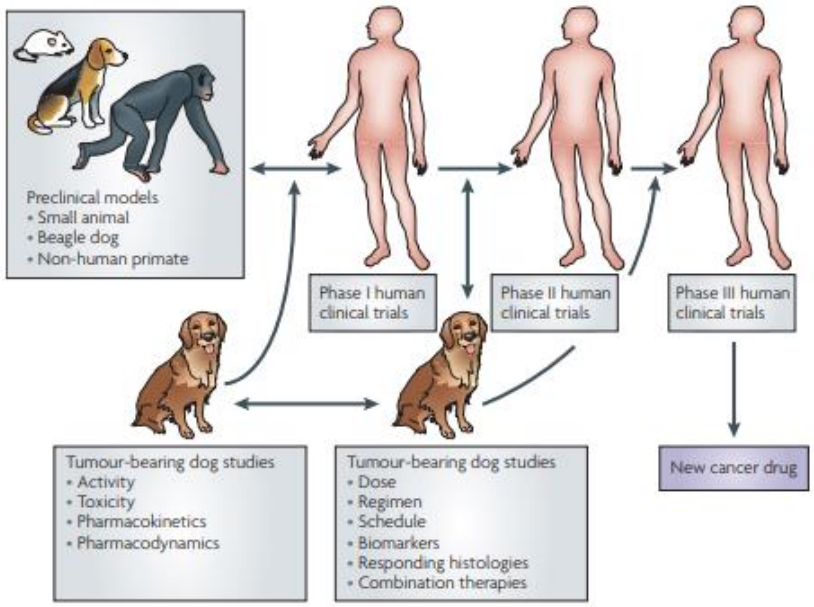


DLBCL; diffuse large B-cell lymphoma, MZL; marginal zone lymphoma, BL; Burkitt lymphoma, PTCL; peripheral T cell lymphoma not otherwise specified, TZL; nodal T-zone lymphoma, LBT; lymphoblastic T-cell lymphoma, FL; follicular lymphoma, MALT; mucosa associated lymphoid tissue lymphoma (extranodal MZL), CLL; chronic lymphocytic leukemia, MCL; mantle cell lymphoma. Adapted from (Ito et al., 2014).

From a drug development perspective, the canine model represents a large and long-lived animal model, evolutionarily more closely related to humans than rodents, that provides a more accurate assessment of the pharmacokinetic/pharmacodynamic (PK/PD) parameters, while determining safety and efficacy of new therapeutic agents and approaches (Lindblad-Toh et al., 2005; Richards & Suter, 2015). Moreover, the relatively fast disease progression rate allows obtaining early conclusions from clinical trials. In fact, a randomized clinical trial in pet dogs requires approximately 1-3 years, whereas a human clinical trial takes about 15

years to be completed. This short timeline allows to integrate the findings of pet trials on human trials, including toxicity, response, pharmacodynamics, dose, regimen, schedule, biomarkers and responding histologies (Marconato et al., 2013) (Figure 4).

Figure 4 -A comparative and integrated approach to cancer drug development.



An optimal drug development path would integrate both preclinical and clinical components of drug development so that questions that emerge in the human clinic could be answered in animals. Translational drug development studies in the pet dog with cancer are optimal for such an integrated approach, being an intermediary between conventional preclinical models (mouse, research-bred dog and non-human primate) and the human clinical trial. Through this integrated approach it is likely that important questions about a new drug candidate can be answered before it enters human studies, for example, toxicity, dose, regimen, pharmacokinetics, pharmacodynamics and activity. Adapted from (Paoloni & Khanna, 2008).

Another main advantage of the canine model is that cNHL is a spontaneously occurring tumor in an immune-competent host, in contrast to murine xenograft or genetically engineered mouse models. This natural occurring cancer setting offers genetic diversity similar to human lymphoma and allows to study biological mechanisms, such as tumor initiation and promotion. Moreover, the pet dog model harnessed by the evolutionary conservation allows to identify similarities between canine and human lymphomagenesis, for example in identifying key ‘driver’ gene mutations common to both species (Richards & Suter, 2015).

In addition, the benefits of the cNHL model extend beyond the biological advantages of a spontaneously occurring tumor in a large animal. Pet dogs share the same living environment as their caregivers, allowing to study environmental risk factors of developing lymphoma (Marconato et al., 2013; Richards & Suter, 2015). For example, an epidemiological study in France demonstrated a correlation between the incidence of cNHL and hNHL and reported a

strong association between cNHL and the distribution of waste incinerators, radioactive waste or other polluted sites (Pastor et al., 2009). Moreover, there is an increased prevalence of lymphoma within specific dog breeds (Gamlem, Nordstoga, & Glattre, 2008) and a breed-specific distribution of B-cell and T-cell lymphomas (Modiano et al., 2005). This in association with the well-organized multi-generational pedigrees kept by many breeders, represents a unique genetic advantage that allows the genetic mapping of lymphoma predisposition genes with strategies that are not possible in humans (Marconato et al., 2013).
The final rationale for using dogs with lymphoma as an animal model relies on the dual benefit concept of this comparative research approach. Improved current health care have promoted the increase dogs lifespan, allowing the diagnosis of late-in-life diseases such as cancer (Paoloni & Khanna, 2007). Lymphoma particularly is one of the most common malignancies in dogs (Marconato et al., 2013). In addition, the social status of dogs as companion animal allows them to benefit from high quality health care and the ethical exploration of translational approaches. Moreover, these initiatives are also motivated by the increasing healthcare standards demanded by pet owners, creating a need for novel cancer therapies in veterinary settings (Henry & Bryan, 2013; Porrello et al., 2006; Weiskopf et al., 2016). Altogether, the use of the cNHL model represents a unique opportunity to strengthen the collaboration between human and veterinary medicine in lymphoma research, that ultimately will lead to advances in the care of people and dogs affected by NHL (Gardner et al., 2016; Richards & Suter, 2015).

1.4. Canine Lymphoma

Lymphomas are among the most commonly diagnosed neoplasias in the dog and represents the most frequently managed neoplasia in veterinary medical oncology (Zandvliet, 2016). The generic term of cNHL comprises a heterogeneous group of lymphoid malignancies that have different cells of origin and biological behaviors (Ito et al., 2014).

1.4.1. Epidemiology and etiology

Lymphoma comprises 83% of all canine hematopoietic malignancies and 7-24% of all canine neoplasias and its frequency is increasing (Crow, 2008; Dobson, Samuel, Milstein, Rogers, & Wood, 2002; Ito et al., 2014; Jemal et al., 2004; Marconato et al., 2013). Lymphoma affects dogs of any age, but occurs most frequently in middle aged to older dogs (median age of 6 to 9 years), with the incidence rate increasing with age from 1.5 cases per 100.000 dogs for dogs

10 years. Moreover, certain breeds show predisposition such as boxers, bull mastiffs, basset hounds, St. Bernards, scottish terriers, airedales and bulldogs. There is no apparent sex predisposition, but intact female dogs seem to have a reduced risk (Villamil et al., 2009; Withrow, Page, & Vail, 2013).

The etiology of this neoplasia has a multifactorial character, emerging from genetic abnormalities, predisposition (breed, in the case of dogs) and environmental exposures (Modiano et al., 2005; Pastor et al., 2009). Although there is no definitive proof of a viral etiology similar to humans (Waugh et al., 2015), the detection of reverse-transcriptase activity in supernatants of lymph node cultures from dogs with lymphomas (Tomley, Armstrong, Mahy, & Owen, 1983), as well as the detection of a gamma-herpes (Epstein-Barr) virus in a proportion of cNHL cases (Chiou, Chow, Yang, Chiang, & Lin, 2005; Huang et al., 2012; Milman, Smith, & Erles, 2011) have supported the theory of a possible viral cause. Despite the association of Helicobacter-infections with the development of gastric mucosa associated lymphoid tissue lymphoma in humans, experimental infections in Beagles showed only the formation of lymphoid follicles and no progression to gastric lymphoma (Rossi et al., 2000). Observational and clinical studies have associated increased cancer risks with primary or acquired immunodeficiencies, autoimmunity, and use of immunosuppressive therapy for organ or stem cell transplantation and/or chronic inflammation (e.g. autoimmunity) (Ponce et al., 2014). There are few evidences supporting this association in the dog. Nevertheless, autoimmune disease (most likely secondary) is frequent in dogs with cNHL (Keller, 1992) and there is a case report of a dog diagnosed with cNHL after cyclosporine treatment (Blackwood, German, Stell, & O'Neill, 2004).

1.4.2. Clinical presentation

cNHL displays several histological subtypes and patients can manifest a wide range of symptoms. The most common clinical presentation of cNHL is multicentric (Ettinger, 2003). Extranodal forms include mediastinal, abdominal (gastrointestinal, hepatic, splenic, renal), cutaneous, ocular, central nervous system and pulmonary lymphoma. The clinical presentation of cNHL can be further complicated by the presence of paraneoplastic syndromes (Zandvliet, 2016).

1.4.2.1. Multicentric lymphoma

Multicentric lymphoma accounts for ±75% of dogs and is characterized by the presence of generalized superficial lymphadenopathy with splenic, hepatic and/or bone marrow involvement (Marconato et al., 2013; Ponce et al., 2010; Vezzali, Parodi, Marcato, & Bettini, 2010). Most dogs with multicentric lymphoma are asymptomatic; however 20% to 40% of dogs will exhibit nonspecific clinical signs such as anorexia, weight loss, vomiting, diarrhea, emaciation, ascites, dyspnea, polydipsia, polyuria and fever (Ettinger, 2003; Marconato et al., 2013).

Multicentric lymphoma classification defined by WHO comprises five stages (Table 1). Sometimes, lymphoma involves only a single lymph node (stage I) or multiple lymph nodes localized in a region of the body (stage II), however generalized lymphadenopathy (stage III), with secondary involvement of liver and/or spleen (stage IV) or blood and/or bone marrow (stage V) are more frequent (Ettinger, 2003; Zandvliet, 2016). A substage may be used for clinical characterization using the suffix *a* when systemic signs are absent and *b* when signs such as fever, weight loss are present (Zandvliet, 2016).

Table 1 – WHO stages for canine multicentric lymphoma.

Stage	
I	Single node or lymphoid tissue in single organ (except bone marrow)
II	Regional involvement of multiple lymph nodes (±tonsils)
III	Generalized lymph node involvement
IV	Stage I-III with involvement of liver and /spleen
V	Stage I-IV with involvement of blood or bone marrow
Substage	
A	Absence of systemic signs
B	Presence of systemic signs (fever, >10% weight loss, hypercalcemia)

1.4.3. Diagnosis and staging

Diagnosis of cNHL should routinely include a complete physical examination, as well as hematological, clinical chemistry profile (Gavazza, Sacchini, Lubas, Gugliucci, & Valori, 2009) and in some cases urinalysis (Di Bella et al., 2013). Bone marrow involvement is observed in up to 55% of dogs (Raskin & Krehbiel, 1989) and cannot be assessed by peripheral blood counts (Martini, Melzi, Comazzi, & Gelain, 2015). Since bone marrow core biopsies or flow cytometry of bone marrow are not routinely performed, cytological of a single bone marrow aspiration sample remains the proved technique to assess bone marrow involvement (Aubry, Spangler, Schleis, & Smith, 2014). However, bone marrow biopsy is considered an invasive procedure, and the result has limited effect on prognosis (unless there

is massive bone marrow involvement) or treatment, as such it is presently not advised to routinely perform a bone marrow biopsy (Vail et al., 2010).

Thoracic and abdominal radiographs are frequently non-specific and only suggestive of lymphoma as a differential diagnosis (Blackwood, Sullivan, & Lawson, 1997). Nonetheless, thoracic radiographs can reveal abnormal findings such as pulmonary infiltrates and lymphadenopathy in 70% of the cases (Starrak, Berry, Page, Johnson, & Thrall, 1997). Moreover, abdominal ultrasonography can be used to evaluate hepatic and/or splenic involvement and abdominal lymphadenopathy; however computed tomography (CT) scan is ideal to assess the disease extent (Zandvliet, 2016). Recently, it was proposed the use of scintigraphy with a radiolabeled peptide nucleic acid–peptide conjugate targeting Bcl2 mRNA for detection of neoplastic lymphocytes in dogs with B-cell lymphoma. Preliminary results reported advantages of using CT scan in the assessment the disease extent and monitoring treatment response (Statham-Ringen et al., 2012).

A sample collected by fine needle aspiration (FNA) analyzed by an experienced pathologist can often suggest a diagnosis of lymphoma. However, histological examination of an excisional biopsy is the best choice for pathologic evaluation as a diagnostic method, since cytological examination of a FNA may be insufficient to complete the lymphoma characterization. An excisional biopsy (removal of a complete lymph node) is preferred, but in many cases an incisional (thru-cut) biopsy is sufficient. Flow cytometry of fine needle aspirates has demonstrated high potential for lymphoma diagnosis and might reduce the need for excisional biopsies (Comazzi & Gelain, 2011; Gelain, Mazzilli, Riondato, Marconato, & Comazzi, 2008; Zandvliet, 2016).

1.4.4. Classification

Classification of malignant lymphoma in dogs is based on anatomic location, histologic criteria and immunophenotypic characteristics.

1.4.4.1. Anatomic classification

Anatomically, lymphomas can be grouped according to the WHO classification (Owen, 1980), with each group having a characteristic clinical presentation (Table 2).

Table 2 – Anatomical classification of cNHL according to WHO.

Anatomic classification	Clinical Presentation	Incidence	Most common immunophenotype
Multicentric	Bilateral and/or symmetrical peripheral lymphadenopathy; absence or presence of tumor metastasis to liver, spleen, tonsil, and bone marrow.	75-80%	B
Alimentary	Gut-associated lymphoid tissue involvement; single or multifocal/diffuse lesions, often with mesenteric lymphadenopathy.	5-7%	T
Mediastinal	Associated with an increase in craniomediastinal lymph nodes, thymus or both. Hypercalcaemia frequently occur as a paraneoplastic syndrome (10-40% of the cases).	5%	T
Cutaneous	Multifocal or generalized skin involvement, may occur involvement of the oral mucosa and extra-cutaneous involvement of lymph nodes, liver, spleen or bone marrow.	6%	T
Extranodal	Occur in any location outside the lymphatic system inclusing eyes, central nervous system, bone, testes, bladder, heart, and nasal cavity.		

1.4.4.2. Histologic classification

Classifications developed for NHL in humans have been widely adapted and used by veterinary pathologists to classify cNHL. The Rappaport classification (Rapport, H, 1966; Teske, Wisman, Moore, & van Heerde, 1994) was one of the earliest classification systems applied to canine malignant lymphomas. However, with the increasing knowledge of the immunologic aspects of malignant lymphomas, a better understanding of maturation and differentiation of lymphoid cells and the advance of chemotherapy, this system was abandoned after the emergence of better classification systems, such as the Lukes-Collins classification (Lukes & Collins, 1974; Teske, Wisman, et al., 1994) in North America and the Kiel classification (Lennert, & Mohri, 1978; Teske, Wisman, et al., 1994) in Europe (Valli et al., 2010). The Kiel classification was easily adapted for canine malignant lymphoma (Valli, et al., 1981).

Later, in an attempt to unify the European and North American classifications, the National Cancer Institute published the working formulation (Robb-Smith, 1982) - a translational system for the existing hNHL classifications, primarily oriented on the clinical outcome and less on cellular criteria (Valli et al., 2010). In addition, the original Kiel classification was revised, and a modified version (Stein et al., 1981) demonstrated to be applicable to canine

malignant lymphomas (Teske, Wisman, et al., 1994) with multiple studies reporting its prognostic utility (Kiupel, Teske, & Bostock, 1999; Teske, Wisman, et al., 1994). Both systems were widely accepted, and the working formulation became largely used in North America with animal studies based on this system (Carter, Valli, & Lumsden, 1986; Fry, Vernau, Pesavento, Brömel, & Moore, 2003; Greenlee et al., 1990), while the modified Kiel system became extensively used in Europe for both humans and animals (Fournel-Fleury et al., 2002; Teske, Wisman, et al., 1994).

However, both classification systems presented major limitations, demonstrating the need for updating all of the previous systems (Valli et al., 2010). This prompted the foundation of the International Lymphoma Study Group (ILSG), which proposed the REAL classification system (Harris et al., 1994). The distinctive features of the ILSG proposal were that lymphomas were classified for the first time as diseases and not simply as cell types, with the diagnostic criteria including: cellular morphology and cell lineage, topography and general biology of each neoplasia that defined it as a specific disease entity (Valli et al., 2010). The REAL system was generally adopted, with a few exceptions, as the revised WHO system of classification (Harris et al., 1999). The validity of this latter system has been proven in lymphoma and human leukemia by the great consensus among pathologists and has been adapted for cNHL. According to this classification, most common subtype of cNHL are diffuse large B-cell lymphomas (54%) (Valli et al., 2010) (Table 3).

Table 3 – Summary of Canine Malignant Lymphoma Revised From the Revised European-American Classification of Lymphoid Neoplasms/ WHO Classification of Lymphoid Neoplasms.

B Cell Neoplasms
Precursor B cell neoplasms
Precursor B lymphoblastic leukemia/lymphoma
Mature (peripheral) B cell neoplasms
B cell chronic lymphocytic leukemia/prolymphocytic
Leukemia/small lymphocytic lymphoma
B cell prolymphocytic leukemia
Lymphoplasmacytic lymphoma
Splenic marginal zone B cell lymphoma
Plasma cell myeloma/plasmacytoma
Extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue type
Nodal marginal zone lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B cell lymphoma
Mediastinal large B cell lymphoma
Burkitt's lymphoma/Burkitt's cell leukemia
Provisional entity: high-grade B cell lymphoma
Burkitt's-like
Primary effusion lymphoma
T Cell and Putative Natural Killer Cell Neoplasms
Precursor T cell neoplasm
Precursor T lymphoblastic
Lymphoma/leukemia
Mature (peripheral) T cell and natural killer cell neoplasms
T cell prolymphocytic leukemia
Large granular lymphocyte leukemia
Aggressive natural killer (NK) cell leukemia
Peripheral T cell lymphomas, unspecified
Adult T cell lymphoma/leukemia
Intestinal T cell lymphoma (+enteropathy associated)
Hepatosplenic γδT cell lymphoma
Subcutaneous panniculitis-like T cell lymphoma
AMycosis fungoides/Sezary syndrome
dAnaplastic large cell lymphoma, T and null cell primary cutaneous type
aPeripheral T cell lymphoma not otherwise specified
pAngioimmunoblastic T cell lymphoma
AAngiocentric T cell lymphoma
d
Adapted from (Valli et al., 2010).

1.4.4.3. Immunophenotypic classification

Immunophenotyping can be performed on a slide-based cytology (Caniatti, Roccabianca, Scanziani, Paltrinieri, & Moore, 1996) or on a histological section of biopsy samples (Milner, Pearson, Nesbit, & Close, 1996) or using flow cytometry (Culmsee, Simon, Mischke, & Nolte, 2001; Gelain et al., 2008). The most regularly used antibodies include CD20, CD21, CD79α and PAX5 as B-cell markers and CD3, CD4 and CD8 as T-cell markers (Caniatti et

al., 1996). For routine patient management, immunophenotypic analysis using two antibodies (typically CD3 and CD79α or CD20) is suitable, however this method can result in up to 20% unclassified lymphomas (Guija de Arespacochaga, Schwendenwein, & Weissenböck, 2007). More recently the use of the PCR-based clonality assay (PARR), a molecular technique that determines the clonality of a population of lymphoid cells by amplifying the DNA encoding the variable regions of the B cell receptors or T, has been proposed as an alternative for immunohistochemistry or flow cytometry. Flow cytometry proved to be superior over PARR, however in the absence of a fresh sample, PARR is an acceptable alternative (Thalheim, Williams, Borst, Fogle, & Suter, 2013).

Regarding the immunologic phenotype, lymphomas may originate from B cells or cells T. About 60% to 80% of cNHL are from B-cell origin and T-cell lymphomas account for 10% to 38%. There may also be lymphomas considered to be mixed, in which both B-cell and T-cell populations exist, that account for approximately 22% of the cases, or null, in which the cells are neither reactive for B-cell or T-cell markers, representing fewer than 5% of the cases (Fournel-Fleury et al., 2002; Greenlee et al., 1990; Ruslander, Gebhard, Tompkins, Grindem, & Page, 1997; Vail et al., 1996; Wilkerson et al., 2005).

1.4.5. Conventional treatment

Given the systemic nature, cNHL requires multiagent chemotherapy to achieve a temporary remission and prolonged survival (Ettinger, 2003; Withrow et al., 2013). Exceptions include lymphomas restricted to a single lymph node or extra-nodal cases in specific organs, in which a local modality, such as surgery or radiotherapy, may be indicated as the first approach. However, when considering a local modality, the pet owner and clinician must be committed to diligent reevaluation due to the possibility of subsequent systemic involvement (Vail, Young, & Pinkerton, 2007).

Considering that the majority of therapy-related studies are directed towards intermediate to high grade multicentric lymphomas and that little is known about the optimal treatment for low-grade and extranodal forms, this review will focus on the treatment options available for intermediate to high grade multicentric lymphomas.

1.4.5.1. Terminology

Treatment response assessment of dogs with lymphoma relies on specific response criteria (Ettinger, 2003). The standardization of these response criteria allows for more consistent and

reliable comparisons of treatment protocols and outcomes. Recently, the Veterinary Cooperative Oncology Group published an integrated guidance from the response assessment criteria established for lymphoma in human using standards available in routine veterinary oncology practices that are simple, repeatable and consistently applicable. These guidelines are intended only for use in dogs with peripheral lymphadenopathy involvement and is not indicated for extranodal disease. In this document there are presented the general disease response definitions (Table 4) (Vail et al., 2010).

Table 4 - General disease response definitions.

Lesion response	Definition
Complete response (CR)	Target lesions: Disappearance of all evidence of disease. All lymph nodes must be non-pathologic in size in the judgment of the evaluator(s). Non-target lesions: Any pathologic lymph nodes must be considered to have returned to normal size in the judgment of the evaluator(s), and no new sites of disease should be observed. Spleen and liver should be considered within normal limits by the evaluator(s).
Partial response (PR)	Target lesions: At least a 30% decrease in the Mean Sum LD of target lesions taking as reference the baseline mean sum LD. Non-target lesions: Not applicable.[a]
Progressive disease (PD)	Target lesions: At least a 20% increase in the Mean Sum LD taking as reference the smallest mean sum LD at baseline or during follow-up (this includes the baseline mean sum LD if that is the smallest on study). The LD of at least one of the target lesions must demonstrate an absolute increase of at least 5 mm compared with its nadir for PD to be defined. For target lesions <10 mm at nadir, an increase in LD of any single previouslyidentified target lesion to >15 mm. Non target lesions: unequivocal progression of existing non-target lesions, in the judgment of the evaluator. (Note: the appearance of one or more new lesions is also considered progression).
Stable disease (SD)	Target lesions: Neither sufficient decrease to qualify for PR nor sufficient increase to qualify for PD. Non-target lesions: Not applicable.[a]

Adapted from (Vail et al., 2010).

1.4.5.2. Chemotherapy

In the case of lymphoma, the fundamental goals of chemotherapy are to induce a complete durable (>6 months) first remission (termed *induction*), to reinduce a remission when the tumor recurs after achieving remission (termed *reinduction*), and, finally, to promote remissions when the cancer no longer respond to induction or reinduction using the initial protocols (termed *rescue*) (Withrow et al., 2013).

Several protocols have been proposed for the treatment of cNHL, and generally, combination chemotherapy protocols have proved to be more effective compared to single-agent protocols.

In fact, induction therapy with single-agent chemotherapy does not normally result in durable remission as standard combination protocols (Withrow et al., 2013).

1.4.5.2.1. Single agent protocols

The most effective agents used as monotherapy against cNHL are doxorubicin (Hahn et al., 1992; Simon et al., 2008), L-asparaginase (MacEwen, Rosenthal, Matus, Viau, & Abuchowski, 1987; Teske, Rutteman, van Heerde, & Misdorp, 1990), vincristine, cyclophosphamide, and prednisolone (R. Chun, Garrett, & Vail, 2000; Garrett, Thamm, Chun, Dudley, & Vail, 2002; Vail et al., 2007). Owing to their ability to induce apoptosis in lymphocytes (Smith & Cidlowski, 2010), synthetic glucocorticoids are widely used in the treatment of cNHL and may be instituted as a single therapy, when conventional chemotherapy is not available. Although most dogs present a good partial to complete response that normally persists for 60–90 days (Bell, Cotter, Lillquist, Sallan, & McCaffrey, 1984; Squire, Bush, Melby, Neeley, & Yarbrough, 1973), this protocol should be considered a palliative treatment, as its use prior to chemotherapy is associated with tumor resistance, leading to shorter periods of remission and survivals (Gavazza, Lubas, Valori, & Gugliucci, 2008; Marconato et al., 2011; Price, Page, Fischer, Levine, & Gerig, 1991; Teske, van Heerde, et al., 1994; Zandvliet, 2016).

Other drugs that have reported activity are often considered second-line agents and include lomustine (Sauerbrey et al., 2007), vinblastine, actinomycin-D, mitoxantrone (Lucroy, Phillips, Kraegel, Simonson, & Madewell, 1998), mustargen, chlorambucil, methotrexate, dacarbazine, 9-aminocamptothecin, ifosfamide, cytosine arabinoside, and gemcitabine.

The Lomustine® (CCNU) can be used as a single agent in the treatment of particular cases, in which the owners seek for a simple protocol but are willing to accept the shorter survival times. CCNU is most commonly used as a rescue therapy for multicentric lymphoma and in the treatment of cutaneous epithelial lymphoma (Moore et al., 1999; Risbon et al., 2006).

Of all of these protocols, doxorubicin as a monotherapy, either as a continuous or an intermittent protocol, appears to be the most effective, however its efficacy is still inferior compared to a doxorubicin-based multi-agent protocol and this alternative should be reserved for treatments with a palliative intent (Higginbotham et al., 2013).

1.4.5.2.2. Combination agent protocols

The selection of combination chemotherapy regimens for cancer treatment aims to delay as much as possible the onset of multidrug resistance (MDR), by using drugs with different mechanisms of action. As such, when designing a combination chemotherapy protocol, each drug in a combination should be effective as a single agent, should not share resistance mechanisms, and ideally, should not result in overlapping toxicities (Saba, Hafeman, Vail, & Thamm, 2009).

COP protocol

The COP protocol consists of a combination of cyclophosphamide (C), vincristine (O - Oncovin) and prednisolone (P). Despite being a well-tolerated and relatively inexpensive, the long-term treatment aspect of this protocol limits its use. This protocol includes a high and low dose dosage and presents remission rates of 60 to 70%, with a median survival of 6 to 7 months (Argyle, Brearley, & Turek, 2008).

CHOP protocol

Presently, the CHOP based protocols represent the most successful therapies, yielding temporary remission (9–11 months) in approximately 85% of dogs (Meuten, 2008; Valerius, Ogilvie, Mallinckrodt, & Getzy, 1997; Withrow et al., 2013). CHOP refers typically to injection-protocols that combine Cyclophosphamide, vincristine (Oncovin), Prednisolone (COP) with doxorubicin (Hydroxydaunorubicin) (CHOP) and/or L-asparaginase (L-CHOP) (Zandvliet, 2016) (Table 5). In fact, just recently, a study has demonstrated that doxorubicin and vincristine strongly decreased the viability of cNHL cell lines, whereas cyclophosphamide induced the highest level of apoptosis (Pawlak, Rapak, Zbyryt, & Obmińska-Mrukowicz, 2014). By incorporating doxorubicin, the CHOP protocol and its derivatives resulted in the highest response rate and longest response durations and form the basis for most currently used treatment protocols for high-grade cNHL. Early proposed protocols consisted of two phases - an intensive induction phase using an intensive protocol that aimed at inducing complete remission, and a following maintenance phase using a less intensive protocol that aimed at maintaining the disease in remission (maintenance phase). It was later shown that a continuous maintenance phase following successful induction, offered no treatment benefit and decreased the probability of attaining a second remission after

relapse (Chun et al., 2000; Lautscham, Kessler, Ernst, Willimzig, & Neiger, 2017), as such the therapy duration was reduced. Although a 6 month protocol, such as the modified protocol of the University of Wisconsin of 25 weeks, is considered the standard of care (Chun et al., 2000; Garrett et al., 2002), shorter protocols (12-week (Simon et al., 2006) and 15-week (Burton, Garrett-Mayer, & Thamm, 2013)) have been reported and have shown similar effectiveness.

A high-dose CHOP protocol has been fairly successful in improving the duration of first remission (Chun et al., 2000). However, toxicity has caused treatment delays or discontinuation in more than 44% of cases, leading to its abandonment. Finally, the association of other cytotoxic drugs with single-agent activity with CHOP-based protocols has not demonstrated significant advantages and most are reserved for subsequent rescue therapy purposes (Withrow et al., 2013).

Table 5 - University of Wisconsin–Madison: 19-week combination chemotherapy protocol for dogs with lymphoma.

Week	Drug/Dosage/Route
1	Vincristine: 0.5-0.7 mg/m^2 IV; Prednisone: 2 mg/Kg PO
2	Ciclofosfamide: 250 mg/m^2 IV* ; Prednisone: 1.5 mg/Kg PO
3	Vincristine: 0.5-0.7 mg/m^2 IV; Prednisone: 1 mg/Kg PO
4	Doxorubicin: 30 mg/m^2 IV; Prednisone: 0.5 mg/Kg PO
5	No treatment
6	Vincristine: 0.5-0.7 mg/m^2 IV
7	Ciclofosfamide: 250 mg/m^2 IV*
8	Vincristine: 0.5-0.7 mg/m^2 IV
9	Doxorrubicin: 30 mg/m^2 IV
10	No treatment
11	Vincristine: 0.5-0.7 mg/m^2 IV
12	Ciclofosfamide: 250 mg/m^2 IV*
13	Vincristine: 0.5-0.7 mg/m^2 IV
14	Doxorrubicin: 30 mg/m^2 IV
15	No treatment
16	Vincristine: 0.5-0.7 mg/m^2 IV
17	Ciclofosfamide: 250 mg/m^2 IV*
18	Vincristine: 0.5-0.7 mg/m^2 IV
19	Doxorrubicin: 30 mg/m^2 IV

If the patient is in complete remission at week 9, treatment continues to week 11. If the patient is in complete remission at week 19, therapy is discontinued and the dog is rechecked monthly for recurrence. A complete blood count should be performed before each chemotherapy treatment—if the neutrophil count is less than 1500 cells/mcL, the clinician should wait 5–7 days and then repeat the count; the drug is administered if the neutrophil count has risen above the 1500 cells/mcL cutoff. * Furosemide is given concurrently with cyclophosphamide to decrease the incidence of sterile hemorrhagic cystitis

1.4.5.2.3. Chemotherapy side effects

Generally, chemotherapeutic regimens designed for pets are well tolerated, presenting less than 5% incidence of severe, life-threatening complications. However, by possessing the lowest therapeutic indices among the different classes of drugs, these agents frequently cause predictable multisystemic toxicities, such as myelosuppression, gastrointestinal disturbances and alopecia (particularly in breeds with continually growing hair coats) (MacDonald, 2009).
Most dogs will experience minimal side effects when treated with appropriate doses of chemotherapy and close monitored. However, depending on the side effect severity, some may require supportive care or a 20% reduction of drug dosage if gastrointestinal side effects or severe neutropenia occur. In addition to common chemotherapy side effects, drugs have varying capacities for unique toxicities including cardiotoxicity (doxorubicin), hepatotoxicity (lomustine), sterile hemorrhagic cystitis (cyclophosphamide), paralytic ileus (vincristine) and hypersensitivity reactions (doxorubicin and L-asparaginase). Lastly, chemotherapy drugs, such as doxorubicin and vincristine, are also potent vesicants (blistering agents) and can produce extensive and severe tissue injury if they leak out of the blood vessels (MacDonald, 2009).
It is important to mention that this relatively good tolerability to chemotherapeutic protocols in the treatment of dogs with lymphoma is reported using low dose intense protocols. Actually, considering the high response rates, it was reasoned that a dose intensification could improve remission and survival rates in dogs with lymphoma. However, the incidence of toxicity was high – 53,9% and 45% of the dogs needed dose reductions and treatment delays, respectively. In addition, 11% of the dogs in this study were hospitalized because of serious acute toxicity. As such, despite the lack of efficacy of the current chemotherapy protocols, it is unlikely that significant improvements can be attained through dose intensifications without increasing the incidence of serious toxicity or death (Sorenmo et al., 2010).

1.4.5.2.4. Rescue protocols

Rescue protocols are indicated when the patient fails to achieve a durable remission in the induction phase of the first-line protocol or relapse following treatment, and include both single-agent and multi-agent protocols. The treatment protocol choice is based on the timing of relapse in relation to the original (first-line) protocol, previously used drugs (e.g. cumulative cardiac toxicity of doxorubicin), and individual clinician's preferences. Regarding the moment of relapse, its occurrence during the first-line protocol typically requires the use

of alternative drugs (meaning drugs not included in the first protocol), while a relapse following completion of the first-line protocol allows to include drugs used in the original protocol (Zandvliet, 2016). Several alternative combination chemotherapeutic rescue protocols have been used to improve survival in dogs with refractory disease, including lomustine, L-asparginase, CCNU, DTIC (Dacarbazine), MOPP (methchlorathamine, vincristine, procarbazine, and prednisone), doxil, and prednisolone alone. Still, these protocols have mostly proven unsuccessful in significantly increasing survival beyond 2 to 3 months, and tend to show more toxicity than first-line protocols (Back et al., 2015; Fahey et al., 2011; Flory et al., 2008; Zandvliet, Rutteman, & Teske, 2013).

1.4.5.3. Radiotherapy

Even though lymphoid cells are sensitive to radiation, radiotherapy represents a second line treatment option for cNHL due to the systemic character of lymphoma (Zandvliet, 2016). In fact, radiotherapy as a monotherapy for multicentric cNHL (2x half-body irradiation, 7 Gy, 4 weeks apart), demonstrated scanty results with objective response in 5/14 dogs of complete and partial response median duration of 102 and 54 days respectively. Moreover, side effects were frequent and particularly serious in dogs with an advanced stage of disease (Laing et al., 1989). Therefore, radiotherapy is more often used in combination with chemotherapy, as an adjuvant and consists of irradiating the whole body, either in a single session of total body irradiation (TBI) or two sessions of half body irradiation (HBI). TBI is associated with severe and potentially fatal, bone marrow depression, which requires autologous bone marrow or peripheral blood stem cell transplantation. Although bone marrow transplantation was already implemented in the dog (Weiden, Storb, Deeg, & Graham, 1979) with promising preliminary results, morbidity and mortality were high (Appelbaum et al., 1986; Deeg et al., 1985). Even though, current work revealed less treatment-related morbidity and mortality (Willcox, Pruitt, & Suter, 2012), TBI has been progressively substituted by two HBI sessions . HBI with a high or low-dose rate has been assessed following conclusion of or within chemotherapy protocol. On one hand, a regular dose-rate HBI - 2x 8 Gy in 2 days, 3-4 weeks apart - performed during or after conclusion of a CHOP chemotherapy protocol lead to a moderate increase in first (median 311 respectively 455 days) and overall (median 486 days respectively 560 days) remission period (Gustafson, Lana, Mayer, & LaRue, 2004; L. Williams et al., 2004). On the other hand, a low-dose rate HBI - 10 cGy/min; 2x 6 Gy, 2 weeks apart – combined with a CHOP-based chemotherapy protocol resulted in lasting remission (410-455 days) and survival (560-684 days) times with tolerable toxicity (Lurie et al., 2009).

1.4.6. Prognosis

Without treatment, cNHL is highly fatal, resulting in death within 4 to 6 weeks (Marconato et al., 2013). For the most common form of intermediate to high grade lymphoma (DLBCL), treatment with the CHOP chemotherapy protocol typically results in a complete remission in up to 95% of patients. Unfortunately, most dogs that achieve remission will eventually relapse or present recrudescence of lymphoma within 6 to 9 months of diagnosis and treatment, resulting from the emergence of MDR clones - tumor clones or tumor stem cells that are naturally more resistant to chemotherapy or that become resistant following exposure to some chosen chemotherapy drugs. The median survival time following treatment with CHOP is approximately 12 months. Dogs that experience the most significant negative prognostic factors, such as stage IV or V, substage b, T-cell immunophenotype, presence of a mediastinal mass, anemia, prolonged pretreatment with corticosteroids and gastrointestinal involvement, are less likely to achieve a complete remission and more likely to have a shorter remission. Indolent lymphomas are associated with longer survival times because of slow progression of disease but are considered less chemoresponsive (Vail et al., 2007).

1.4.7. Future goals and challenges

Even though our understanding on the genetics, molecular biology, and diagnosis of cNHL has increased substantially over the past 25 years, this has had little effect on treatment and has only slightly improved prognosis. Chemotherapy still remains the mainstay for the treatment of cNHL and regardless of the numerous published chemotherapeutic protocols, it seems we have reached a stalemate concerning what this treatment modality has to offer in standard settings (Zandvliet, 2016). The 12 month median survival barrier and the 20% to 25% 2 years survival rates demonstrate an urgent and unmet need in veterinary medicine to develop new treatment strategies for refractory disease (Back et al., 2015; Fahey et al., 2011; Flory et al., 2008; Zandvliet et al., 2013). Further advances in remission and survival times await the development of new methods of delivering or targeting traditional chemotherapeutic drugs, new generation of chemotherapeutic drugs, or novel nonchemotherapeutic treatment modalities. Mechanisms of avoiding or eliminating MDR, enhancing tumor apoptosis, tumor ablation, and immune-system reconstitution, as well as novel immunomodulatory therapies for lymphoma, are all promising areas of research in both human and veterinary medicine.

1.4.8. Immunotherapy

After decades of weakening or even eliminating the patient's immune system with chemotherapy, now the trend is to harness the ability of the immune system to eradicate cancer (Klingemann, 2018). Work from the last two decades has finally brought immunotherapy into the forefront of cancer treatment, with demonstrable clinical success for aggressive tumors where other therapies had failed (Anderson & Modiano, 2015). The field of veterinary immunotherapy holds similar promise for companion animals with cancer, and several efforts have been made in order to develop veterinary specific immunotherapies. In the nearby future, it is hoped that tumor immunotherapy will become a valid therapeutic tool in veterinary oncology, along with chemotherapy, radiotherapy and surgery (Zandvliet, 2016). Both passive and active modalities have been used to generate therapeutic anti-tumor immune responses in cNHL. Passive immunotherapy involves the transfer of biological reagents, such as mAbs or antigen-specific adaptive immune cells, into the cancer patient. Whereas, active immunotherapy seeks to elicit an anti-tumor response from the patient's own immune system, typically through vaccination (Mellman, Coukos, & Dranoff, 2011).

1.4.8.1. Passive immunotherapy: Antibodies

The use of a mAbs targeting the human surface antigen CD20 (Rituximab®), expressed on B-lymphocytes has revolutionized the treatment of B-cell lymphoma by significantly improving disease-free interval and overall survival, with minimal toxicity (Ito et al., 2015; Motta et al., 2010). Rituximab is a chimeric antibody and was the first US Food and Drug Administration (FDA) approved mAb for the treatment of human cancer, being used for the treatment of most B-cell NHL and subtypes of acute lymphocytic leukemias (Chames, Van Regenmortel, Weiss, & Baty, 2009; Dimitrov & Marks, 2009; Waldmann, 2003). This immunotherapy provided significant enhancements in the efficacy of treatment versus existing non-mAb therapies, increasing the rate of durable remissions from 30% to 60% (Ito et al., 2015). As a result, since the approval of rituximab in 1997, multiple antibody-based therapies have been developed for human lymphoma .

Although immunotherapy plays a major role in the treatment of many human B-cell malignancies, its role in cNHL is still limited. Whereas immunohistochemistry demonstrated the presence of CD20 in cNHL tissue samples (Jubala et al., 2005; Kano et al., 2005), Rituximab® failed to bind (Impellizeri, Howell, McKeever, & Crow, 2006). Interestingly, in 1992, prior to FDA approval of Rituximab, the United States Department of Agriculture (USDA) approved the licensing of MAb 231 for use in cNHL. MAb 231 consists of a murine-

derived mAb that showed both *in vitro* (Rosales, Jeglum, Obrocka, & Steplewski, 1988) and *in vivo* activity and served as adjuvant therapy following remission induction with chemotherapy (Jeglum, 2009).

A pilot study of anti-human leukocyte antigen (HLA)-DR mAbs in dogs with lymphoma was undertaken to verify the suitability of a canine model to address therapeutically relevant endpoints prior to a full trial in dogs, and ultimately human investigation. *In vitro* studies demonstrated that L243, a murine IgG1 anti-HLA-DR, binds to normal and malignant canine lymphocytes and induces apoptosis in cNHL cells. Moreover, L243 was administered safely to normal dogs and dogs with lymphoma, and bound to malignant cells in nodal tissue. Preliminary evidence of transient disease stabilization was observed in a subset of dogs with advanced-stage lymphoma following L243 immunotherapy. hL243γ4P (IMMU-114), a humanized IgG4 anti-HLA-DR, currently under evaluation preclinically for human trials, also shown to bind malignant canine lymphocytes, and safety and pharmacokinetic data from the administration of IMMU-114 to normal dogs indicated similar behavior to L243 in these assessments. These findings provide a rationale for the use of dogs with lymphoma in safety and efficacy evaluations of anti-HLA-DR mAbs for both veterinary and human applications (Stein et al., 2011).

Considering the success achieved with Rituximab in human medicine, several studies focused on developing canine anti-CD20 antibodies. Interestingly, although the proposed rituximab binding epitopes are conserved between human and canine CD20, the canine mAb does not induce direct apoptosis of tumor cells. Studying the differences in the mechanism of action between human and canine anti-CD20 mAbs may yield insights to improve the engineering of mAbs. Despite these differences, each of the canine anti-CD20 mAbs has been shown to have diagnostic or clinical potential (Anderson & Modiano, 2015; Ito et al., 2015).

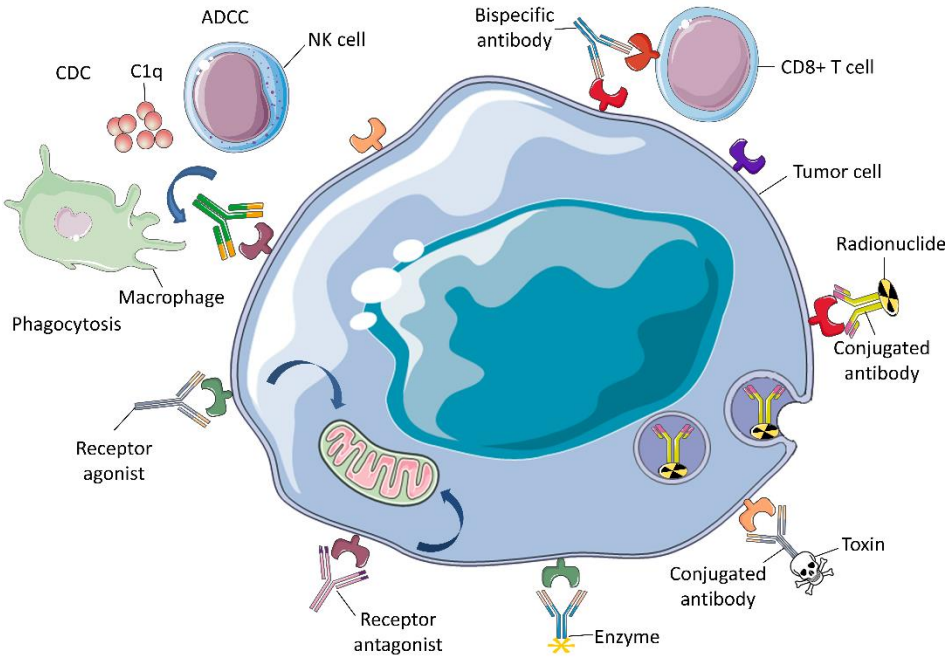
A novel anti-canine CD20 mAb (6C8) that recognized the extracellular domain of canine CD20 and showed high-affinity binding to canine CD20 in solution and its native conformation on canine B-cells was developed. This mAb promoted phagocytosis of B-cell lymphoma cells by macrophages, but in its current framework did not induce direct cytotoxicity or complement dependent cytotoxicity (Ito et al., 2015). Likewise, a new anti-CD20 mAb (NCD1.2) that binds both human and canine CD20 has been developed, in order to strengthen human-canine comparative model. NCD1.2 binds to clinically derived canine cells including B-cells in peripheral blood and in different histotypes of B-cell lymphoma. V_H and V_L genes from the NCD1.2 hybridomas were cloned and packaged as single chains (scFv) into a phage-display library Recombinant anti-CD20 scFv were identified and selected as a possible useful tool for evaluation in bioconjugate-directed anti-CD20 immunotherapies in

comparative medicine (Jain et al., 2016). Moreover, a canine anti-CD20 mAb has been fully approved by USDA for clinical usage in dogs with B-cell Lymphoma and is currently being commercialized in the United States and Canada. Treatment with AT-004 anti-canine CD20 was subject to a prospective randomized clinical trial and preliminary results suggest an improved median progression-free survival of dogs with B cell lymphoma (Killick, Stell, & Catchpole, 2015). Futhermore, another mAb, anti-CD47, has proved to increase the innate anti-tumor immune response of numerous human leukemias and lymphomas xenograft models in both *in vitro* and *in vivo* settings (Chao et al., 2010; Majeti et al., 2009). Preliminary results propose that anti-CD47 may also present these therapeutic effects on the canine B-cell lymphoma field (Anderson & Modiano, 2015). In addition, AT-005 (Aratana Therapeutics, Del Mar, CA, USA), a speciated mAb targeting CD52 on T cells, has received conditional USDA approval for the treatment of T cell lymphoma and is currently being evaluated in clinical trials (Klingemann, 2018).

The development of technology to speciate antibodies has led to a variety of clinical trials in companion dogs since "caninization" of antibodies is crucial when approaching canine patients with cancer. With this goal in mind, work groups have already presented the technique to generate "caninized" antibodies, namely, a canine anti-EGFR IgG and nowadays is also a service offered by companies (Singer et al., 2014).

The success of mAbs in human medicine strongly encourages veterinary medicine to develop similar therapeutics for our pets. Despite their potential, little speciated antibodies have been established for veterinary use or fewer were tested in veterinary clinical trials. mAbs have the capacity to treat a diversity of hematopoietic and solid tumors, do not need to be a personalized product and manufacturing techniques are well established, so that cost is not a major obstacle. Therefore, mAb therapy represents one of the most promising immunotherapy strategies in veterinary settings (Anderson & Modiano, 2015) (Figure 5).

Figure 5 – Antibody therapeutics for cancer.



Targeting mAbs to the tumor can result in the destruction of the tumor cells by antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity. Similarly, targeting cytokines or immunomodulatory molecules either by bispecific scFv or antibody–ligand fusion proteins to the tumor modulates the immune response against the tumor. In addition, antibody–ligand fusion proteins can induce apoptosis to targeted cells as well as bystander cells by, for example, presenting FasL. A more direct approach to kill the targeted cell is the conjugation of cytotoxic drugs, toxins or radionucleotides to the mAbs. The direct targeting approach requires the homogenous expression of antigen in the tumour cell population; depending on the radionucleotides used, however, radioimmunoconjugates can exert bystander effects and kill surrounding cells which do not express the antigen. The antibody-directed enzyme prodrug therapy approach specifically aims at causing bystander effects by targeting enzymes to the tumor cell and delivering a prodrug that is locally converted to a chemotherapeutic agent by the targeted enzyme.

1.4.8.2. Passive immunotherapy: Adoptive T-cell transfer

There is significant evidence suggesting the efficacy of cell-based therapies, such as, allogeneic transplantation, donor-lymphocyte infusion, and therapies using *ex vivo* expanded T and NK cells, such as lymphokine-activated killer cells, cytokine-induced killer cells, or CD3/CD2843 –expanded cells (Brody, Kohrt, Marabelle, & Levy, 2011).

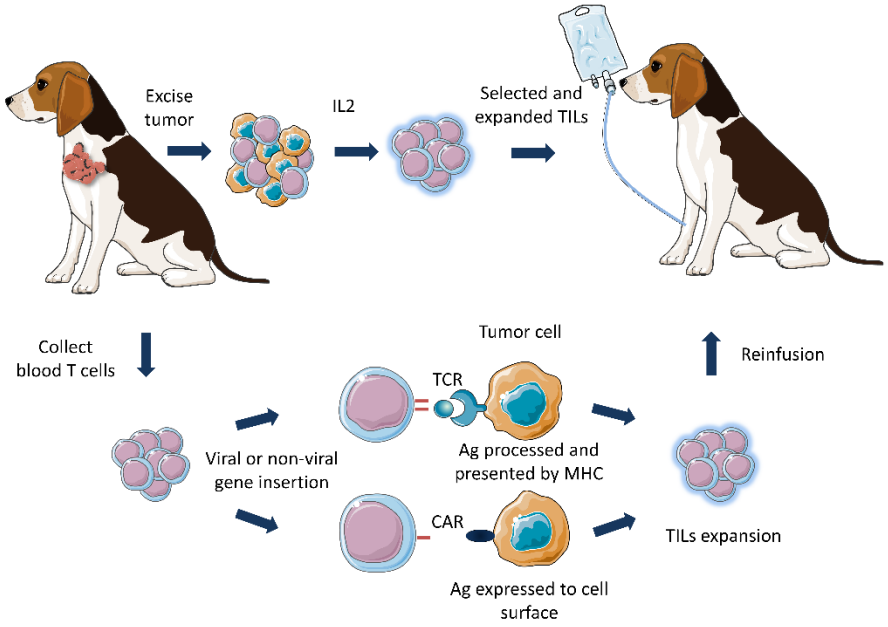
Adoptive cell therapy (ACT) consists of a treatment that uses a cancer patient's own T lymphocytes with anti-tumor activity, expanded *in vitro* and reinfused into the patient (Figure 6). This immunotherapeutic option represents the most effective treatment for patients with metastatic melanoma inducing visible cancer regression in approximately 50% of patients. ACT is also associated with clinical improvement in selected patients with post-transplant lymphoproliferative diseases caused by Epstein–Barr virus infection. Moreover, recent studies have reported that normal lymphocytes can be converted into lymphocytes with anti-cancer

activity through genetic modification caused by retroviruses that encode T-cell receptors. These data represented a possibility of extending ACT immunotherapy to patients with a large diversity of cancer types (Rosenberg, Restifo, Yang, Morgan, & Dudley, 2008).

As dogs possess a fully intact immune system and genetic similarity to humans, tumor-infiltrating lymphocytes (TILs) therapy in dogs has the potential to inform human trials and investigate the use of this therapy in other cancers, such as hematopoietic malignancies. In a recent clinical trial by O'Connor et al., non-specific, autologous T cells isolated from dogs with NHL were propagated *ex vivo* using a novel artificial antigen presenting cell system prior to reinfusion (O'Connor et al., 2012; O'Connor & Wilson-Robles, 2014a). The infused T cells persisted for greater than 49 days and trafficked to secondary lymphoid organs, demonstrating that the adoptive transfer of autologous T cells is safe in canine patients. Adoptive immunotherapy using non-specific autologous T-cells was demonstrated to be viable and effective in improving first remission and overall survival periods in dogs with multicentric lymphoma (Gavazza et al., 2013).

TIL efficacy is based on T cells capacity to identify tumor antigens, consequently this therapy reports poor outcome when treating solid tumors with a low mutation character. Therefore, gene-transfer methods were established to apply artificial anti-tumor receptors into normal T cells collected from patient's peripheral blood. In order to identify known tumor antigens expressed in a MHC (Major histocompatibility complex) molecule setting, TCR (T cell receptor) engineered T cells are produced expressing one α and one β chain (Rosenberg & Restifo, 2015). Chimeric antigen receptors (CARs) consist of artificial receptors formed by a tumor-antigen specific single-chain antibody variable fragment linked to an intracellular signalling domain and co-stimulatory molecules. CARSs don't depend on patient APCs (Antigen presenting cell) to present antigen and do not require to be syngeneic to patient's immune system, because they are non-MHC class limited molecules (Anderson & Modiano, 2015). Canine T cells expressing a HER2 (Human epidermal growth factor receptor 2)-specific CAR have been produced and showed anti-tumor activity in vitro targeting HER2+ canine osteosarcoma cells (Mata et al., 2014). Ongoing studies aim to develop canine CARs against B cell lymphoma and other malignancies (Anderson & Modiano, 2015).

Figure 6 - Approaches to adoptive T cell therapy.



In the top scheme, a resected tumor sample is digested into a single cell suspension and cultured in the presence of IL-2 to select for naturally occurring tumor-infiltrating lymphocytes (TILs). The TILs are then expanded, tested for anti-tumor activity, and reinfused into the patient. In the bottom scheme, autologous T cells are harvested from the blood and either a transgenic TCR or a chimeric antigen receptor (CAR) is introduced by viral or non-viral transduction. TCRs are capable of recognizing a specific tumor antigen presented in the context of an MHC molecule. CARs are MHC-independent and capable of directly recognizing an antigen on the tumor cell surface. Following transduction, the transgenic T cells are expanded and reinfused.

1.4.8.3. Passive immunotherapy: Immunomodulators

Cytokine therapy aims to enhance immune responses and tumor control in a variety of spontaneous oncologic diseases. In human medicine, modest success has been obtained with a low-dose IL-2 therapy delivered subcutaneously, with few side effects (Atzpodien & Kirchner, 1991; Burns et al., 2000, 2003; Dudek et al., 2008; Lissoni et al., 1994; Miller et al., 1997) Additionally, subcutaneous GM-CSF (Granulocyte-macrophage colony-stimulating factor) therapy boosts cell-mediated immune responses and improves anti-idiotype vaccines efficacy in human lymphoma (Bendandi et al., 1999). In canine patients, IL-2 delivered subcutaneously, intralesionally, by inhalation and via liposome–DNA complexes encoding IL-2 gene, as a monotherapy or in combination with other modalities, promoted regression in dogs with oral melanoma, soft tissue sarcoma, squamous cell carcinoma and pulmonary metastases from osteosarcoma (Dow et al., 1998, 2005; Khanna et al., 1997; Moore, Theilen, Newell, Madewell, & Rudolf, 1991; Otter et al., 1999; Thamm et al., 2003). Likewise, in dogs with oral melanoma, combination therapy including GM-CSF delivered intralesionally, either

via liposome–DNA complexes or via GM-CSF secreting transgenic xenogeneic cells, resulted in regression (Dow et al., 1998; Finocchiaro, Fiszman, Karara, & Glikin, 2008).

A phase I study enrolling 15 dogs with B-cell lymphoma tested a therapy with a combination of autologous tumour antigen-coated microbeads (large multivalent immunogen - LMI) with cytokine therapy including IL-2 and GM-CSF, following induction of remission with conventional chemotherapy. Results demonstrated no significant toxicity, no adverse effects in disease-free interval and half of the animals presented quantifiable delayed-type hypersensitivity reactions to intradermal LMI, suggestive of a specific cell-mediated immune response (Henson et al., 2011).

1.4.8.4. Active immunotherapy: Vaccines

Several attempts to use vaccines as a treatment for cNHL have been made (Figure 7) (Zandvliet, 2016). In the initial studies, Freund's adjuvant was added to lymphoma cell extracts lysates. Despite de fact that these early studies reported some treatment benefit (Crow et al., 1977), this was later atributted to the use of the Freund's adjuvant (Re et al., 1980).

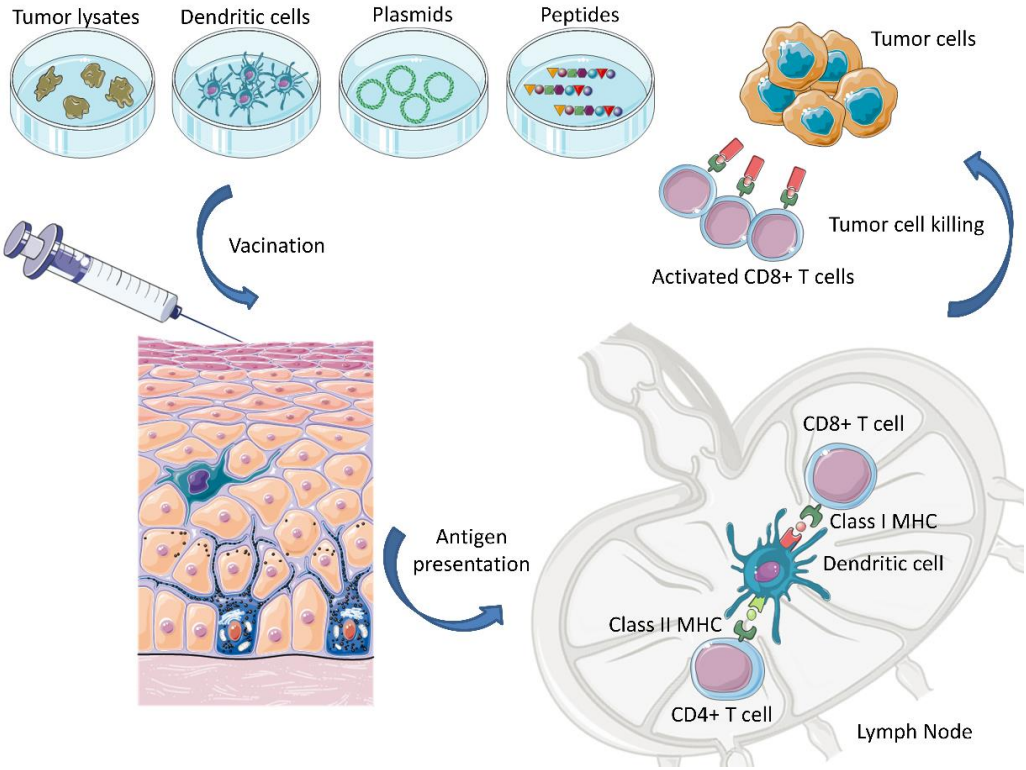
Jeglum *et al.* described an intralymphatic administration of an autologous tumor vaccine following induction of remission with chemotherapy thus results were conflicting (Jeglum, Winters, & Young, 1989; Jeglum, Young, Barnsley, & Whereat, 1988; Jeglum et al., 1986).

Recently, an autologous vaccine consisting of hydroxylapatite ceramic powder with autologous heat shock proteins (HSP) purified from affected lymph node biopsy is currently available. On one hand, HSPs resultant from tumor cells, including gp96, hsp90, hsp70, calreticulin, hsp110, and hsp170, present strong immunogenicity (Marconato et al., 2014). The chaperone function of HSPs allows their combination with immunogenic tumor specific peptides (HSPPC), exposing the host to a large repertoire of tumor associated antigens (TAA) for immunization. On the other hand, hydroxylapatite vehicles and HSPPCs functions as an adjuvant. In order to reproduce the tumor heterogeneity, each vaccine is produced for each dog. Vaccination protocol consists of four administrations within four weeks and one injection a month for four months in combination with dose-intense chemotherapy. Preliminary results showed that the autologous vaccine is effective in prolonging overall survival and the time to progression in dogs with DLBCL and multicentric indolent B-cell neoplasia, without increasing treatment toxicity (Marconato et al., 2014, 2015).

In a recent clinical trial, autologous CD40-activated B-cells loaded with total RNA from autologous lymphoma cells were administered to 19 dogs with NHL as an adjuvant, following induction of a complete response with chemotherapy. Vaccination stimulated an anti-tumor

immune response and increased a lasting second remission rate, although median time to disease progression and overall survival did not differ between groups (Sorenmo et al., 2011). Moreover, a new approach targeting canine telomerase reverse transcriptase via a DNA-vaccine is reported. As telomerase confers immortality to cells, telomerase reverse is overexpressed in cancer cell lines and in several tumors and undetectable in the majority of normal tissues, establishing a possible target for translational cancer immunotherapy. A DNA-vaccine targeting canine telomerase reverse transcriptase can induce an immune response against telomerase in dogs with multicentric lymphoma, and conventional chemotherapy seems not to alter the immunotherapy effects (Peruzzi et al., 2010). The combination of this vaccine and chemotherapy (COP-protocol) resulted in a durable immune response, as well as prolonged survival in dogs with B-cell lymphoma (Gavazza et al., 2013).

Figure 7 - Vaccination strategies and challenges.



Vaccines utilize a variety of strategies to activate the immune system against tumor associated antigens (TAAs), including tumor cell lysates or peptide antigens, dendritic cells (DCs) activated with TAAs, and DNA plasmids designed to produce TAAs. The TAAs must then be presented by functional antigen presenting cells to T cells capable of recognizing the TAA. Once activated, T cells must travel to the tumor and induce tumor cell death. T cell tolerance to TAAs, dysfunctional antigen presentation, T-cell exhaustion induced by checkpoint inhibitors (such as PD-1), and immunosuppressive cells in the tumor microenvironment may all result in suppression of the immune response and variable patient responses to vaccination.

1.4.8.5. Active immunotherapy: Checkpoint blockage

There are several obstacles to the effective immunotherapy of cancer that may limit the success of treatment of cancer patients with minimal disease or may promote merely transient tumor responses. Some of these complications are tumor cell related, though the major result from a series of negative immunoregulatory elements (i.e. Tregs, myeloid-derived suppressor cells, tumor-associated macrophages, vascular endothelial growth factor, TGFβ, IL-10) that are normally responsible for the prevention of self-reactive destructive immune responses that lead to autoimmune disease (Guo et al., 2013; Pardoll, 2012).

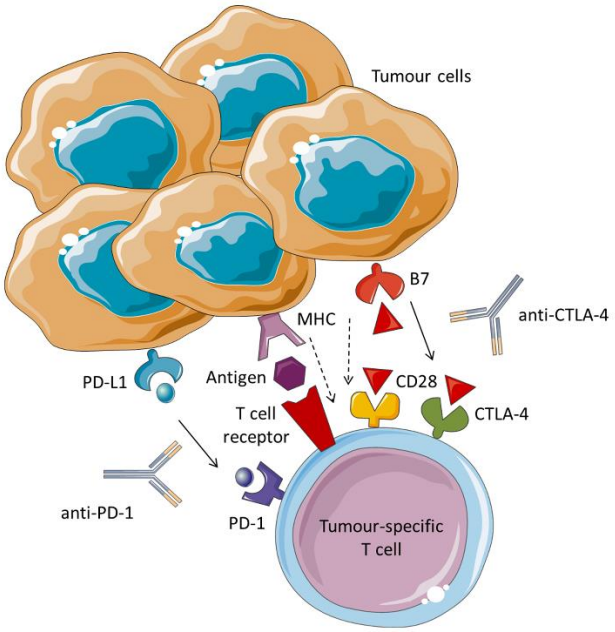
These immunoregulatory mechanisms correlate with poor clinical success in cancer patients and limit the outcome of vaccines in preclinical models. Therefore, a focus of recent preclinical tumor immunology has been to study various types of checkpoint blockade, either to improve vaccine-induced immunity or to reveal an underlying antitumor immune response. One approach is the use of mAbs directed to these checkpoints, and there is considerable preclinical evidence demonstrating the effect of agonistic antibodies specific for 4-1BB and OX4 mAbs or antagonistic mAbs specific for co-inhibitory receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed-death 1 (PD-1) (Armand, 2015; Berzofsky et al., 2004; Pardoll, 2012).

Immune checkpoint inhibitors mAbs therapy, such as antibodies against CTLA-4 and PD-1 mAbs, ligand PD-L1, have attracted notable attention due to reports in human phase-III clinical trials of inducing clinical responses in 20%–65% of patients with a diversity of tumors; a small subclass of patients have achieved complete and long-lasting remissions (Anderson & Modiano, 2015; Sharma & Allison, 2015). Briefly, binding of checkpoint receptors on the surface of T cells by their associated ligands (B7-1 and B7-2 for CTLA-4, PD ligand 1 [PD-L1] and PD-L2 for PD-1) promotes downregulation of T-cell function. Therefore, these molecules limit tumor´s ability to promote anergy or fatigue of activated T cells, by reactivating tumor-specific T cells (Figure 8) (Anderson & Modiano, 2015).

Canine clinical trials with checkpoint inhibitors have yet to be conducted. Nevertheless, expression of canine PD-L1 has been reported on a variety of canine tumor types, including mastocytoma, melanoma and renal cell carcinoma (Maekawa et al., 2014). A preliminary study suggests that anti-PD-L1 might play a significant role in the treatment of dogs with tumors expressing PD-L1, by demonstrating that treatment of canine tumor infiltrating lymphocytes with this molecule improved IFN-γ production (Maekawa et al., 2014). Moreover, canine CTLA-4 has also been described and cloned (Shin et al., 2007) Even though anti-CTLA-4 has not yet been established, an agonistic recombinant canine CTLA has been

efficiently used to promote tolerance in a transplant model (Graves et al., 2009), suggesting that the mechanism of action of CTLA-4 in dogs is similar to humans and that CTLA-4 checkpoint blockage could represent a novel immunotherapy for canine cancer. Hematologic malignancies such as lymphoma are likely targets for this type of treatment. Several clinical trials of checkpoint blockade have been performed in HM, with promising preliminary results suggesting the therapeutic benefit of this approach, namely results of PD-1 blockade in Hodgkin lymphoma (HL) (Armand, 2015).

Figure 8 - Mechanism of action of immune checkpoint inhibitors.



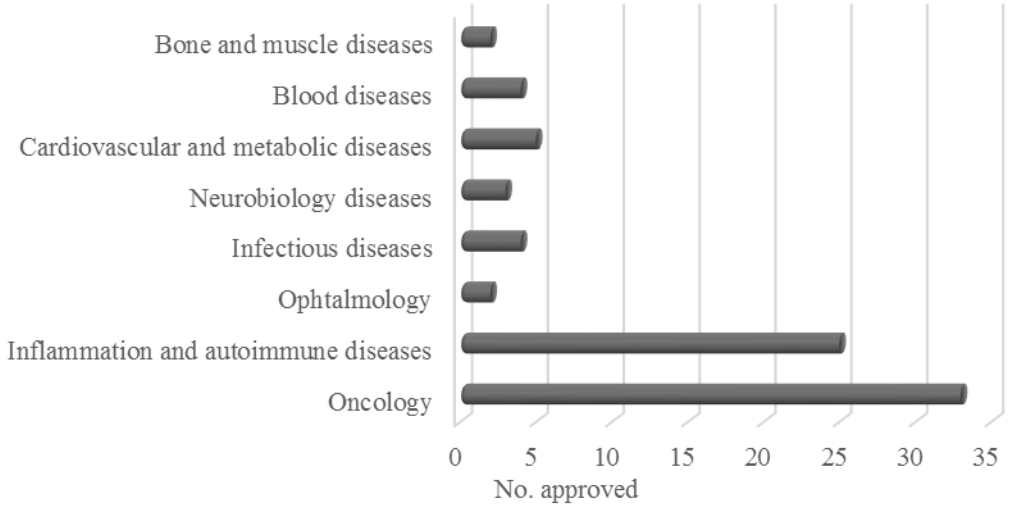
PD-1 is expressed on activated T cells and when it binds to its ligand PD-L1 on tumor cells leads to T cell exhaustion. CTLA-4 competes with CD28 (costimulatory T cell molecule) for B7 ligands (CD80 and CD86 that are not shown in the figure) and upon activation decreases T cell proliferation as well as activity. Blockade of CTLA-4 (by anti-CTLA-4) and PD-1 (anti-PD-1) or PD-L1 stimulates effector T cells to produce antitumor responses. PD-1: programmed death-1, PD-L1: programmed death-ligand 1, MHC: major histocompatibility complex, TCR: T cell receptor, and CTLA-4: cytotoxic T lymphocyte antigen.

1.4. Antibodies: The New Era of Biologics

The discovery of hybridoma technology, described by Kohler and Milstein in 1975, and the subsequent ability to develop mAbs, initiated a paradigm shift in antibody research and their clinical development (Köhler & Milstein, 1975). Yet, despite representing a major breakthrough in antibody-based therapy, early clinical studies demonstrated that these unmodified murine mAbs presented properties that limited their use in a clinical context. One of the most important shortcomings was the high immunogenic character of these mAbs that

resulted in the production of anti-mouse antibodies and limited their therapeutic utility. Furthermore, murine mAbs demonstrate decreased serum half-life and inability to elicit human effector responses (Aires da Silva, Corte-Real, & Goncalves, 2008; Hwang & Foote, 2005). To overcome these limitations, murine mAbs were manipulated by recombinant DNA technology to turn their constant regions into human variants, which led to a reduction in immunogenicity while promoting an efficacy optimization, thus improving the grounds for the development of antibody-based therapies (Aires da Silva et al., 2008; Jones, Dear, Foote, Neuberger, & Winter, 1986). Since then, a total of 74 antibody-based molecules have been approved for a wide range of indications, of which 17 mAbs have been approved for the treatment of cancer by the FDA and 15 by the European Medicines Agency (EMA) (Figure 9). In addition, a similar number of antibody candidates have reached late phase clinical trials, clearly demonstrating the potential of these biologic molecules as therapeutic agents (Strohl, 2017).

Figure 9 – Approved mAbs and Fc fusion proteins for therapeutic use.



Adapted from (Strohl, 2017).

1.5.1. Antibody structure

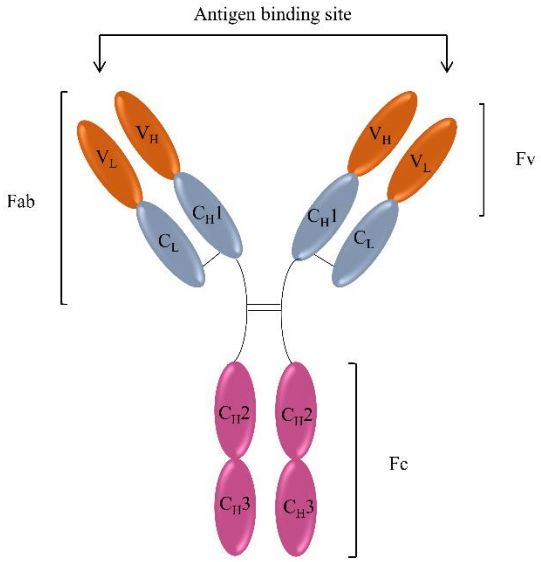
Antibodies, also named as immunoglobulins (Igs), are heterodimeric glycoproteins produced by B cells during the adaptive immune response. In mammals, the antibody basic structure consists of two identical heavy chains (H) and two identical light chains (L) in a Y-shaped format. The L chains belong to kappa (κ) or lambda (λ) subtypes and H chains to α, δ, ε, γ or μ isotypes. Antibodies are divided in five different classes or isotypes: IgA, IgD, IgE, IgG and IgM, based on sequence and length of heavy-chain constant domains, each presenting a

specific structure and role in immunological processes. Due to its high prevalence in human serum, importance for immune response and excellent specificity, IgG represents the leading isotype being used in immunotherapy (Schroeder & Cavacini, 2010; Williams & Barclay, 1988).

In IgG class (Figure 10), each heavy chain is constituted by three constant domains (CH1, CH2 and CH3) and one variable domain (VH), while the light chain consists of a single constant (CL) and a variable domain (VL). The fragment antigen-binding (Fab) is composed by the variable domains positioned into antibody N-terminus and the CL and CH1 regions. A flexible sequence (hinge region) links this fragment to the CH2 and CH3 domains, components of the fragment crystallizable (Fc). Several inter-domain disulfide bonds along with the highly conserved intra-domain bonds maintain antibody integrity (Schroeder & Cavacini, 2010; Williams & Barclay, 1988).

On one hand, the variable domains promote antibody specificity and affinity towards antigen mainly through three hypervariable loops, collectively known as complementary determining regions (CDRs). The conformation of the VH and VL chain CDRs result in six hypervariable loop structures (H1, H2, H3, L1, L2 and L3) that form the antigen-binding site. These domains also present four relatively conserved β-sheet strands, the framework sequences, which act as scaffolds that support the CDR loops. On the other hand, the Fc region plays a major role in mediating antibody effector functions through complement and gamma Fc receptor (FcγR) binding. Furthermore, this IgG region is responsible for the prolonged antibody half-life through a recycling mechanism dependent on neonatal Fc receptor binding (FcRn) (Schroeder & Cavacini, 2010; Turner, 1998; Williams & Barclay, 1988).

Figure 10 – Antibody IgG structure.



Antibody molecule is divided into three major fragments: two fragment antigen-binding (Fabs) and one fragment crystallizable (Fc). Each Fab comprises a variable fragment (Fv), constituted by antibody variable domains (VH and VL), one heavy-chain constant domain (CH1) and one constant light-chain variable domain (CL). Fc region comprises two heavy-chain constant domains (CH2 and CH3), represented in pink, responsible for complement and Fc receptor binding. The hinge region is located between CH1 and CH2.

1.5.2. Antibody mechanisms of action

In cancer therapy, the main purpose of antibody treatment is to promote the direct or indirect destruction of cancer cells and a number of strategies have been successfully employed. MAbs can bind to target cancer cells and directly promote signaling-induced death or can mediate an anti-tumor immune response by promoting antibody-dependent cellular cytotoxicity (ADCC) and inducing complement-dependent cytotoxicity (CDC) (Makkouk & Weiner, 2015). In ADCC responses, antibodies bind to target cells and the antibody Fc domains engage with FcγRs on the surface of effector cells, such as macrophages and natural killer cells. These immune cells cause phagocytosis or lysis of the target cell. In CDC responses, antibodies promote directly target cell death through the development of a complement chain membrane attack complex. Furthermore, mAbs based therapies can also block growth-promoting pathways in the tumor stroma, such as angiogenesis or can directly modulate the activity of anti-tumor adaptive immune cells by blocking inhibitory signals responsible for limiting T cell activation (Vacchelli et al., 2014). mAbs that interfere with the inhibitory signals are called checkpoint blockade mAbs. Likewise, mAb variable regions are also used to retarget immune effector cells towards cancer cells through the use of bispecific

or trispecific mAbs that recognize cancer cells with one arm and activating antigens on immune effector cells with the other arm (Gleason et al., 2014; Huehls, Coupet, & Sentman, 2015; Kontermann & Brinkmann, 2015).

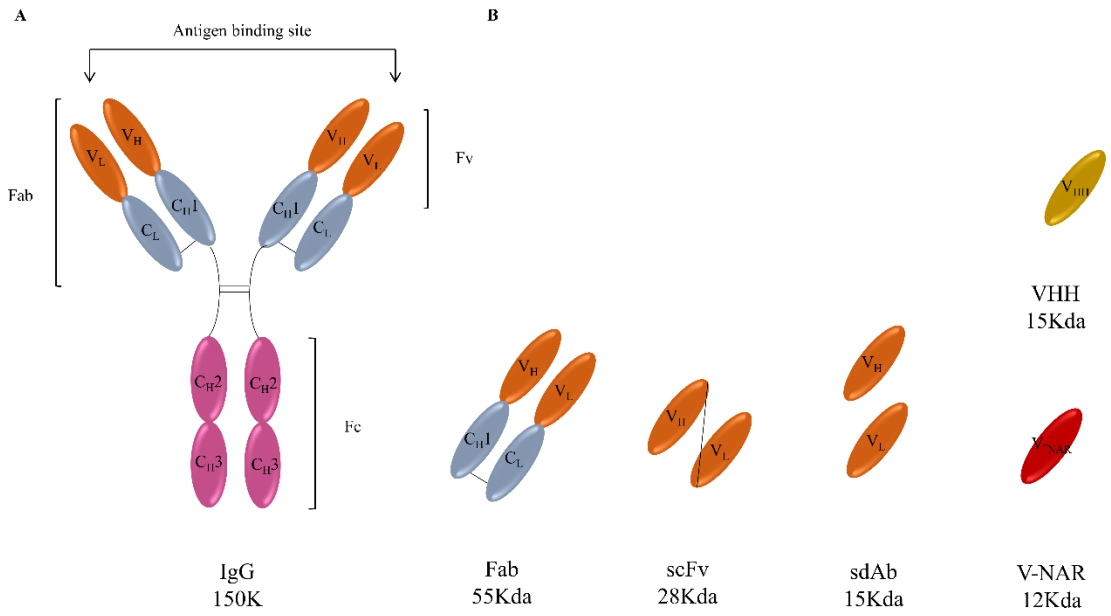
Moreover, mAb based therapeutic approaches have been developed in which the immunoglobulin moiety serves for the selective *in vivo* pharmacodelivery of bioactive payloads such as cytotoxic drugs, bispecific antibodies, radionucleotides or cytokines to sites of disease, thereby sparing healthy tissues (Waldmann, 2003). Radio-immunoconjugates deliver radioisotopes to the cancer cells, whereas antibody–drug conjugates deliver highly potent toxic drugs to the cancer cells.

1.5.3. Antibody Fragments and single domain antibodies

Most marketed antibodies consist of a full-length IgG molecule. By providing a long half-life and effector functions, these molecules have been presenting a quite successful application in therapeutics. However, this conventional antibody format present some drawbacks that limit their clinical use and there is a range of therapeutic applications in which other antibody formats may be more appropriate. On one hand, due to high molecular weight (~150 kDa), IgG antibodies are known to penetrate poorly into densely packed tissues, to have an impaired reaching of difficult targets and to present a slow clearance rate. In addition, in some conditions, the long antibody serum half-life results in poor contrast in imaging applications. On the other hand, inappropriate activation of Fc receptor-expressing cells sometimes leads to massive cytokine release with subsequent toxic effects. To address these major issues, smaller antibody scaffolds such as the Fab or the single chain variable fragment (scFv) or single-domain antibody (sdAb) are emerging as alternative therapeutic agents (Figure 11) (Hudson, 1998; Presta, 2003).

Fab (~50 kDa) and scFv (~30 kDa) can be obtained through the cleavage of the IgG molecule with papain or recombinant technology (Bird et al., 1988; Hudson, 1999; Hudson & Souriau, 2003; Skerra & Plückthun, 1988). Both fragments are stable and exhibit properties similar to the whole antibody molecule. Fab contains the variable and CH1 domains of an antibody, whereas scFv is constituted by a VL-VH pair connected by a flexible peptide linker, in order to improve antibody folding and stability. The use of a short linker (typically 5 amino acids), allows to combine two scFv molecules and form a stable non-covalent dimer, named diabody (Db, ~50 kDa) (Holliger & Hudson, 2005).

Figure 11 - Schematic representation structure of a conventional IgG antibody and antibody fragments of biotechnological and clinical interest.



(A) IgG antibodies comprise a pair of identical heavy and light chains linked by disulphide bonds. Light chains contain one constant domain (CL) and one variable domain (VL), while heavy chains contain three constant domains (CH1, CH2 and CH3) and one variable domain (VH). The variable domains of both the heavy and light chains are responsible for the antigen-binding site of the molecule. The Fc constant region recruits effector functions of the immune system. Constant light (CL) and heavy (CH) chain domains are represented in orange and gray. Variable light (VL) and heavy (VH) chain domains are represented in green. B) The engineering of antibody fragments that can be generated from an intact conventional IgG: antigen-binding fragment (Fab), single-chain Fv fragment (scFv), heavy and light single domains antibodies (VL and VH sdAbs) and natural camelid variable domain (VHH) and shark variable domains (V-NAR).

One of the most promising alternatives to conventional IgG´s are sdAbs. sdAbs are the smallest functional antigen-binding fragments of an antibody that can be isolated from conventional IgGs or obtained from naturally occurring antibodies devoid of light chain that were discovered in two types of organisms, the camelids (camels and llamas) and cartilaginous fish (wobbegong and nurse shark) (Aires da Silva et al., 2004; Greenberg et al., 1995; Hamers-Casterman et al., 1993; Holliger & Hudson, 2005; Holt, Herring, Jespers, Woolven, & Tomlinson, 2003; Muyldermans, 2001). Due to their small size, sdAbs show improved tissue penetration and are able to reach targets not easily accessible by conventional antibodies, such as enzyme active sites or canyons in receptors molecules. Moreover, similarly to Fab and scFv, sdAbs lack the Fc domain of a full IgG antibody, presenting a low nonspecific uptake in tissues that highly express Fc receptors. Additional important advantages include their high stability, low immunogenicity, and lower manufacturing cost. Furthermore, sdAbs can be easily attached to other proteins, peptides, small molecules or nanoparticles by simple molecular biology or chemical procedures (Holliger & Hudson, 2005).

We have been showing that rabbit derived sdAbs can be developed against several targets and that these minimal scaffolds show great potential for therapeutic applications (Goncalves & Aires-Da-Silva, 2008; Volker et al., 2016). The unique ontogeny of rabbit B cells promotes vastly distinctive antibody repertories rich in *in vivo* pruned binders of high diversity, affinity and specificity (Weber, Peng, & Rader, 2017). Furthermore, rabbits are evolutionarily distant from mice and rats, so epitopes that are not immunogenic in rodents can be recognized by rabbit mAbs, increasing the targetable epitopes and facilitating the generation of mAbs that cross react with other species (Weber et al., 2017) - a key aspect for clinical translational.
Currently, 20–25% of the mAbs in clinical development for cancer and non-cancer indications are recombinant human antibodies derived from phage display libraries or from transgenic mice. This past year, five antibody "fragments" (scFv) were reported in clinical phase 2/3 (Iezzi, Policastro, Werbajh, Podhajcer, & Canziani, 2018).

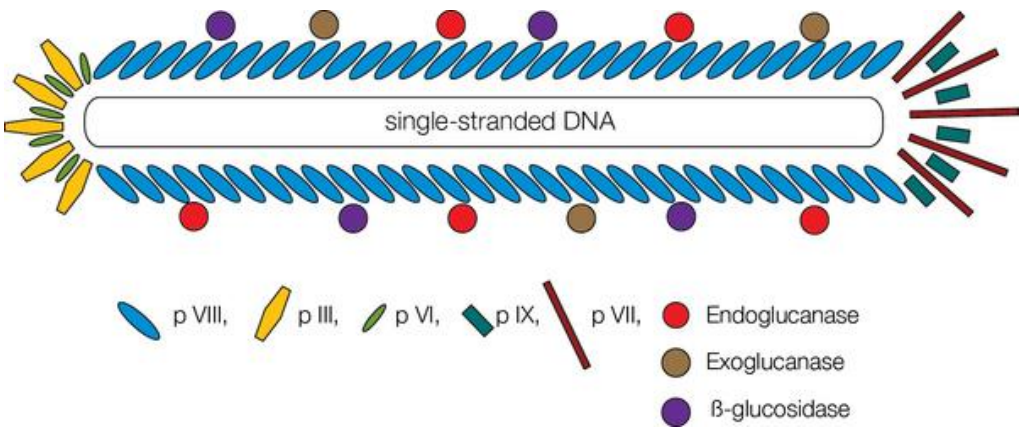
1.5.4. Phage Display and immune libraries tailoring

Antibody development relies on display technologies for the selection of therapeutic candidates. These *in vitro* platforms englobe three main phases: generation of a large repertoire of individual clones (library); display and selection rounds towards the target or desired feature, and functional screening and characterization of selected molecules. The principal display technologies are ribosome and mRNA/cDNA display, phage display and cell display (Galán et al., 2016). Phage display is one of the most effective molecular diversity techniques and is a well-established technology for *in vitro* selection of antigen-specific antibodies from recombinant antibody libraries. This technology is based on a direct linkage between phage phenotype and its encapsulated genotype, which leads to the successful presentation of antibody libraries on the filamentous bacteriophages (phages) particle surface (Bazan, Całkosiński, & Gamian, 2012).
Phage display libraries can be successfully developed using a naïve or immunized source or developed synthetically or semi-synthetically. A naïve library is generated from the natural immune repertoire of a donor, which variability relies only in sources of sequence variation during B cell maturation. The main characteristic of a naïve library is the unbiased nature of the large repertoire. An immune library refers to antibody repertoires originated from a donor immunized with a desirable antigen or disease infected donors. Although this library approach narrows the repertoire spectrum, these antibody libraries mainly produce good affinities antibodies with high clonal diversity due to *in vivo* somatic hypermutation that contributes towards affinity maturation. More recently, antibody libraries were developed synthetically

by replacing one or more of the CDR loops with artificial elements. The resulting antibody repertoire offers an highly diverse repertoire with unnatural variability that evades biases and redundancies of natural *in vivo* formed antibodies. A key subset of synthetic libraries is the production of semi-synthetic libraries. The main difference between semi- and fully synthetic libraries is the source of the diversity. In semi-synthetic libraries, the diversity is largely obtained from natural sources whereby the genes encoding the CDR are isolated. As such, the diversity is still natural, taking advantage of the maturation processes of antibodies *in vivo*, whereas in fully synthetic libraries the unarranged and randomized V-gene segments are synthetically assembled ex vivo normally by polymerase chain reaction (Ch'ng, Choong, & Lim, 2016; Frenzel et al., 2017).

In the case of immunized libraries, cDNA is generated from the mRNA extracted from the spleen or bone marrow of an animal immunized with the desirable antigen. The heavy and light chain genes are amplified by polymerase chain reaction (PCR) and the antibody fragment inserted into one of the encoding genes for surface coat proteins. In general, M13 and fd-based display continue to be the most widespread antibody phage display selection tools (Harel Inbar & Benhar, 2012) and with a few exceptions (pioneering work done at the Scripps Research Institute (Kang, Barbas, Janda, Benkovic, & Lerner, 1991) where Fab was displayed on p8), all known antibody phage display repertoires are fused to the minor phage coat protein p3 (Bradbury & Marks, 2004), which is involved in the bacterial infection process through the F pilus and is present in 3–5 copies per phage (Holliger & Riechmann, 1997) and not to the major phage coat protein p8 which was found to be less efficient, though more prevalent with 2700 copies per phage (Figure 12) (Kretzschmar & Geiser, 1995).

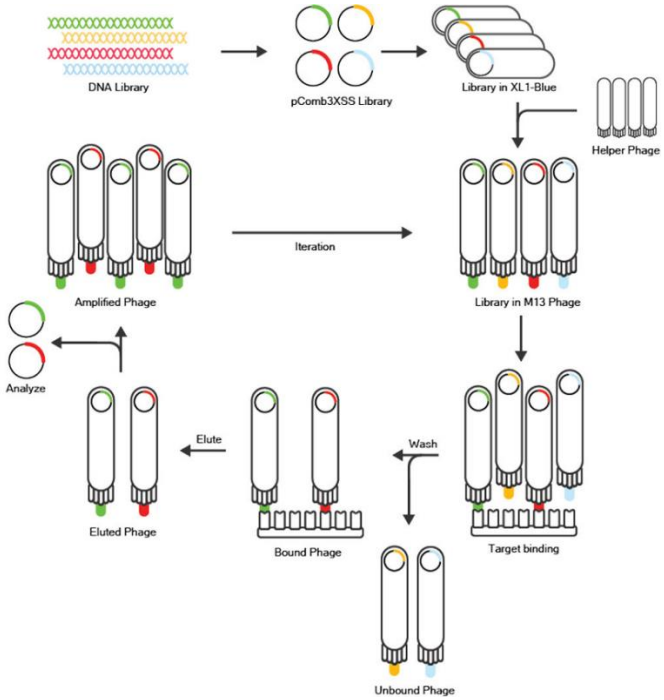
Figure 12 – Schematic of the M13 filamentous phage structure.



Schematic representation of the phage structure showing how the five structural proteins are arranged around its ssDNA genome. Legend labeling the structural proteins. Adapted from iGEM.

The phagemid DNA is then transformed into competent *E. coli* cells and, after expression, the coat protein fusions are integrated into new phage particles assembled in the bacteria. This enables phage particles to be replicated as ssDNA in the presence of a helper phage. Helper phages provide the necessary viral tools for the initiation and packaging of phagemid DNA into phage particles. Helper phages contain a defective origin of replication thus limiting their own packaging ability to produce more helper phage molecules. This characteristic is important as it reduces the ability of helper phage to replicate resulting in dominant phagemid DNA production (Hoogenboom et al., 1998). Phage particles are harvested from the infected cells and positive clones with desired affinities selected by a process known as biopanning, whereas phages of interest are eluted, enriched by reinfection and re-growth in bacteria (Figure 13) (Smith, 1985).

Figure 13 – Overview of the creation of the recombinant M13 phages carrying affinity body library and phage display biopanning.



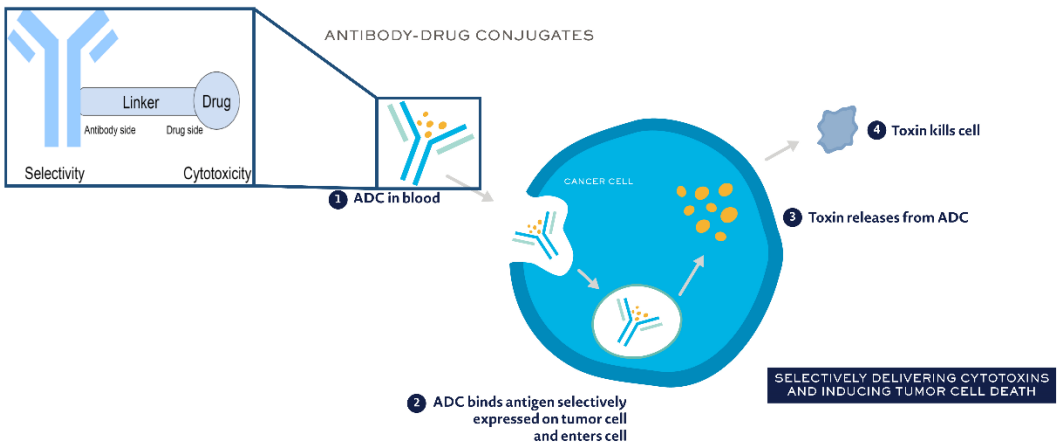
Phage display cycle with phagemid. A library of DNA sequences with random variations of the protein of interest (POI) displayed on the pIII coat protein is cloned into a phagemid vector. After transformation of *E. coli* cells and subsequent infection with helper phages, the phage library is created. Using an immobilized target molecule, rounds of selection and amplification are performed until phages with the highest affinity are isolated. DNA sequencing can be used to identify the phages, and/or directed evolution can be used to create new libraries for panning. Adapted from iGEM.

1.5.5. Single domain antibodies as targeting moieties for cancer therapy

Antibody-drug conjugates (ADCs) are an emerging novel class of anticancer treatment agents that combine the tumor selectivity, pharmacokinetics and biodistribution properties of antibodies with the cytotoxic potency of small molecules (Figure 14) (Sievers & Senter, 2013). This concept was first envisioned by Paul Ehrlich in the early 20th century who reasoned that if a compound could be made to selectively target a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity (Ehrlich, 1913). Hence, such molecules named "magic bullets" would be created that only killed the targeted organism. As antibody-based therapies were developed, the idea that antibodies could be suitable moieties for the creation of these “magic bullets” gained scientific evidence.

By attaching cytotoxic effector molecules to antibodies, ADCs are designed to improve the therapeutic index window of anticancer drugs as it promotes the selective delivery and uptake of the cytotoxic drug to the cancer cells to which the antibody binds, restricting its effect on normal cells. Additionally, ADCs draw on the *in vivo* distribution properties of the antibodies to reduce systemic toxicity of the cytotoxic agent. At the same time, ADCs enhance the anti-tumor activity of the unmodified antibodies (Wu & Senter, 2005).

Figure 14 - General mechanism of action of ADCs.



The antibody of an ADC binds to tumor associated antigen. Upon binding, the ADC-antigen complex is internalized into the cancer cell. During the degradation of the complex, the released payload exerts its effects on the intracellular target leading to apoptosis. Adapted from Abbvie and (Chen & Cao, sem data).

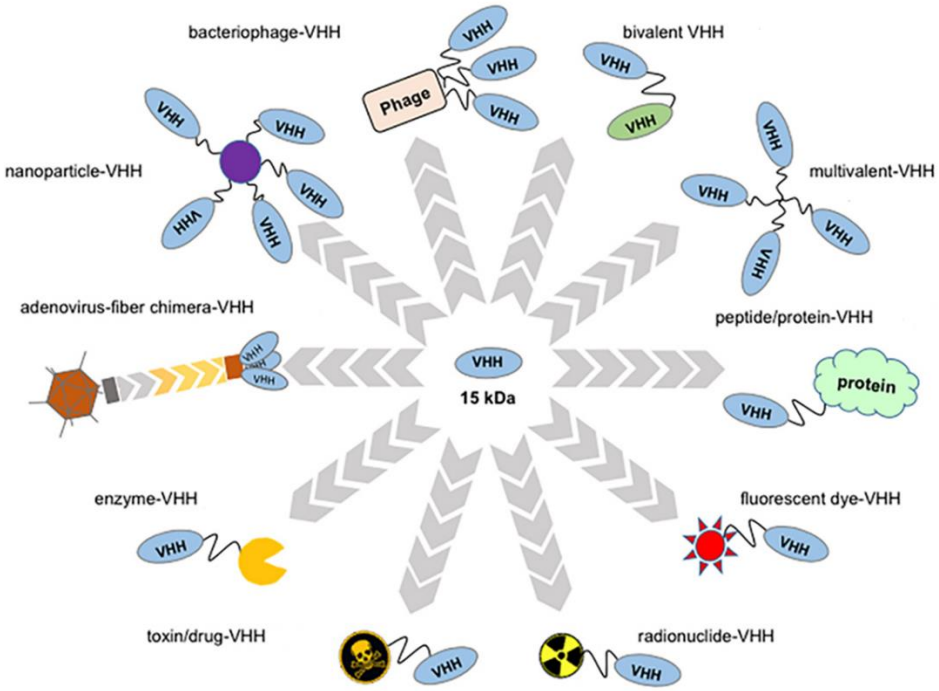
The first generation of ADCs consisted of a mAb conjugated with clinically approved anticancer chemotherapeutic agents such as vinblastine, mitomycin, methotrexate and doxorubicin (Perez et al., 2014). Unexpectedly, the clinical trials demonstrated that these ADCs exhibited limited clinical sucess, possibly due to the low potency of the payload (Elias

et al., 1994; Petersen, DeHerdt, Schneck, & Bumol, 1991; Yang & Reisfeld, 1988). In turn, the second generation of ADCs resort to more potent payloads, namely calicheamicin, auristatin and maytansin analogs. This along with the optimization of the pharmacokinetics of the conjugation linker and the development of fully human in order to solve immunogenicity problems, led to the approval of the first ADC - gemtuzumab ozogamicin (Sievers et al., 2001). As a result, in 2000, Gemtuzumab ozogamicin (MylotargR) received accelerated approval for the treatment of refractory acute myeloid leukemia (AML). Unfortunately, ten years later, gemtuzumab ozogamicin was withdrawn from the US market due to lack of clinical benefit (Petersdorf et al., 2009). Several issues have been associated with the limited clinical efficacy, including poor stability of the acid-labile conjugation linker, heterogeneous drug loading (approximately 50% of the antibodies are unconjugated) and sensitivity to multidrug resistance pumps that are often overexpressed in AML. More recently, two novel ADCs were approved by the FDA for the treatment of Hodgkin lymphoma and anaplastic large-cell lymphoma, brentuximab vedotin (AdcetrisR), and HER2 positive breast cancer, trastuzumab emtansine (KadcylaR) (Burris et al., 2011; Younes et al., 2010). These ADCs showed improved linker stability and pharmacokinetics. Though this recent clinical success has led to an impressive expansion of the clinical ADC pipeline, with more than 20 products being currently in clinical trials, many challenges remain related to ADC design and development, including the identification of ideal targets, antibody format, as well as linker-payload combination.

Most ADCs on the market and in clinical development are comprised of the complete IgG antibody, which is likely due to its favorable pharmacokinetics properties when compared to antibody fragments (Kim & Kim, 2015). Nonetheless, there is also growing interest in using antibody fragments, such as sdAbs, as targeting agents for the delivery of therapeutic and imaging agents. As a matter of fact, the use of these small biomolecules may be the ideal format for cancer therapy as they present higher total tumor uptake, improved tumor to blood ratio and faster clearance rate than intact IgG or Fab (Chames et al., 2009). Moreover, a short serum half-life may be desirable in cases where the risk of toxicity in healthy tissues increases with prolonged exposure (Tijink et al., 2009).

Owing to their solubility, stability, independent folding and versatility properties, sdAbs have been recently exploited as excellent frameworks for the development of novel diagnostic and therapeutic carriers, including toxins, radionuclides, dyes, peptides, proteins, nanostructures, phage and virus (Figure 15).

Figure 15 – sdAb-associated strategies in targeting tumors and tumor accessory cells.



VHH-associated strategies in targeting tumors and tumor accessory cells. Top, clockwise: bivalent bi-specific VHH; multivalent, high-avidity mono-VHH molecules; VHH fusions ranging from vascular penetration peptide-VHH to engineered hu-Fab and albumin-binding domains; fluorescent dye fusions, for example, one spontaneously crossing the blood–brain barrier; radionuclide-VHHs; toxin-VHH theragnostics; chromogenic enzyme fusions: here an alkaline phosphatase-VHH may be applied in ELISA, dot blot, and transferred protein identification in western blot; oncolytic virus; VHH decorated nanoparticles for therapeutics delivery and in facilitating photothermal therapy; bacteriophage engineered to display VHH and deliver targeted therapeutics may also be developed for signal amplification in ELISA assays. Adapted from (Iezzi et al., 2018).

Several payloads have been investigated for the development of drug-delivery nanobodies, however only two immunotoxins made use of cytotoxic compounds for cancer targeted therapy. Behdani and colleagues reported the development of a VEGFR2-specific nanobody, conjugated to the truncated form of pseudomonas exotoxin A (PE-A) that provided efficient inhibition of tumor cell growth (Behdani et al., 2013). More recently, a humanized CD7 nanobody-based immunotoxins coupled with a derivative of the same PE-A toxin exhibited promising anti-T-cell acute lymphoblastic leukemia potential (Yu et al., 2017). As such, active research has been focusing on the development of promising highly potent payloads.

In the area of natural products these efforts have led to the discovery of extremely potent cytotoxic compounds, which could be considered for ligand based pharmaco-delivery applications. These agents include potent tubulin agents, such as maytansinoids, dolastatins and cryptophicins (all strong inhibitors of tubulin polymerization with IC50 in the picomolar

range), but also duocarmycins (highly cytotoxic minor groove binders and DNA-alkylating agents). Despite these drugs failed to demonstrate clinical benefits on their own, demonstrating that the increase of the potency by itself does not increase the therapeutic index of chemotherapy, they represent attractive payloads for the preparation of ADCs (Aguiar et al., 2018).

As the field of antibody fragments expands, novel and promising structures are being developed with unique and valuable characteristics. The combination of these innovative moieties with potent payloads may surpass many of the drawbacks associated with the use of IgGs and small molecules as targeting agents (Holliger & Hudson, 2005). In addition, the interdisciplinary and synergetic investigation between chemistry, immunology and biotechnology may trigger a new era of ADC. As a result, the emergence of these biomolecules may represent a paradigm shift in cancer treatment as it can finally fulfill the promise of the magic bullet reasoned in the early 20th century.

1.5. Aims and Thesis outline

Considering the great clinical potential that sdAbs as targeting moieties may represent in cancer treatment, this work aimed to develop a novel sdAb-based drug delivery system for canine NHL, which serves as an animal model of human NHL. However, the development of ADCs requires the careful selection of an appropriate antigen target, the screening of the most promising highly potent cytotoxic drugs and the development of stable linkers that can selectively release the cytotoxic payload. As such, to achieve the proposed objective, a strategic, multidisciplinary approach composed by a series of milestones was planned to tackle all these issues (Figure 16). Furthermore, to overcome limitations associated with conventional preclinical models, we aimed to validate the naturally occurring canine B-cell lymphoma as an animal model for the development of immunotherapeutic strategies for NHL.

Objectively, the main goals of this project can be summarized as follows:

I - Characterization of the immune status of dogs diagnosed with B cell NHL (Chapter 2):

Despite the potential of using the dog as an animal model for the development of immunotherapies, efforts to develop such strategies for veterinary applications have been hampered by several limitations. Importantly, the canine immune system has not been sufficiently studied and characterized compared to the human immune system. We know relatively little, for example, about lymphocyte subtypes and expression and regulation of their receptors, or about canine cytokines that support their function. In part, this lack of information is due to the limited availability of canine-specific reagents to characterize immune system components. This also implies that there are no predictive tests, to ascertain which dog may benefit from what type of immunotherapy. Moreover, tumors in dogs have not been as well characterized as their human counterparts with respect to genotype and phenotype. As such, the main goal of this chapter was to contribute to fill in this gap. Within this context, considering that high quality basic, clinical and translational cancer research requires prompt access to well-preserved biological samples, throughout this project multiple samples of dogs diagnosed with lymphoma were collected and used for the construction of a cNHL biobank. This samples supported the execution of the studies that allowed to characterize the immune status of dogs diagnosed with B cell NHL.

II - Development of recombinant antibodies for the treatment and diagnosis of canine NHL (Chapter 3):

Our research plan aimed at developing recombinant antibodies against cNHL, a promising animal model of immunotherapeutical approaches for NHL. Within this context, a comprehensive strategy was implemented to guarantee a successful project outcome, by developing sdAbs against canine CD20, a well-known clinically validated target, and by developing novel sdAbs against cNHL antigens using an innovative strategy.

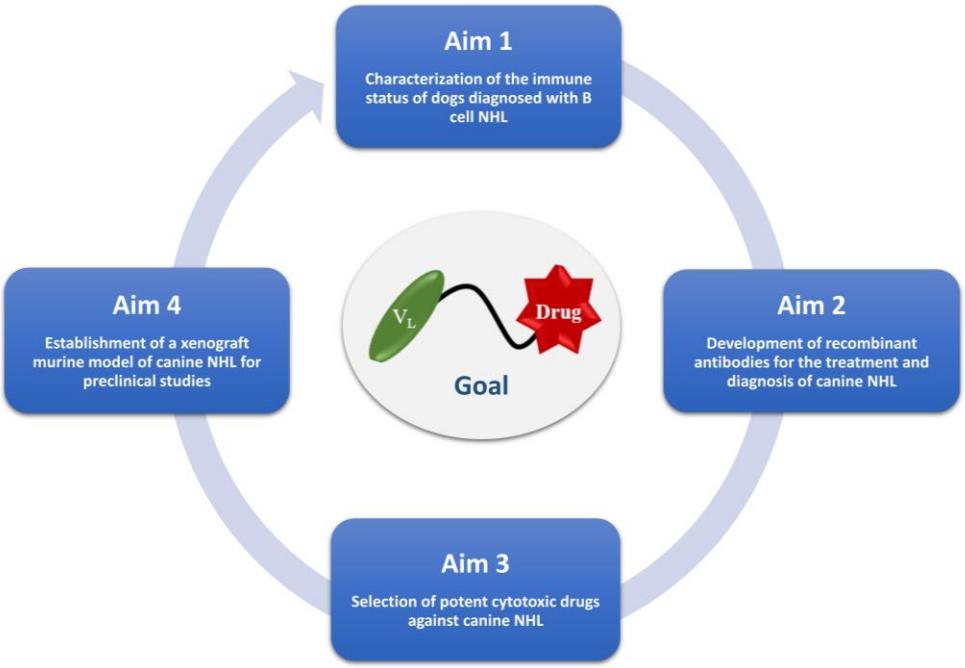
III - Selection of potent cytotoxic drugs against canine NHL (Chapter 4),

One of the most important challenges when developing an ADC is the selection of a suitable payload. With that in mind, in this chapter we aimed to screen a panel of cytotoxic drugs with potent anti-tumor properties against cNHL. Panobinostat, a HDAC inhibitor revealed to be the most promising molecule and was further characterized under chapter 4. In the future, we expect to couple this molecule with our antibody candidates in order to develop an ADC.

IV - Establishment of a xenograft murine model of canine NHL for monitoring tumor progression and treatment response in preclinical studies (Chapter 5).

The integration of cNHL in comparative studies has been limited by the lack of suitable mouse models for preclinical studies. To overcome these limitations, we aimed to establish and characterize a localized subcutaneous bioluminescent canine DLBCL xenograft mouse model. This model will be a valuable preclinical tool for veterinary applications, while contributing to comparative oncology.

Figure 16 – Thesis at a glance.



To properly address and discuss the above-mentioned objectives, this thesis has been divided in six chapters. The first Chapter consists of a detailed state of the art literature review. Chapter 2 to 5 were based in scientific manuscripts that were either published in international peer reviewed journals or are currently in preparation for submission. Finally, the last Chapter integrates the results presented in each of the previous Chapters with a complete discussion and conclusion. Importantly, results presented under Chapter 3 were subject to intellectual property protection through a European Patent submission.

Chapter 2

Canine multicentric lymphoma exhibits systemic and intratumoral cytokine dysregulation

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Adapted from a manuscript submitted for publication

2.1. Abstract

NHL is among the most common neoplasias in dogs and humans. Owing to remarkable similarities with its human counterpart, the 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.

2.2. 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 & Wilson-Robles, 2014b; 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 & 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 chimeric antigen receptor 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 & 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 (Y.-H. 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 & 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 & 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, Dawe, Leifer, & Brown, 1982; Dutta, Novilla, & Bumgardner, 1978; Weiden et al., 1974). Furthermore, a dysfunction in humoral response of dogs with lymphoma was also identified, suppressed antibody responses to sheep red blood cells and to primary and secondary immunization with bacteriophage, being reported (Medleau, Dawe, & Calvert, 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, auto-immune diseases, such as immune-mediated thrombocytopenia, have been also associated with an 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 determine 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.

2.3. Material and methods

2.3.1. Biological samples

- **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.

- **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 -80ºC. Additionally, PBMC were isolated by Ficoll gradient method (Biocoll Separating Solution, BioChrom®, Fisher Scientific, New Hampshire, USA) and following cell viability was assessment, aliquots of 5 x 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 -80ºC 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 x 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.

- **Control groups**

Whole blood samples were collected from 9 canine healthy donors and processed for serum 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 at the end of a non-related study. Lymph node samples were fined cut and stored at -80ºC in RNAlater®.

2.3.2. 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 6). 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 (1ng), 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. 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 & Schmittgen, 2001).

Table 6 - 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-17[a]	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-GCTGTAACTGTCAGGCCATGTTT RV-TGTTTTGTCACTCTCCTCTCTCCA	140	Henriques *et al*. (2016) [a]
RLP27	NM_001003102	FW-TCGTCAACAAGGATGTCTTCAGAG RV-TCTTGCCAGTCTTGTACCTCTCCT	96	Henriques *et al*. (2016) [a]

[a] (Henriques et al., 2016) [b] (Peters, Helps, Calvert, Hall, & Day, 2005) [c] (Schmitz, Garden, Werling, & Allenspach, 2012)

2.3.3. Multiplex cytokine immunoassay

Milliplex® MAP magnetic bead panel based on Luminex® xMAP® technology (CCYTOMAG-90K, 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, Romano, Matassa, & De Guise, 2014). The minimum detectable concentrations for the four cytokines according to the manufacturer was 12.2 pg/ml. Significance of differences of the median value of each serum cytokine concentration between cNHL group and control group was analyzed.

2.3.4. 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%.

2.4. Results

2.4.1. 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 7.

Table 7 - cNHL patient clinical characteristics and corresponding tissue and blood samples.

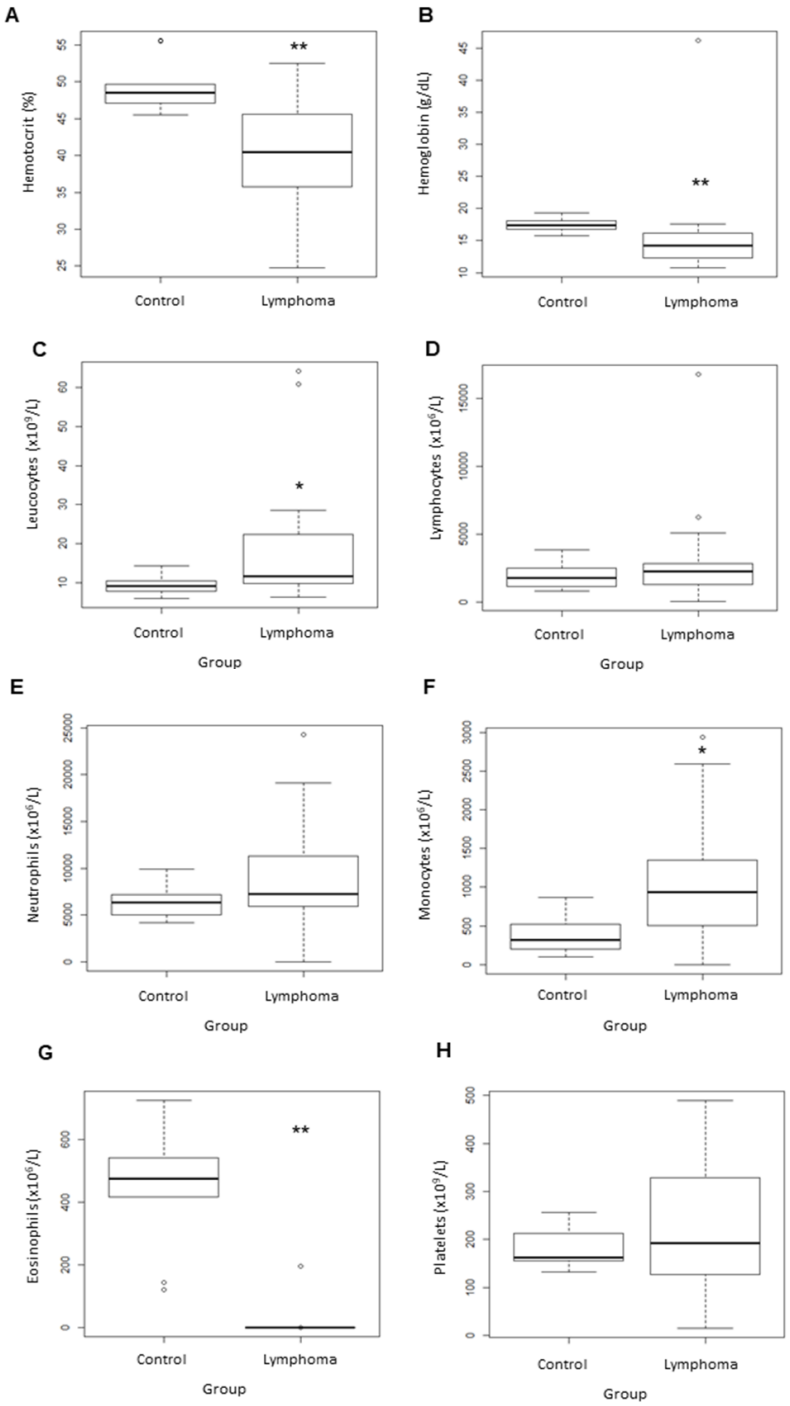
Patient (n=22)	Age (Years)	Sex*	Breed	WHO Classification	Tumor sample[a]	PBMC sample[b*]	Serum sample[c]	CHOP protocol[d]
1	12	M	Schnauzer	DLBCL	+	+	+	-
2	15	M	Mixed-breed	PTCL	+	-	+	-
3	8	M	Labrador	DLBCL	+	+	+	+
4	12	M	Golden Retriever	Blastic NK Lymphoma	+	+	+	+
5	6	M	Rottweiller	DLBCL	+	+	+	+
6	6	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

2.4.2. 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 (Figure 17).

Figure 17 - Differences in blood cell counts of cNHL group compared to the control group.

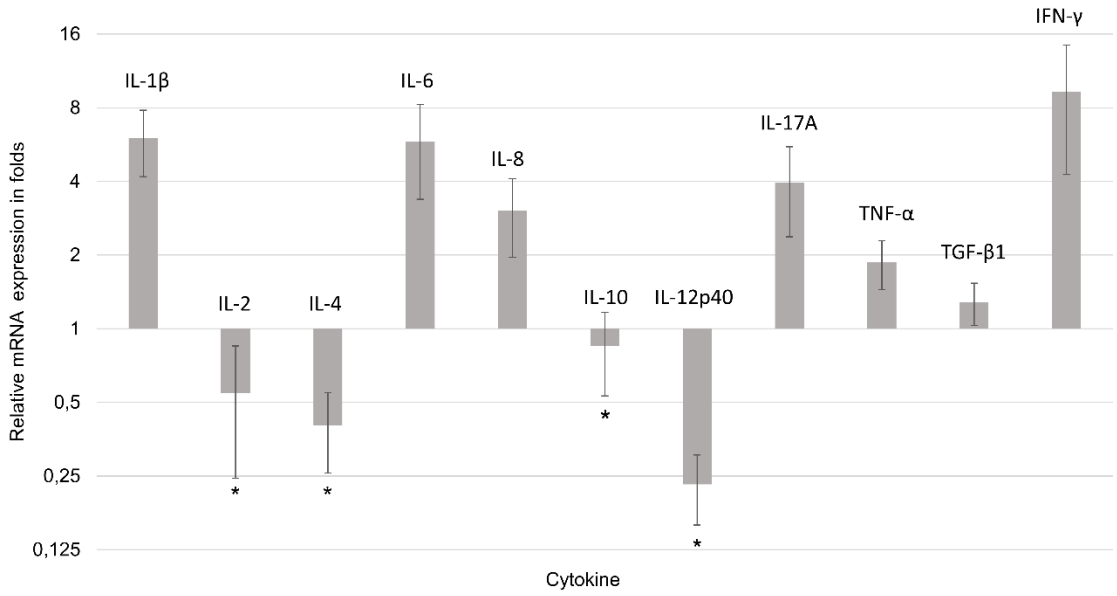


Data of hematological parameters, including hemoglobin, hematocrit, leucocytes, lymphocytes, neutrophils, monocytes, eosinophils and platelets are presented as box and whiskers (**A**-**H**). * $p < 0,05$; ** $p < 0,001$.

2.4.3. 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 cytokine mRNA expression in naïve cNHL patients, compared to healthy donor lymph nodes samples. As shown in Figure 18, 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.

Figure 18 - Relative cytokine mRNA expression in cNHL tumor tissue.



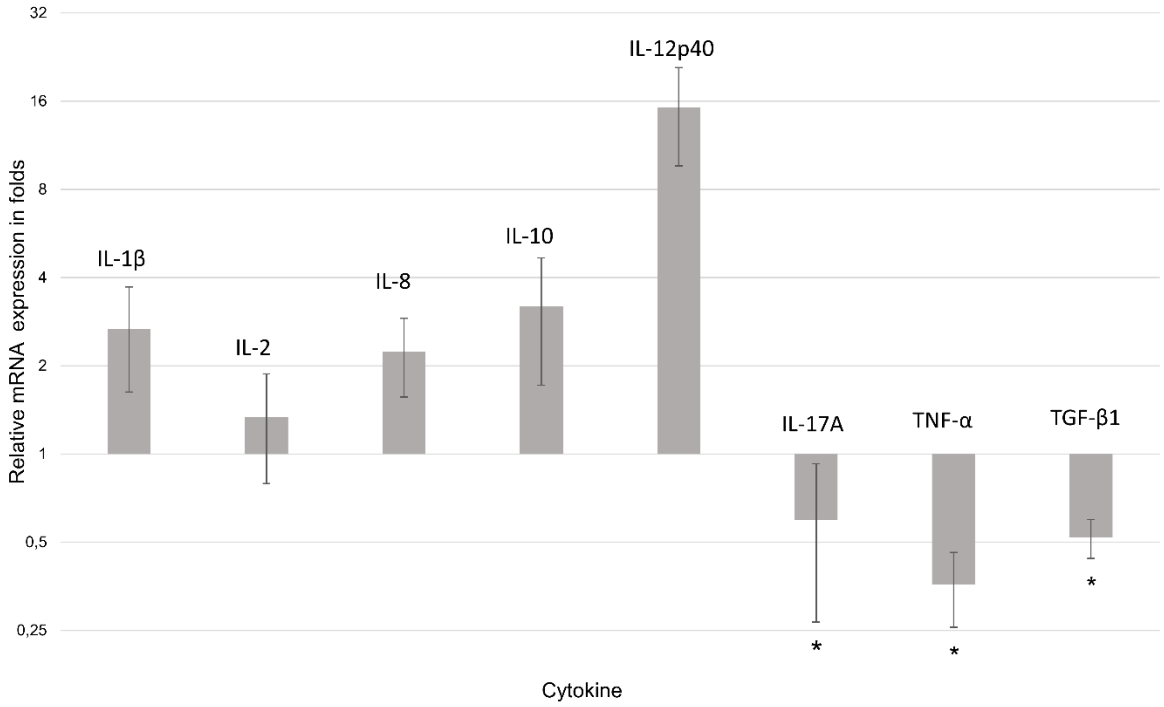
Results are expressed as a fold difference between mean ± SEM of mRNA expression level in cNHL affected lymph nodes samples and a control group with healthy donors. * $p < 0,05$.

2.4.4. 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 Figure 19, 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.

Figure 19 - Relative cytokine mRNA expression in cNHL circulating PBMC.



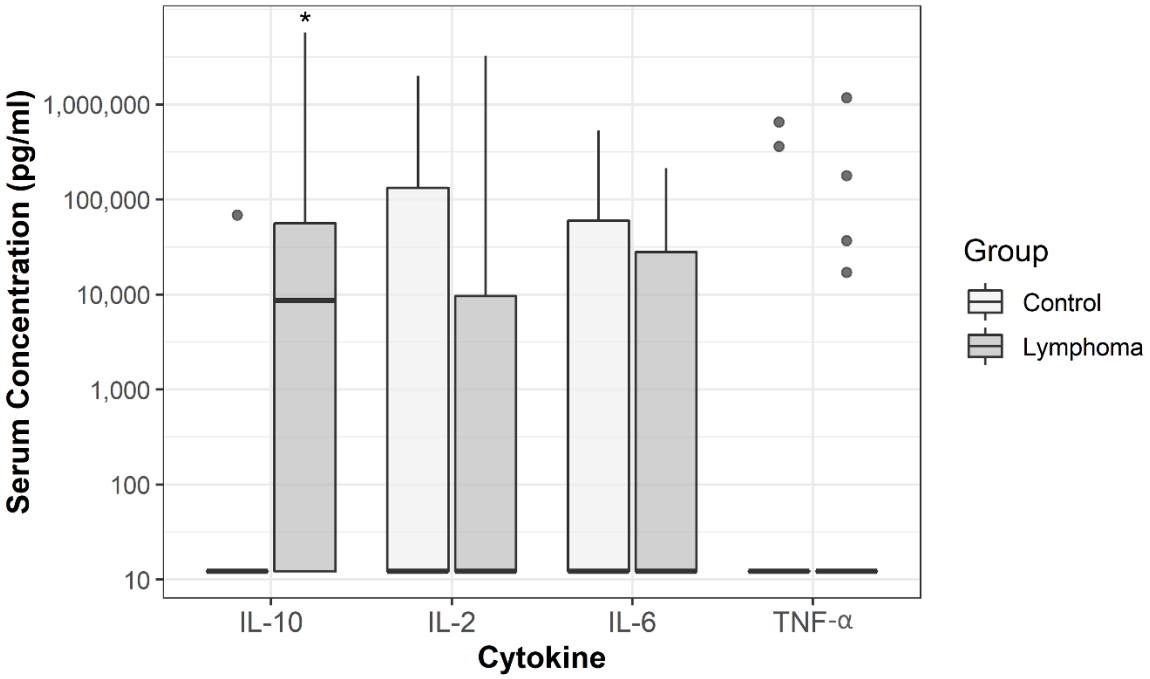
Results are expressed as a fold difference between mean ± SEM of mRNA expression level in cNHL PBMC and a control group with healthy donors. * $p < 0,05$.

2.4.5. 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 between sera concentration of IL-2, IL-6 and TNF-α of cNHL and control groups were found (Figure 20). 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.

Figure 20 - Differences in serum cytokine concentrations of cNHL group compared to control group.



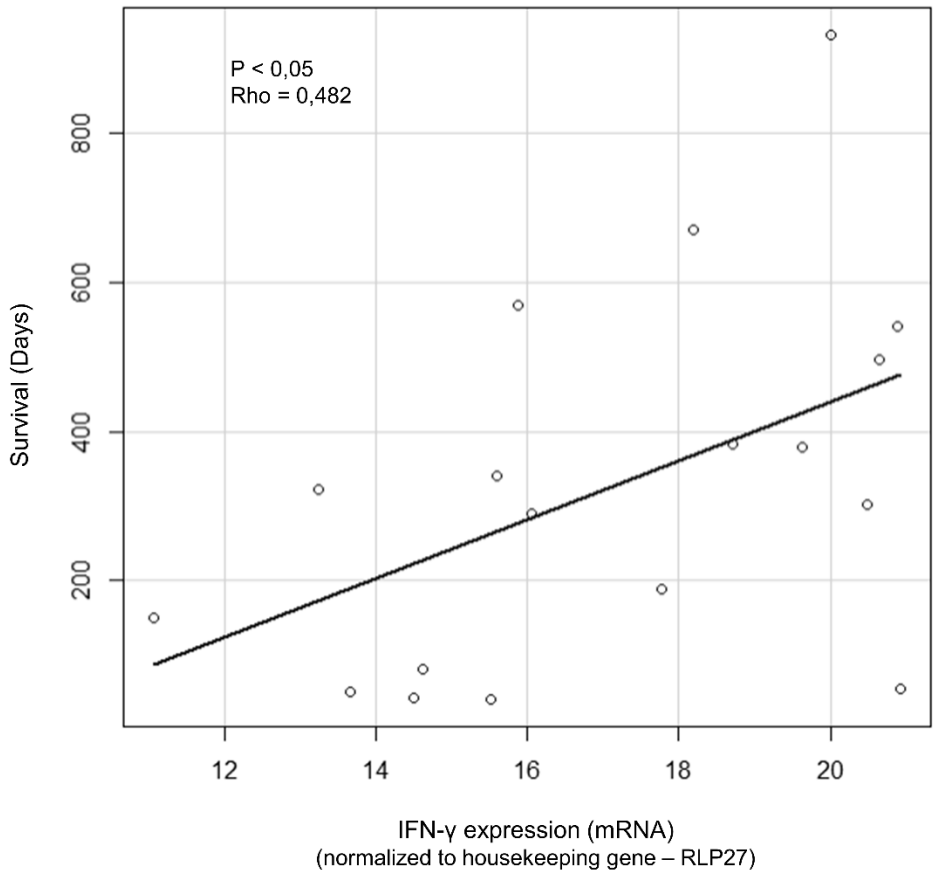
Data are presented as box and whiskers and a log10 scale was used for the y axis for an optimal visualization of results. * $p < 0,05$.

2.4.6. 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) (Figure 21).

Figure 21 - 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 RLP27.

2.5. 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 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 & 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, Wasmer, Rau, & Krebs, 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, Rochon, Franza, & Aebersold, 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, Munoz, Fitting, Misset, & Carlet, 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 & 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 an 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, Apollonio, & Ramsay, 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, Sanchez-Aguilera, & Piris, 2008; Hsu, Waldron, Hsu, & Hough, 1993; E. A. Jones, Pringle, Angel, & Rees, 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, Marincola, & Young, 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., sem data). 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, Biller, Lana, Bachand, & Dow, 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, Thamm, Eickhoff, Avery, & Dow, 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 & 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. Lastly, a major drawback of this work was the inability to perform multiplex immunoassay on tumors extract due to kit specifications.

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.

Chapter 3

Development of engineered recombinant antibodies for diagnosis and treatment of cNHL

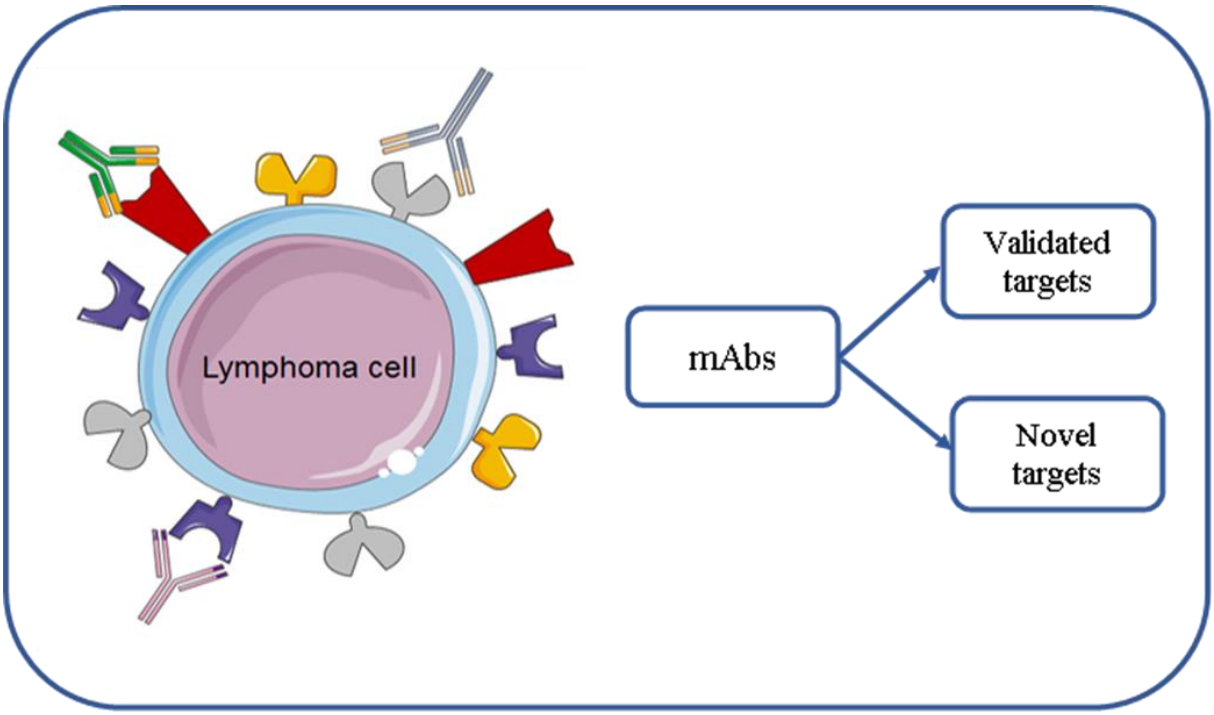
Preface

mAbs are leading agents in modern anti-cancer therapy, but have their use limited by the paucity of clinically relevant models for validation. Rodent models are the conventional preclinical test-bed, however they do not correlate with clinical success rates, demonstrating an urgent need for innovative pre-clinical models. Naturally occurring tumors in dog mimic features of those observed in humans, difficult to reproduce with other models. This represents a unique opportunity to study the complex immune interactions while also addressing long-term efficacy and toxicity of cancer immunotherapies. hNHL is one of the leading causes of cancer-related death. The use of a mAb targeting CD20 in combination with conventional chemotherapy has revolutionized the treatment of B-cell lymphoma by significantly improving disease-free interval and overall survival. However, regardless of the elected therapy, the mortality rate is still high. Therefore, there is a continuing need to develop novel therapeutic strategies, of which mAbs is one of the most promising. Canine lymphoma and human NHL share many histopathological, molecular, genetic and clinical features, making the dog an excellent animal model to explore novel therapeutic molecules and approaches.

Within this context, under this chapter, we aimed to develop recombinant antibodies for cNHL treatment and diagnosis, as an animal model of hNHL. For that purpose, two divergent strategies were explored for the generation of mAbs for cNHL – a validated target-led discovery method and a platform for the identification of therapeutic mAbs directed to novel targets. The first approach relies on the conventional antibody development process that starts with the identification and characterization of a biological target. This discovery path has led to the isolation of the majority of antibody therapeutics that today are used in the clinic and

remains a reliable option. However, as the number of validated targets diminishes, and the target space becomes increasingly competitive with the development of many biosimilar and biosuperior candidates, alternative strategies are gaining favor. Indeed, it has become clear that the identification of the next generation of first-in-class antibody drugs against novel targets to address unmet medical needs will be crucial for the success of biotherapeutics. Considering the many strengths presented by each approach, we aimed to pursue both by developing sdAbs against canine CD20, a well-known clinically validated target, and by developing novel sdAbs against cNHL antigens using an innovative methodology (Figure 22).

Figure 22 – Chapter research plan.



Our research plan aimed at developing recombinant antibodies against cNHL, a promising animal model of immunotherapeutical approaches for NHL. Within this context, a comprehensive strategy was implemented to guarantee a successful project outcome, by developing sdAbs against canine CD20, a well-known clinically validated target, and by developing novel sdAbs against cNHL antigens using an innovative strategy.

Characterization of the canine CD20 as a therapeutic target for comparative passive immunotherapy

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Adapted from a manuscript in preparation

3.1.1. Abstract

Since its approval 20 years ago, the anti-CD20 monoclonal antibody (mAb) rituximab has changed the treatment landscape for patients with B-cell NHL and established a new standard care. By prolonging survival in a range of these diseases, rituximab has reduced mortality and improved the clinical prognosis for many patients with B-cell NHL. As a result, veterinary community have been pursuing similar immunotherapeutic approaches for canine B-cell NHL, a common disease that shares remarkable similiarlities with its human counterpart and remains essentially incurable. Moreover, comparative medicine is leveraging the spontaneous canine NHL as an highly translational model to investigate the clinical potential of novel immunotherapies, complementing conventional murine studies that have failed to predict results in human clinical trials. Hence, to surpass the lack of cross reactivity of rituximab to the canine CD20 epitope, a few 1st generation mAbs targeting canine CD20 were recently developed and one has obtained clinical approval. However, these mAbs have presented disappointing scientific and clinical results, demonstrating that the discovery of an effective antibody for the treatment cNHL may require a more complex and innovative approach.

Within this context, we conducted an investigation on canine CD20 sequence gene and its protein and gene expression in a cNHL biobank. Furthermore, we report the use of a novel

strategy for the generation of recombinant anti-CD20 monoclonal antibodies that bind both human and dog epitopes. Data obtained under gene and protein expression studies on lymphoma and normal canine cells demonstrated an overexpression of this receptor on canine lymphoma cells. Additionally, a new sequence of canine CD20 was identified in our biobank samples, diverging from previous published sequences. Importantly, by successfully developing a highly diverse library of rabbit sdAbs against a novel canine CD20 sequence and by coupling subtractive antibody selection rounds on whole-cells with a high-throughput screening, we were able to identify a panel of sdAbs with high binding activity and specificity to canine and human CD20. In conclusion, these results provide new data validating the canine CD20 as a potential target for veterinary immunotherapeutic strategies. Furthermore, the exploitation of these sdAbs may become a useful tool for the development of novel therapeutic alternatives for comparative oncology.

3.1.2. Introduction

Antibody-based therapies represent one of the most promising and exciting fields in modern anti-cancer therapy. Due to their high specificity, antibodies have the ability to selectively and efficiently target a single molecule and promote the direct or indirect destruction of tumor cells (Neves & Kwok, 2015). However, despite the success of recent clinical breakthroughs, including checkpoint inhibitors and engineered T cells, only a small percentage of patients durably respond to immunotherapy and/or do not present toxicity signs (Park et al., 2016). As such, the challenge now is to extend the range of patients that benefit from immunotherapy while minimizing treatment-related adverse events. To address this, it is crucial to identify factors predictive of response that may help to properly select patients for treatment, identify rational combination therapies, and define progression and resistance (Klevorn & Teague, 2016). This is particularly critical when developing immunotherapies considering that the patient's immune system is expected to be as significant as tumor-related aspects when determining response and toxicity (Park et al., 2016).

Advances in cancer immunotherapy relies on faithful predictive preclinical investigation and rodent models have been the foundation of preliminary basic research and safety assays (Malaney et al., 2014). However, these induced-tumor models underrepresent the heterogeneity and complex interaction between the human immune cells and cancers. In fact, laboratory mice rarely develop spontaneous tumors, are housed under specific-pathogen free conditions that markedly influences immune development, and incompletely model key characteristics of the tumor/immune microenvironment, creating challenges for clinical

translation. As a result, these murine models have failed to correlate with clinical success rates (Biemar & Foti, 2013; Kohnken et al., 2017; Kola & Landis, 2004). Thereby, the use of alternative animal models is pivotal to bridge the translational gap between murine models and human clinical studies and the dog represents a powerful resource of models for cancer immunotherapy research. Dogs are an appealing outbred combination of companion animals that experience spontaneous cancer development in the setting of an intact immune system (Park et al., 2016). In addition, naturally occurring tumors in dogs present many clinical, pathological, immunologic, molecular, diagnostic and therapeutic similarities to those observed in humans, that are difficult to reproduce in other models (Gardner et al., 2016; Pinho et al., 2012; Ranieri et al., 2013; Rowell et al., 2011). This allows studying the complex immune interactions during the course of treatment while also addressing long-term efficacy and toxicity of cancer immunotherapies (Park et al., 2016).

In the last decades, the scientific community has been reporting cases of therapeutic success using mAbs in the treatment of NHL in humans. The use of a monoclonal antibody targeting the surface antigen CD20 (Rituximab®) in combination with chemotherapy regimen CHOP has revolutionized the treatment of B-cell lymphoma by significantly improving disease-free interval and overall survival, with minimal toxicity (Ito et al., 2015; Motta et al., 2010). Even though current therapy strategies have significantly improved the prognosis of patients diagnosed with NHL, the rate of mortality is still high (Molina, 2008; Zappasodi et al., 2015), ranking 6th as the most common cause of cancer-related death in the United States (US) (Kong et al., 2015; Siegel et al., 2015). Consequently, there are current research lines focused on improving anti-CD20 modalities and the use of canine Diffuse Large B-cell and Marginal Zone lymphomas, that share a similar molecular fingerprint to the human counterpart, as an animal model is one of the most promising strategies (Ito et al., 2014; Mudaliar et al., 2013). cNHL represents an heterogeneous group of malignancies that are among the most common neoplasias of dogs. Despite a wide variation in clinical presentations and histological subtypes, most dogs present with generalized lymphadenopathy (multicentric form) and intermediate to high-grade lymphoma, more commonly of B-cell origin. Without treatment, this disease is associated with high mortality (Marconato et al., 2013), requiring prompt chemotherapy to achieve temporary remission and prolonged survival. Still, cure is rarely achieved and the majority of dogs relapse with lethal, drug-resistant disease (Vail et al., 2007; Zandvliet, 2016). Thus, there is an urgent and unmet need in veterinary medicine to develop new treatment strategies for refractory disease.

Although immunotherapy plays a major role in the treatment of human B-cell malignancies, its role in canine lymphoma is still limited. Whereas immunohistochemistry using antibodies

that recognize the CD20 intracellular domains, demonstrated the presence of CD20 in canine lymphoma tissue samples (Jubala et al., 2005; Kano et al., 2005). Rituximab® and other anti-human and anti-mouse antibodies specific to the extracellular domains failed to bind to canine CD20, even though the proposed binding epitopes are conserved between human and canine CD20 (Impellizeri et al., 2006). For that reason, technology to speciate antibodies is essential when developing similar passive immunotherapy strategies for canine cancer patients. Furthermore, these efforts were also driven by the emergence on the drug discovery landscape of a pet therapeutic industry that aimed to meet increasing healthcare standards demanded by pet owners and a large clinical need (Sinha, 2014). Indeed, anti-CD20 mouse mAbs that can target canine CD20 have been reported (Ito et al., 2015; Jain et al., 2016; Rue et al., 2015). In addition, an anti-CD20 mAb (Blontress®) has been approved for canine B-cell lymphoma by the US Department of Agriculture and are commercially available in the USA and Canada. Nevertheless, the 1st generation of these mAbs are demonstrating suboptimal therapeutic efficacy, substantially inferior to results reported in human patients. As a result, the company is currently working on 2nd and 3rd generation mAbs (Klingemann, 2018; Sinha, 2014). Interestingly, what initially seemed like a relatively straightforward approach to develop a canine mAb therapy, revealed itself to be a challenging task. In fact, the development of an effective anti-CD20 passive immunotherapy for cNHL may require a comprehensive target validation and a better understanding of antibody effector functions present in the canine immune system.

Within this context and to fulfill these gaps, we conducted for the first time an investigation on canine CD20 sequence gene and its protein and gene expression in a cNHL biobank. Furthermore, we report the use of a novel strategy for the generation of recombinant anti-CD20 monoclonal antibodies that bind both human and canine epitopes. Through this approach we intend to contribute for the development of more effective immunotherapies for cNHL, while adding value to comparative oncology.

3.1.2. Material and Methods

3.1.2.1. Biological samples

- **Canine Multicentric Lymphoma Biobank**

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 after node biopsy was performed. 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 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. All sample collection was conducted with 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 extraction of plasma, serum, DNA (Dneasy Blood & Tissue, Qiagen, Hilden, Germany) and mrna (Rneasy Protect Animal Blood System, Qiagen) that were stored at -80ºc. Additionally, PBMC were isolated by Ficoll gradient method (Biocoll Separating Solution, biochrom®, Fisher Scientific, New Hampshire, USA), cell viability was assessed and for storage, aliquots of 5 x 10^6 cells were suspended in 90% FBS (Gibco, Life Technologies, Paisley, UK) and 10% 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 -80ºc 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 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 x 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.

- **Control group**

Due to ethical concerns, sterile biopsy lymph nodes samples were collected, fined cut and stored at -80ºC in RNA later™ from a healthy control group of dogs housed at the animal facility of Faculty of Veterinary Medicine - Universiteit Utrecht.

- **Cell lines and culture**

The canine B-cell lymphoma cell line CLBL-1 was provided by Dr. Barbara Rütgen (University of Vienna, Austria). The human Burkitt's lymphoma Raji cell line, the human T lymphocyte cells and the human cell line HEK293T cell line (appropriated for ectopic expression of mammalian proteins) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CLBL-1, Raji and Jurkat cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Gibco) and penicillin 100 U/ml/streptomycin 0.1 mg/ml (Gibco). 293T cell line was cultured in Dulbecco Modified Eagle Medium (DMEM) medium supplemented with 10% FCS (Gibco) and penicillin 100 U/ml/streptomycin 0.1 mg/ml (Gibco). All cell lines cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 (T75-tissue culture flasks, Greiner Bio-One, Kremsmünster, Austria).

3.1.2.2. Characterization of canine CD20 expression in a canine multicentric lymphoma biobank

- **Immunochemistry**

A representative area of each lymph node was selected and tissue sections of 3µm thickness were mounted on glass slides (Star Frost adhesive glass slides, Thermo Scientific, Rockford, USA), deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. Except for anti-CD3 antibody, all protocol steps were carried out using the Novolink Polymer Detection System (Novocastra, Leica Biosystems, Newcastle, UK), according to the

manufacturer's instructions. The antigen retrieval step was performed by microwave treatment (3 × 5 min) in Tris–EDTA buffer (pH 9.0). After endogenous peroxidase blocking and treatment with protein block solution (Protein Block Solution-Kit NovoLink™), tissue sections were incubated 1 h at room temperature with the following primary antibodies: mouse monoclonal anti-human anti-CD79 αcy (clone HM57, diluted 1:150; Dako) and mouse anti-human PAX5 (clone 1EW, 1:100, LEICA). In turn, CD3 immunostaining included endogenous peroxidase blocking by incubating the slides in 3% hydrogen peroxide in water for 30 min followed by antigen retrieval in Tris–EDTA buffer (pH 9.0), as described above. Blocking was performed with blocking solution (PBS + 0.1Tween + 5% goat serum + 2.5% Bovine Serum Albumin (BSA)), for 1 h at room temperature followed by incubation with rabbit polyclonal anti-human CD3 (1:200; Dako, Glostrup, Denmark) for 2 h. The peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (diluted 1:100, Dako) was used as secondary antibody. For all antibodies, the staining was developed by incubating the slides with the substrate diaminobenzidine (DAB kit, Zytomed Systems, Berlin, Germany). Staining without the primary antibody and staining with the isotype-matched irrelevant monoclonal antibody were used as negative controls. Human tonsil and dog spleen sections were used as positive control tissues. All slides were independently subjected to blind scoring by two independent pathologists. Discordant interpretations were further debated and settled using a multiobserver microscope.

- **Relative quantification of canine CD20 expression by real-time qPCR**

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 8. Despite the DNAse treatment, and to preclude genomic DNA amplification, primers covered putative exon–exon junctions. Optimization experiments and efficiency assessments for each amplification system were previously performed (data not shown). 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 8). qRT-PCR was performed using the 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 (1ng), 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. 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 & Schmittgen, 2001).

Table 8 - Detailed primers and conditions used for reverse PCR and real-time PCR assays for CD20 analysis.

Gene	Accession number	Sequence (5'–3')	Amplicon size (bp)	Application
CD20	NM_001048028.1	FW-GGGCCCAGGCGGCCATGACAACACCCAGAAATTCA RV-CCTGGCCGGCCTGGCCAGGGATGCTGTCGTTTTCTATT	894	RT-PCR*
		FW-TGTCTATGCGCCCATCTGTATAA RV-TTTTCCTTTGACCAAACTCTTCCT	124	qRT-PCR*
RLP27	NM_001003102	FW-TCGTCAACAAGGATGTCTTCAGAG RV-TCTTGCCAGTCTTGTACCTCTCCT	96	qPCR*

*RT-PCR – Reverse Transcriptase Polymerase Chain Reaction qRT-PCR – Real-Time Polymerase Chain Reaction

- **Analysis of canine CD20 protein expression by immunoblotting**

Lymph node cells were thawed and washed twice with PBS. Total protein was extracted using RIPA lysis buffer (25mM TrisHCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodiumdeoxycholate, 0.1% SDS) supplemented with fresh protease inhibitors (Roche) and phosphatase inhibitors cocktail (Sigma). Samples were quantified by the Bradford method. CD20 receptor expression was demonstrated by Western blot analysis. Total protein extract samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, proteins were incubated with Anti-CD20 antibody (polyclonal, rabbit, 1:500 dilution, Thermo Fisher Scientific, Rockford, IL, USA) and then with Peroxidase-AffiniPure

Anti-Rabbit IgG antibody (polyclonal, goat, 1:10000 dilution, Jackson ImmunoResearch, PA, USA) as a secondary antibody. Proteins were visualized using Luminata Forte Western HRP (Merck Millipore, Darmstadt, Germany) and acquired using the ChemiDoc XRS+ imaging system (Bio-Rad, California, USA). Beta-actin expression were used as protein loading control using an Anti-βactin antibody (AC-15 clone, mouse, 1:10000 dilution, Sigma-Aldrich) as a primary antibody and an Anti-Mouse IgG antibody (polyclonal, goat, 1:7500 dilution, Jackson ImmunoResearch) as a secondary antibody. Bioinformatic tools using Image Lab (Biorad) were used to quantify CD20 expression and demonstrate differences between samples.

- **Sequence analysis of the canine CD20 gene**

The canine CD20 was cloned by RT-PCR. For that purpose, mRNA was isolated from lymph nodes samples of the canine lymphoma biobank and control group using Rneasy Mini Kit (Qiagen GmbH; Hilden, Alemanha). To eliminate possible contaminant DNA, total RNA samples were subjected to DNAse treatment, using RNase-free DNase Set (Promega; Wood Hollow road, Madison, E.U.A.), following the manufacturer's instructions. Thereafter, first-strand cDNA was synthesized using Transcriptor High Fidelity (Roche) following the manufacturer's instructions and used as a template for RT-PCR using primers presented in Table 8, designed at the ends of reported sequence of canine CD20. Sequencing was performed by GATC. Translation to amino acid sequences, multiple sequence alignment and phylogenetic analysis were performed using the "Vector NTI" software. Canine CD20 protein secondary structure prediction was made using the InterPro platform on UniProt.

- **Statistical analysis**

All data are expressed as mean ± standard error of mean (SEM). All statistical analyses were carried out using R-software. Normality test was performed using the Shapiro-wilks test. The distribution of CD20 ct values passed the normality test and lymphoma and control groups were therefore compared using Welch Two Sample t-test. The significance level was set at 5%.

3.1.2.3. Generation of monoclonal antibodies against canine CD20

- **Cloning of canine CD20**

The canine CD20 was cloned by RT-PCR from lymph node samples of a canine multicentric biobank case (B2) following the conditions previously mentioned above. PCR products were digested with SfiI (Roche) then purified and cloned into the FUGW at SfiI sites. Then, the ligated product was transformed into *E.coli* BL21 competent cells via thermal shock. 10 colonies were picked and PCR colony was performed using primer cCD20-F and primer cCD20-R (Table 8).

- **Transfection of canine CD20 into HEK 293T cells**

The HEK 293T cells (ATCC) were transfected with plasmid DNA using the FuGENE® HD (Roche), according to the manufacturer's instructions. HEK 293T cells were grown as monolayers in DMEM supplemented with 10% FCS and 3 µg of pFUGW vector CD20 was added. The HEK 293T transfected cells were incubated for 48 hours. Canine CD20 transient expression was confirmed by immunoblotting for canine CD20 expression (Thermo Scientific) and GFP staining with flow cytometry analysis. In parallel, a HEK 293T^{hCD20} cell line was simultaneously generated for cross-reactivity analysis purposes, using identical methodology.

- **Rabbit immunization**

A New Zealand White rabbit was immunized with HEK 293T cells transfected with canine CD20 vector. The injections were administrated subcutaneously at 2–3 week intervals during 4 months.

- **Rabbits serum titer against canine CD20**

To evaluate the serum specificity and titer, before each immunization blood was harvested from the marginal ear vein for serum isolation. Serum was analyzed by enzyme-linked immunosorbent assay (ELISA) and immunoblotting using CLBL-1 cell line, Raji cell line and lymphoma primary cells and Peroxidase-conjugated goat anti-rabbit antibody (Jackson Immune Research) as secondary antibody. HEK 293T cells with canine CD20 vector, CLBL-

1 cells, Raji cells and HEK 293T cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, proteins were probed with rabbit serum (dilution 1:5000) and then with HRP conjugated anti-rabbit mAb as a secondary antibody. As a control, we used pre-bleed sera.

- **sdAbs immune library construction**

Five days after the final boost, spleen and bone marrow were harvested separately for total RNA using TRI Reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized using Transcriptor High Fidelity (Roche) following the manufacturer's instructions. PCR was performed to amplify the heavy-chain variable (VH) and light-chain variable regions (VL) from the rabbit's cDNA using the set of primers presented at Table 9. PCR products encoding a library of antibody fragments (sdAbs) were then gel purified, restriction digested with *Sfi*I and cloned into pCOMB3xSS. Subsequently, the ligated product was transformed into electro competent cells via electroporation and the library was tittered. To confirm library insert efficiency and diversity, PCR colony was performed using primer RSC-F (5´GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC 3') and primer RSC-B (5'GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG 3').

Table 9 - Nucleotide sequences of primers used for the construction of a sdAbs library.

	VH* Forward Primers sequences (5′–3′)
SDVH1-F	GGGCCCAGGCGGCCCAGTCGGTGGAGGAGTCCRGG
SDVH2 -F	GGGCCCAGGCGGCCCAGTCGGTGAAGGAGTCCGAG
SDVH3-F	GGGCCCAGGCGGCCCAGTCGYTGGAGGAGTCCGGG
SDVH4-F	GGGCCCAGGCGGCCCAGSAGCAGCTGRTGGAGTCCGG
	VH* Reverse Primers sequences (5′–3′)
SDG-R	CCTGGCCGGCCTGGCCACTAGTGACTGAYGGAGCCTTAGGTTGCCC
	VL* Forward Primers sequences (5′–3′)
RSCVK1-F	GGGCCCAGGCGGCCGAGCTCGTGMTGACCCAGACTCCA
RSCVK2-F	’GGGCCCAGGCGGCCGAGCTCGATMTGACCCAGACTCCA
RSCVK3-F	GGGCCCAGGCGGCCGAGCTCGTGATGACCCAGACTGAA
RSCλ-F	GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGTCGCCCTC
	VL* Reverse Primers sequences (5′–3′)
SDVLj10-B	CCTGGCCGGCCTGGCCTTTGATTTCCACATTGGTGCC
SDVLj0-B	CCTGGCCGGCCTGGCCTAGGATCTCCAGCTCGGTCCC
SDVL42j0-B	’CCTGGCCGGCCTGGCCTTTGACSACCACCTCGGTCCC
SDVLλ-B	CCTGGCCGGCCTGGCCGCCTGTGACGGTCAGCTGGGTCCC

*VH – Heavy Chain variable domain, VL – Light Chain variable domain

3.1.2.4. Selection of recombinant monoclonal antibodies against canine CD20

- **Selection of anti-CD20 sdAbs by Phage Display**

To display anti-CD20 sdAbs on M13 phage, sdAbs library was infected with M13 helper phage (10^{12} plaque-forming units/ml) during 1 h at 37 °C and grown overnight. Phages were precipitated, and titer was determined. The phage library displaying sdAbs was panned using a subtractive cell phage display protocol as described in Basbas studies, including a negative selection on HEK 293T cells followed by a positive selection on CLBL-1 cells (Barbas, Burton, Scott, & Silverman, 2004; Popkov, Rader, & Barbas, 2004). Three rounds of phage display panning were performed in order to select the best candidates.

- ***In vitro* binding and expression studies**

To express and select anti-CD20–sdAbs, phagemid DNA encoding selected anti-CD20 sdAbs was cloned into pT7-PL (pT7-peptide leader) vector and transformed into *E. coli* strain BL21. Library insert efficiency was confirmed by colony PCR. The colonies obtained were picked and incubated overnight at 30°C on 100 µl of Super Broth (SB) medium containing Overnight Express™ Autoinduction System 1 (Novagen®) and 100 µg/ml of Ampicilin. Next day, 40 µl of BugBuster (Roche) containing anti-protease cocktail-EDTA free inhibitors (Roche) were added and incubated for 30 minutes at 4°C. Next, the plates were centrifuged at 1200 rpm and the supernatant was used to perform an ELISA assay. Three different parameters were analyzed: binding of antibodies to the antigen, expression level and unspecific binding. To evaluate the binding to the antigen (CD20), plates with the total CLBL-1 extract attached were used, washed with PBS and blocked with BSA 3% for 1 hour at 30°C. Then, plates were washed and the supernatant containing VLs was incubated for 1 hour at 30°C. Plates were washed and incubated with anti-HA-HRP antibody (Roche) was added. After 1 hour of incubation, plates were washed, ABTS (Roche) substrate solution was added and optical density at 405 nm was measured at different time points. To evaluate the level of expression, the same process was carried out, except for the antigen coating. To evaluate the unspecific binding, in turn of the antigen, BSA 3% was added. Rabbit serum and anti-CD20 antibody were used as positive controls. BL21 cell extracts and SB medium were used as negative controls.

- **Sequencing of anti-CD20 sdAbs**

Sequencing was performed by GATC Biotech AG (Ebersberg, Germany) using the pComb3x ATG primer (5’ GGG CCC AGG CGG CCA TGA CAA CAC CCA GAA ATT CA 3’). To translate to amino acid sequences and to evaluate homology, the Vector NTI Advance 10 software (Thermo Fisher Scientific) was used.

3.1.3. Results

3.1.3.1. Characterization of canine CD20 expression in a canine multicentric lymphoma biobank

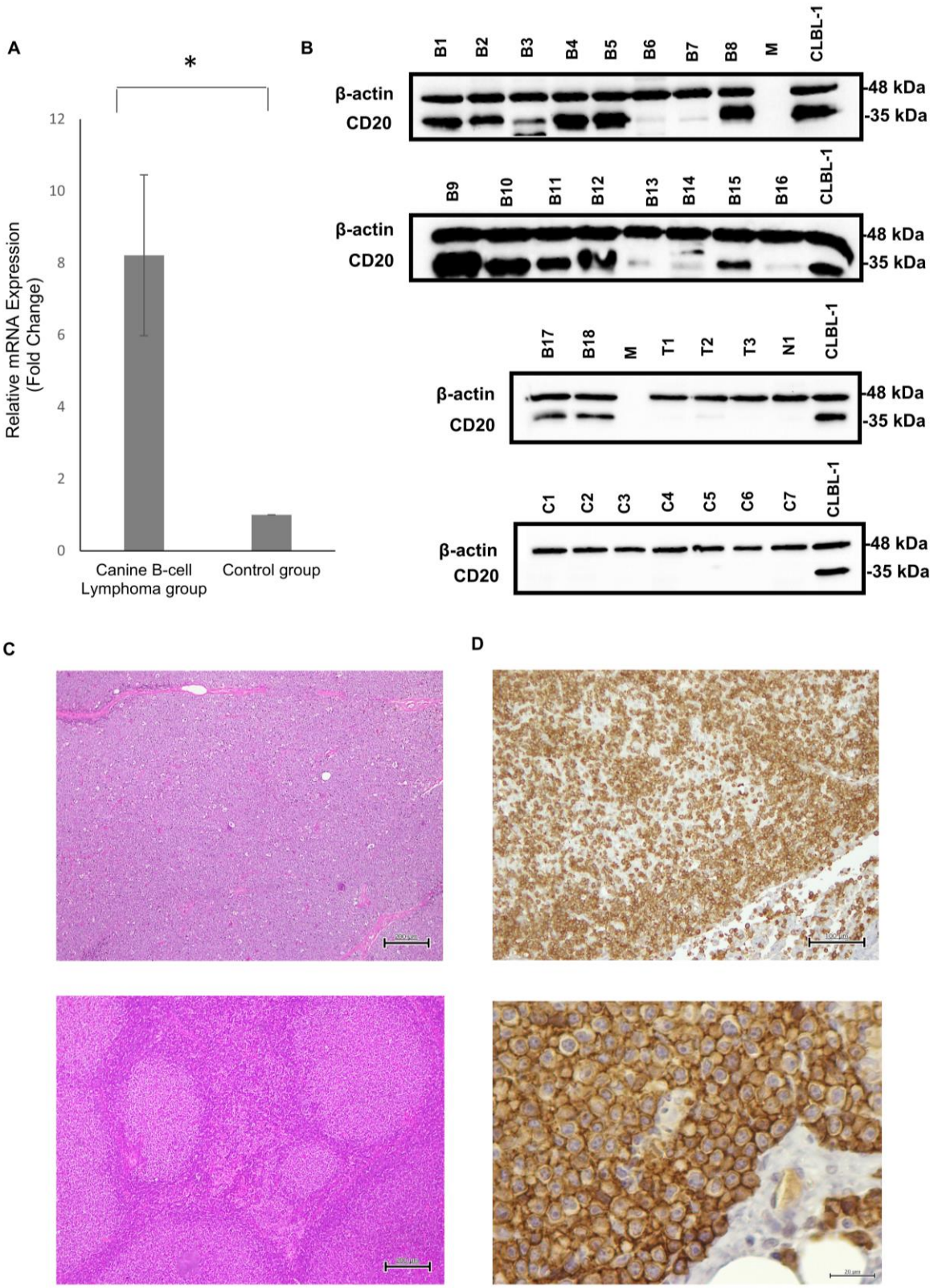
The selection of tumor antigens suitable for antibody targeting and immunotherapy demands an extensive investigation of tumor and normal tissue expression (Scott, Allison, & Wolchok, 2012). CD20 is highly expressed by over 95% of B cell lymphocytes throughout their development, from the pre-B cell stage until their final differentiation into plasma cells, but is absent on the hematopoietic stem cell (Kosmas, Stamatopoulos, Stavroyianni, Tsavaris, & Papadaki, 2002). Thereby, CD20 is a lineage specific receptor, being expressed in both healthy and the majority of malignant B-cells (Einfeld, Brown, Valentine, Clark, & Ledbetter, 1988). Its expression has been demonstrated to be heterogeneous between and within different subtypes of the human B-NHL and has been considered a predictor of response to rituximab containing treatment in patients with B-cell lymphomas (Prevodnik, Lavrenčak, Horvat, & Novakovič, 2011). As such, a comprehensive analysis of the expression of CD20 in both healthy and B-cell lymphoma diagnosed dogs might also have a critical role in the development of a similar canine anti-CD20 immunotherapy. Therefore, to further characterize CD20 gene and protein expression in canine lymphoma, relative quantification of gene expression by qRT-PCR and western blot analysis was performed.

Following histopathological and immunochemistry analysis by a veterinary pathologist, canine lymphoma cases were divided in three different groups: B-cell lymphoma, T-cell lymphoma and non-B non-T cell lymphoma. CD20 immunophenotype was confirmed in all samples before gene and protein expression analysis (Figure 23C).

Overall, qRT-PCR demonstrated that mRNA expression level of canine CD20 was significantly higher in B-cell lymphoma samples when compared with healthy lymph nodes and non-B-cell lymphoma samples, including T-cell and non-B and non-T cell lymphoma samples ($p < 0,05$). This increment was quantified as 8.2 ± 2.23 times fold, validating the CD20 mRNA overexpression in canine B-cell lymphoma samples (Figure 23A). Regarding CD20 protein expression by western blot analysis, all B-cell lymphoma samples demonstrated a CD20-positive phenotype. Moreover, CD20 expression was found to be heterogeneous on B-cell lymphoma samples. On the contrary, healthy samples presented a scanty CD20 expression, as well as T-cell and non-B and non-T cell lymphoma samples (Figure 23B). These findings corroborated the qRT-PCR data, demonstrating a canine CD20 overexpression

in lymphoma samples and validating this receptor as an important lymphoma immunotherapy target.

Figure 23 - Characterization of canine CD20 expression in a canine multicentric lymphoma biobank.



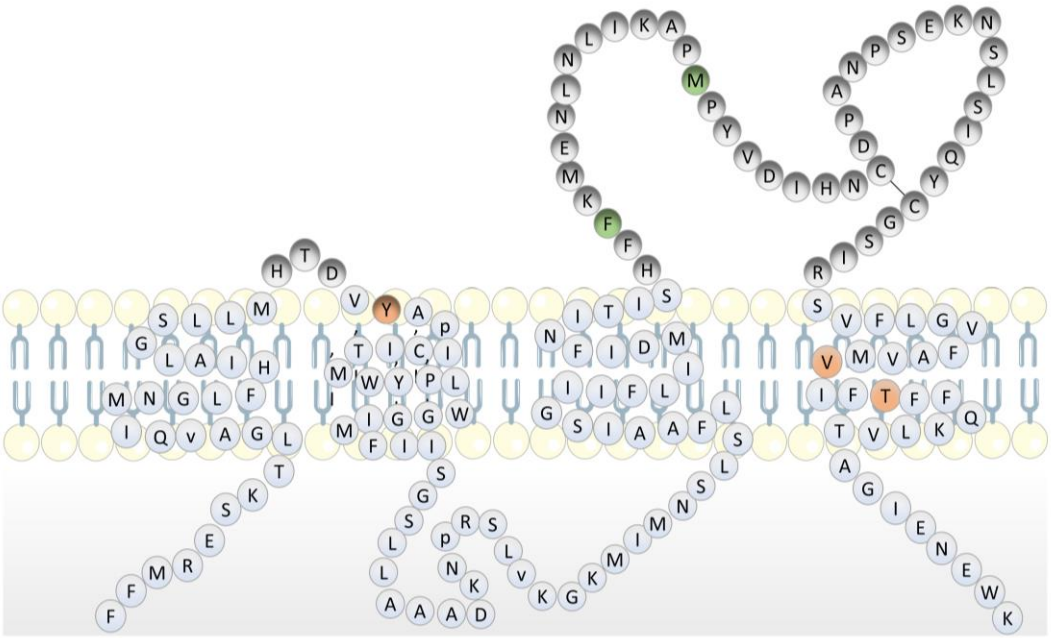
(**A**) Relative cytokine mRNA expression in cNHL tumor tissue. Results are expressed as a fold difference between mean ± SEM of mRNA expression level in cNHL affected lymph nodes samples and a control group with healthy donors. * $p < 0,05$. (**B**) Total protein was extracted from lymph nodes samples from cNHL biobank and healthy donor samples and CD20 expression were assessed by western blotting. β-actina was used for loading control. Representative blots are shown. B1-B18 refers to B-cell lymphoma samples, T1-T3 to T-cell lymphoma samples, N1 to a non-B non-T cell lymphoma and C1-C7 to control samples. (**C**) Upper panel – Diffuse growth pattern of large lymphocytes of a canine DLBCL (H&E, x40). Bottom panel – Nodular growth pattern of closely packed lymphocytes of a follicular lymphoma of a canine patient (H&E, x40). (**D**) Imunohistochemistry of a canine PTCL showing positivity in virtually 100% of the tumor cells (anti-CD3 antibody, Gill's hematoxylin, x100). Bottom panel – Immunohistochemistry of a canine DLBCL showing positivity in virtually 100% of the tumor cells (anti-CD20 antibody, Gill's hematoxylin, x 200).

3.1.3.2. Sequence analysis of the canine CD20 gene

The canine CD20 amino acid sequences exhibit 73 and 68% sequence similarities with human (X12530) and mouse (AB045181) CD20 sequences, respectively, suggesting that the CD20 receptor might play a role in the immune system similar to other mammals. In fact, the canine *CD20* was predicted to contain four membranes spanning domains, three intracellular domains including the N - and C – terminal domains and two extracellular loops as the human CD20 (Riley & Sliwkowski, 2000; Tedder et al., 1989). Moreover, potential differences in the coding sequence of the canine CD20 gene might explain the absence of cross-reaction between the two species, especially if these genetic alterations occur in the specific binding domains. The full-length of canine CD20 cDNAs was sequenced using canine mRNA from our biobank as a template. The full-length of the cDNA sequence of canine *CD20* was 894 bp encoding 298 amino acids. Lymphoma lymph nodes, PBMCs and lymph nodes from healthy donors were analyzed and presented an analogous CD20 sequence. This canine sequence diverged by eight mutations when compared with previous published sequences (Ito et al., 2015; Kano et al., 2005), of which six resulted in amino acid substitution and two corresponded to a silence mutations. As shown in Figure 24, the majority of the coding differences found are positioned on CD20 cytoplasmic and transmembrane portions, while differences in residues 147 and 159 are located in the larger extracellular loop. Importantly, our canine CD20 sequence presented an 82.2% similarity with human CD20 gene,

demonstrating that identified mutations resulted in a higher resemblance with human CD20 gene.

Figure 24 - Sequence analysis of the canine CD20 gene in a canine lymphoma biobank based on UniProt database prediction.



Position	Kano et al., 2005	Ito et al., 2015	Obtained sequence
77	Cysteine	Tyrosine	Tyrosine
147	Phenylalanine	Leucine	Phenylalanine
159	Methionine	Isoleucine	Methionine
198	Leucine	Valine	Valine
201	Alanine	Threonine	Threonine
273	Glycine	Glutamate	Glutamate

Different amino acid residues between the canine CD20 sequences. The aminoacid residues that differ from the sequence reportated by Kano et al. 2005 are indicated in orange, while differences from the sequence reported by Ito at. 2015 are indicated in green. Detailed information about position and aminoacid residue is shown in the table.

3.1.3.3. Development of monoclonal antibodies against canine CD20

- **Generation and screening of high-titered antisera against canine CD20**

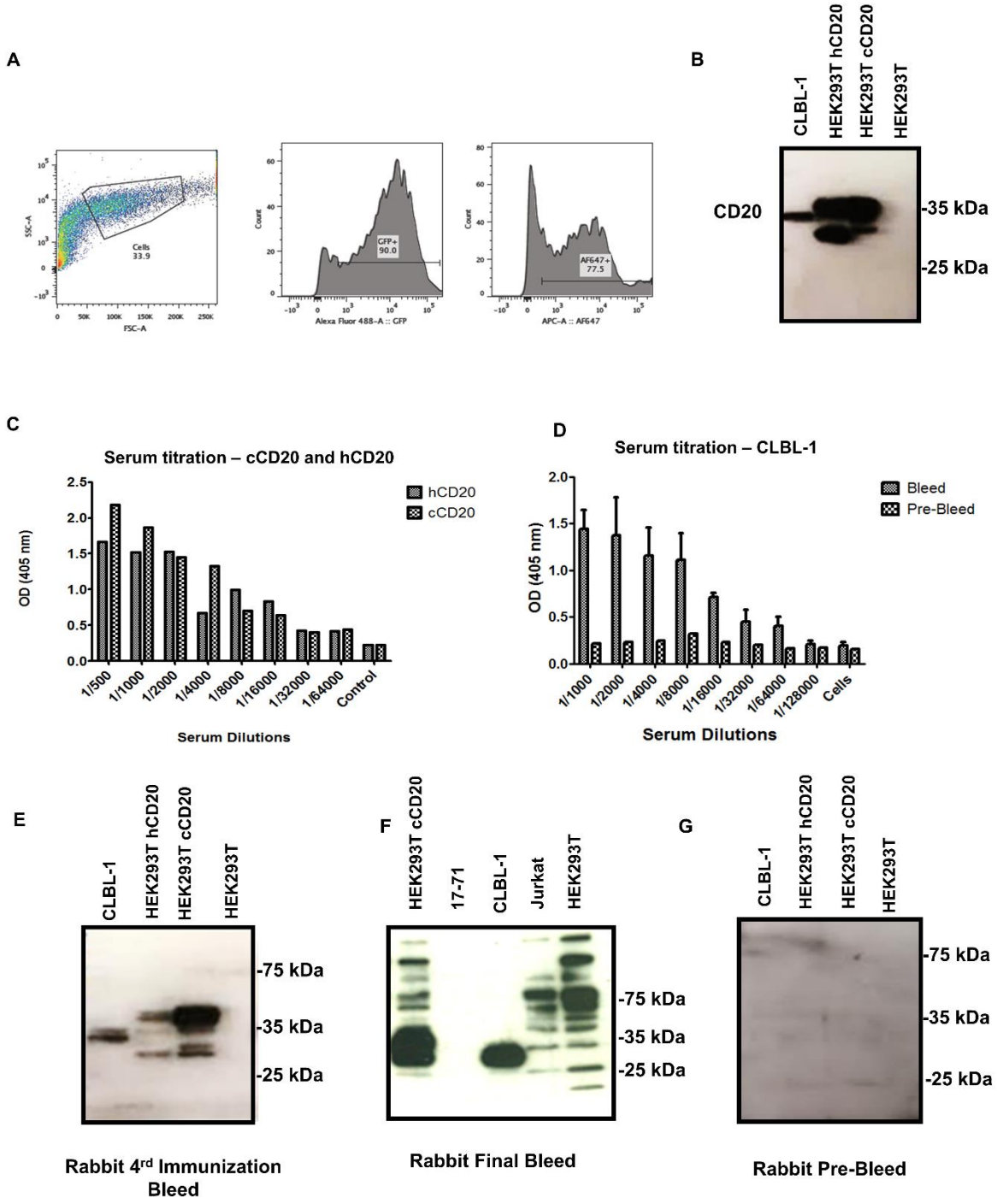
The HEK 293T cell line was transfected with a pFUGW vector encoding canine CD20 and GFP reporters, as described in the material and methods section, for rabbit immunization. While flow cytometry analysis confirmed GFP expression in HEK 293T^{cCD20} cell line (Figure 25A), immunoblotting analysis established CD20 expression (Figure 25B). In parallel, HEK 293T^{hCD20} was simultaneously generated for cross-reactivity analysis purposes (data not shown).

The canine CD20 antiserum was successfully produced from a female New Zealand white rabbit immunized with HEK 293T cells transfected with canine CD20 vector. Antibody titers and specificity were monitored by western blot and ELISA. As shown in Figure 25C, cell ELISA assays showed that final bleed serum recognized both HEK 293T^{cCD20} and HEK 293T^{hCD20} cells contrarily to pre-bleed serum, confirming cross-reactivity against human CD20. Moreover, these data demonstrated that immunizations resulted in a strong immune response with a high serum titer (1:60.000). Similar results were obtained using CLBL-1 cells, that express CD20 in its native form (Figure 25D).

To further test binding specificity of rabbit serum against canine CD20 and human CD20, sera was evaluated by immunoblotting. After four immunizations, rabbit sera specifically recognized the 33 kDa canine CD20 protein expressed by CLBL-1 cells and HEK 293T cells transfected with canine CD20 vector and the 35 kDa human CD20 protein expressed by HEK 293T cells transfected with human CD20 (Figure 25E). Importantly, western blot assays demonstrated that, with the course of immunizations, rabbit sera started presenting weaker signs against unspecific proteins expressed by HEK 293T cells and Jurkat (Figure 25F). These results were essential for the design of the phage display screening. In agreement, rabbit pre-bleed, harvested before starting immunizations, did not show any signal against immunized cell or control cells (Figure 25G).

Altogether, these results allowed to confirm a high antisera titer against canine and human CD20, indicating a specific B-cell reaction and assuring a high-quality antibody gene library. As a result, the animal was sacrificed and the bone marrow and the spleen - major repositories of plasma cells that secretes antibodies - were extracted for total RNA preparation and cDNA synthesis.

Figure 25 - Generation and screening of high-titered antisera against canine CD20.



(**A**) Histogram of HEK 293T^{cCD20} cell line construction, representing GFP expression analysis by FACS. (**B**) Canine and human CD20 phenotype was confirmed by western blotting analysis. Total protein extracts of both cell lines were probed with an anti-CD20 antibody using HRP-conjugated goat anti-rabbit Fc polyclonal antibody as secondary antibody. CLBL-1 extract, a canine B-cell lymphoma cell line with confirmed CD20 expression, was used as a positive control and HEK293T as a negative control. (**C**) Titration and binding activity of serum antibodies corresponding to pre-bleed and final bleed. Antisera from the immunized rabbit was analyzed for binding activity to both HEK 293TcCD20 and HEK 293ThCD20 cell lines by cell ELISA using HRP-conjugated goat anti-rabbit Fc polyclonal antibody as secondary antibody. Data were obtained by Abs measurement at 405 nm with reference a

492 nm. Values represent mean±standard deviation of triplicates (n=3) for each condition, and similar results were obtained in two independent experiments. (**D**) The same experiment was carried out to analyze binding activity to CLBL-1 cell line. (**E**) Immunized rabbit serum specificity was analyzed by Western blotting. Total protein extracts of HEK 293T^{cCD20}, HEK 293T^{hCD20}, CLBL-1 and HEK 293T cell lines were probed with the serum extracted during the 4th immunization. HRP-conjugated goat anti-rabbit Fc polyclonal antibody was used as secondary antibody. Representative blots are shown. Protein profiles recognized by rabbit polyclonal revealed specificity of the immune response. (**F**) Immunized rabbit final bleed specificity was analyzed by Western blotting. Total protein extracts of HEK 293T^{cCD20}, CLBL-1, 17-71 (canine B-cell lymphoma cell line not expressing CD20), Jurkat (T-cell leukemia cell line) and cell HEK 293T cell lines were probed with the final serum. HRP-conjugated goat anti-rabbit Fc polyclonal antibody was used as secondary antibody. Representative blots are shown. Protein profiles recognized by rabbit polyclonal revealed specificity of the immune response, as well as, an unspecific response against other targets. (**G**) Immunized rabbit pre-bleed specificity was analyzed by Western blotting, as mentioned above in panel E. No binding activity to protein extracts before the start of rabbit immunizations was confirmed.

- **Construction of a rabbit anti-CD20 sdAb phage display library**

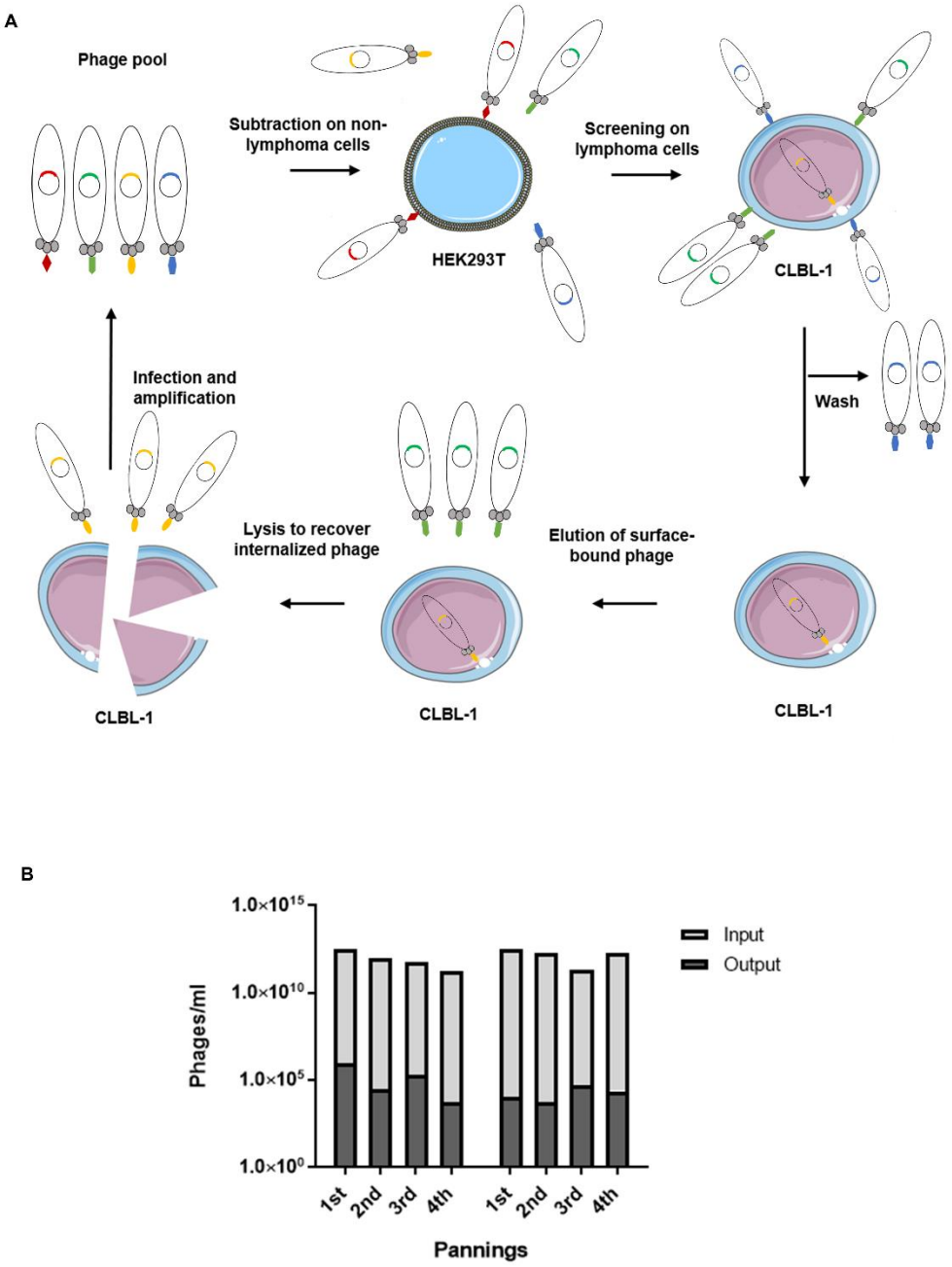
A rabbit sdAb library was constructed using RNA from spleen and bone marrow cells of a rabbit previously immunized with HEK 293T cells transfected with canine CD20 vector. For that purpose, specific oligonucleotide primers covering all known variable rabbit antibody family sequences were used to amplify VH and VL gene segments (Table 9). The recombinant phagemid pComb3X containing the VL sdAb genes was transformed into *E. coli* ER2538 cells to yield 2,25 x 10^6 and 6,42 x 10^6 individual clones for bone marrow and spleen, respectively. Rabbit repertoire presented an efficiency of 100% in all libraries.

- **Selection of recombinant monoclonal antibodies against canine CD20**

Selection of specific sdAbs for the CD20 receptor were performed using a subtractive cell phage display as described in the material and methods section. This cell phage display screening protocol was based on the previously Carlos Barbas studies, in which it is reported a novel whole-cell selection protocol with negative and positive selection steps (subtractive phage display) designed to remove antibodies reacting with common antigens (Popkov et al., 2004). Phage display conditions implemented herein are summarized in Figure 26A. VL library was selected for phage display screening due to high diversity presented by VL repertoire compared to rabbit VH repertoire. Other advantages of the rabbit VL repertoire

includes its higher diversity and a larger complementarity-determining region (CDR)-L3 loop length, when compared to its mice and human counterpart. (Knight, 1992; Mage, Lanning, & Knight, 2006; Ros et al., 2004; Sehgal, Johnson, Wu, & Mage, 1999; Zhang, Liu, Guan, Wang, & Yuan, 2017). Briefly, four pannings were performed with a subtractive selection including a negative and a positive selection. To promote the elimination of non-specific antibodies, a negative selection was performed using the HEK 293T cell line, which do not express the CD20. In addition, the CLBL-1 cell line was used for the positive selection due to its stable expression of CD20 receptor. Over the course of selection, stringency was improved by increasing the number of washes in order to collect the sdAb-phage clones with greater target affinity or specificity. Different elution methods were performed to select for binders and internalized antibodies (Figure 26A). As shown in Figure 26B, the phage display rounds resulted in a lower number of phages in the output titers compared to the input titers ($\sim 10^{11\text{-}12}$ to $\sim 10^{5\text{-}4}$ pfu). Furthermore, the biopannings profile indicates that the Phage Display protocol successfully lead to the enrichment for CD20 specific antibodies-phages.

Figure 26 – Phage display selection of highly specific anti-CD20 sdAbs.



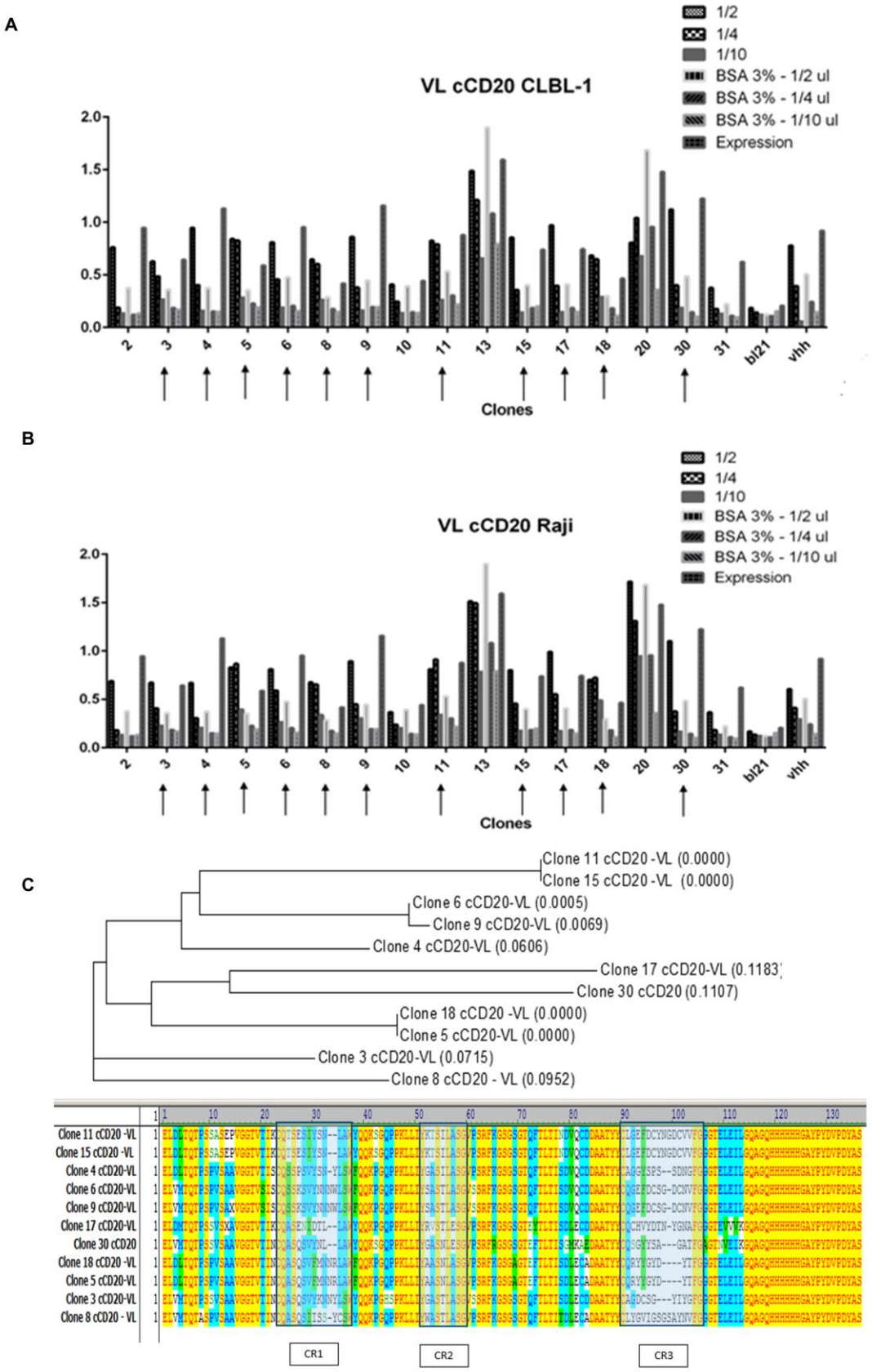
(**A**) Schematic representation of subtractive whole-cell phage display. The antibody-phage library was initially subtracted on cell not expressing CD20 (HEK293T cells), and then the free phages were screened on cells harbouring CD20 receptor (CLBL-1 cells). A washing step was performed to remove all the non-specific and unbound phage. Cell surface bound phage were removed by tripsin elution, while the internalized phages were retrieved by cell lysis. The isolated phage particles were then amplified and used for subsequent panning rounds. (**B**) Graphic representation of amplified and eluted phage titers of each round of phage display selections. sdAbs with binding (binders) or internalization (internalizers) properties are shown. The titers were determined by virions or plaque forming units per milliliter measurement.

To further select the best candidates based on expression and binding features, the last panning was cloned into a pT7-PL vector. This vector possesses a peptide leader, which promotes the sdAbs expression to be directed to the periplasmic space, allowing to evaluate the expression level of sdAbs. Following, an high throughput screening was performed on a total of 528 clones, in which multiple ELISA assays were carried out to evaluate expression and binding to the protein extract from CLBL-1 cells. This study allowed to select 12% of clones that revealed the stronger signal regarding binding to protein cell extract and expression. Clone binding and expression activity were further tested and confirmed by ELISA (Figure 27A), which allowed to select the 11 best candidates. In addition, to evaluate clones cross-reactivity against human CD20, ELISA assays were performed using Raji extract. As shown in Figure 27B, all selected clones presented cross-reactivity against human CD20.

- **Sequencing of anti-CD20 sdAbs**

To evaluate anti-CD20 sdAbs coding sequence, selected clones were sequenced by Sanger sequencing and sequence alignment and phylogenetic analysis were performed using the "Vector NTI" software., as shown in Figure 27C.

Figure 27 - Selection of recombinant monoclonal antibodies against canine CD20.



(**A**) Relative binding activity of sdAbs. The lead candidates of high throughput selection were analyzed by ELISA to evaluate binding activity against CLBL-1 protein extracts and BSA and expression levels. HRP-conjugated anti-HA mAb was used as secondary antibody. Results were measured by optical density at 405 nm. BL21 extracts and a non-related VHH antibody were used as negative controls. (**B**) The same experiment was carried out to evaluate lead candidates binding activity against Raji protein extract. (**C**) Sequence homology and diversity analysis of the eleven lead candidates.

3.1.4. Discussion

Comparative medicine within the one world health framework offers an important platform with complex cross-species models that allows the research of novel therapeutic agents and approaches for human diseases (Gardner et al., 2016). Therefore, collaborative interdisciplinary efforts are being made in order to contribute for the development of innovative immunodiagnostic and immunotherapeutics models bringing canine and human diseases together (Park et al., 2016). Cancer remains a major cause of mortality and morbidity and its devastating consequences also affects our companion animals (Baioni et al., 2017; Fitzmaurice et al., 2017). Approximately 1 in 4 dogs will, at some stage in their life, develop a type of neoplasia and canine lymphoma is one of the most common neoplasias (Dorn, Taylor, Chaulk, & Hibbard, 1966; Dorn, Taylor, & Schneider, 1970; Dorn, Taylor, Schneider, Hibbard, & Klauber, 1968; Kidd, 2008; Merlo et al., 2008; Teske, 1994). Chemotherapy still remains the mainstay for the treatment of canine lymphoma and regardless of the numerous published chemotherapeutic protocols, it seems we have reached a stalemate concerning what this treatment modality has to offer in standard settings (Argyle & Pecceu, 2016). The 12 month median survival barrier and the 20% to 25% 2 years survival rates demonstrate an urgent and unmet need in veterinary medicine to develop new treatment strategies for refractory disease (Vail et al., 2007). In the last decade, the scientific community has been reporting cases of therapeutic success using mAbs in the treatment of lymphoma in humans (Adler & Dimitrov, 2012). One of the most successful examples has been the application of the monoclonal antibody Rituximab towards a growing range of disease indications (Chames et al., 2009). Rituximab is a chimeric monoclonal antibody with humanized constant chains fused recombinantly to murine variable domains (Griffin & Morley, 2013). Since the approval of rituximab in 1997 five other mabs have been approved by the FDA for the treatment of B-cell NHL and HL, including two anti-CD20 agents; tositumomab and ibritumomab; which are full-length mouse monoclonal antibodies that are conjugated to a radiochemical carrier (Cang,

Mukhi, Wang, & Liu, 2012). Although, therapeutic options for humans with NHL have improved remarkably and some of the more aggressive forms of lymphoma may at times be cured with combination chemo/immunotherapy, almost all patients with low-grade lymphoma and approximately 50% of patients with high-grade lymphoma die of this disease (Molina, 2008; Zappasodi et al., 2015). Few chemotherapy agents were recently incorporated into the treatment of lymphomas and the interest over the last years has mainly focused on the development of targeted compounds, such as mAbs (Stathis & Ghielmini, 2012).

The successful introduction of antibody-based proteins into the arsenal of therapeutics was highly promoted by the innovation in the production and engineering of antibodies (Chiu & Gilliland, 2016). The application of recombinant or synthetic biologic allows the construction of the most appropriate bio-conjugated to a specific platform, whether by using conventional antibodies with functional Fc domains, by developing antibody-drug conjugates that deliver targeted radiation or toxins or by constructing recombinant nanobodies that are suitable to selected manipulation (Jain et al., 2016). The latter have rapidly become one of the new-generation of antibodies and are a promising alternative to conventional mAbs.

We have been showing that rabbit derived sdAbs can be developed against several targets and that these minimal scaffolds show great potential for therapeutic applications (Corte-Real et al., 2005; da Silva et al., 2012). sdAbs are presently the smallest functional antibody fragment, only consisting of an antibody heavy-chain or light-chain variable domains (VH or VL). The small size of these fragments allows to improve tumor penetration and accessibility to targets not easily reached by IgGs (Aires da Silva et al., 2008; Goncalves & Aires-Da-Silva, 2008; Volker et al., 2016). In addition to the reduced size, complementary-determining regions (CDRs; antigen-binding regions) of sdAbs can be easily engineered to develop specific and high-affinity binders. Moreover, sdAbs also present excellent properties such as high stability, solubility, low immunogenicity and low manufacturing cost (Aires da Silva et al., 2008; Volker et al., 2016).

In our approach we explore the properties of these recombinant molecules for the development of rabbit sAbs towards canine CD20 protein, with cross-reactivity against human CD20. Our strategy comprised (i) the characterization of canine CD20 mRNA and protein expression and sequence analysis on a canine multicentric lymphoma biobank; (ii) the construction of a canine anti-CD20 library by amplifying the variable genes from cDNA derived from mRNA from the spleen and bone marrow of a rabbit immunized with HEK 293T with canine CD20 vector cells (iii) the selection of specific sdAbs for the canine CD20 receptor were performed using a subtractive whole-cell phage display followed by a high throughput screening.

To the best of our knowledge, we conducted for the first time a comprehensive target characterization of the canine CD20. This data is crucial to determine if tumor associated antigen specificity will guarantee high response rates and low toxicity (Scott et al., 2012). Data obtained under gene and protein expression studies on lymphoma and normal canine cells demonstrated an overexpression of this receptor on canine lymphoma cells, validating the canine CD20 as a potential target for veterinary immunotherapeutic strategies. Furthermore, when designing a passive immunotherapy similar to rituximab other clinical challenges must be considered, such as treatment resistance (Tsai, Hernandez-Ilizaliturri, Bangia, Olejniczak, & Czuczman, 2012). In fact, in human medicine only ~50% of patients with follicular lymphoma (FL) respond to initial treatment with single-agent rituximab (McLaughlin et al., 1998) and the majority of responders eventually become refractory to rituximab (Davis et al., 2000). Thus, there is a need to understand both the determinants responsible for *de novo* rituximab sensitivity as well as the mechanisms leading to acquired resistance.

On one hand, a proven predictor of innate rituximab sensitivity is CD20 surface expression in rituximab–naïve B cells (Meerten, Rijn, Hol, Hagenbeek, & Ebeling, 2006), demonstrating the importance of CD20 expression analysis before implementing an anti-CD20 immunotherapy. Indeed, lymphomas present heterogeneous levels of CD20 expression according to lymphoma types. For example, CD20 expression is higher in FL and lower in CLL (Chronic Lymphocytic Leukemia), which may explain the lower response rate to rituximab of CLL cell lines (Meerten et al., 2006). Acquired rituximab resistance following therapy exposure has likewise been associated with reduced levels of CD20, that are correlated with CD20 mRNA levels, suggesting transcriptional-level control (Czuczman et al., 2008; Small, McLeod, & Richards, 2013). Therefore, by characterizing gene and protein CD20 expression levels on a canine lymphoma naïve patient biobank, we hope to contribute for the research of predictive factor of response and possible mechanism of resistance that can overtime limit therapy responses, essential for the successful implementation of a canine anti-CD20 antibody-based therapy.

In addition, we have identified, for the first time, non-conservative amino acid differences on the canine CD20 protein sequence. Considering that these differences were observed in all canine lymphoma biobank, CLBL-1 cell line and healthy donors' lymph nodes samples, we believed that this might be the most predominant sequence of the canine CD20 to present. Curiously, non-conservative amino acid differences recognized promoted a greater resemblance to human CD20 counterpart.

The human CD20 is a nonglycosylated 30 to 35-kDa integral membrane non-glycosylated phosphoprotein (Perosa et al., 2009). The 16-kb gene encoding human CD20, consists of 8 exons that have been mapped to chromosome 11 (11q12-q13) and belong to the MS4A (membrane spanning 4A) gene family (Henry et al., 2010). The physiological ligand and exact biological function remains unclear, although it is proposed that it may be involved in calcium signaling downstream of B cell antigen receptor activation (Boross & Leusen, 2012; Walshe et al., 2008). Initial analysis of CD20 deficient mice showed no immune-deficient phenotype (O'Keefe, Williams, Davies, & Neuberger, 1998), however, more recently CD20 deficient mice and humans were found to exhibit decreased T-independent immune responses (Kuijpers et al., 2010). Although the crystal structure of CD20 has not been defined, the protein is predicted to be a tetraspan molecule with intracellular termini and two extracellular loops of 9 and 43 residues spanning from aa 72 to 80 and from aa 142 to 184, respectively (Polyak & Deans, 2002; Teeling et al., 2006). The larger extracellular loop, particularly nearby or between residues A170 and S173, contains the epitope recognized by rituximab and most other anti-CD20 mAbs (Polyak & Deans, 2002). Moreover, the membrane orientation and topology of CD20 was confirmed using antisera generated against peptides near the amino and carboxyl termini and by proteolytic digestion of extracellular regions (Polyak, Tailor, & Deans, 1998). Rituximab binding was found to be imposed by both amino acid sequence and quaternary structure, justifying the heterogeneity in the fine specificity of CD20 monoclonal antibodies (Polyak & Deans, 2002). While Rituximab binding is inhibited by reduction and alkylation of CD20, demonstrating that the epitope recognition is conformational (Ernst et al., 2005). Alanine-170 and Proline-172 were found to be critical determinants for extracellular CD20 epitopes, as their mutation in human CD20 abolished the binding of CD20 mAbs. Despite the proposed epitope(s) of rituximab in human CD20 to be relatively conserved in canine CD20, including residues 170 and 172, rituximab does not bind to canine CD20 (Impellizeri et al., 2006; Jubala et al., 2005). This might indicate that there are additional mechanisms involved in the lack of cross-reactivity, such as the structural formation of the large extracellular loop of canine CD20, which determine the binding avidity and function of anti-CD20 antibodies to canine CD20 in this region (Ito et al., 2015). On one hand, the lack of cross-reactivity of human anti-CD20 mAbs against other species, as mouse and dog, has made it challenging to improve mechanisms and pharmacology associated with targeting this antigen in naturally occurring animal models of B-cell lymphoma (Ito et al., 2015). On the other hand, studying the differences in the mechanism of action between human and canine anti-CD20 mAbs may yield insights to improve the engineering of mAbs (Anderson & Modiano, 2015).

Finally, we used a novel strategy for the generation of recombinant anti-CD20 monoclonal antibodies against this novel coding sequence described herein, that bind canine CD20 and present cross-reactivity with human CD20 Indeed, the development and the implementation of anti-CD20 mAbs as a successful therapeutic strategy for canine lymphoma has revealed itself to be a challenging task, considering that the reported therapeutic efficacy of these mAbs demonstrated to be suboptimal and substantially inferior to results reported in human patients. Within this context, we described herein the development of recombinant mAbs against canine CD20 through the construction of a sdAb library from an immunized rabbit coupled with a phage display screening of the library on native-like receptor-expressed on cells. The rabbit antibody repertoire has been used for decades in diagnostic applications in the form of polyclonal antibodies. Now it also holds great promise as a source for generation of therapeutic mAbs (Rader et al., 2000). The unique ontogeny of rabbit B cells promotes vastly distinctive antibody repertories rich in *in vivo* pruned binders of high diversity, affinity and specificity (Weber et al., 2017). Furthermore, rabbits are evolutionarily distant from mice and rats, so epitopes that are not immunogenic in rodents can be recognized by rabbit mAbs, increasing the targetable epitopes and facilitating the generation of mAbs that cross react with other species (Weber et al., 2017) - a key aspect for clinical translational. Rabbit immunizations with HEK 293T cells transfected with canine CD20 vector resulted in a specific and selective high-titered antiserum against canine and human CD20. This strong and specific B-cell response allowed the construction of a high-quality antibody gene library. In addition, phage display screening of the library on native-like receptor-expressed on cells might have potentiated the diversity of antibodies againrformed by phage display, resulted in a specific pool of sdAbs-phages and high-throughput screening selected the best candidates.

The availability of sdAbs against canine CD20, which binds both the large extracellular loop of canine and human CD20, offers a platform for translational immunotherapy investigation. In fact, the use of rabbit derived sdAbs allows to fully caninize the molecule and to promote ADCC responses after fusion to canine Fc constant regions as a traditional approach, or to develop more sophisticated recombinant molecules such as antibody-conjugates and bispecific antibodies.

In conclusion, our work provides new data validating canine CD20 as a promising target for immunotherapeutic strategies for veterinary settings, while contributing to comparative oncology. Future studies will determine canine CD20 expression value as a predictive factor of anti-CD20 immunotherapy. Moreover, we report herein a panel of novel sdAbs that recognize both canine and human CD20, that may become a useful tool for exploring the development of novel therapeutic alternatives for comparative oncology. Future studies are

necessary to test the therapeutic potential of these sdAbs in *in vitro* and *in vivo* experimental models.

A novel strategy to develop tumor-specific antibodies for a sdAb-based drug delivery system

3.2.1. Abstract

Cancer is among the leading causes of death worldwide and is still one of the most challenging diseases for the pharmaceutical industry to effectively treat. With four ADCs already on the market and over 70 ADCs undergoing clinical studies, ADCs are becoming one of the most rapidly growing classes of human therapeutics for cancer. As a result, pharmaceuticals companies and academia are intensely focused in the development of novel highly specific and potent ADCs. However, the complexity of ADC components, conjugation methods, off-target toxicities still pose challenges for the strategic design of antibody-drug conjugates to achieve their fullest therapeutic potential. Tumor bearing murine models fail to predict ADC's clinical activity and tolerability, demonstrating an urgent and unmet need for better animal models for a fast, successful, and cost-effective drug development. Naturally occurring tumors in client-owned dogs closely recapitulate their human counterparts, making the dog an excellent animal model to explore novel therapeutic molecules and approaches. Using the potential of rabbit derived sdAbs technology, we aim to develop a highly selective and potent new anti-cancer ADC therapeutic molecule. Within this context, this work explores a novel strategy of tumor-specific antibodies selection against canine lymphoma, for the development of a drug delivery system. For that purpose, a highly diverse library of rabbit sdAbs against primary canine NHL cells has been successfully constructed, to ensure the presence of antibodies against any potentially relevant target. By coupling subtractive antibody selection rounds on whole-cells and a high-throughput screening, we have selected novel sdAbs targeting cNHL. In conclusion, the data presented herein validates a novel strategy for mAb selection and contribute for the development of a promising mAb-based therapy for NHL.

3.2.2. Introduction

In 2015 alone, cancer was responsible for 8.8 million deaths worldwide, second only to cardiovascular diseases as the leading cause of death (Fitzmaurice et al., 2017). Owing to population growth, ageing, and the adoption of lifestyle behaviors associated with increased cancer risk, this number is expected to rise by about 70% over the next 20 years (Fitzmaurice et al., 2017; Torre et al., 2016). These are impressive numbers that make cancer one of the major challenges of modern society. As such, the development of effective cancer treatments remains one of the biggest unmet clinical need of today's biomedical research and pharmaceutical development. Conventional chemotherapy and radiotherapy appear to have a potent effect to kill tumor cells, but they also eliminate healthy cells. Therefore, massive attention went to the development of more effective curable options by targeted cancer therapy, of which monoclonal antibodies represents one of the most promising (Hu, Liu, & Muyldermans, 2017).

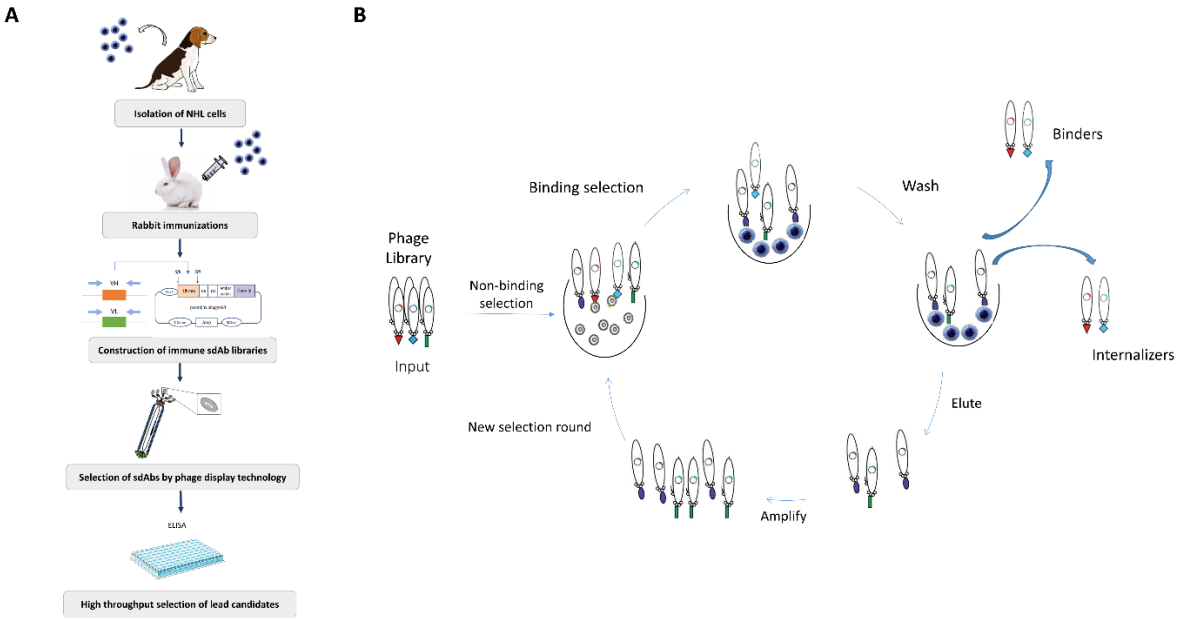
Biological therapies play an increasing role in cancer treatment, however the number of naked antibodies showing clinical efficacy as single agent remains limited. One way to enhance therapeutic potential of antibodies is to conjugate them to small molecule drugs. This combination is expected to couple the benefits of highly potent drugs on one hand and selective binders of specific tumor antigens on the other hand. Nevertheless, the design of an ADC is complex, requiring thoughtful combination of antibody, linker, and drugs in the context of a target and a defined cancer indication (Sassoon & Blanc, 2013). Notably, of the 2016 Nice Insight CDMO Outsourcing Survey respondents that have biologic drugs in their pipelines, 57% are developing ADCs and 56% are developing vaccines, compared to 51% with naked mAbs under evaluation (That's Nice, 2016). Another source states that 182 companies around the world have ADCs in their pipeline (Zhao, 2017). However, despite the increasing interest and investiment made in the last years, only four ADCs have been licensed to date (Drake & Rabuka, 2018). In fact, there are plenty examples of drugs that demonstrated potential in early pre-clinical stages but have failed to progress or have been terminated abruptly. Many of these failures were due to toxicity and incomplete characterization (Donaghy, 2016). So, it has become clear that conventional preclinical studies using tumor-bearing murine models are not suitable in predicting ADC's clinical activity and tolerability. The main problem in this case is that the human tumor target is not commonly expressed in the animal host murine tissues. Consequently, the ADC molecule distributes exclusively to the tumor xenograft, resulting in high antitumor activity and complete tumor regression. However, when these ADC agents with strong preclinical activity enter clinical phases,

similar antitumor responses are unfortunatelly not observed, or non-target toxicity in normal tissue restricts dose-escalation to levels not particularly active. As such, for this promising platform to evolve successfully, lessons from past ADC history and critical re-evaluation of preclinical and clinical results must be undertaken (Tolcher, 2016). Naturally occurring tumors in client-owned dogs closely recapitulate their human counterparts, making the dog an excellent animal model to explore novel therapeutic molecules and approaches (Ito et al., 2014; Marconato et al., 2013). By providing realistic opportunities to better evaluate therapeutic index, ADC mechanisms of action and ADCs off target toxicities, pet clinical trials can revolutionize this therapeutical field and contribute for the clinical success of these molecules. However, the development of a drug delivery system for comparative oncology settings requires a careful selection of targets, that present suitable caracteristics for ADC targeting in both species.

Within this context, the present study aims to validate a novel strategy of tumor-specific antibodies selection against cNHL for the development a drug delivery system for the treatment of B-cell malignancies. We have been showing that rabbit derived sdAbs can be developed against several targets and that these minimal scaffolds show great potential for therapeutic applications (Corte-Real et al., 2005; da Silva et al., 2012; Goncalves & Aires-Da-Silva, 2008). sdAbs are presently the smallest functional antibody fragment, only consisting of an VH or VL, which improves their tumor penetration and accessibility to targets not easily reached by IgGs (Aires da Silva et al., 2008; Corte-Real et al., 2005; Popkov et al., 2004; Volker et al., 2016). In addition to the reduced size, complementary-determining regions of sdAbs can be easily engineered to develop specific and high-affinity binders. Moreover, sdAbs also present excellent properties such as high stability, solubility, low immunogenicity and low manufacturing cost (Aires da Silva et al., 2008; Volker et al., 2016). Owing to their advantageous properties, these scaffolds are being explored as targeting moieties for drug delivery systems. sdAbs can readily be linked genetically to Fc-domains, other antibodies fragments, peptide tags, or toxins and can be conjugated chemically at a specific site to drugs, radionuclides, photosensitizers, and nanoparticles. These properties make them particularly suited for specific and efficient targeting of tumors *in vivo*.

With that in mind, a highly diverse library of rabbit sdAbs against primary canine NHL cells has been successfully constructed, to ensure the presence of antibodies against any potentially relevant target. By coupling a subtractive antibody selection rounds on whole-cells and a high-throughput screening, we selected sdAbs targeting NHL antigens in order to develop a promising antibody-based therapy for NHL, while contributing to comparative oncology (Figure 28).

Figure 28 – Schematic illustration of research methodology.



Representation of a new strategy of NHL-specific antibodies selection. (**A**) An immune sdAb library was generated and selected by a subtractive whole-cell phage display. Rabbits were immunized with canine primary NHL cells and sdAb repertoire was constructed from B cell sources and cloned into a phage display vector system. Highly specific anti-NHL sdAbs were selected by phage display. (**B**) Schematic outline of the whole-cell phage display. Four rounds of biopanning were performed against the intact CLBL-1 cells, a canine B-cell lymphoma cell line. Binders were eluted using tripsin and internalizers were obtained after cell lysis using tween 20. After the third round of panning, eluted phages were subjected to a subtraction round against isolated lymph node cells from healthy donors (round 4).

3.2.3. Material and methods

3.2.3.1. Biological samples

- **Canine Multicentric Lymphoma Biobank**

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 after node biopsy was

performed. 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 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. All sample collection was conducted with 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 extraction of plasma, serum, DNA (Dneasy Blood & Tissue, Qiagen, Hilden, Germany) and mrna (Rneasy Protect Animal Blood System, Qiagen) that were stored at -80ºc. Additionally, PBMC were isolated by Ficoll gradient method (Biocoll Separating Solution, biochrom®, Fisher Scientific, New Hampshire, USA), cell viability was assessed and for storage, aliquots of 5 x 10^6 cells were suspended in 90% FBS (Gibco, Life Technologies, Paisley, UK) and 10% 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 -80ºc 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 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 x 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.

- **Control group**

Whole blood and sterile biopsy lymph nodes samples were harvested from a control group and processed as previously described.

- **Cell lines and culture**

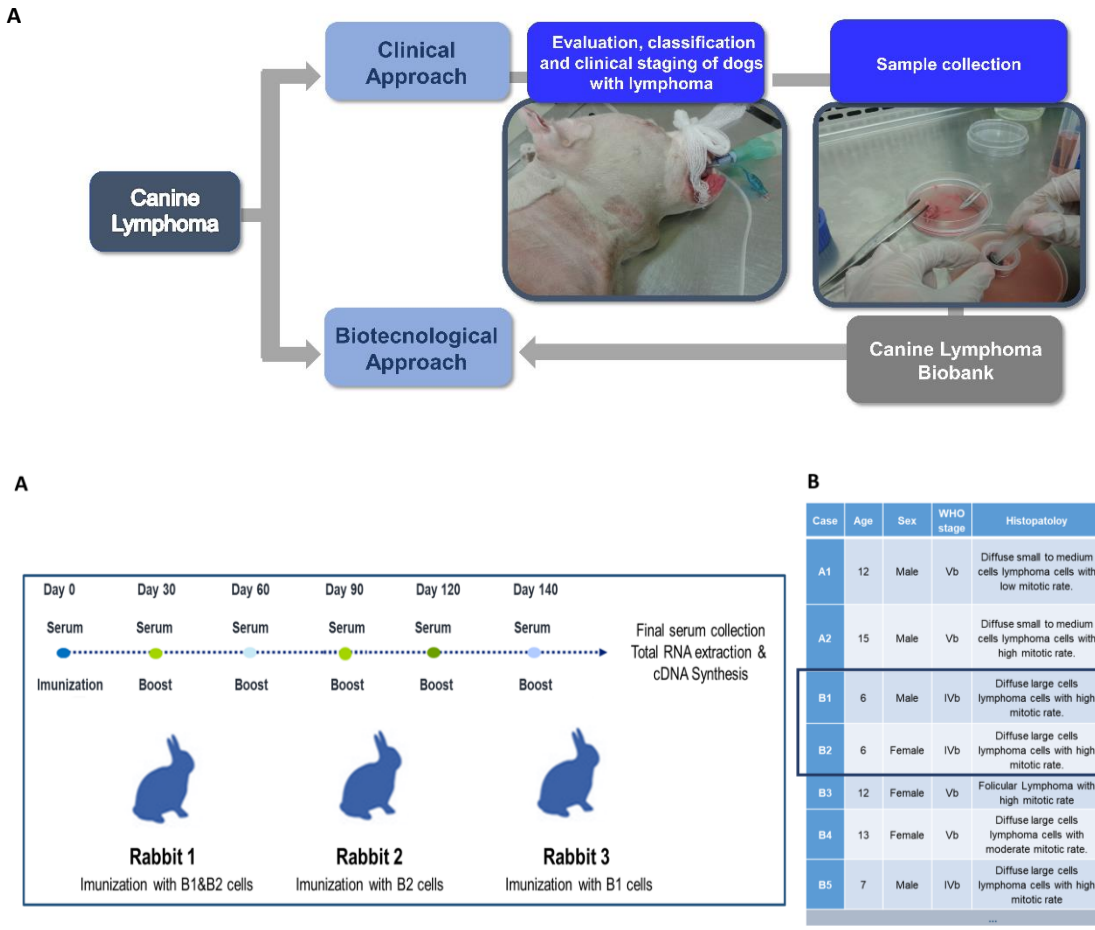
The canine B-cell lymphoma cell line CLBL-1 was provided by Dr. Barbara Rütgen (University of Vienna, Austria). The human Burkitt's lymphoma Raji cell line, the human T lymphocyte cells and the human cell line HEK293T cell line (appropriated for ectopic expression of mammalian proteins) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). CLBL-1, Raji and Jurkat cell lines were maintained in RPMI-1640 medium (Gibco) supplemented with 10% FCS (Gibco) and penicillin 100 U/ml/streptomycin 0.1 mg/ml (Gibco). 293T cell line was cultured in DMEM medium supplemented with 10% FCS (Gibco) and penicillin 100 U/ml/streptomycin 0.1 mg/ml (Gibco). All cell lines cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 (T75-tissue culture flasks, Greiner Bio-One, Kremsmünster, Austria).

3.2.3.2. Generation of monoclonal antibodies against canine NHL

- **Rabbit immunization**

Three female New Zealand White rabbit were immunized with 1 x 10^7 of cNHL primary cells from our biobank, as described in Figure 29. For that purpose, cells were thawed, washed in PBS and after confirmation of cell viability, resuspended in 1ml of PBS. The injections were administrated subcutaneously at 2 week intervals during 4 months.

Figure 29 – Rabbit immunizations.



Case	Age	Sex	WHO stage	Histopatoloy	immunohistoc hemistry
A1	12	Male	Vb	Diffuse small to medium cells lymphoma cells with low mitotic rate.	B-cell Lymphoma
A2	15	Male	Vb	Diffuse small to medium cells lymphoma cells with high mitotic rate.	T-cell Lymphoma
B1	6	Male	IVb	Diffuse large cells lymphoma cells with high mitotic rate.	B-cell Lymphoma
B2	6	Female	IVb	Diffuse large cells lymphoma cells with high mitotic rate.	B-cell Lymphoma
B3	12	Female	Vb	Folicular Lymphoma with high mitotic rate	B-cell Lymphoma
B4	13	Female	Vb	Diffuse large cells lymphoma cells with moderate mitotic rate.	B-cell Lymphoma
B5	7	Male	IVb	Diffuse large cells lymphoma cells with high mitotic rate	B-cell Lymphoma
...					

(**A**) Under chapter 2, a canine lymphoma biobank was constructed with samples collected from lymph nodes collected from dogs submitted at the oncology unit of the FMV-UL's teaching Hospital. Dogs diagnosed with multicentric lymphoma were followed and staged using the WHO system. In order to develop a rabbit antibody repertoire for the generation of antibodies against canine lymphoma antigens, we immunized rabbits using canine lymphoma primary cells from our biobank. (**B**) Three *New Zeland White* female rabbits were immunized with canine lymphoma primary cells from the biobank for 4 months, every 2-3 weeks. After thawing and celular viability evaluation, canine lymphoma cells (B1 e B2) were resuspended in PBS and injected subcutaneously. Before each imunization, blood was harvested from the marginal ear vein for serum isolation and serum titer evaluation by ELISA. (**C**) Description of cases included on our canine lymphoma biobank. Samples collected from B1 and B2 cases where used to immunize the rabbits.

- **Rabbits serum titer against canine cNHL**

To evaluate the serum specificity and titer, before each immunization blood was harvested from the marginal ear vein for serum isolation. Serum was analyzed by ELISA and

immunoblotting using CLBL-1, Raji and Jurkat cell lines and cNHL primary cells from the biobank. Peroxidase-conjugated goat anti-rabbit antibody (Jackson Immune Research) as secondary antibody. HEK 293T cells with canine CD20 vector, CLBL-1 cells, Raji cells and HEK 293T cells were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, proteins were probed with rabbit serum (dilution 1:5000) and then with HRP conjugated anti-rabbit mAb as a secondary antibody. As a control, we used pre-bleed sera.

- **sdAbs immune library construction**

Five days after the final boost, spleen and bone marrow were harvested separately for total RNA using TRI Reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized using Transcriptor High Fidelity (Roche) following the manufacturer's instructions. PCR was performed to amplify the VH and VL from the rabbit's cDNA using the set of primers presented at Table 9 in sub-chapter 3.1. PCR products encoding a library of antibody fragments (sdAbs) were then gel purified, restriction digested with *Sfi*I and cloned into pCOMB3xSS. Subsequently, the ligated product was transformed into electro competent cells via electroporation and the library was tittered. To confirm library insert efficiency and diversity, PCR colony was performed using primer RSC-F and primer RSC-B. Phage library sequencing was performed by GATC Biotech AG (Ebersberg, Germany) using the pComb3x ATG primer. To translate to amino acid sequences and to evaluate homology, the Vector NTI Advance 10 software (Thermo Fisher Scientific) was used.

3.2.3.3. Selection of recombinant monoclonal antibodies against canine cNHL

- ***Selection of anti-cNHL sdAbs by Phage Display***

To display anti-cNHL sdAbs on M13 phage, sdAbs library was infected with M13 helper phage (10^{12} plaque-forming units/ml) during 1 h at 37 °C and grown overnight. Phages were precipitated, and titer was determined. The phage library displaying sdAbs was panned using a whole-cell phage display protocol on CLBL-1 cells as described by Barbas (Barbas et al., 2004; Popkov et al., 2004). Four rounds of phage display panning were performed in order to select the best candidates. A subtractive strategy was used for the forth panning using primary lymph node and circulating PBMC from a healthy donor.

- ***In vitro* binding and expression studies**

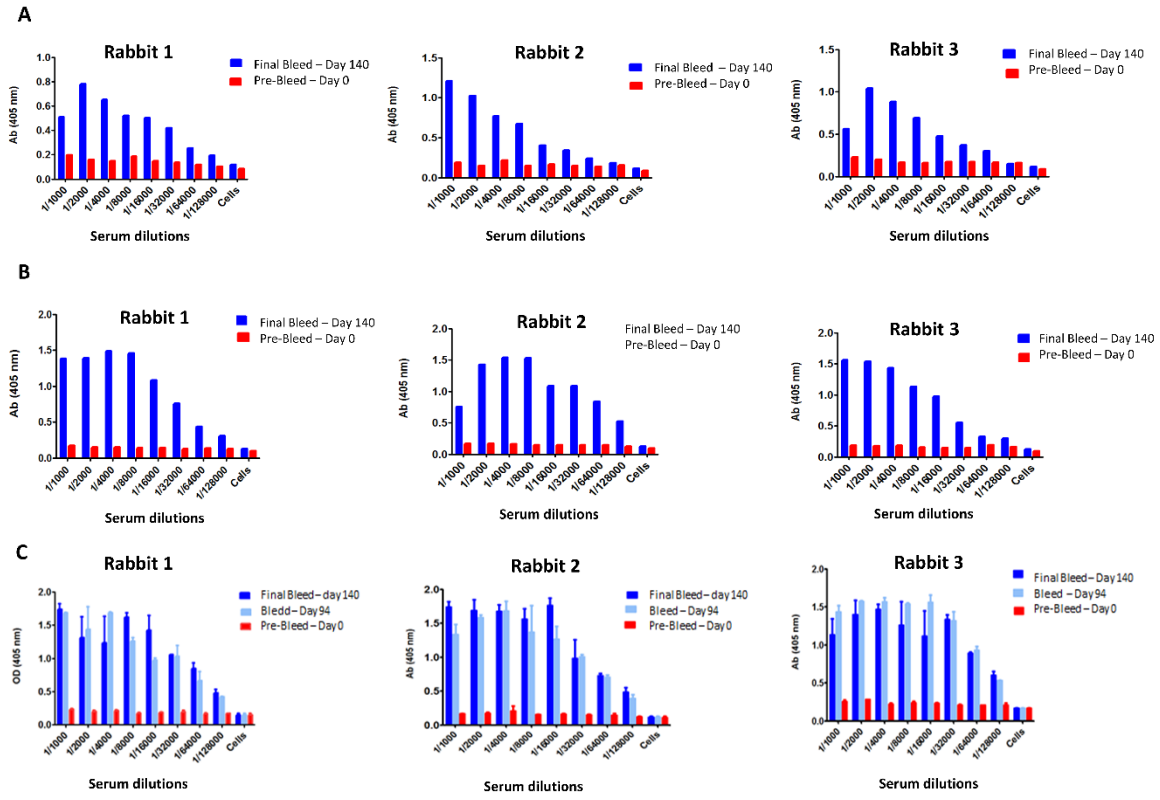
To express and select anti-cNHL–sdAbs, phagemid DNA encoding selected anti-CD20 sdAbs was cloned into pT7-PL (pT7-peptide leader) vector and transformed into *E. coli* strain BL21. Library insert efficiency was confirmed by colony PCR. The colonies obtained were picked and incubated overnight at 30°C on 100 µl of Super Broth (SB) medium containing Overnight Express™ Autoinduction System 1 (Novagen®) and 100 µg/ml of Ampicilin. Next day, 40 µl of BugBuster (Roche) containing anti-protease cocktail-EDTA free inhibitors (Roche) were added and incubated for 30 minutes at 4°C. Next, the plates were centrifuged at 1200 rpm and the supernatant was used to perform an ELISA assay. Three different parameters were analyzed: binding of antibodies to the antigen, expression level and unspecific binding. To evaluate the binding to the cNHL antigens, plates were coated with CLBL-1 extracts, washed with PBS and blocked with BSA 3% for 1 hour at 30°C. Then, plates were washed and the supernatant containing VLs or VHs was incubated for 1 hour at 30°C. Plates were washed and incubated with anti-HA-HRP antibody (Roche) was added. After 1 hour of incubation, plates were washed, ABTS (Roche) substrate solution was added and optical density at 405 nm was measured at different time points. To evaluate the level of expression, the same process was carried out, except for the antigen coating. To evaluate the unspecific binding, in turn of the antigen, BSA 3% was added. Rabbit serum and anti-CD20 antibody were used as positive controls. BL21 cell extracts and SB medium were used as negative controls.

3.2.4. Results

3.2.4.1. Generation and screening of high-titered antisera against canine cNHL

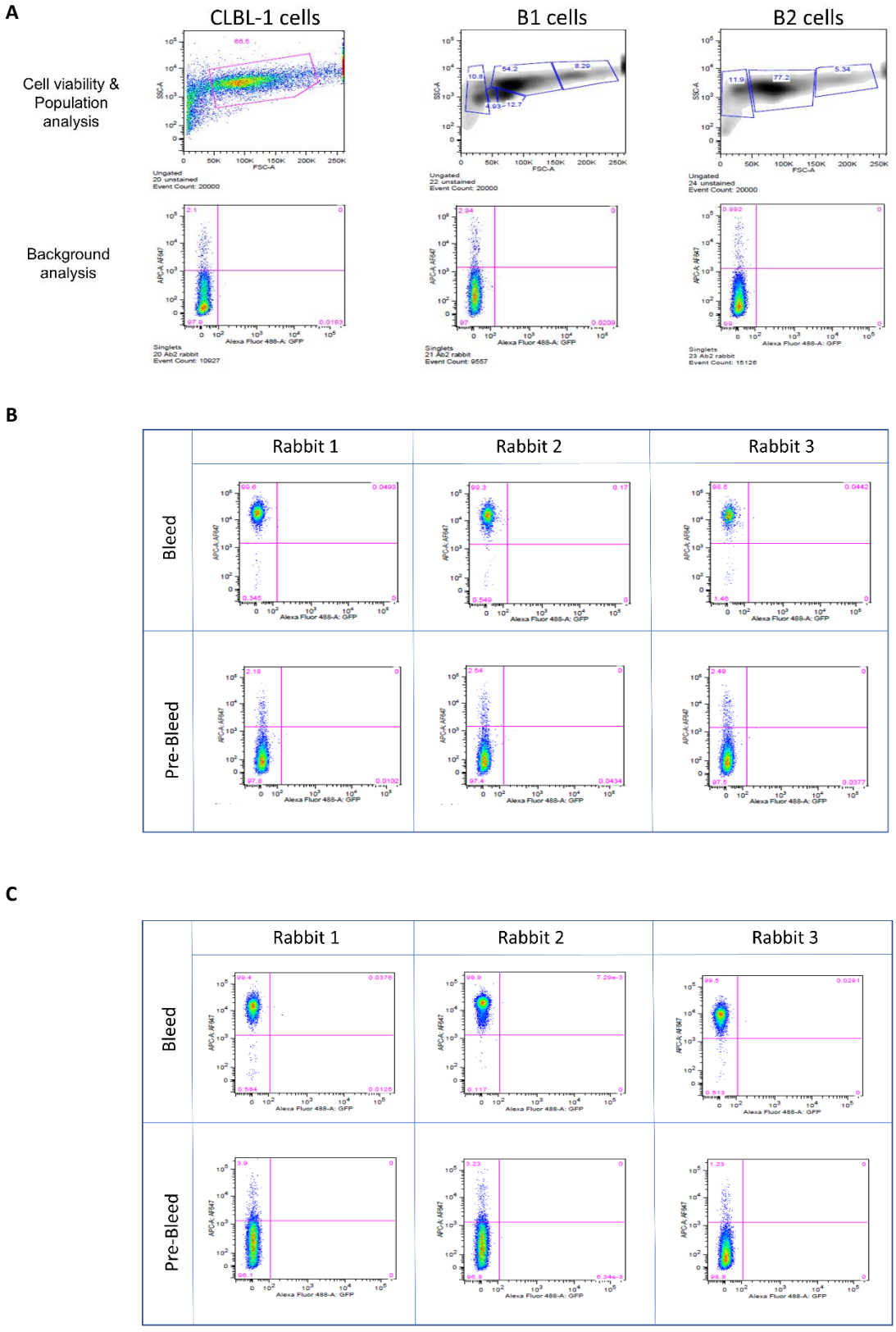
A highly specific sera against multiple cNHL antigens was successfully produced from three female New Zealand white rabbit immunized with cNHL primary cells from our canine lymphoma biobank, as described previously in the Material and Methods section. Antibody titers and specificity were monitored by ELISA, FACS and western blot analyses. As shown in Figure 30, cell ELISA assays showed that all three rabbits final bleed serum presented an highly specific and selective response against our biobank cNHL primary cells (Figure 30A and 30B) and CLBL-1 cell line (Figure 30C), a canine B-cell lymphoma. Furthermore, these data demonstrated that immunizations resulted in a strong immune response with a high serum titer (1:60.000), contrarily to pre-bleed serum. FACS analysis confirmed these results (Figure 31).

Figure 30 – ELISA analysis of high-titered antisera against canine cNHL.

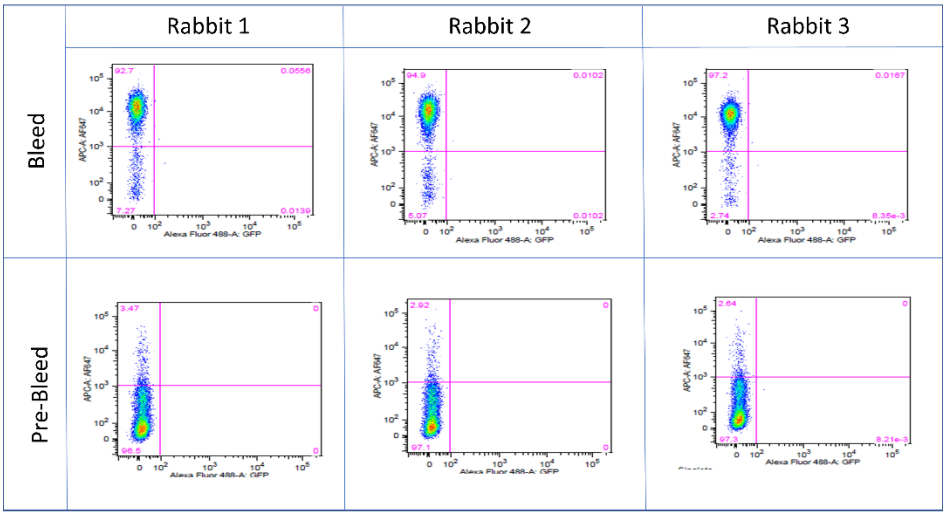


(**A**) Rabbit #1 Rabbit #2 and Rabbit #3 sera analysis by cell ELISA for binding activity to B1 primary lymphoma cells from our biobank. All rabbits showed a selective and specific immunitary response against B1 cells. (**B**) Rabbit #1 Rabbit #2 and Rabbit #3 sera analysis by cell ELISA for binding activity to B2 primary lymphoma cells from our biobank. All rabbits showed a selective and specific immunitary response against B2 cells. (**C**) Rabbit #1, rabbit #2 and rabbit #3 serum analysis by cell ELISA for binding activity to CLBL-1, a canine B-cell lymphoma line. All rabbits showed a selective and specific immunitary response against CLBL-1.

Figure 31 - FACS analysis of high-titered antisera against cNHL.



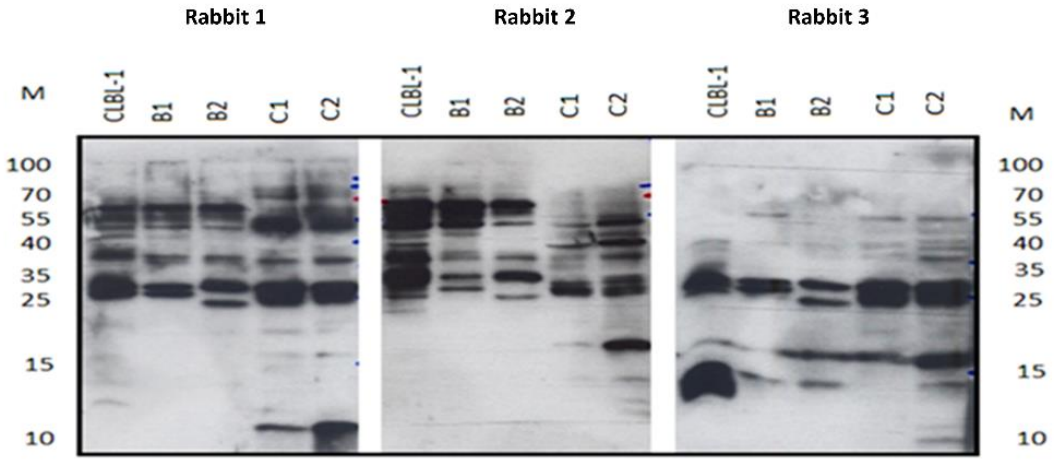
D



(**A**) Cell viability and population analysis and background evaluation. (**B**) Rabbit #1, rabbit #2 and rabbit #3 serum analysis by FACS for binding activity to B1 primary lymphoma cells from our biobank. (**C**) Rabbit #1, rabbit #2 and rabbit #3 serum analysis by FACS for binding activity to B2 primary lymphoma cells from our biobank. (**D**) Rabbit #1, rabbit #2 and rabbit #3 serum analysis by FACS for binding activity to CLBL-1, a canine B-lymphoma cell line. All rabbits showed a selective and specific immunitary response against CLBL-1, B1 and B2 cells.

To evaluate the target protein profile recognized by the rabbit polyclonal sera, western blot analysis was carried out on cNHL cells and canine PBMCs from healthy donors. As shown in Figure 32, all three rabbits sera presented a distinct target profile. Moreover, receptors recognized on cNHL primary and CLBL-1 extracts were similar and differences between epitopes recognized in cNHL lymphocytes and PBMC from a healthy donor were demonstrated, revealing potential tumor-specific epitopes detected by rabbit polyclonal antibodies. In agreement, rabbit pre-bleed, harvested before starting immunizations, did not show any signal against immunized cells or control cells. These results enabled a better design of the phage display screening.

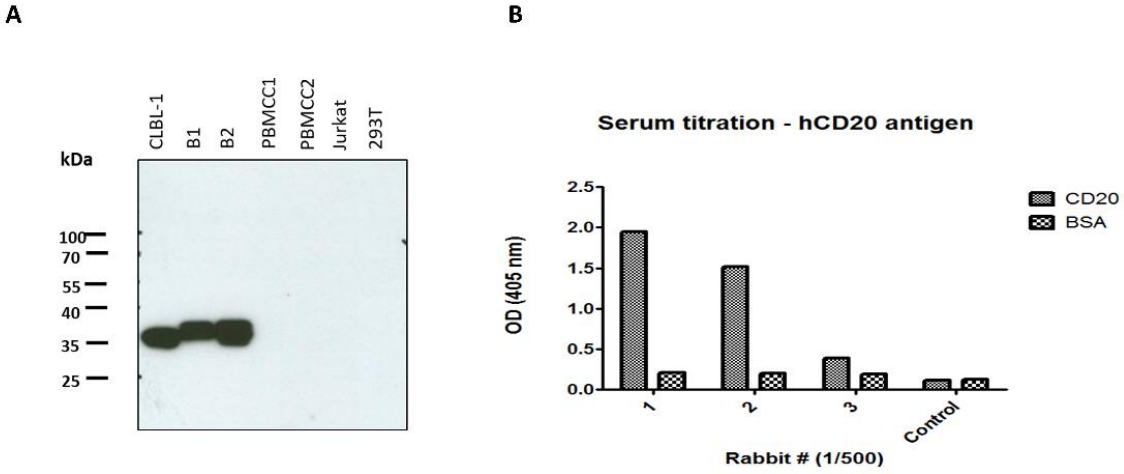
Figure 32 - Evaluation of receptors recognized by rabbit policlonal sera.



Protein profiles analysis by WB of lymphoma primary cells (B1 and B2 cells from our biobank), PBMC from healthy dogs (C1 and C2) and positive control cell line – CLBL-1. Representative blots are shown. Results allowed to identify potential receptors recognized by rabbits sera.

Finally, to confirm that tumor-specific targets were recognized by polyclonal sera, ELISA analysis was performed using the CD20 receptor, a well-known immunotherapy target. These results showed that all three rabbits presented an immune response against CD20, however rabbit 1 presented the highest immune titer (Figure 33). Based on these results, rabbit 1 was selected as the most promising source of highly specific antibodies against cNHL and was therefore sacrificed for bone marrow and spleen extraction, further used for total RNA preparation and cDNA synthesis.

Figure 33 – Tumor targeting validation.



(**A**) Evaluation of CD20 expression by Western blotting analysis on CLBL-1 cell line, lymphoma primary cells (B1 and B2 cells from our Biobank), PBMC from healthy dogs (PBMC1 and PBMC2) and negative control cell lines – Jurkat and 293T. This analysis confirmed CD20 expression in our cNHL biobank samples (B1 and B2), as well as on CLBL-1 cell line, whereas CD20 expression was not detected on PBMC from healthy donors and negative control cell lines such as Jurkat and 293T (**B**) Evaluation of rabbit polyclonal sera binding activity against human CD20 by ELISA. These results showed that all three rabbits presented an immune response against CD20, however rabbit 1 presented the highest immune titer.

3.2.4.2. Construction of a rabbit anti-cNHL sdAb phage display library

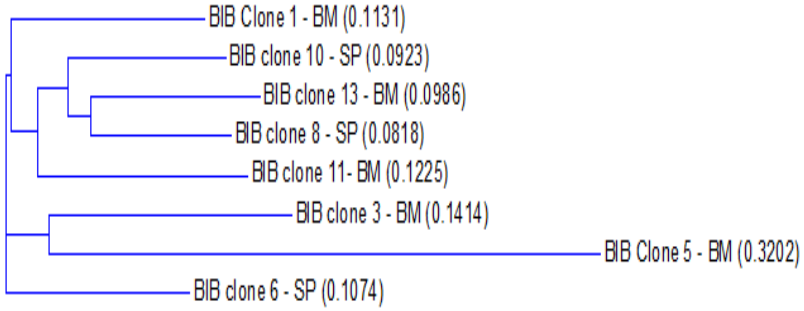
A rabbit sdAb immune library was successfully constructed using RNA from spleen and bone marrow cells of a rabbit previously immunized with cNHL primary cells from our biobank. For that purpose, specific oligonucleotide primers covering all known variable rabbit antibody family sequences were used to amplify VH and VL gene segments. The recombinant phagemid pComb3X containing the sdAb genes was transformed into *E. coli* ER2538 cells (Table 10). Rabbit repertoire presented an efficiency ranging between 90% and 100%. Sequence analysis confirmed library diversity (Figure 34).

Table 10 - Construction of a rabbit anti-cNHL sdAb phage display library.

Library	Bone Marrow	Spleen
VH	1 ,145x10^9 (100%)	4,51 x10^9 (100%)
VL	2,1 x10^7 (90%)	3,6x10^6 (100%)

Library diversity and insert efficiency results are presented for VH and VL libraries of each B cell source.

Figure 34 – Sequence analysis of phage libraries.



Homology tree obtained from the homology analysis of selected the clones.

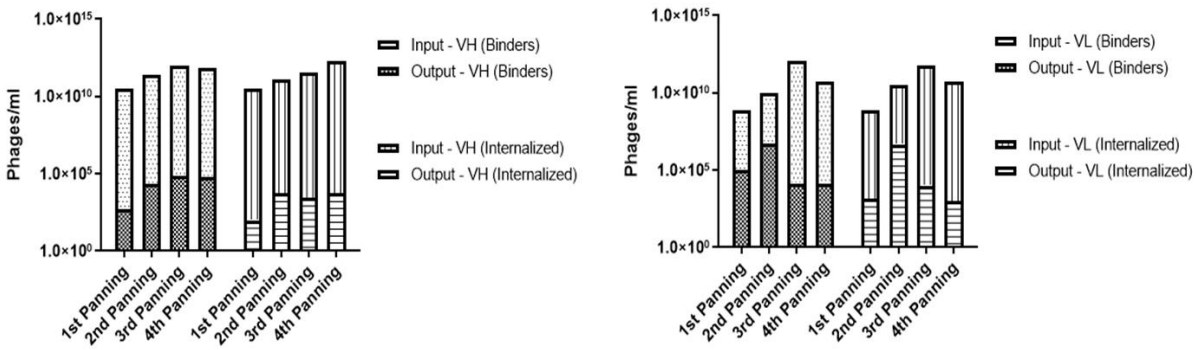
3.2.4.3. Selection of recombinant sdAbs against cNHL

Selection of specific sdAbs targeting cNHL epitopes were performed using a whole cell phage display as described in the material and methods section. This cell phage display screening protocol was based on the previously Carlos Barbas studies, in which it is reported a novel whole-cell selection protocol designed to select mAbs recognizing surface epitopes, that potentially internalize (Popkov et al., 2004). The last panning consisted of a subtractive screening on control cells, in order to remove sdAbs reacting with common antigens. Phage display conditions implemented herein are summarized in Table 11. Over the course of selection, stringency was improved by increasing the number of washes in order to collect the phage clones with greater target affinity or specificity. Different elution methods were performed to select for binders and internalized antibodies. As shown in Figure 35, the phage display rounds resulted in a lower number of phages in the output titers compared to the input titers (~$10^{11\text{-}12}$ to ~$10^{3\text{-}4}$ pfu). Furthermore, the biopannings profile indicates that the phage display protocol successfully led to the enrichment for highly specific cNHL antibodies-phages.

Table 11 - Subtractive phage display conditions used in each panning.

	1º Panning	2º Panning	3º Panning	4º Panning
Conditions	- 5 x 10^6 cells (CLBL-1) - 3 x wash - Trypsin 5 min 37ºC - TWEEN 20 10 min	- 5 x 10^6 cells (CLBL-1) - 5 x wash - Trypsin 5 min 37ºC - TWEEN 20 10 min	- 5 x 10^6 cells (CLBL-1) - 10 x wash - Trypsin 5 min 37ºC - TWEEN 20 10 min	- Subtractive panning - 5x10^6 cells (CLBL-1; Lymph node) - 10 x wash - Trypsin 5 min 37ºC - TWEEN 20 10 min

Figure 35 – Subtractive whole-cell phage display selection.



Graphic representation of amplified and eluted phage titers of each round of phage display selections. sdAbs with binding (binders) or internalization (internalizers) properties for each VH and VL library are shown. The titers were determined by plaque forming units per milliliter measurement.

To further select the best candidates based on expression and binding features, the last panning was cloned into a pT7-PL vector. This vector possesses a peptide leader, which promotes the sdAbs expression to be directed to the periplasmic space, allowing to evaluate the expression level of sdAbs. Following, an high throughput screening was performed on a total of 744 clones of each VH and VL biopanning outputs, in which multiple ELISA assays were carried out to evaluate expression and binding to the protein extract from CLBL-1 cells. Clones that revealed the stronger signal regarding binding to protein cell extract and expression were selected. This allowed to identify 15,3 % and 9,7% of binders clones and 19,1% and 16,9% of clones that internalize, corresponding to VH and VL respectively.

3.2.5. Discussion

Naturally occurring tumors in client-owned dogs are closely similar to their human counterparts, creating an excellent opportunity to incorporate pet dogs into the preclinical development path of cancer therapeutics. The use of pet dogs as animal models aims to surpass many of the bottlenecks associated with conventional models such as pharmacokinetic / pharmacodynamics relationships, dose/regimen, expected clinical toxicities, and ultimately the potential for biologic activity, while also provides access to innovative cancer treatments to dogs (Garden, Volk, Mason, & Perry, 2018; Park et al., 2016). NHL remains one of the most common and deadly neoplasias in both dogs and humans. As a result, several initiatives have been focusing on the development of therapeutic strategies with mutually benefiting purposes, such as immunotherapies in the form of mAbs (Ito et al., 2014; Marconato et al., 2013). As a result, a few mAbs directed against CD20 and CD52 were approved for the treatment of cNHL, however these naked mAbs have presented disappointing scientific and clinical results, demonstrating that the discovery of an effective antibody for the treatment cNHL may require a better understanding of antibody effector functions present in the canine immune system or the exploration of other ways of promoting a tumoral cytotoxic effect (Klingemann, 2018). By using antibodies as targeting vehicules of potent payloads, ADCs may circumvent the lack of antitumor efficacy presented by naked antibodies when used on their own (Kim & Kim, 2015). Furthermore, the development of an ADC-based therapy for cNHL may also open perspectives for the use of the dog as a promising preclinical model to bridge the gap between conventional murine studies and clinical trials. Neverthertheless, the research of comparative immunotherapies has been limited by the scarcity of information regarding tumor epitopes common to both cNHL and hNHL (Park et al., 2016). This limitation is particularly relevant when developing ADCs, since target selection is the first

and most important determining factor for a successful ADC, directly affecting its efficacy, therapeutic window and toxicity profile. The ideal characteristics of a useful ADC antigen include up regulated expression in the tumor; internalization of antigen *via* endocytosis in the presence of ligand and its recycling back to the plasma membrane and homogeneous antigen expression in the tumor microenvironment with low antigen abundance in healthy tissues (Sau, Alsaab, Kashaw, Tatiparti, & Iyer, 2017). Characteristics not easily found in most cancer targets. Moreover, target features that have not been investigated to date in comparative clinical oncology settings.

Target-based screening has been the discovery platform of most therapeutic mAbs. Nonetheless, the decline in the number of validated targets and the intense competition around target space, demonstrated an urgent and unmet need for alternative strategies for the identification of new targets for the development of innovative mAb-based therapies and the maximization of their clinical efficacy (Gonzalez-Munoz, Minter, & Rust, 2016).

Within this context, the present study aims to validate a novel strategy of tumor-specific antibodies selection against canine lymphoma for the development a drug delivery system for the treatment of B-cell malignancies. This approach relies on the use of intact cancer cells to display cancer-restricted antigens that can then be captured using multiple antibody-based technologies. Addressing the drug discovery process from this angle — the antibody / therapeutic activity side — provides an opportunity to discover new targets and target epitopes, use the antibody as a tool to validate the target, and rapidly evaluate the functional characteristics of the antibody itself as a possible therapeutic agent (Loo & Mather, 2008).

For that purpose, a highly diverse library of rabbit sdAbs against primary canine NHL cells were successfully constructed, to ensure the presence of antibodies against any potentially relevant target. The rabbit antibody repertoire has been used for decades in diagnostic applications in the form of polyclonal antibodies. Now it also holds great promise as a source for generation of therapeutic mAbs (Rader et al., 2000). The unique ontogeny of rabbit B cells promotes vastly distinctive antibody repertories rich in *in vivo* pruned binders of high diversity, affinity and specificity (Weber et al., 2017). Furthermore, rabbits are evolutionarily distant from mice and rats, so epitopes that are not immunogenic in rodents can be recognized by rabbit mAbs, increasing the targetable epitopes and facilitating the generation of mAbs that cross react with other species (Weber et al., 2017) - a key aspect for clinical translational. Rabbit immunizations with isolated B-cell canine lymphoma primary cells from our biobank resulted in a specific and selective high-titered antiserum against canine epítopes. This strong and specific B-cell response allowed the construction of a high-quality antibody library. Following, a novel strategy of phage display screening on whole cells was used to select

sdAbs against membrane embedded target antigens in their natural environment and conformation to antibody-bearing phages, resulting in an enriched pool of sdAbs-phages (Stark, Venet, & Schmid, 2017). This technique was performed using two distinct steps of phage recovery in order to select both phages that bind to the cell membrane and internalize. Finally, an high throughput screening allowed to select the best sdAbs candidates targeting cNHL regarding binding activity and expression properties. The obtained data showed that these selected sdAbs have great promise. In fact, recently a final *in vivo* selection on a human and canine NHL murine model confirmed that these sdAbs populations were indeed highly selective and specific against NHL. With this novel approach the repertoire of targetable NHL tumor receptors may be expanded, while simultaneously confirming the availability of the epitope *in vivo* and generating new antibodies for targeting. In the future, we expect to use these promising sdAbs as moieties for the development of a novel ADC for NHL, by coupling a potent payload to the selected sdAbs.

Chapter 4

The histone deacetylase inhibitor panobinostat is a potent antitumor agent in canine diffuse large B-cell lymphoma

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4.1. Abstract

NHL is one of the most common causes of cancer-related death in the United States and Europe. Although the outcome of NHL patients has improved over the last years with current therapies, the rate of mortality is still high. A plethora of new drugs is entering clinical development for NHL treatment; however, the approval of new treatments remains low due in part to the paucity of clinically relevant models for validation. Canine lymphoma shares

remarkable similarities with its human counterpart, making the dog an excellent animal model to explore novel therapeutic molecules and approaches.

Histone deacetylase inhibitors (HDACis) have emerged as a powerful new class of anti-cancer drugs for human therapy. To investigate HDACi antitumor properties on canine diffuse large B-cell lymphoma, a panel of seven HDACi compounds (CI-994, panobinostat, SBHA, SAHA, scriptaid, trichostatin A and tubacin) was screened on CLBL-1 canine B-cell lymphoma cell line. Our results demonstrated that all HDACis tested exhibited dose-dependent inhibitory effects on proliferation of CLBL-1 cells, while promoting increased H3 histone acetylation. Amongst all HDACis studied, panobinostat proved to be the most promising compound and was selected for further *in vitro* and *in vivo* evaluation. Panobinostat cytotoxicity was linked to H3 histone and α-tubulin acetylation, and to apoptosis induction. Importantly, panobinostat efficiently inhibited CLBL-1 xenograft tumor growth, and strongly induced acetylation of H3 histone and apoptosis *in vivo*. In conclusion, these results provide new data validating HDACis and, especially, panobinostat as a novel anti-cancer therapy for veterinary applications, while contributing to comparative oncology.

4.2. Introduction

It has become evident that the importance of small companion animals in the "One Medicine" concept goes beyond their role as reservoirs for infectious diseases and their contribution to human health through the human-companion animal bond. Pet dogs in particular are excellent models for the study of spontaneous degenerative, neoplastic, autoimmune and allergic disorders whose pathophysiology closely resemble their human counterparts (Sundberg & Schofield, 2009; Takashima & Day, 2014). Indeed, when compared with other animal models, the canine model presents unique advantages: diseases are naturally occurring in immune-competent hosts; the size of the animals allows testing therapeutic approaches similar to the ones used in humans; disease mapping and pharmacogenomics are simplified by the organization of dogs into isolated populations with reduced genetic variation (breeds); the relatively fast disease progression rate allows obtaining early conclusions from clinical trials; and the social status of dogs as companion animal allows them to benefit from high quality health care and the ethical exploration of translational approaches (Ito et al., 2014; Marconato et al., 2013; Rowell et al., 2011). Cancer is among the leading causes of death in both dogs and humans. Therefore, efforts towards bringing together veterinary and human medicine for the comparative research of cancer are being pursued (Fürdös, Fazekas, Singer, & Jensen-Jarolim, 2015). These initiatives are also motivated by the increasing healthcare standards

demanded by pet owners, originating the need for novel cancer therapies in veterinary settings (Henry & Bryan, 2013; Porrello et al., 2006; Weiskopf et al., 2016). One of the most common neoplasias in both species is NHL, an heterogeneous group of cancers characterized by the proliferation of malignant lymphocytes (Fisher & Fisher, 2004; Seelig et al., 2016). Human NHL is the sixth most common cancer in the United States, and its incidence nearly doubled since the early 1970s (Fisher & Fisher, 2004; Siegel et al., 2015). Human NHL represents 90% of all lymphomas and 85–90% of cases arise from B lymphocytes. This group of malignancies usually develops in the lymph nodes, but can occur in almost any tissue, ranging from the more indolent FL to the more aggressive DLBCL and Burkitt's lymphoma (Shankland et al., 2012). Even though current therapeutic options have resulted in improved response rates, the mortality rate is still high (Molina, 2008; Zappasodi et al., 2015). Moreover, the toxicity of conventional chemotherapy often limits its efficacy. Therefore, there has been an increasing interest in the design and development of novel target-specific molecules over the past years (Kong et al., 2015). Owing to shared molecular, incidence, genetic, histopathologic and clinical features, canine lymphoma has been proposed as a comparative animal model for the research of novel therapeutic agents and approaches for human NHL (Gardner et al., 2016; Pinho et al., 2012; Ranieri et al., 2013; Rowell et al., 2011). Canine lymphoma displays several histological subtypes and patients can manifest a wide range of symptoms. However, most suffer from generalized lymphadenopathy (multicentric form) and are diagnosed with intermediate to high-grade lymphoma, more commonly of B-cell origin. Without treatment, the disease has high mortality (Marconato et al., 2013), requiring prompt chemotherapy to achieve temporary remission and prolonged survival. Yet cure is rarely achieved and the majority of dogs relapse with lethal, drug-resistant lymphoma. Thus, there is an urgent need to develop new treatment strategies in veterinary medicine for refractory disease (Vail et al., 2007).

Histone deacetylase inhibitors (HDACis) have emerged as an highly efficient new class of anti-cancer drugs (Chun, 2015). Histone deacetylases (HDACs) catalyze the deacetylation of histones (and other acetyl-lysine-containing proteins), leading to chromatin condensation and transcriptional repression (Bannister & Kouzarides, 2011). By inhibiting deacetylating enzymes activity, HDACis regulate aberrant deacetylation and modify gene expression in cancer cells, culminating in cytotoxicity (Mackmull et al., 2015; H. Miles Prince, 2010; W. S. Xu, Parmigiani, & Marks, 2007). Other putative mechanisms of action include cell cycle arrest, DNA repair inhibition, apoptosis induction and angiogenesis inhibition (Khan & La Thangue, 2012). Hematological malignancies seem to be particularly sensitive to HDACis (Ageberg, Rydström, Relander, & Drott, 2013). In fact, these agents have shown single-agent

activity against T-cell lymphomas, cutaneous T-cell lymphomas, mantle cell lymphomas, and Hodgkin disease (Zain, 2012). To date, four HDACis have been approved for cancer therapy by the FDA – vorinostat, romidepsin, belinostat and panobinostat (Stahl et al., 2016). Considering the high efficacy presented by HDACis in targeted human cancer therapy, we conducted the first investigation on their antitumor properties using a canine B-cell lymphoma model. For this purpose, a panel of seven HDACis was initially tested on the well characterized CLBL-1 canine B-cell lymphoma cell line (Rütgen et al., 2010, 2012) and panobinostat was identified as the most promising compound. Panobinostat was therefore deeply investigated and showed strong *in vitro* and *in vivo* antitumor properties.

4.3. Material and methods

4.3.1. Cell lines and reagents

The canine CLBL-1 (Rütgen et al., 2010, 2012) and 17-71 (Steplewski, Jeglum, Rosales, & Weintraub, 1987) (kindly provided by Dr. Steven Suter, College of Veterinary Medicine, NC State, Raleigh, North Carolina, USA) B-cell lymphoma cell lines were cultured in RPMI-1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco) and penicillin 100 U/ml plus streptomycin 0.1 mg/ml (Gibco). Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 (T75-tissue culture flasks, Greiner Bio-One, Kremsmünster, Austria). The HDAC Inhibitor Set II, which includes CI-994, Panobinostat (LBH589), SAHA, SBHA, Scriptaid, Trichostatin A and Tubacin, was purchased from Sigma-Aldrich (St. Louis, MO, Cat # EPI009). HDACi stock solutions were prepared at 5 mg/ml, except for CI-994 (10 mg/ml) and SBHA (50 mg/ml) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20ºC. Panobinostat for the *in vivo* studies was purchased from Selleckchem (Houston, TX, Cat # S1030).

4.3.2. Cytotoxic assay

To determine the effect of HDACis on CLBL-1 and 17-71 cell proliferation, a cell viability assay was performed using the Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland). Briefly, cells were seeded at a density of 6 x 10^4 cells/well in 96-well plates in 200 µl of culture medium plus 10% heat-inactivated FCS, and subjected to increasing concentrations (2.5 nM to 20 µM) of the HDAC Inhibitor Set II library or vehicle (DMSO at

0.2% final concentration). After 24 h of treatment, cell viability and proliferation were assessed using the WST-1 reagent, following the manufacturer's instructions. Absorbance at 450 nm was measured using a plate reader (BMG LABTECH GmbH, Germany). Two replicate wells were used to determinate each data point and three independent experiments were carried out in different days. Best-fit IC50 values were calculated using GraphPad Prism software (version 5.00; San Diego, CA, USA), using the log (inhibitor) vs response (variable slope) function.

4.3.3. Immunoblotting

After HDACis treatment, cells were harvested, washed twice with PBS and lysed using RIPA lysis buffer (25mM TrisHCL pH 7.6, 150mM NaCl, 1% NP-40, 1% sodiumdeoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche). Protein extract samples were quantified using the Bradford method (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Total protein extract samples were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. After blocking, proteins were probed with the following primary antibodies: anti-acetylhistone H3 (Lys9, Lys14) antibody (polyclonal, rabbit, 1:2500 dilution, Thermo Fisher Scientific, Rockford,IL, USA), anti-histone H3 (polyclonal, rabbit, 1:1000 dilution, Thermo Fisher Scientific), and then with Peroxidase-AffiniPure Anti-Rabbit IgG antibody (polyclonal, goat, 1:10000 dilution, Jackson ImmunoResearch, PA, USA) as a secondary antibody. Proteins were detected by chemiluminescence using Luminata Forte Western HRP (Merck Millipore, Darmstadt, Germany) and acquired using the ChemiDoc XRS+ imaging system (Bio-Rad).

4.3.4. Evaluation of caspase-3/7 activity

Caspase-3 and 7 activity levels were measured using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA). For this purpose, CLBL-1 cells were seeded and treated with 1-20 nM panobinostat as mentioned above. After 24 h of treatment, 100 μL of each cell suspension were transferred into a white 96-well plate, and then mixed with 75 μL of Caspase-Glo 3/7 reagent by orbital shaking for 30 s. Subsequently, the mixture was incubated at room temperature for 30 min, allowing complete cell lysis, stabilization of proluminescent substrate cleavage by caspases, and accumulation of luminescent signal. The resulting luminescence

was measured using the GloMax-Multi+ Detection System (Promega). Three independent experiments were carried out in different days.

4.3.5. Evaluation of apoptotic cell death

The percentage of apoptotic cells after panobinostat treatment was assessed by flow cytometry using the Guava Nexin Assay (Merck Millipore, Darmstadt, Germany), according to manufacturer's instructions. This assay relies on Annexin V-PE and 7-AAD staining to distinguish between viable (Annexin V/7-AAD double negative), early-apoptotic (Annexin V positive/7-AAD negative), and late-apoptotic/dead cells (Annexin V/7-AAD double positive). Briefly, CLBL-1 cells were seeded and treated with 1- 20 nM panobinostat. After 24 h of treatment, cells were collected, centrifuged at 500 g for 5 min, and resuspended in PBS containing 2% FBS. Next, 50 µL of each cell suspension were stained with an equal volume of Guava Nexin reagent for 20 min, at room temperature, protected from light. Sample acquisition and analysis were performed in a Guava easyCyte 5HT flow cytometer using the Nexin software module (Merck Millipore). Three independent experiments were carried out in different days.

4.3.6. Immunofluorescence microscopy

CLBL-1 cells (6 x 10^4) were treated with increasing concentrations of panobinostat as described above. After 24 h treatment, cells were fixed in methanol/acetone (1:1) at -20ºC for 20 min, washed in PBS, incubated with 0.1% Triton X-100 for 15 min, washed, blocked with BSA 1%/PBS TWEEN 0,2% for 30 min, washed and probed with anti-α-tubulin antibody (clone DM1A, 1:200 dilution, Sigma Aldrich) or anti-Acetyl-Tubulin antibody (clone 6-11B-1, 1:200 dilution, Sigma Aldrich) in PBS for 1 h at room temperature. Then, cells were washed with PBS for 10 min and incubated with anti-mouse IgG alexa 594 secondary antibody (polyclonal, goat, 1:300 dilution, Invitrogen) in PBS for 1 h at room temperature. Nuclear staining was obtained by mounting with DAPI Vectashield (Vector Labs, CA, USA). Cells were visualized using an Olympus IX-50 inverted microscope (Olympus Portugal, Lisbon, Portugal) equipped with Ludl Bio-Point filter wheels, and a 12-bit PCO (Kelheim, Germany) Sensicam QE cool CCD (Ludl Electronic Products, New York, NY, USA). Integrated control of the filter wheel and image acquisition was achieved by Image-Pro Plus 4.5 and Scope- Pro 3.1 (Media Cybernetics, Rockville, MD, USA). Settings for image acquisition (camera exposure time, filters and time interval) were determined by custom-made

macros. Images were collected with Olympus 10x or 100x plan objectives (Numerical Aperture = 0.95 and 1.4, respectively).

4.3.7. Xenograft studies

All animal-handling procedures were performed according to EU recommendations for good practices and animal welfare, and approved by the Animal Care and Ethical Committee of the Veterinary Medicine Faculty. Female 6–8-wk-old SOPF/SHO SCID mice (Charles River Laboratory) were maintained in microisolation cages under pathogen-free conditions. Mice were allowed to acclimatize for at least two weeks prior to the start of the experiment. Suspension of 1 x 10^6 CLBL-1 cells in PBS with matrigel® (Corning, NY, USA, Cat # 354248) (1:1) were injected subcutaneously into the dorsal interscapular region to induce tumors. When tumors reached a minimum volume of 100 mm^3, mice were randomly assigned to one of three groups: not treated (vehicle only, n=5), 10 mg/kg panobinostat (n=5) and 20 mg/kg panobinostat (n=5). The tested doses (Catalano et al., 2012; Crisanti et al., 2009; Imai et al., 2016) and the vehicle selection (2% DMSO + 48% PEG300 + 2% Tween 80 + ddH_2O) (Hennika et al., 2017) were based on previously published studies and following manufacturer's recommendation. Treatment consisted of intraperitoneal injections 5 days per week, over two weeks. Tumor volume and body weight was measured three times per week. Tumor volume was calculated as $(width)^2$ x length. Compound activity was determined by tumor growth inhibition (TGI). TGI was determined as the percent change in tumor volume of treated over control animals (%T/C). In the end of the study, tumor samples were finely cut, and either stored at -80ºC in RNAlater™ (Invitrogen, Life Technologies, Paisley, UK) or formalin-fixed. Main organs including the liver, kidney, lung, spleen and intestine were collected and formalin-fixed. For immunoblotting and caspases 3/7 detection, RNA later™ (Invitrogen) preserved tumor samples were thawed and processed using tissueLyser II (Qiagen, Hilden, Germany) for tissue disruption, and RIPA lysis buffer supplemented with fresh protease inhibitors (Roche) for total protein extraction. Samples were quantified using Bradford method (Bio-Rad Protein Assay Dye Reagent Concentrate) according to the manufacturer's instructions, and then evaluation of H3 histone acetylation was carried out as described above. Additionally, tumor protein extracts (15 μg) were used for caspase activity measurement, using the Caspase-Glo 3/7 Assay.

4.3.8. Histology

Tissues, including tumors, were fixed in 10% buffered formalin were embedded in paraffin, using a Leica tissue processor. Four µm sections were cut from paraffin blocks and stained with H&E. Sections were mounted onto superfrost ultra plus slides (Menzel-Glaser, Braunschweig, DE), for immunohistochemistry.

4.3.9. TUNEL staining

Apoptotic cells were quantitated in tumor tissue sections, excluding areas of necrosis, using the transferase mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick-end labeling (TUNEL) assay (ApopTag® Red *In Situ* Apoptosis Detection kit; Merk Millipore, Darmstadt, Germany), following the manufacturer's instructions. Specimens were then counterstained with Hoechst 5 µg/ml, for 10 min, at room temperature. Finally, slides were rinsed and a glass coverslip was mounted using Fluoromount-GTM mounting media (Beckman Coulter Inc., Fullerton, CA). Specimens were examined by fluorescence microscopy using an AxioScope.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were acquired under 630x magnification, using an AxioCam HRm camera with the ZEN 2012 software (Blue Edition, version 1.1.2.0). Only areas with dense tumor cell mass displaying similar cell density were considered. Quantitation of TUNEL-positive cells was performed using Image J software (http://rsbweb.nih.gov/ij/). Apoptosis frequency was expressed as the number of TUNEL-positive cells per field.

4.3.10. Statistical Analysis

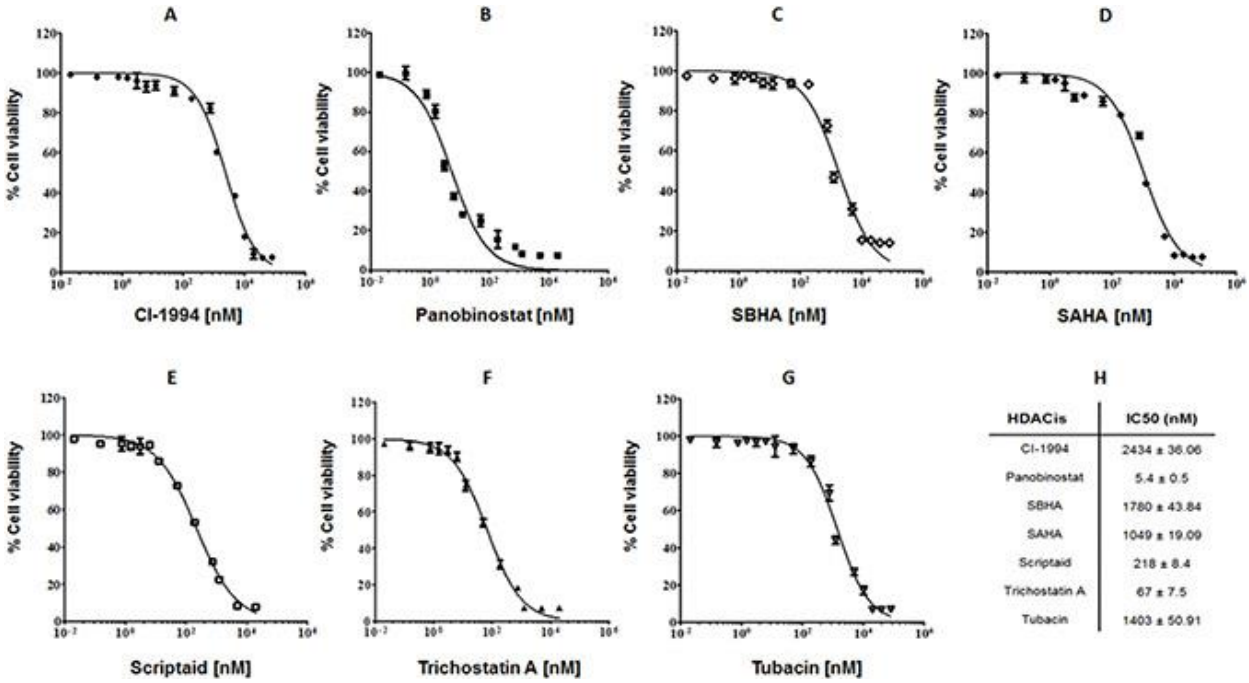
All data are expressed as mean ± SEM from at least three independent experiments. For *in vitro* and *in vivo* assays, statistical significances were determined using two-tailed Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

4.4. Results

4.4.1. HDACis suppress cell proliferation and present cytotoxic effects on canine lymphoma

Aiming to evaluate the potential cytotoxic effects of HDACis on canine lymphoma we have tested a panel of seven compounds with HDACi activity - CI-994, panobinostat, SBHA, SAHA, scriptaid, trichostatin A and tubacin - in the well-characterized CLBL-1 cell line. CLBL-1 was selected for our study as it is the well-known canine cell line that faithfully represents DLBCL, reproducibly inducing tumors and preserving its phenotype in the xenotransplantation setting (Rütgen et al., 2010, 2012; Weiskopf et al., 2016). The effect of the tested compounds on cell viability was measured using the WST-1 reagent as described in material and methods section. As shown in Figure 36, all tested HDACi compounds exhibited dose-dependent inhibitory effects on the proliferation of CLBL-1 cells. On the contrary, no evidence of toxicity was detected for vehicle-treated cells. The data obtained clearly demonstrated that panobinostat (IC_{50} = 5.4 ± 0.5 nM), scriptaid (IC_{50} = 218 ± 8.4 nM) and trichostatin A (IC_{50} = 67 ± 7.5 nM) exhibited the higher antiproliferative and cytotoxic activity (Figure 36). The remaining HDACis (CI-994, SBHA, SAHA and tubacin) demonstrated a lower susceptibility to interfere with CLBL-1 proliferation and showed IC_{50} values in the µM range (Figure 36).

Figure 36 - HDACis present cytotoxicity effect on canine B-cell lymphoma.

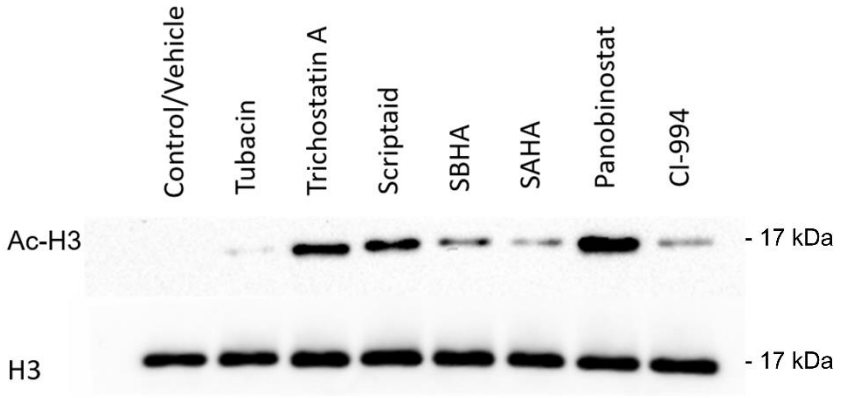


CLBL-1 cells (6×10^4) were subjected to the indicated concentrations of HDACis - CI-994, panobinostat, SBHA, SAHA, scriptaid, trichostatin A and tubacin (**A-G**). After 24 h treatment, cell viability and proliferation were evaluated with WST-1 reagent. Two replicate wells were used to determinate each data point and three independent experiments were carried out in different days. Best-fit IC50 values of each HDACis were calculated using the log (inhibitor) vs response (variable slope) function (**H**).

4.4.2. HDACi cytotoxicity is associated with histone acetylation

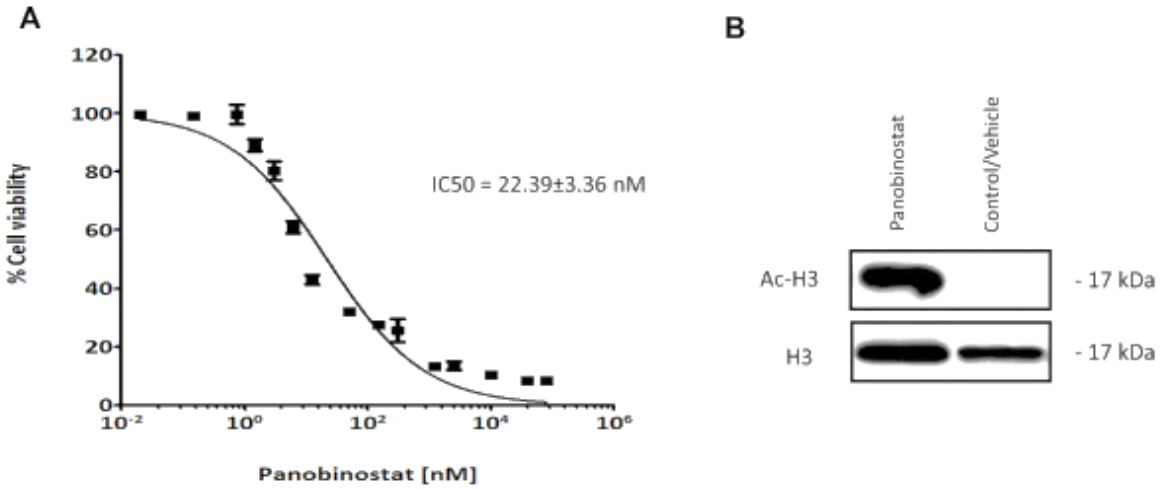
The primary molecular mechanism of HDACis action is to modify the acetylation status of core histone proteins, leading to chromatin remodeling with consequent alteration in gene expression and cell differentiation. Therefore, to elucidate the mechanism of action of HDACis in the CLBL-1 cell line, we evaluated the acetylation status of H3 histone protein by western blot analysis. As shown in Figure 37, immunoblot analysis demonstrated that CLBL-1 cells presented an hyperacetylation status of the H3 histone protein following 24 h treatment with 20 µM of HDACis, when compared with control vehicle treated cells. Importantly, the H3 histone acetylation levels were consistent with cytotoxic effects of the different HDACis and the compounds that showed the higher potency (Figure 36) promoted the higher effect on acetylation status (Figure 37). Considering the strong anti-proliferative activity and high degree of histone acetylation induction, panobinostat demonstrated to be the most promising therapeutic molecule. To confirm the strong activity in canine B-cell lymphoma, a different cell line, namely 17-71, was also tested with panobinostat. Again, the data obtained (Figure 38) demonstrated that panobinostat presents a similar activity profile and histone acetylation induction as shown in CLBL-1. Panobinostat presented therefore a consistent and potent anti-tumor effect against canine DLBCL and was selected for further characterization.

Figure 37 - Cytotoxic effect of HDACis correlates with histone acetylation.



CLBL-1 cells (6×10^4) were exposed to 20 µM of HDACi compounds. After 24 h treatment, cells were harvested for total protein extraction and acetylation of H3 histones were assessed by western blotting with anti-acetyl-histone H3 polyclonal antibody (Ac-H3). DMSO was used as vehicle control and loading was controlled with anti-histone H3 polyclonal antibody (H3). Representative blots are shown.

Figure 38 - Panobinostat presents cytotoxicity on 17–71 canine lymphoma cells and is correlated with histone acetylation.

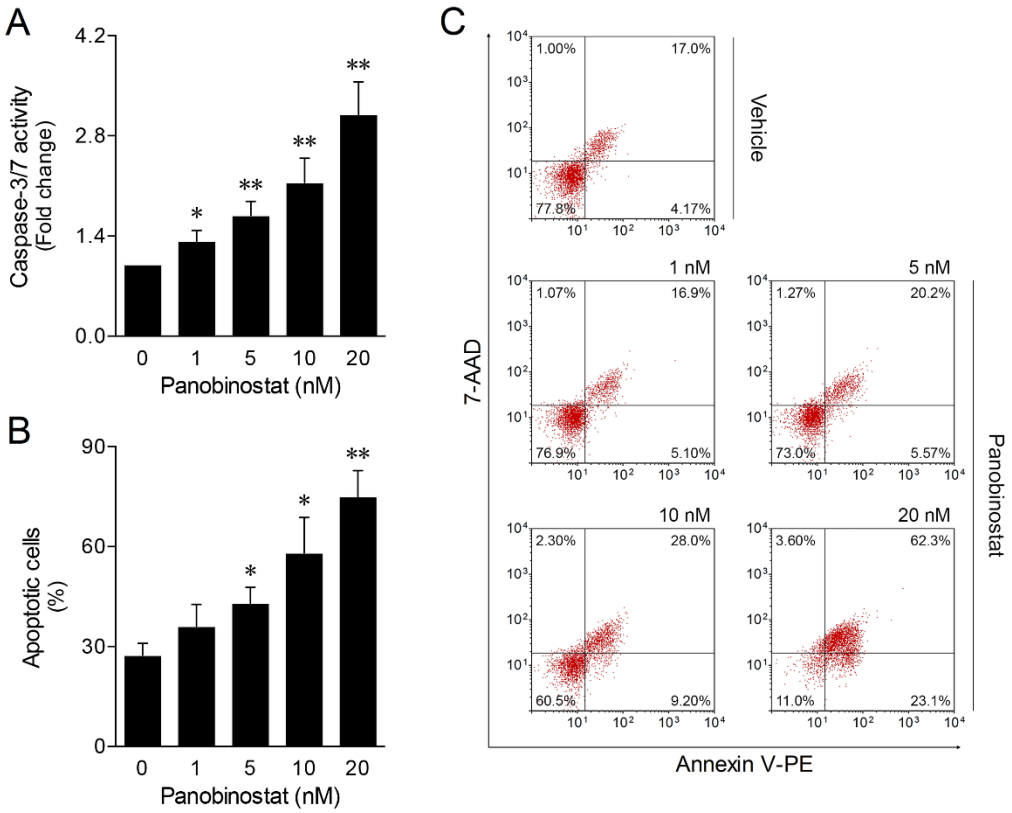


(**A**) 17–71 cells (6×10^4) were subjected to the indicated concentrations of panobinostat. After 24 h treatment, cell viability and proliferation were evaluated with WST-1 reagent. Two replicate wells were used to determinate each data point and three independent experiments were carried out in different days. Best-fit IC50 values were calculated using the log (inhibitor) vs response (variable slope) function. (**B**) 17–71 cells (6×10^4) exposed to 20 µM were harvested for total protein extraction and acetylation of H3 histones were assessed by western blotting with anti-acetyl-histone H3 polyclonal antibody (Ac-H3). DMSO was used as vehicle control and loading was controlled with anti-histone H3 polyclonal antibody (H3). Representative blots are shown.

4.4.4. Panobinostat cell death is associated with apoptosis induction

To clarify the nature of CLBL-1 canine lymphoma cell death induced by panobinostat, we measured the levels of caspase-3 and -7 activities and Annexin V/7-AAD staining following 24 h treatment. As shown in Figure 39A, panobinostat treatment induced high levels of caspase activity in a dose-dependent manner. The maximum level of caspase-3/7 activity was seen at 20 nM. Accordingly, a higher percentage of apoptotic cell death was also determined at 20 nM by flow cytometry analysis of Annexin V/7-AAD staining (Figure 39B and 39C). These results are in agreement with the cell viability and proliferation data upon panobinostat treatment, indicating that the cytotoxic activity of panobinostat in the CLBL-1 cell line is consistent with the induction of apoptosis.

Figure 39 - Panobinostat induces apoptosis on canine lymphoma.

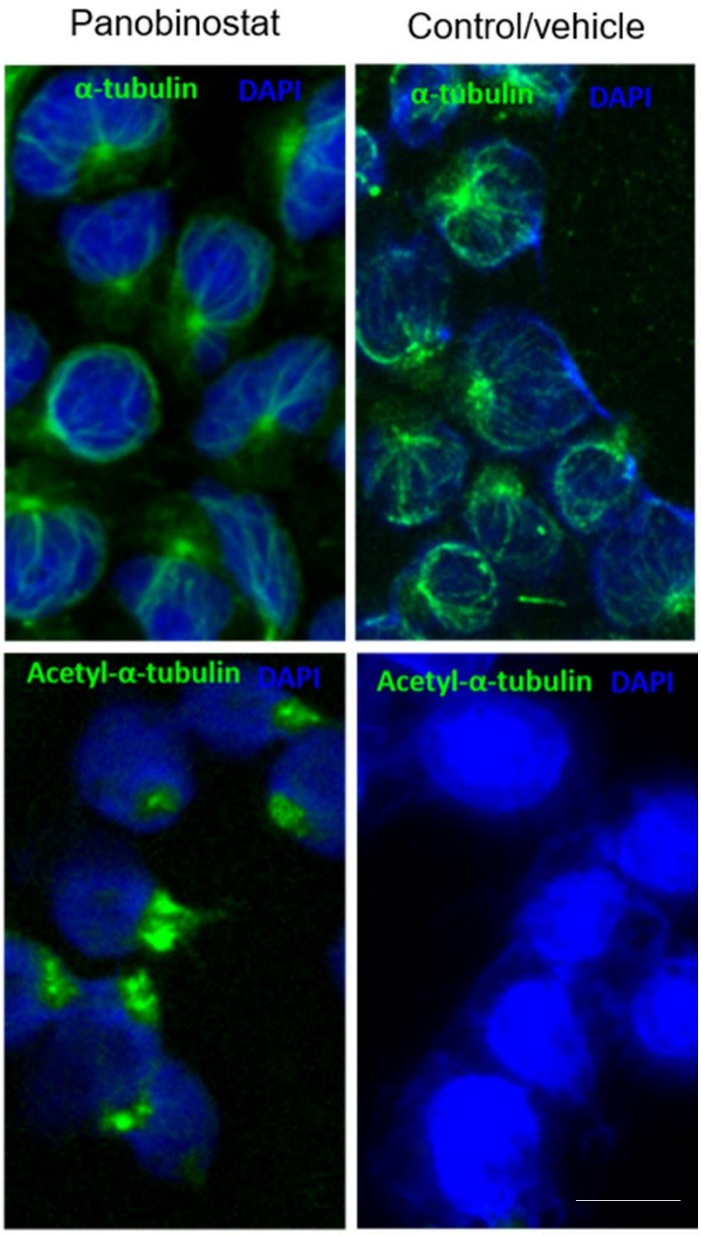


CLBL-1 cells (6×10^4) were subjected to the indicated concentrations of panobinostat. DMSO was used as vehicle control. After 24 h treatment, cells were harvested for apoptosis studies. **(A)** Caspase-3/7 activity was determined using the Caspase-Glo 3/7 assay. Results are expressed as mean ± SEM fold-change to vehicle control cells. **(B)** The percentage of apoptotic cells was determined according to Annexin V/7-AAD (Guava Nexin Assay) staining. Results are expressed as mean ± SEM. **(C)** Representative flow cytometry plots of cells stained for Annexin and 7-AAD are shown. *$p < 0.05$ and ** $p < 0.01$ from vehicle control cells.

4.4.3. Panobinostat cytotoxicity is linked to α-tubulin acetylation

The activity of HDACis extends beyond chromatin remodeling, and has been associated with the acetylation of non-histone proteins, such as the α-tubulin. The hyperacetylation of α-tubulin results in the stabilization of microtubules and subsequent cytotoxicity (Bolden, Peart, & Johnstone, 2006; H. M. Prince & Prince, 2009). Therefore, to further characterize the mechanism of action of panobinostat, we have assessed the levels of acetylated tubulin (ac-tubulin) in the CLBL-1 cells by immunofluorescence labeling. As shown in Figure 40, immunofluorescence studies revealed an α-tubulin immunolabeling throughout the cytoplasm of both vehicle and panobinostat treated cells, evidencing a normal microtubule network, radiating from the perinuclear microtubule-organizing center. On the contrary, acetylated α-tubulin immunofluorescence studies demonstrated a clear labelling of cells after panobinostat treatment for 24 h, whereas vehicle treated cells exhibited a low basal α-tubulin acetylation (Figure 40). Thus, panobinostat treated cells displayed robust acetylation of α-tubulin with bundling and increased density of acetylated microtubules radiating from the perinuclear region.

Figure 40 - Panobinostat cytotoxicity is linked to α-tubulin acetylation.

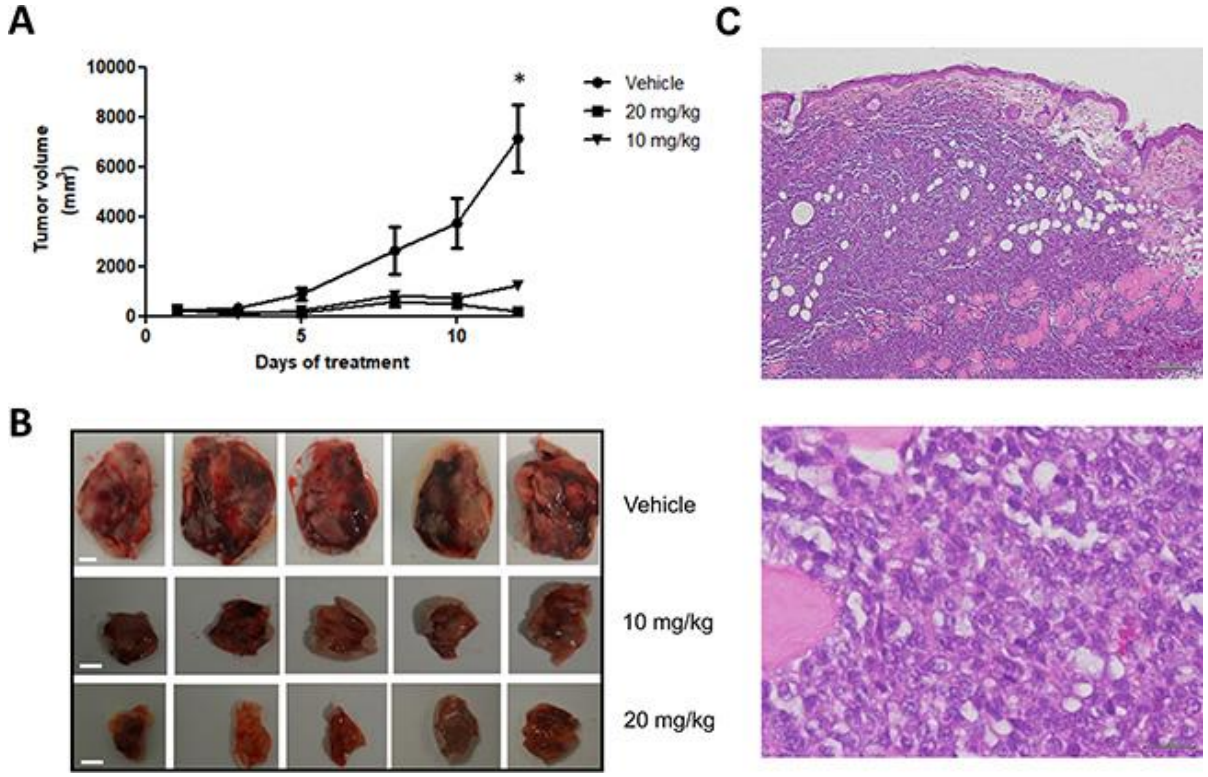


CLBL-1 cells were treated with 10 nM of panobinostat for 24 h and then microtubules were visualized by immunofluorescence labeling using antibodies against tubulin and acetylated tubulin (ac-tubulin) using the appropriate excitation and emission filters as described in the material and methods section. Representative microphotographs with tubulin and ac-tubulin labeling (green) and DAPI stained-nuclei (blue) at 100× magnification are shown. Scale bar, 5 μm.

4.4.5. Panobinostat induces tumor regression in a canine lymphoma xenograft mouse model

The antitumor effect of panobinostat on canine B-cell lymphoma was further tested in a *in vivo* murine xenograft model. CLBL-1 suspensions (1×10^6 cells) were injected subcutaneously into the dorsal interscapular region of SOPF/SHO SCID mice. When tumors reached ~100 mm^3, mice were randomized into three groups: not treated (controls/vehicle only, n = 5), panobinostat at 10 mg/kg (n = 5) and panobinostat at 20 mg/kg (n = 5) as described in the material and methods section. After two weeks of treatment, panobinostat at 10 mg/kg and 20 mg/kg doses inhibited tumor growth by 82.9% and 97.3%, respectively, when compared to vehicle control treated mice ($p < 0.05$) (Figure 41A and 41B). All xenografts were nodular; infiltration was macroscopically evident, and tumors were highly adherent to surrounding tissues. Microscopically (H&E staining) tumors showed infiltration of the dermis, hypodermis, muscular paniculus and muscle striated by large lymphoid cells of indistinct cytoplasm and nucleus containing several little evident nucleoli (Figure 41C). Moreover, pathologic evaluation described a high degree of necrosis and mitotic activity. These pathological features are characteristic of an high grade lymphoma (Rütgen et al., 2012). No histological alterations were identified in main organs examined (data not shown). Nevertheless, it is important to mention that in the majority of treatment groups, the animals lost an average of 5-10% of their starting body weight, and that some mice from the 20 mg/kg treatment group presented skin dehydration.

Figure 41 - Panobinostat treatment strongly inhibits *in vivo* tumor growth in a canine NHL tumor xenograft murine model.



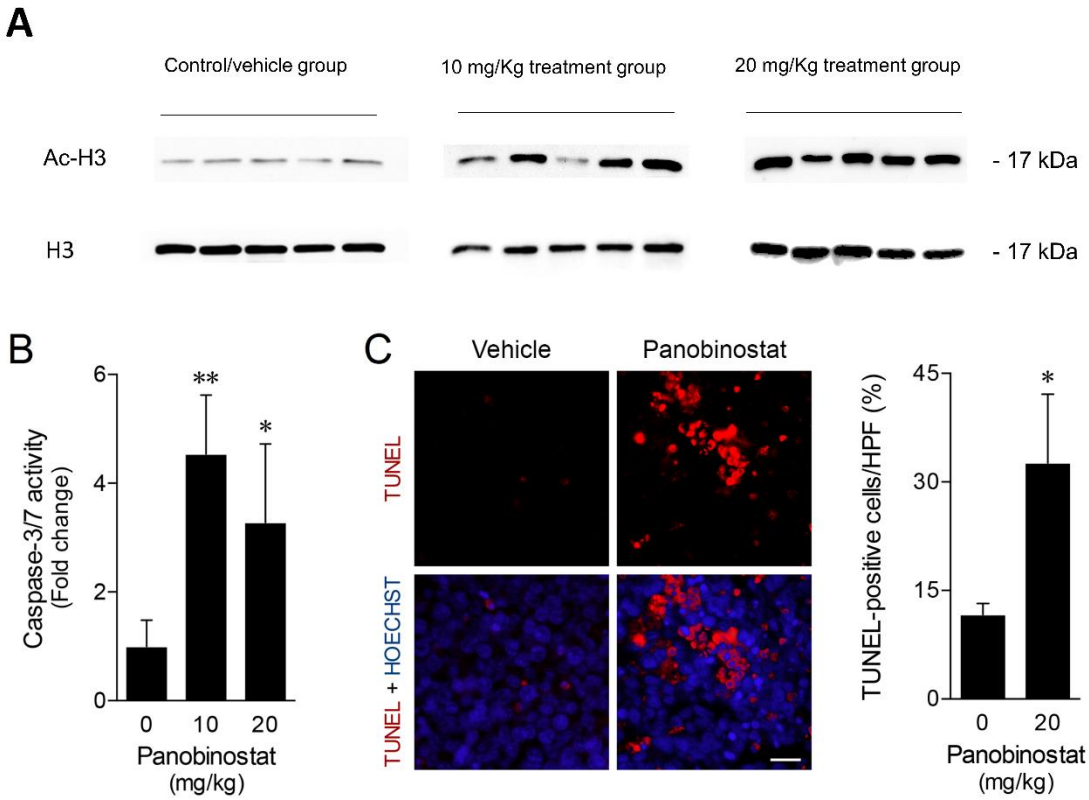
SOPF/SHO SCID mice (6-8 weeks-old) were injected subcutaneously with 1×10^6 CLBL-1 cells in a matrigel suspension. When tumors reached ~100 mm^3, mice were randomized into three treatment groups: not treated (controls/vehicle only), panobinostat at 10 mg/kg and panobinostat at 20 mg/kg (n = 5 per group). Mice were treated with intraperitoneal injections for 2 weeks, 5 days per week. **(A)** Tumor volumes were measured prior to drug administration and after the initiation of therapeutic interventions, three times a week, using a caliper and calculated as $(width)^2 \times length$ (±SEM). The tumor growth curve showed that both treatment groups had a statistically significant tumor growth inhibition compared to vehicle group. *$p < 0.05$ when compared to the vehicle control treatment. **(B)** Representative images of xenografted tumors were captured at the end of 2 weeks of therapy. Scale bar =5 mm. (**C**) - Representative images of the hematoxylin and eosin (H&E) stained xenograft tumor sections. Upper panel - magnification = 20x, scale bar = 200 μm. Bottom panel - magnification = 400x, scale bar = 20 μm.

4.4.6 Panobinostat *in vivo* effects on canine lymphoma xenograft tumors

To evaluate the panobinosat effects *in vivo*, the acetylation status of the H3 histone protein was evaluated in the tumor xenograft samples by western blot analysis. As shown in Figure 42A, the data obtained demonstrated an increase of H3 histone acetylation on treated tumor samples, compared with control/vehicle treated samples. These differences were strongly evident in 20 mg/kg treated tumor samples. To further characterize panobinostat mechanism

of action, apoptosis was evaluated according to caspase activity in tumor protein extracts and TUNEL analysis in histological sections. Consistent with the *in vitro* observations, caspase-3/7 activity was significantly increased up to 4-fold in tumors from treated animals (Figure 42B). Taking that into consideration, TUNEL analysis of *in vivo* tumors was performed after selection of fields without apparent necrosis by the pathologist. Tumor sections from mice receiving panobinostat treatment demonstrated an increase of TUNEL-positive cells as compared to vehicle treated tumors ($p < 0.05$). As shown in Figure 42C, panobinostat treatment at 20 mg/kg was associated with an increase up to 30% of TUNEL-positive cells. Altogether, these results demonstrate that panobinostat exhibits strong antitumor activity in a canine B-cell lymphoma xenograft murine model.

Figure 42 - Panobinostat promotes strong *in vivo* anticancer activity.



(**A**) H3 histone acetylation in total protein extracts from xenograft tumor samples were evaluated by western blot with anti-acetyl-histone H3 polyclonal antibody. Loading was controlled with H3 histone using anti-histone H3 polyclonal antibody. Representative blots are shown. (**B**) Caspase-3/7 activity was evaluated in tumor protein extracts using the Caspase-Glo 3/7 assay. Results are expressed as mean ± SEM fold-change to vehicle treated tumors. (**C**) Apoptosis was evaluated in tumor sections and quantified according to TUNEL-positive cells per high power-field (HPF) (left panel). Representative microphotographs of TUNEL analysis (630× magnification), with apoptotic cells (red)

and Hoechst stained-nuclei (blue) are shown (right panel). Scale bar, 15 μm. Results are expressed as mean ± SEM. *$p < 0.05$ and ** $p < 0.01$ from vehicle control treatment.

4.5. Discussion

Lymphoma is responsible for significant morbidity and mortality in pet dogs. Its remarkable similarities to human NHL prove clinically realistic opportunities to explore therapeutic protocols that may translate to human clinical trials (Ito et al., 2014; Marconato et al., 2013). To the best of our knowledge, this is the first study exploring HDACis antitumor therapeutic properties for treatment of canine B-cell lymphoma. HDACis have demonstrated anticancer efficacy in both *in vitro* and *in vivo* studies across a range of malignancies, and most impressively in hematological cancers. Consequently, they have undergone a rapid phase of clinical development either as a monotherapy or in combination with other anticancer agents (Chun, 2015; Dickinson, Johnstone, & Prince, 2010). Indeed, four HDACis have been approved by the FDA for cutaneous T-cell lymphoma and multiple myeloma treatment - vorinostat, romidepsin, belinostat and panobinostat (Stahl et al., 2016). Regarding the veterinary clinical setting, it has been previously reported by Kisseberth *et al.* the *in vitro* effect of two HDACis, OSU-HDAC42 and SAHA, on a panel of canine cancer cell lines, including canine T-cell lymphoma (Kisseberth, Murahari, London, Kulp, & Chen, 2008). In this study, the authors showed that the HDACis induced cytotoxicity, histone acetylation and apoptosis when cells were treated under μM doses (Kisseberth et al., 2008). In another study, Wittenburg *et al*. also described that the HDACi valproic acid sensitizes human and canine osteosarcoma cells to the anti-tumor effects of the topoisomerase-II inhibitor doxorubicin. The pharmacokinetics and pharmacodynamics features of this combination treatment was further assessed during a Phase I clinical trial in tumor-bearing dogs, demonstrating the safety and clinical utility of this class of drugs for veterinary oncological applications (Wittenburg, Gustafson, & Thamm, 2010).

Within this context, in the present study we aimed to investigate the HDACis antitumor properties on canine diffuse large B-cell lymphoma. For this purpose, a panel of seven HDACis was initially tested on the well-characterized CLBL-1 canine B-cell lymphoma cell line. The antitumor properties of the tested compounds observed here is in line with previous studies from other authors, both in hematological [37–40] and solid malignancies in other species (Bluethner et al., 2007; Catalano et al., 2012; Edwards, Li, Atadja, Bhalla, & Haura, 2007; Fortunati et al., 2010; Haefner et al., 2008). Indeed, all seven HDACis exhibited dose-dependent inhibitory effects on the proliferation of CLBL-1 cells. Nevertheless, and

importantly, with our study we were able to identify three HDACis (panobinostat, trichostatin A and scriptaid), which presented a high cytotoxic activity under the nM range. Moreover, our data demonstrated that the entire panel of HDACis evaluated was able to induce histone H3 acetylation on the CLBL-1 canine lymphoma cell line. Importantly, the levels of acetylated histone status were correlated with the IC_{50} values of the HDACis tested.

Based on the potent cytotoxic activity and induced histone acetylation, panobinostat revealed to be the most promising HDACi against canine B-cell lymphoma (CLBL-1 and 17-71) and was selected for further *in vitro* and *in vivo* studies. Panobinostat is a pan-HDACi, with low nanomolar concentration and high inhibitory activity against all Class I, II and IV HDACs both *in vitro* and *in vivo*, which has undergone extensive preclinical and clinical scrutiny (Prince & Prince, 2009). Panobinostat appears to be at least 10-fold more effective *in vitro* against Class I, II and IV HDACs when compared with vorinostat, the first FDA approved HDACi (Cea et al., 2013). A marked anti-tumor activity of panobinostat has been demonstrated across a broad range of cancer cell lines from hematologic malignancies, including cutaneous T cell lymphoma (CTCL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma, HL, as well as in solid tumors such as breast, colon, prostate and pancreatic cancer (Cea et al., 2013; Prince & Prince, 2009). Importantly, despite its strong antitumor activity at nanomolar concentrations, panobinostat only resulted in apoptosis of normal human cell lines at far greater concentrations, indicating a selective toxicity for cancer cells (Anne, Sammartino, Barginear, & Budman, 2013). In addition, other potential uses for panobinostat include maintenance therapy, its use as a potential radio-sensitizing agent and, combinatory therapy with other immunomodulatory drugs (Prince & Prince, 2009).

Noteworthy, the results presented herein have shown that the high efficacy of panobinostat against canine diffuse large B-cell lymphoma at sub-nanomolar concentrations was comparable to what has been described in several human lymphoid malignancies, where IC_{50} doses range from 5.6 to 31.5 nM (mean 11.2 nM) (Smith et al., 2009).

Several studies also report the involvement of non-histone protein acetylation in a diverse array of cellular processes including protein traffic, apoptosis and cell motility (Catalano et al., 2012; Singh et al., 2010). Therefore, to further investigate HDACi acetylation of non-histone proteins, we evaluated the effects of panobinostat on tubulin acetylation. Our data demonstrated that low doses of panobinostat (5 nM) on CLBL-1 cells led to tubulin acetylation, a marker of microtubule stabilization, and bundle formation.

Apoptosis activation through either the extrinsic or the intrinsic pathway has been shown to be one of the main mechanisms by which HDACis induce tumor cell death (Carew, Giles, &

Nawrocki, 2008). In this context, activation of caspases is a known feature of HDACi-induced apoptosis (Carew et al., 2008; Fulda & Debatin, 2006). In agreement with this, apoptotic cell death of CLBL-1 canine lymphoma cells after panobinostat treatment was strongly evidenced at 20 nM by the activation of caspase-3 and -7, and by the subsequent increase in the percentage of apoptotic cells. These results confirmed that the CLBL-1 canine lymphoma cell line used in this study was a good model to investigate the effects of HDACis and panobinostat. Additionally, it is worth mentioning that the effects of panobinostat observed in this work are attained at concentrations achievable in patient plasma, and are thus highly relevant to the clinical set (Catalano et al., 2012). Indeed, in a Phase I clinical study on intravenous panobinostat, Cmax reached up to 200 nM (Giles et al., 2006) and preliminary results (Beck et al., 2004) from an ongoing Phase I pharmacokinetic study on oral panobinostat, conducted in patients with solid tumors and hematologic malignancies, a steady state Cmax ranged from 15 to 35 nM.

Finally, in order to evaluate the pre-clinical efficacy of panobinostat, we performed *in vivo* studies using a SCID xenograft model implanted with CLBL-1 canine lymphoma cells. Animal data confirmed the antitumor properties of panobinostat, showing that the drug significantly induces apoptosis, while impairing tumor growth. The maximum TGI effect was strongly evident at both 10 mg/kg and 20 mg/kg (TGI of 82.9% and 97.3%, respectively). Furthermore, enhanced H3 histone acetylation levels on tumor samples confirmed that *in vivo* tumor growth inhibition was correlated with panobinostat mechanism of action as observed in the *in vitro* studies. It is also important to mention that panobinostat treated groups, namely animals from the 20 mg/Kg treated group, presented mild toxicity signs, similarly to those reported in previously published studies (Catalano et al., 2012). As stated by Subramanian and collaborators, HDACis are mostly well tolerated, however, due its potent and wide spectrum HDAC inhibition, panobinostat is associated with significant dose-limiting toxicities (Subramanian, Bates, Wright, Espinoza-Delgado, & Piekarz, 2010). No previous clinical studies have been performed in canine patients using panobinostat and other HDACi with similar toxicity profile, such as the FDA-approved vorinostat and romidepsin (Andreu-Vieyra & Berenson, 2014). For this reason, following preclinical validation of panobinostat, it will be important in the future to carry toxicity studies in dogs using single-agent and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) combination protocols.

In conclusion, the anti-cancer activity of panobinostat demonstrated herein validates HDACis as a novel cancer therapy for canine B-cell lymphoma. Translational and clinical studies will determine the clinical utility and safety of panobinostat as a single/adjuvant agent for the treatment of canine lymphoma. In addition, this work opens up perspectives in comparative

oncology as it validates the naturally occurring canine B-cell lymphoma model for translational HDACis research.

Chapter V

Establishment of a bioluminescent canine B-cell lymphoma xenograft model for monitoring tumor progression and treatment response in preclinical studies

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5.1. Abstract

Canine DLBCL is one of the most common cancers in dogs which shares remarkable similarities with its human counterpart, making the dog an excellent model for the investigation of novel therapeutic agents. However, the integration of canine lymphoma in comparative studies has been limited due in part by the lack of suitable xenograft mouse models for preclinical studies. To overcome these limitations, we established and characterized a localized subcutaneous bioluminescent canine DLBCL xenograft mouse

model. The canine CLBL-1 cell line expressing the luciferase and green fluorescent protein reporters was generated and used to establish the xenograft tumor model. A pilot study was first conducted with three different cell densities (0.1×10^6, 0.5×10^6 and 1×10^6 cells) in SCID mice. All mice presented homogeneous tumor induction within eight days after subcutaneous injection, with a 100% engraftment efficiency and no significant differences were observed among groups. The tumors were highly aggressive and localized at the site of inoculation and reproduced histological features and immunophenotype consistent with canine DLBCL. Importantly, xenograft tumors were detected and quantified by bioluminescent imaging. To assess response to therapy, a therapeutic study with a histone deacetylase inhibitor, panobinostat, was performed. The results demonstrated that panobinostat (20 mg/kg) efficiently inhibited tumor growth and that bioluminescent imaging allowed the monitorization and quantification of tumor response to therapy. In summary, this study provides a bioluminescence canine DLBCL model that offers high engraftment efficiency, preservation of tumor features, and noninvasive monitoring of tumor progression, validating the model as a promising preclinical tool for both veterinary and human medicine.

5.2. Introduction

NHL is a leading cause of cancer-related death in the United States and Europe, and its incidence continues to increase (Teras et al., 2016; Tilly et al., 2015). NHL encompasses a heterogeneous group of malignancies that usually originates in the lymph nodes, but can occur in almost any tissue, resulting from the neoplastic transformation of B and T lymphocytes (Shankland et al., 2012). DLBCL is the most common subtype of NHL, comprising approximately 30-58% of all NHL cases (Tilly et al., 2015). DLBCL is an aggressive form of lymphoma that is initially chemoresponsive, showing favorable responses to frontline R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone) immunochemotherapy. However, 10-15% of DLBCL patients are primarily refractory to this treatment and approximately 20-25% relapse after the initial response, with poor survival rates after current salvage therapy regimens (Sehn & Gascoyne, 2015). As such, new therapeutic agents and approaches are urgently needed. A multitude of new drugs are entering clinical development for NHL treatment; nevertheless the approval of new therapies remains low due in part to the scarcity of clinically relevant models for validation (Younes, 2011). Canine DLBCL, one of the most common neoplasias in dogs, shares genetic, biological, molecular and clinical similarities with its human counterpart, making the dog an excellent animal model to explore novel therapeutic molecules and approaches for human

DLBCL (Curran et al., 2017; Ferraresso et al., 2017; Mudaliar et al., 2013; Richards et al., 2011; Richards & Suter, 2015). Moreover, dogs diagnosed with lymphoma are frequently treated with anthracycline based chemotherapy regimens, similarly to human DLBCL patients, providing realistic opportunities to explore therapeutic protocols that may translate to human clinical trials (Richards et al., 2011). These initiatives are also encouraged by the increasing healthcare standards demanded by pet owners, creating the need for novel cancer therapies designed for veterinary applications (Henry & Bryan, 2013; Porrello et al., 2006; Weiskopf et al., 2016). Still, the integration of canine lymphoma as an animal model for clinical validation of therapeutics has been limited partially by the lack of suitable cNHL mouse models for preclinical research (Richards & Suter, 2015). In fact, even though comparative oncology studies provide unique information not easily provided by conventional preclinical models, the use of the tumor-bearing dog model for innovative drug development requires previous controlled toxicokinetic studies in laboratory animals (LeBlanc, Mazcko, & Khanna, 2016).

Mouse models have been critical tools for studying the biology and genetics of cancer as well as for predicting efficacy and for evaluating toxicity of anti-cancer therapeutics (Morton & Houghton, 2007; Yee, Ignatenko, Finnberg, Lee, & Stairs, 2015). Indeed, the discovery that tumor tissue could be xenografted into T-cell deficient nude athymic (nu/nu) mice (Pelleitier & Montplaisir, 1975), and later into B-cell-deficient and T-cell-deficient severe combined immunodeficient (scid/scid) mice (Paine-Murrieta et al., 1997), started a new era for experimental studies in oncology, allowing the routine and efficient transplantation and propagation of human tumor tissues in mice. In fact, many human xenograft tumor models have been established, especially for human lymphoma, resulting in the identification of therapeutic molecules that continue to lead clinical cancer management as chemotherapy treatments (Kohnken et al., 2017).

Despite the increasing investment in canine lymphoma research, there is a paucity of validated, well characterized and widely disseminated canine lymphoma preclinical models. Possibly due to the low number of available well-characterized canine lymphoid cell lines, the majority of *in vivo* canine lymphoma models described represent T-cell lymphoid malignancies (Kisseberth et al., 2007; Nadella et al., 2008; Umeki et al., 2013). Indeed, CLBL-1 cell line is the only canine cell line that faithfully represents DLBCL, reproducibly inducing tumors and preserving its phenotype in the xenotransplantation setting (Rütgen et al., 2010, 2012; Weiskopf et al., 2016). Within this context, CLBL-1 xenograft mouse models are the most reliable preclinical tool of canine B-cell lymphoma. Although previous studies [11, 24] paved the way for the development of canine B-cell lymphoma mouse models many

questions remained to be answered regarding tumor engraftment efficiency, reproducibility and the potential to be used for bioluminescent (BLI) monitoring. Aiming to overcome such limitations, we established and characterized a new localized subcutaneous bioluminescent canine CLBL-1 DLBCL xenograft mouse model that easily allows monitoring tumor progression and treatment response in preclinical studies.

5.3. Material and methods

5.3.1. Cell culture and reagents

The canine CLBL-1 B-cell lymphoma cell line previously established by Dr Barbara Rütgen, (Department of Pathobiology, University of Veterinary Medicine, Vienna, Austria) (Rütgen et al., 2010, 2012) was cultured in RPMI-1640 medium (Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated FCS (Gibco) and penicillin 100 U/ml plus streptomycin 0.1 mg/ml (Gibco). Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 (T75-tissue culture flasks, Greiner Bio-One, Kremsmünster, Austria).

5.3.2. Construction of a CLBL-1$^{GFP+luciferase+}$ stable cell line

For *in vivo* live imaging, a CLBL-1$^{GFP+luciferase+}$ stable cell line was generated using a lentiviral system encoding firefly luciferase and green fluorescent protein (GFP) reporters. CLBL-1 cells were transduced with luciferase-2A-GFP lentiviral particles (Amsbio Cat#LVP020), according to the manufacturer's protocol and as previously described (da Silva et al., 2012). Briefly, 5×10^6 CLBL-1 cells were resuspended in 100 µl of lentiviral particles ($1x10^7$ IFU/ml) and subject to spinoculation method (O'Doherty, Swiggard, & Malim, 2000). After 6h, medium was changed for fresh complete RPMI and after 24h an equal amount of fresh medium was added. At 72h, transduction efficiency was assessed by FACS and GFP positive cells were sorted, using FACSAria IIu sorter (BD Biosciences), and maintained in the same culture medium supplemented with gentamycin 50 µg/ml (Gibco) for 7 days to avoid contamination. After 4 weeks in culture, CLBL-1$^{GFP+luciferase+}$ cells were subjected to a second sort to ensure a stable GFP expressing cell line and cultured as previously described above. Two weeks after the second cell sort and two months following the cell line maintenance, GFP fluorescence was confirmed by FACS analysis. In addition, luciferase activity was confirmed using a luciferase assay kit (Promega, Wisconsin, USA) according to the manufacturer´s protocol. As a control, non-transduced CLBL-1 cells were analyzed in

parallel. To confirm that no alteration of cellular physiology occurred during the construction of CLBL-1$^{GFP+luciferase+}$ cell line, we compared growth patterns of both parental and transduced cell lines using a cell doubling time assay as previously described by *Rütgen et al, 2010* (Rütgen et al., 2010). Finally, CLBL-1$^{GFP+luciferase+}$ cell line was authenticated by short tandem repeat testing and compared to the parental CLBL-1 cell line (Eurofins Genomics, Ebersberg, Germany).

5.3.3. Mouse and breeding conditions

All animal-handling procedures were performed according to EU recommendations for good practices and animal welfare, and approved by the Animal Care and Ethical Committee of the Veterinary Medicine Faculty. Female 6–8-wk-old SOPF/SHO SCID mice (Charles River Laboratory) were maintained in microisolation individually ventilated cages under pathogen-free conditions (Tecniplast, Buguggiate, Italy, Boxunsfeu model, with H14 Hepa Filter e Prefilter sheet for Smart Flow). Mice were allowed to acclimatize for at least two weeks prior to the experiment start. Mice were kept on a 12h light: 12h dark cycle. Room temperature was maintained at 24–26°C. Food pellets and water were sterilized and provided *ad libitum.*

5.3.4. Establishment of a localized subcutaneously bioluminescent canine DLBCL xenograft model

To establish the bioluminescent xenograft model, a pilot tumor induction study was first conducted with three different cell densities. For that, nine SCID mice were randomly assigned to three different groups, according to the cell density used for inoculation: group I - 1×10^6 cells (n=3), group II - 0.5×10^6 cells (n=3) and group III - 0.1×10^6 cells (n=3). Suspensions of CLBL-$^{1GFP+Luciferase+}$ cells in PBS with matrigel® (Corning, NY, USA, Cat # 354248) (1:1) were injected subcutaneously into the dorsal interscapular region to induce tumors. Tumor volume and body weight were measured three times per week. Tumor volume was calculated as $(width)^2 \times length$ from electronic caliper measurements. Tumor endpoints criteria included tumor volume diameter superior to 1.5 cm and/or signs of marked changes in locomotion and posture, difficulties in accessing or ingesting food and drink, weight loss ≥15%, signs of pain (grimace scale ≥1). The tumor load in the mice was also analyzed by weekly BLI with IVIS system (Xenogen Corp., Alameda, CA) as described below. After two weeks of tumor development, animals reached a humane endpoint and were sacrificed,

necropsy was performed by a veterinary pathologist. Tumor and main organs including the liver, kidney, lung, spleen, and intestine were harvested and formalin-fixed.

5.3.5. *In Vivo* Bioluminescence Imaging

In vivo BLI was conducted on a cryogenically cooled IVIS system (Xenogen Corp., Alameda, CA) using LivingImage acquisition. Prior to BLI imaging, mice received a 150 mg/kg intraperitoneal injection with D-Luciferin (Xenolight, potassium salt). D-Luciferin was purchased from PerkinElmer´s and was dissolved to 15 mg/ml in PBS, filter-sterilized. Fifteen minutes after substrate injection, animals were anesthetized by intraperitoneal injection with a mixture of medetomidine (1mg/Kg) and ketamine (75 mg/Kg). A photographic image of the animal was taken in the chamber under dim illumination, followed by acquisition and overlay of the pseudocolor image representing the spatial distribution of photon counts produced by active luciferase within the animal. An integration time of 1 min with a binning of 100 pixels was used for luminescent image acquisition. Acquired images were analyzed using Living Image Software version 4.5.5 (Xenogen Corp.). Signal intensity was quantified as the sum of all detected photon counts within the region of interest after subtraction of background luminescence measured at the dorsal trunk.

5.3.6. Histopathological Analysis

Tissues, including tumors, were fixed in 10% buffered formalin and embedded in paraffin using a Leica tissue processor. Three µm sections were cut from paraffin blocks and stained with hematoxylin & eosin (H&E). Sections were mounted onto superfrost ultra plus slides (Menzel-Glaser, Braunschweig, DE) for immunohistochemistry.

5.3.7. Imunohistochemistry analysis

A representative area of each tumor was selected and tissue sections of 3µm thickness were mounted on glass slides (Superfrost glass slides, Thermo Scientific, Braunschweig, Germany), deparaffinized with xylene and hydrated in a graded ethanol series to distilled water. All protocol steps were carried out using the Novolink Polymer Detection System (Novocastra, Leica Biosystems, Newcastle, UK), according to the manufacturer's instructions. The antigen retrieval step was performed by microwave treatment (5 min at 900 watts plus 15 min at 650 watts) in Tris–EDTA buffer (pH 9.0). The system's Peroxidase

Block Solution and Protein Block Solution were used sequentially to block endogenous peroxidase and to prevent unspecific labelling, respectively. Tissue sections were incubated 30 min at room temperature with two antibodies: polyclonal rabbit anti-human CD20 (Thermo Fisher Scientific), diluted 1:200, and rabbit polyclonal anti-human CD3 (Dako, Glostrup, Denmark), diluted 1:400. For all antibodies, labelling was developed by incubating the slides with the system´s chromogen, diaminobenzidine (DAB), and hydrogen peroxide as substrate. Nuclear background staining was performed with Gill's hematoxylin (30 seconds). Labelling without the primary antibody was used as negative control. Dog lymph node sections were used as positive control.

5.3.8. Assessment of therapeutic response in the bioluminescent mouse model of canine DLBCL

To validate the bioluminescent canine DLBCL xenograft model for preclinical studies and its potential to investigate the utility of BLI in monitoring response to therapy, a therapeutic study was conducted with panobinostat, a HDACi. For this purpose, ten SCID mice were injected subcutaneously into the dorsal region with suspensions of 1×10^6 cells of CLBL-$1^{GFP+Luciferase+}$ cells in PBS with matrigel® (1:1) to induce tumors. When tumors reached a minimum volume of 100 mm^3, mice were randomly assigned to one of the two groups: control group (vehicle only, n=5) and treatment group (20 mg/kg panobinostat, n=5). Vehicle (2% DMSO + 48% PEG300 + 2% Tween 80 + ddH_2O) and treatment dose selection were based on our previous studies (Dias et al., 2018). Panobinostat (Selleckchem, Houston, TX, Cat # S1030) stock solutions were prepared at 67 mg/ml in DMSO (Sigma-Aldrich) and stored at -20ºC. Treatment consisted of intraperitoneal injections 5 days per week, over two weeks. Tumor volume and body weight was measured three times per week. Tumor volume was calculated as $(width)^2 \times length$. Compound activity was determined by TGI. TGI was determined as the percent change in tumor volume of treated over control animals (%T/C). At the end of the study, all animals were examined using *in vivo* bioluminescence imaging, as described above, and were sacrificed for necropsy examination by a pathologist. Tumor and main organs, including the liver, kidney, lung, spleen and intestine, were collected and formalin-fixed.

5.3.9. Statistical analysis

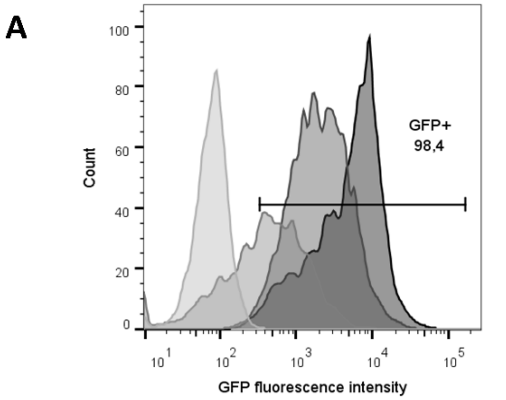
All data was expressed as mean ± standard error of mean (SEM). Analysis was performed using Prism 5 (Graphpad Software). All data normality was analyzed using Shapiro-Wilk's test. For *in vitro* assays, statistical significance of results was determined by One-way ANOVA followed by Tukey Multiple Comparison test to compare individual groups. The distribution of the *in vivo* assays results did not pass the normality test. Therefore, groups were compared using the Mann–Whitney U-test.; p values < 0.05 were considered statistically significant.

5.4. Results

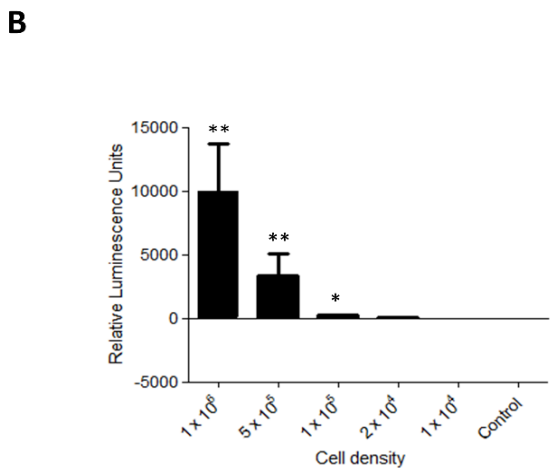
5.4.1. Generation of a stable CLBL-1$^{GFP+luciferase+}$ cell line

The CLBL-1 cell line (Rütgen et al., 2010, 2012) was transduced with a bicistronic lentiviral vector, as described in the material and methods section, to generate a stable canine DLBCL cell line expressing both firefly luciferase and GFP reporters for bioluminescence and fluorescence detection. The CLBL-1 cell line was selected for our study because it is the only canine cell line that faithfully represents DLBCL, reproducibly inducing tumors and preserving its phenotype in the xenotransplantation setting (Dias et al., 2018; Rütgen et al., 2010, 2012; Weiskopf et al., 2016). As shown in Figure 43A, a stable CLBL-1$^{GFP+luciferase+}$ cell line was generated after two cycles of cell sorting. The luciferase activity was confirmed using a luciferase assay kit. As shown in Figure 43B, luciferase activity of CLBL-1$^{GFP+luciferase+}$ was confirmed and it was correlated with cell density. In contrast, no luciferase activity was observed for the parental CLBL-1 cells.

Figure 43 - Generation of a stable CLBL-1$^{GFP+luciferase+}$ cell line.



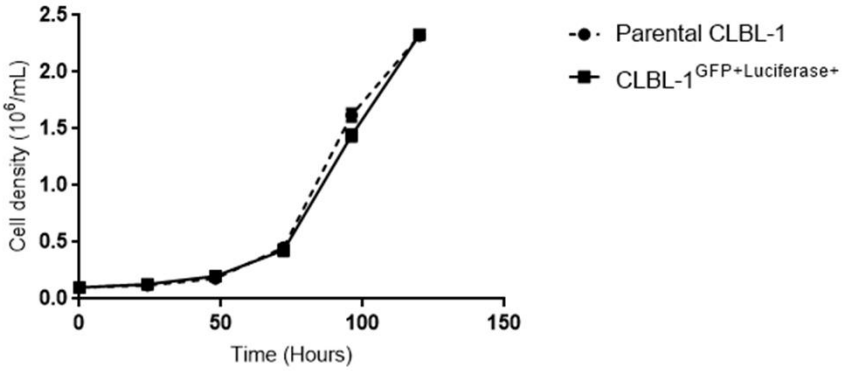
	SAMPLE NAME	% GFP
	CLBL-1	0,2
	First sort CLBL-1$^{GFP+Luciferase+}$	59,3
	Second sort CLBL-1$^{GFP+Luciferase+}$	97,9
	Stable line CLBL-1$^{GFP+Luciferase+}$	98,4



(A) Histogram of CLBL-1$^{GFP+luciferase+}$ cell line construction, representing GFP expression analysis after the first and second cell sort. CLBL-1 cells were transduced with lentiviral particles encoding GFP-luciferase reports. After 72 h, GFP positive cells were sorted, using FACSAria IIu sorter (BD Biosciences), and cultured in RPMI medium. After 4 weeks in culture, cells were subjected to a second sort to ensure a stable GFP expressing cell line. The phenotype of the final CLBL-1$^{GFP+luciferase+}$ stable cell line that was used in further assays, is represented by the right-side histogram. **(B)** Luciferase activity was analyzed in the CLBL-1$^{GFP+luciferase+}$ cell line using the parental cell line as a control (Control 1 – 1×10^4 cells and Control 2 - 1×10^6 cells). Indicated cell densities of both cells lines were lysed, incubated with D-luciferin and luminescence was measured. Results are expressed as mean ± SEM. *$p < 0.05$ and ** $p < 0.01$ from control cells.

Importantly, the evaluation of growth patterns through a cell doubling time assay confirmed that the stable CLBL-1$^{GFP+luciferase+}$ cell line exhibited a similar doubling time compared to the CLBL-1 parental cell line (26.45 hour doubling time for CLBL-1$^{GFP+luciferase+}$, versus 26.52 hours for the parental CLBL-1 cell line) (Figure 44).

Figure 44 - Growth curves of the CLBL-1$^{GFP+luciferase+}$ cell line versus the parental CLBL-1 cell line.

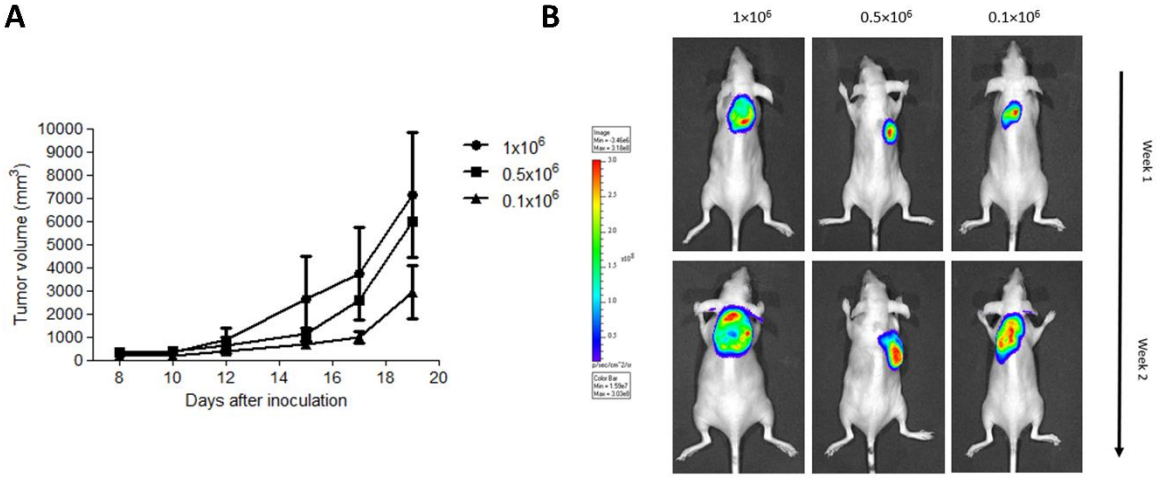


The cells were grown for 5 days in the regular cell culture conditions. The total numbers of cells over time are plotted in a logarithmic scale. Both cell lines showed similar growth patterns and doubling times.

5.4.2. Establishment of a subcutaneously bioluminescent canine DLBCL mouse model

To develop the subcutaneous bioluminescent canine DLBCL xenograft mouse model a pilot study was first performed with SCID mice inoculated with three different cell densities. For that, mice were randomly assigned to three distinct groups (n=3) according to the number of cells administered (group I = 1×10^6 cells, group II = 0.5×10^6 cells and group III = 0.1×10^6 cells). Suspensions of CLBL-1$^{GFP+luciferase+}$ cells were inoculated subcutaneously into the dorsum of SCID mice. All xenograft mice, regardless of cell density, presented tumor development at the site of injection eight days after cell inoculation. Importantly, tumors were established with a success rate of 100% (n=9) (Figure 45A). All tumors were efficiently monitored and quantified by BLI. No significant differences in tumor growth were observed between groups. As shown in Figure 45B, the BLI signal obtained confirmed tumor induction and allowed for tumor growth monitoring. In addition, it is important to mention that apart from tumor formation, no clinical abnormalities were observed in any of the transplanted mice during the experimental study.

Figure 45 - Establishment of a bioluminescent mouse model of canine DLBCL.



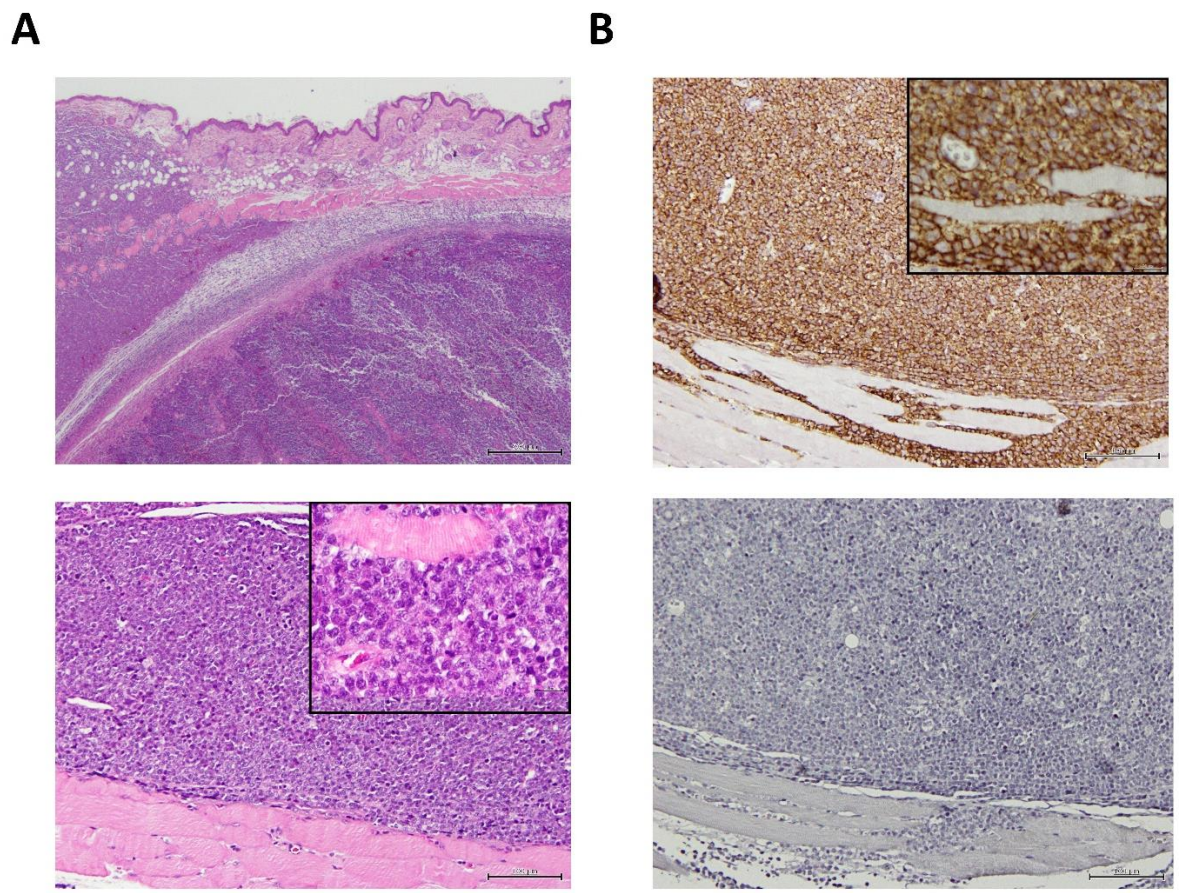
SOPF/SHO SCID mice (6-8 weeks-old) were injected subcutaneously with CLBL-1$^{GFP+Luciferase+}$ cells using three different cell densities (1×10^6 cells (n=3), 0.5×10^6 cells (n=3) and 0.1×10^6 cells (n=3)) in a matrigel suspension. **(A)** Tumor volumes were measured three times a week, using a caliper and calculated as $(\text{width})^2 \times \text{length}$ (±SEM). There were no significant differences in tumor size between groups. **(B)** Bioluminescent imaging was performed to monitor tumor development. Prior to BLI imaging, mice received an intraperitoneal injection with D-Luciferin. Fifteen minutes after substrate injection, animals were anesthetized and subjected to *in vivo* imaging. Representative images of bioluminescence imaging at the end of the first and second week are shown.

5.4.3. Characterization of xenograft tumor histopathological features

To assess macroscopic and microscopic characteristics of the bioluminescent canine DLBCL xenograft model, necropsy and histopathological evaluation were by a veterinary pathologist. Macroscopically, all xenografts were located in the injection site, the dorsal interscapular region. Tumors were nodular, soft and hemorrhagic and highly adherent to subcutaneous tissue and underlying muscle. No gross macroscopic alterations were identified in the main organs examined. Microscopically, tumors corresponded to compact infiltration of the dermis, hypodermis, muscle panniculus and skeletal muscle by large lymphoid cells with indistinct cytoplasmic borders, finely distributed nuclear chromatin and inconspicuous nucleolus (Figure 46A). There were extensive areas of necrosis and mitotic activity was considered intermediate (six to seven mitosis per high power field). These microscopic features are characteristic of a medium to high grade lymphoma (Rütgen et al., 2012).

Immunohistochemistry analysis of the xenograft tumor using CD20 and CD3 labelling was positive for CD20 in virtually 100% of the tumor cells confirming the phenotype of the CLBL-1$^{GFP+luciferase+}$ cell line (Figure 46B). Importantly, all these characteristics were consistent with data obtained for the parental CLBL-1 xenograft model (Dias et al., 2018; Rütgen et al., 2010, 2012).

Figure 46 - Histopathological characteristics of the CLBL-1$^{GFP+Luciferase+}$ cell line as a xenograft tumor in SOPF/SHO SCID mice.

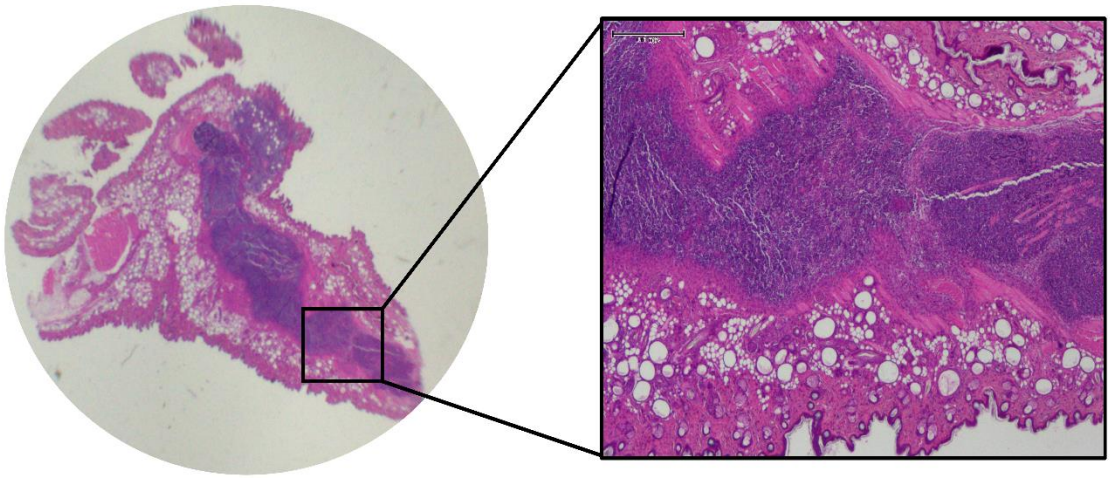


Mouse interscapular region. Xenograft CLBL-1$^{GFP+Luciferase+}$ tumor. **(A)** Upper panel - Compact infiltration of the dermis, hypodermis, muscle panniculus and skeletal muscle by large lymphoid cells (H&E, 20x). Bottom panel – Magnification of the tumor showing lymphoid cells with indistinct cytoplasmic borders and finely distributed nuclear chromatin and inconspicuous nucleolus. A muscle fiber is surrounded by tumor cells in the insert (H&E, 100x, insert 400x). **(B)** Upper panel – Immunohistochemistry for B-cells showing positivity in virtually 100% of the tumor cells (anti-CD20 antibody, Gill's hematoxylin, 100x, insert 400x). Bottom panel – Immunohistochemistry for T-cells, showing that tumor cells were negative for this marker (anti-CD3, Gill's hematoxylin, 100x).

5.4.4. Evaluation of bioluminescence canine DLBCL xenograft model for non-invasive monitoring tumor progression and response to therapy

Aiming to evaluate the suitability of the established bioluminescent canine DLBCL xenograft model for monitoring tumor progression and therapeutic responsiveness, we conducted an *in vivo* therapeutic study using panobinostat, a HDAC inhibitor. We have recently investigated antitumor properties of HDAC inhibitors for the treatment of canine DLBCL (Dias et al., 2018). Among a panel of HDAC inhibitors studied, panobinostat proved to be the most promising compound showing strong *in vitro* and *in vivo* antitumor properties against canine DLBCL. Therefore, panobinostat was selected to test the treatment response in the bioluminescent CLBL-1$^{GFP+luciferase+}$ xenograft model. Considering that in the pilot study all cell densities presented the same engraftment efficiency (100%), the 1×10^6 cell density was used to establish the xenograft model and the therapeutic study, allowing the further comparison with the xenograft model established using parental CLBL-1 cell line (Dias et al., 2018). As expected and consistent with the pilot study, all inoculated mice presented tumors eight days after inoculation. When tumors reached ~100 mm^3, mice were randomized into two groups: control group (not treated/vehicle, n=5) and treated group (panobinostat at 20 mg/kg, n=5). After two weeks of treatment, panobinostat at 20 mg/kg dose inhibited tumor growth by 93.3% when compared to vehicle control treated mice ($p < 0.05$) (Figure 48A). This tumor growth inhibition was similar to data gathered from the panobinostat efficacy study performed on the xenograft model using the parental CLBL-1 cell line (Dias et al., 2018). In addition, besides macroscopic dimensions, xenograft tumors of the panobinostat treated group presented identical histopathological characteristics to the xenograft tumors of the control group (Figure 47).

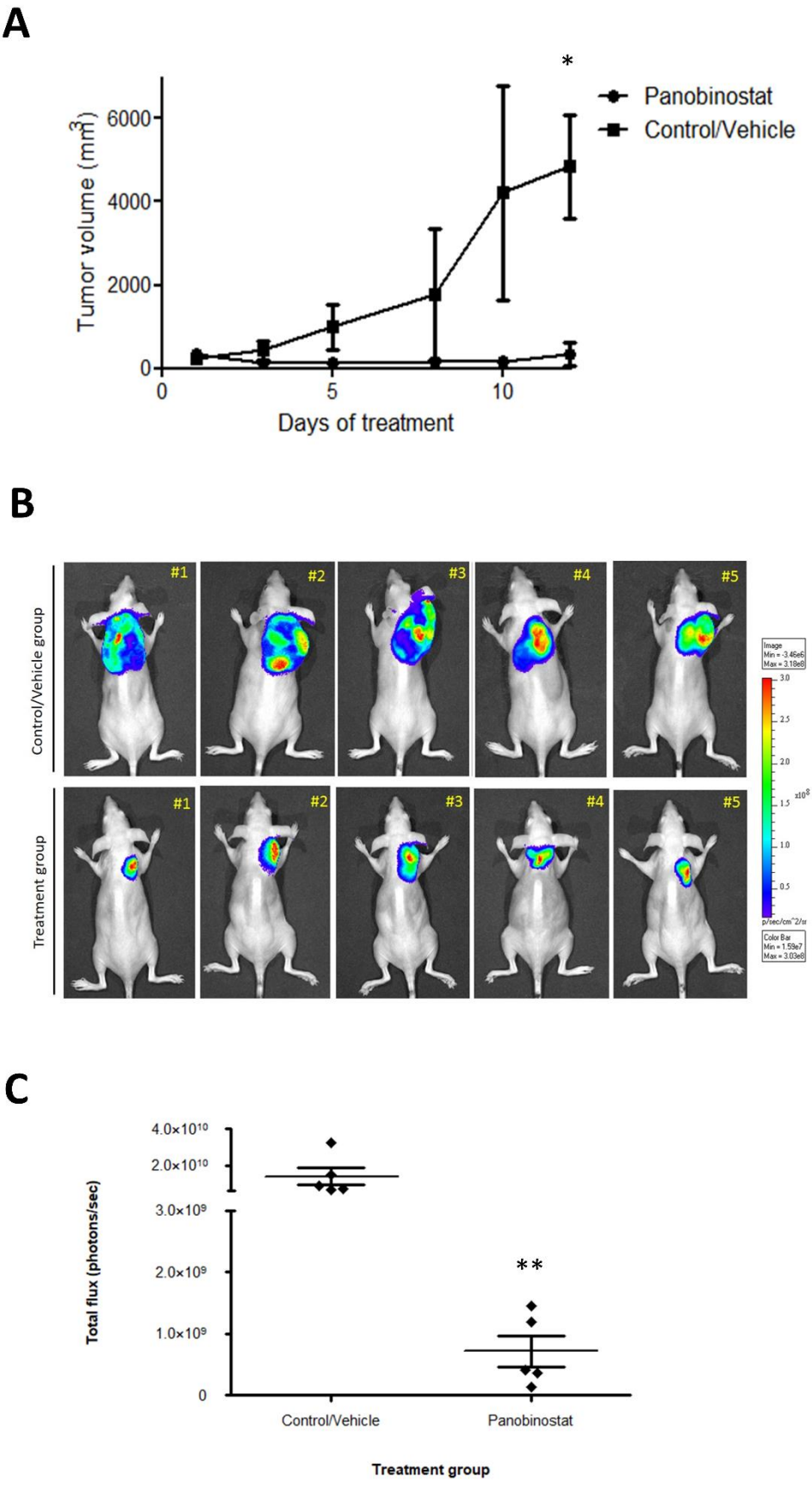
Figure 47 - Histopathological characteristics of the CLBL-1$^{GFP+Luciferase+}$ xenograft tumor in SOPF/SHO SCID mice after panobinostat treatment.



Mouse interscapular region. Xenograft CLBL-1$^{GFP+Luciferase+}$ tumor. **(A)** Compact infiltration of the dermis, hypodermis, muscle panniculus and skeletal muscle by large lymphoid cells (H&E, 20x). **(B)** Magnification of the tumor showing lymphoid cells with indistinct cytoplasmic borders and finely distributed nuclear chromatin and inconspicuous nucleolus. A muscle fiber is surrounded by tumor cells in the insert (H&E, 100x, insert 400x).

To validate the bioluminescence model for the detection of a therapeutic response, we quantified the photon signal intensity from BLI and statistically examined the differences between the two groups. As shown in the representative photographs (Figure 47B) and in the BLI measurements (Figure 47C) we can observe a significant lower BLI signal, up to 20-fold, in treated mice compared to untreated control mice ($p < 0.01$). Thus, the BLI signal obtained showed to be extremely suitable for visualization of tumor localization in mice and to monitor the tumor response to the therapeutic molecule.

Figure 48 - Assessment of therapeutic response of the bioluminescent canine DLBCL xenograft mouse model.



SOPF/SHO SCID mice (6-8 weeks-old) were injected subcutaneously with 1×10^6 of CLBL-$1^{GFP+Luciferase+}$ cells. When tumors reached ~100 mm^3, mice were randomized into two treatment groups: not treated (controls/vehicle only) and panobinostat at 20 mg/kg (n = 5 per group). Mice were treated with intraperitoneal injections for 2 weeks, 5 days per week. **(A)** The tumor growth curve showed that treatment group had a statistically significant tumor growth inhibition compared to the vehicle group (*$p < 0.05$). **(B)** Bioluminescent imaging was performed to monitor therapeutic response. Fifteen minutes after D-Luciferin substrate injection, animals were anesthetized and subjected to *in vivo* imaging. Representative images of bioluminescence imaging at the end of the therapeutic assay are shown. **(C)** Quantitative analysis of photon counts derived from CLBL-$1^{GFP+Luciferase+}$ xenograft mice between control/vehicle mice and mice receiving panobinostat treatment. The treated mice group presented a significant lower BLI signal, up to 20-fold, compared to untreated control mice; **$p < 0.01$ when compared to the vehicle control treatment.

5.5. Discussion

DLBCL is the most common canine aggressive B-cell lymphoma worldwide, sharing similar biological, behavioral, genetic, and molecular characteristics with the human counterpart (Aresu, 2016; Ferraresso et al., 2017; Mudaliar et al., 2013) Despite having good response to multiagent chemotherapy, curative treatment remains elusive for most dogs (Marconato et al., 2011). As such, collaborative efforts are being made to integrate naturally occurring canine lymphoma into novel cancer treatment studies, in order to improve the treatment for dogs diagnosed with lymphoma, while accelerating therapeutic development for human lymphoma (Ito et al., 2014; Marconato et al., 2013). However, the integration of canine lymphoma in comparative studies has been limited due in part by the lack of validated, well-characterized and widely disseminated canine lymphoma models for preclinical research (Richards & Suter, 2015).

To date, few *in vivo* canine lymphoma models have been described (Ito et al., 2011; Kisseberth et al., 2007; Nadella et al., 2008; Rütgen et al., 2012; Umeki et al., 2013). This is mainly due to the low number of available well-characterized canine lymphoid cell lines. In fact, canine hematopoietic cell lines have been historically difficult to establish and most cell lines are of T-cell origin (Richards & Suter, 2015; Seiser et al., 2013). Therefore, the majority of canine lymphoma xenograft murine models described until now represent T-cell lymphoid malignancies (Kisseberth et al., 2007; Nadella et al., 2008; Umeki et al., 2013). Notably, only four of the available cell lines are reportedly of B-cell origin, including the GL-1 cell line derived from a dog with B-cell acute lymphoblastic leukemia (Nakaichi et al., 1996); the 17-71, a B-cell cell line not initially phenotyped and that does not express typical B-cell

lymphoma markers (Steplewski et al., 1987); 3132, a cell line that probably is not of B-cell origin despite initial reports of surface immunoglobulin (Strandstrom & Rimaila-Parnanen, 1979) and CLBL-1, the only available cell line that has been well-characterized both *in vitro* and *in vivo* (Dias et al., 2018; Roode et al., 2016; Rütgen et al., 2010, 2012; Weiskopf et al., 2016). As a matter of fact, CLBL-1 appears to be the only exclusive cell line that faithfully represents DLBCL, reproducibly inducing tumors and preserving its phenotype in the xenotransplantation setting (Rütgen et al., 2010, 2012). Primary canine DLBCL xenografts have also been described and are a possible alternative approach, however, these tumors only form when implanted intraperitoneally into conditioned NOD *scid* gamma (NSG) mice and their variability in growth makes therapeutic evaluation challenging (Ito et al., 2011; Weiskopf et al., 2016).

The CLBL-1 cell line tumorigenicity, genomic stability, histological and morphological properties were initially reported on a xenograft murine model (Rütgen et al., 2012). For that purpose, CLBL-1 cell line was subcutaneously injected in the right and left flank of $Rag2^{-/-}\gamma c^{-/-}$ mice. This model was highly tumorigenic, and all mice demonstrated liver, spleen, bone marrow, ovaries and uterus lymphoma involvement. This was the first study demonstrating that CLBL-1 canine lymphoma cell line develops multicentric lymphoma as observed in canine patients, making it a highly stable tool for B-cell lymphoma research in veterinary and human medicine. Nevertheless, it revealed certain shortcomings related to heterogeneous clinical presentation and inability to monitor disease progression through non-invasive methods (Rütgen et al., 2012). A similar *in vivo* study with CLBL-1 in murine xenograft model has also been reported by Weiskopf *et al*. to study the synergy of the antitumor activity of blocking CD47 and anti-CD20 immunotherapy. This work established xenograft models of disseminated, intra-abdominal and subcutaneous disease into NSG mice, paving the way for the development of bioluminescent canine B-cell lymphoma mouse models. However, many questions remained to be answered regarding tumor engraftment efficiency, reproducibility and BLI monitoring (Weiskopf et al., 2016).

To overcome these limitations, we aimed at establishing and characterizing a localized subcutaneous bioluminescent xenograft mouse model of canine DLBCL, which would easily allow monitoring of tumor progression and treatment response in preclinical studies. For this purpose, we established a SCID xenograft model of canine DLBCL by subcutaneously implanting CLBL-$1^{GFP+Luciferase+}$ cells. The development of a stable cell line of CLBL-1 expressing luciferase and GFP, allows monitoring and quantifying the disease progression noninvasively. Currently, bioluminescent imaging (BLI) is one of the most widely used techniques to track target cells *in vivo*, especially hematopoietic cell lines that disseminate

widely in their hosts as xenografts (Xu et al., 2016). This technique relies on the fluorescent signal produced by the chemical reaction between the luciferase and its substrate (D-luciferin), as such it is highly specific and sensitive, allowing to visualize, quantify and monitor in real-time the tumor development (Close, Xu, Sayler, & Ripp, 2010). CLBL-$1^{GFP+ Luciferase+}$ cell line implanted in SCID mice induced highly aggressive tumors, with rapid tumor growth that requires close monitoring to avoid tumor burden. Three different cell densities for tumor establishment were tested (0.1×10^6, 0.5×10^6 and 1×10^6 cells) and all presented homogeneous tumor development within eight days after injection, with a 100% engraftment success rate. There were no significant differences in tumor growth curve between different cell density groups.

Histological and immunohistochemical analysis revealed that xenograft tumors retained similar histological characteristics and B-cell and T-cell markers expression, compared to original CLBL-1 cell line xenografts (Rütgen et al., 2010, 2012). Finally, the CLBL-$1^{GFP+Luciferase+}$ model, comparable to the parental CLBL-1 model, demonstrated a high consistency in disease progression with tumor onset occurring after 8 days of inoculation in all animals, providing an intervention window of two weeks that allows the rapid screening of a plethora of therapeutic molecules.

In order to confirm whether the bioluminescent canine DLBCL xenograft model could be a reliable preclinical tool for drug investigation, we conducted a therapeutic study. We have recently investigated antitumor properties of a panel of seven HDAC inhibitors for the treatment of canine DLBCL. Amongst all HDAC inhibitors studied, panobinostat proved to be the most promising compound and was selected for further *in vitro* and *in vivo* investigation. This potent HDACi demonstrated strong antitumor properties against a CLBL-1 xenograft canine tumor growth, as it efficiently inhibited tumor growth (Dias et al., 2018). As such, panobinostat was selected to test the treatment response in our established bioluminescent model. The results presented herein, demonstrated that treatment with panobinostat (20mg/kg) efficiently inhibited tumor growth and consequently reduced the BLI signals, up to 20-fold, when compared with the control mice. Thus, the BLI measurements obtained with the established bioluminescence xenograft model were extremely suitable for visualization of the tumor localization in the mice, but also highly useful for the quantitative detection of the tumor load and response to therapy.

In conclusion, in this study we established and characterized a novel localized subcutaneous bioluminescent canine DLBCL xenograft model that offers high engraftment efficiency, preservation of relevant tumor features and reproducible tumor growth. This model established with CLBL-$1^{GFP+Luciferase+}$ cells can be therefore efficiently used to monitor non-

invasively and quantitatively the outgrowth of canine DLBCL, and be a valuable preclinical tool for veterinary applications, while contributing to comparative oncology.

Chapter VI

Final conclusions and future perspectives

The current landscape of cancer research is facing a profound transformation with the introduction of immune-oncology as the fourth pillar for cancer therapy. Not only have immunotherapies resulted in unprecedented clinical responses, rapid drug development and several first-in-class approvals from the FDA in the past few years, but the advent of such innovative therapies is also revolutionizing treatment paradigms and algorithms in current oncology and hemato-oncology practice (Kelly, 2018). As a result, clinical and translational research need to adapt to a rapidly changing scenario to effectively translate novel concepts into sustainable and accessible therapeutic options for cancer patients (Golan, Milella, Ackerstein, & Berger, 2017). Historically, the success rate in developing new oncology drugs has been surprisingly low as compared to other areas of medicine, particularly in late phases of development. The complexities and challenges of the new era of immune-oncology strongly emphasize the need to identify new strategies, models and paths to develop fast, successful, and cost-effective therapies (Golan et al., 2017; Ventola, 2017).

Advances in cancer immunotherapy relies on faithful predictive preclinical investigation and rodent models have been the foundation of preliminary basic research and safety assays (Malaney et al., 2014). However, these induced-tumor models underrepresent the heterogeneity and complex interaction between the human immune cells and tumors. In fact, laboratory mice rarely develop spontaneous tumors, are housed under specific-pathogen free conditions that markedly influence immune development, and incompletely model key characteristics of the tumor/immune microenvironment, creating challenges for clinical translation. As a result, these murine models have failed to correlate with clinical success rates (Biemar & Foti, 2013; Kohnken et al., 2017; Kola & Landis, 2004). Thereby, the use of alternative animal models is pivotal to bridge the translational gap between murine models and human clinical studies.

The inclusion of a canine model in the drug development path of cancer immunotherapies is being widely recognized as a valid solution to overcome several hurdles associated with conventional preclinical models (Decker et al., 2017). Dogs with naturally occurring tumors are highly translational models that represent an opportunity to investigate the clinical potential of novel immunotherapies in a comprehensive manner. By complementing murine studies and human clinical trials, dogs allow monitoring the "scaling up" effects of a therapeutic approach that depends on complex interactions between tumor and immune cells, while assessing long-term efficacy and toxicity (Park et al., 2016). Taken together, these features may allow the establishment of solid foundations to rapidly translate the results obtained from canine patients to human patient management, with benefits for both species (Barutello et al., 2018). However, the implementation of such canine clinical trials is far from being an easy quest. It requires multiple organized efforts to validate the canine model, which still lacks a thorough characterization of the canine immune system and its effector cells and molecules, the evaluation of common tumor epitopes, the development of canine-specific/cross-reactive agents and the establishment of preclinical models for veterinary oncological settings (Klingemann, 2018).

Driven by the great success achieved with the application of immunotherapies in the treatment of hNHL and by the remarkable similarities of canine lymphoma to its human counterpart, canine lymphoma has been one of the main focus of comparative research regarding the development of immunotherapeutic approaches for dogs (Ito et al., 2015; Jain et al., 2016; Marconato et al., 2015; O'Connor & Wilson-Robles, 2014b; Weiskopf et al., 2016). These initiatives are also motivated by the great clinical demand to improve the poor prognosis of dogs diagnosed with cNHL, considering that the majority of dogs, regardless of the treatment options used, relapse with terminal, drug-resistant disease (Vail et al., 2007). 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 & Dow, 2015). Nevertheless, the reported therapeutic efficacy of these mAbs is suboptimal and substantially inferior to results reported in human patients, demonstrating an urgent and unmet need to develop effective immunotherapeutic strategies for cNHL (Klingemann, 2018).

Within this context, the present work was designed to establish an innovative and promising research line that enables the development of a novel sdAb-based drug delivery system for NHL, and the characterization of the immune system of dogs diagnosed with cNHL and validation of an animal model of human NHL.

In addition to tackling many of the limitations associated with the canine model, this work aimed to usher-in a new generation of targeted cancer therapies. For that purpose, a multidisciplinary strategy addressing several distinct complemental objectives was carefully designed. Altogether achieving the proposed objectives would allow the construction of a solid framework that offers a platform that could both support current research and promote further developments. Therefore, the present work comprised six different chapters with specific goals that have ultimately contributed to the main objective of the study.

The first chapter begins by covering a general overview of the challenges and prospects of today's cancer immunotherapies and the role that comparative medicine might play in solving the limitations brought by this rapidly expanding field. The state of art of both human and canine NHL and the rationale behind the use of the canine model to bridge the translational gap between murine preclinical studies and human clinical trials were also addressed, followed by a review of currently available immunotherapies for cNHL that demonstrates the potential contained in these therapeutic options. Finally, a brief synopsis of antibody therapeutics was presented, highlighting the latest advances in antibody development, the advantages of antibody fragments and the promising use of sdAbs as targeting moieties for cancer therapy. By shedding some light on these subjects, the intricate scope of our work was unraveled, allowing a better understanding of its outline and specific goals. The following chapters (2, 3, 4 and 5) focused on the main achievements and the implications of these findings.

Considering that new knowledge holds the key for innovation, basic and applied research go hand in hand when it comes to exploring the full potential of cNHL model. As mentioned above, much is still unknown about the cNHL immune status and it has become clear that insights into the immune system of these animals can propel the discovery of truly effective immunotherapies, either by unveiling new mechanisms/receptors or by improving the clinical efficacy of previous therapies. With that in mind, chapter 2 reports an effort to gain a better understanding of the systemic and intratumoral cytokine dysregulation/dysfunction in canine lymphoma. For that purpose, considering that high quality basic, clinical and translational cancer research requires prompt access to well-preserved biological samples, a canine lymphoma biobank was established with samples collected from several diagnosed dog patients. These samples were used to assess the cytokine mRNA profiles of circulating and intratumoral environments in multicentric canine lymphoma. Furthermore, blood count analysis and the comparison of putative cytokine serum levels of dogs diagnosed with lymphoma with those of healthy control dogs were also performed. Overall, it was concluded that, similarly to its human counterpart, local and systemic dysregulation in cytokine response

might be involved in cNHL pathogenesis and tumor survival promotion, progression and immune escape. By confirming and extending previous investigations, our work contributed to identify potential cytokine candidates to be used not only for diagnostic and prognostic purposes but also as targets for therapeutic intervention either by cytokine inhibition and/or immunomodulatory strategies. Notably, preliminary studies have reported that chemotherapy protocols, while prompted complete tumor responses, appeared to fail to restore immune response in cNHL, possibly reducing its ability to re-establish control over residual disease (Axiak-Bechtel et al., 2014). Also, active immunotherapy strategies suh as cancer vaccines have also proven to be successful in mounting an immune response and improving survival in dogs with NHL, when in combination with conventional chemotherapy (Marconato et al., 2014, 2015; Peruzzi et al., 2010). These findings strengthen the concept that harnessing the immune system with immunotherapeutic approaches might be the long-waited solution to improve the prognosis of cNHL. Importantly, the successful construction of a 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 current and future research studies. This is will be particulary advantageous for the investigation of common targets in human and canine NHL for the development and application of immunotherapeutic approaches. Indeed, these samples were used for the evaluation of the gene expression of several markers: CD19, CD21, CD22, CD74, and CD80; and assessment of their potential as immunotherapy targets for canine B-cell lymphoma. This study was conducted under this thesis project and resulted in the master thesis of André Almeida.

The work described in chapter 3 explored the development of recombinant antibodies for cNHL treatment and diagnosis. In the last decade, the scientific community has been reporting cases of therapeutic success using mAbs in human lymphoma treatment, encouraging the establishment of similar therapeutic options in veterinary settings (Adler & Dimitrov, 2012). However, the recently approved mAb-based therapies have presented disappointing scientific and clinical results, demonstrating that the discovery of an effective antibody for the treatment of cNHL may require a more complex and innovative approach. We have been showing that rabbit derived sdAbs can be developed against several targets and that these minimal scaffolds show great potential for therapeutic applications (Goncalves & Aires-Da-Silva, 2008; Volker et al., 2016). sdAbs are presently the smallest functional antibody fragment, only consisting of a VH or VL domain, which improves their tumor penetration and accessibility to targets not easily reached by complete IgGs (Aires da Silva et al., 2008; Corte-Real et al., 2005; Popkov et al., 2004; Volker et al., 2016). In addition to the reduced size, complementary-determining regions (CDRs; antigen-binding regions) of sdAbs can be easily engineered to develop

specific and high-affinity binders. Moreover, sdAbs also present excellent properties such as high stability, solubility, low immunogenicity and low manufacturing cost (Aires da Silva et al., 2008; Volker et al., 2016). Importantly, rabbit sdAbs also benefit from the unique ontogeny of rabbit B cells that promotes vastly distinctive antibody repertories rich in *in vivo* pruned binders of high diversity, affinity and specificity (Weber et al., 2017). Furthermore, rabbits are evolutionarily distant from mice and rats, so epitopes that are not immunogenic in rodents can be recognized by rabbit mAbs, increasing the targetable epitopes and facilitating the generation of mAbs that cross react with other species (Weber et al., 2017) - a key aspect for clinical translational.

As such, it has become clear that rabbit recombinant antibody fragments will become part of the new-generation of mAbs and that cNHL, as an animal model to its human counterpart, could could serve as a proof of concept and propel the development of novel immunotherapies strategies.

Nevertheless, a critical question remained: should we develop antibodies against a validated target or should we pursuit novel immunotherapeutic targets? On one hand, the majority of antibody therapeutics have been isolated from target-led drug discovery, where many years of target research preceded drug program initiation. On the other hand, as the search for validated targets becomes more challenging and target space becomes increasingly competitive, alternative strategies are gaining favor (Minter, Sandercock, & Rust, 2017). Considering the many strengths presented by each approach, we aimed to pursue both, as described in chapter 3, by developing sdAbs against canine CD20, a well-known clinically validated target in human NHL, and by developing novel sdAbs against other specific cNHL antigens using an innovative strategy.

The anti-CD20 mAb Rituximab has revolutionized the treatment of B-cell malignancies and is now used in a growing range of clinical settings (Ito et al., 2015; Motta et al., 2010). This unprecedented success has not only substantially changed the mindset of the clinical community about the ability of mAb to improve outcomes but has also catalyzed the interest in the pharmaceutical industry to develop the next generation of anti-CD20 mAbs (Alduaij & Illidge, 2011; Chames et al., 2009). A few 1st generation mAbs targeting canine CD20 were recently developed and one has obtained clinical approval, as previously mentioned. However, unsatisfactory clinical results have highlighted the need for 2nd and 3rd generation mAbs and an urgent demand to gain better understanding of antibody effector functions present in the canine immune system (Ito et al., 2015; Jain et al., 2016; Klingemann, 2018; Rue et al., 2015; Sinha, 2014). With this in mind, we have conducted the first comprehensive target characterization of canine CD20 using our cNHL biobank. Data obtained under gene

and protein expression studies on lymphoma and normal canine cells demonstrated an overexpression of this receptor on canine lymphoma cells, validating the canine CD20 as a potential target for veterinary immunotherapeutic strategies. Additionally, a new sequence of canine CD20 was identified in our biobank samples, diverging from previous published sequences. Furthermore, we reported the use of a novel strategy for the generation of recombinant anti-CD20 monoclonal antibodies that bind to the canine receptor and presents cross-reactivity against the human receptor. This resulted in the selection of a panel of novel sdAbs, that may become useful tools for exploring the development of novel therapeutic alternatives for comparative oncology.

Although the addition of Rituximab has altered the therapeutic landscape and improved prognosis of hNHL, the mortality rate is still high. Therefore, considerable room for improved outcome remains, including the use of novel strategies that act synergistically with CHOP–based protocols to provide greater tumor specificity and less nonspecific toxicity (Marconato et al., 2015). Among mAb-based therapies, ADCs are considered one of the most promising strategies, combining the tumor selectivity, pharmacokinetics and biodistribution properties of antibodies with the cytotoxic potency of small molecules (Diamantis & Banerji, 2016). The selection of the target antigen is the first and most important determining factor for a successful ADC, directly affecting its efficacy, therapeutic window and toxicity profile. The ideal characteristics of a useful ADC antigen include up regulated expression in the tumor; internalization of antigen *via* endocytosis in the presence of ligand and its recycling back to the plasma membrane and homogeneous antigen expression in the tumor microenvironment with low antigen abundance in healthy tissues (Sau et al., 2017). Therefore, CD20 targeting does not gather all the recommended criteria to be considered a valuable ADC target, despite its upregulation in cancer cells and low expression in normal cells, as the majority of anti-CD20 mAbs do not internalize upon binding (Klein et al., 2013). Recent studies indicate that, depending on the linker and drug combination, this mechanism may not be essential and ADCs can be cleaved extracellularly or via other mechanisms, therefore presenting a bystander killing effect (Staudacher & Brown, 2017). Nevertheless, during the development of sdAbs against novel cNHL targets, we focused in selecting sdAbs with binding and internalization properties, in order to develop a highly selective and potent new anti-cancer ADC therapeutic molecule.

To overcome the limitations of current discovery platforms, we have explored a novel selection strategy of tumor-specific antibodies against canine lymphoma that relies on displaying cancer-restricted antigens that can then be captured using multiple antibody-based technologies. Approaching the drug discovery process from this angle — the

antibody/therapeutic activity side — provides an opportunity to discover new targets and target epitopes, use the antibody as a tool to validate the target, and rapidly evaluate the functional characteristics of the antibody itself as a possible therapeutic agent (Loo & Mather, 2008).

For that purpose, a highly diverse library of rabbit sdAbs against primary canine NHL cells has been successfully constructed, to ensure the presence of antibodies against any potentially relevant target that is overexpressed in the disease setting. By coupling subtractive antibody selection rounds on whole-cells and a high-throughput screening, we have selected a panel of novel sdAbs targeting cNHL.

The obtained data showed that these selected sdAbs have great promise. In fact, recently a final *in vivo* selection on a human and canine NHL murine model confirmed that these sdAbs populations were indeed highly selective and specific against NHL. With this novel approach the repertoire of targetable NHL tumor receptors may be expanded, while simultaneously confirming the availability of the epitope *in vivo* and generating new antibodies for targeting. In the future, we expect to use these promising sdAbs as tools for the development of a novel ADC for NHL, by coupling a potent cytotoxic drug payload to the selected sdAbs.

Among the main components of an ADC – payload, linker and antibody – the payload is an important and crucial constituent of the therapeutic ADC molecule. In broad terms, payloads for ADCs can range from small cytotoxic molecules to protein toxins, enzymes, other proteins and radionucleotides (Strohl & Strohl, 2012). In the first generation of ADCs, researchers used clinically approved chemotherapeutics with known clinical profiles like doxorubicin, methotrexate, and 5-flurouracil as payloads, however, this ADC class was underwhelming due to the insufficient potency of the payload and/or high toxicity as a consequence of the instability of the ADC and systemic loss of the drug (Dan et al., 2018; Vankemmelbeke & Durrant, 2016). Since then, the development of second-generation ADCs focused on improving linker stability. Moreover, it has resorted to more potent (100–1000-fold) tubulin-targeting agents such as the auristatins and maytansinoids, or DNA-targeting agents such as the calicheamicins. That has led to the relatively recent US FDA approval of two microtubule-inhibiting ADCs: brentuximab vedotin (SGN-35, Acetris®) for Hodgkin and anaplastic large cell lymphoma and ado-trastuzumab emtansine (T-DM1, Kadcyla®) for metastatic HER2-positive breast cancer. Regardless, the second-generation of ADCs are not without its shortcomings. Notably, the heterogeneity of the final ADC with respect to drug–antibody ratio (DAR) due to stochastic coupling strategies, the narrow therapeutic index, the limited penetration of solid tumors and the development of resistances, continue to drive the field toward further improvements. As such, third generation ADCs focused on site-specific

conjugation to ensure homogenous ADCs with well-defined DAR. At the same time, efforts are being made to explore payloads with novel modes of action, with special focus on agents that present activity against nonproliferating cancer cells, which would widen the target area to include tumor-initiating cells and possibly overcome resistances (Vankemmelbeke & Durrant, 2016).

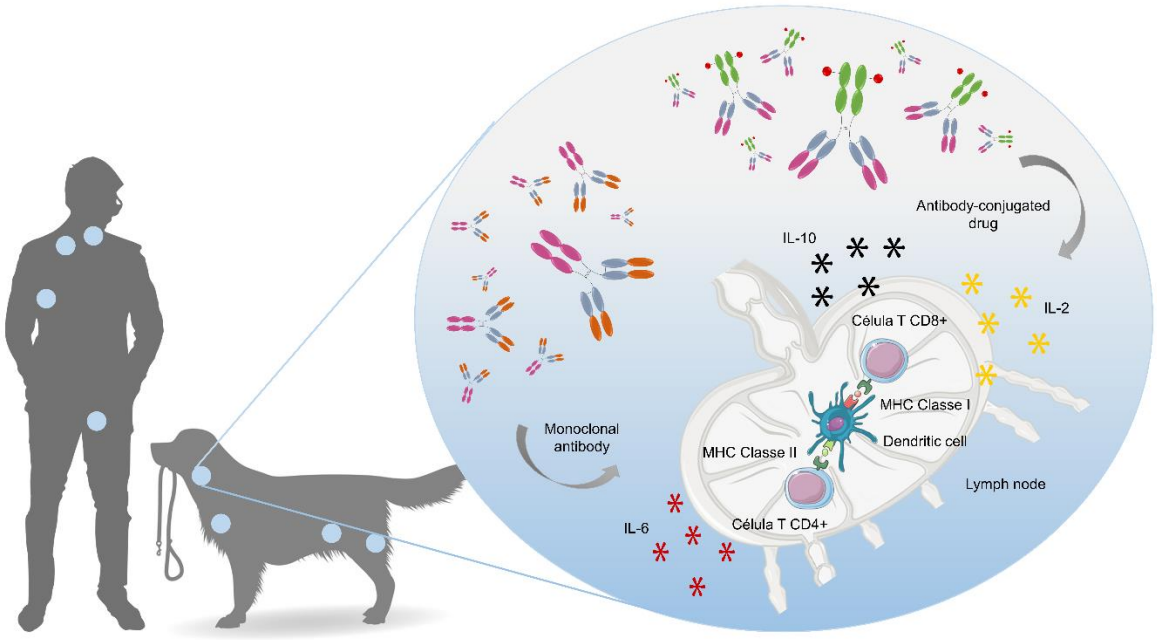
Within this context, a screening of multiple drugs, acting via different mechanisms, was conducted to assess their cytotoxic effect on cNHL, aiming to identify potential payloads to be coupled with our sdAbs. This allowed the recognition of a few highly potent drugs, among which HDACi were particularly effective and gained special interest due to the high efficacy they also present in targeted human cancer therapy, namely hematopoietic tumors. Ultimately, this culminated on a pioneer investigation of their antitumor properties using a canine B-cell lymphoma model, which is described under chapter 4. A panel of seven HDACis was initially tested on the well characterized CLBL-1 canine B-cell lymphoma cell line and panobinostat was identified as the most promising compound. Panobinostat was therefore thoroughly characterized and showed strong *in vitro* and *in vivo* antitumor properties.

The execution of this work prompted the establishment of a murine xenograft model of canine B-cell lymphoma using CLBL-1 cell line, required to test the antitumor effect of panobinostat *in vivo*. Despite recognizing all the limitations of conventional murine models, there is no question that these models remain fundamentally important and should not be discarded mainly to conduct preliminary screening and pre-clinical studies. In fact, the cost, reproducibility, well-characterized attributes, and wide biologic variation of these model systems still render them extremely valuable for proof-of-concept studies (Decker et al., 2017). Therefore, this model proved to be highly valuable as a stable tool for B-cell lymphoma research. However, it also presents opportunities for improvement. In light of this, under chapter 5, we described the establishment and characterization of a novel localized subcutaneous bioluminescent cNHL xenograft model that offers high engraftment efficiency, preservation of relevant tumor features and reproducible tumor growth. This model established with a stable CLBL-1 expressing firefly luciferase and GFP reporters proved to be a rather efficient non-invasive and quantitative method to monitor the outgrowth of cNHL. a. Thus, it represents an innovative preclinical tool for comparative medicine.

As conclusion, the approach taken during the course of this thesis allowed not only the characterization of new potential therapeutic antibodies for lymphoma treatment, but also the establishment of a solid platform for the acceleration of translational research of novel immunotherapeutic approaches for comparative oncology. The successful construction of a cNHL biobank allowed the characterization of the immune status of dogs diagnosed with

NHL with the investigation of the cytokine response present in the disease setting and the evaluation of the expression of potential targets for immunotherapeutic strategies, while guarantees the availability of properly cryopreserved samples for ongoing and future projects. Furthermore, the methodologies applied to identify potential targets, select and characterize the best therapeutic antibody, characterize a highly potent payload and to develop and characterize a bioluminescent model for *in vivo* testing, resulted in an innovative workflow that can be easily translated to other drug development research projects. In addition, this work opens up perspectives in comparative oncology as it contributes for the validation of the naturally occurring canine B-cell lymphoma model for translational immune-oncology research. As a result, this work has created an avenue to develop fast, successful, and cost-effective immunotherapies for comparative oncology (Figure 49).

Figure 49 - Canine lymphoma project.



Overall, our research group is particularly interested in the development of innovative mAb-bases therapies. By focusing on therapeutic areas with a high unmet medical need, such as cancer, we aim to provide solutions that improve patient's quality of life and prolong lives. At the core of our oncology programme is the validation of canine lymphoma as an animal model for immunotherapeutic approaches in comparative medicine. Notably, the Canine Lymphoma Project provides an integrated drug discovery platform that maximize interdisciplinary cooperation and leverage commonalities across humans and dogs, for the development of novel immunotherapies against non-Hodgkin lymphoma, benefiting both species. Currently, this work is actively investigating new immunotherapies that mobilize the patient's own immune system to combat cancer and mAb-

based drug delivery systems that selectively direct potent payloads to the site of the disease and promote cancer cells death.

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