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Article

IL-1R8 Downregulation and Concomitant *TLR7* and *TLR9* Up-regulation Are Related to the Pathogenesis of Canine Diffuse Large B-Cell Lymphoma

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Abstract: Diffuse large B-cell lymphoma (DLBCL) is the most common haematological malignancy in humans and dogs. Several studies disclosed some similarities between the two species, including the constitutive activation of NF- κB pathway as a fundamental underlying pathogenetic mechanism. In humans, downregulation of *IL-1R8* is implicated in DLBCL development, but its role in dogs has not been explored so far. To gain insight into the pathogenesis of this tumor in dogs, we evaluated the mRNA and protein expression of IL-1R8 in 12 hyperplastic lymph nodes obtained from dogs not bearing tumors and from 50 dogs with DLBCL. Moreover, we analysed through qRT-PCR the expression of *TLR7*, *TLR9*, *MYC*, and *p52* genes that are known to be involved in the *IL-1R8* regulatory network. *IL-1R8* and *p52* were downregulated in DLBCLs compared to control lymph nodes ($p<0.001$), while a higher expression of *TLR7*, *TLR9* and *MYC* was observed in tumors ($p<0.01$). Immunohistochemistry confirmed gene expression results, revealing a significantly lower IL-1R8 staining score in DLBCLs compared to control lymph nodes ($p<0.0001$). Taken together, these results suggest that IL-1R8 downregulation may represent one of the mechanisms driving DLBCL pathogenesis in dogs, mainly through dysregulation of the Toll-like/Interleukin receptors signalling cascade and the aberrant activation of classical NF-κB pathway.

Keywords: dog, diffuse large B-cell lymphoma, *IL-1R8*, *TLR7*, *TLR9*, qRT-PCR

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most frequent non-Hodgkin lymphoma subtype worldwide, accounting for approximately 40% of all human cases [1]. The name, DLBCL, stems from the presence of large neoplastic B-cells, two or three times larger than red blood cells, and the diffuse pattern, destroying nodal and, in some cases, extra nodal tissues [1]. Similarly, DLBCL is the most common canine aggressive lymphoproliferative tumor, being characterized by a heterogeneous clinical course that cannot be deciphered by morphology and immunophenotype alone [2-5]. The disease is aggressive and, although some dogs achieve long-term remission, the majority succumbs to DLBCL [6].

In human DLBCL, the neoplastic transformation of B-cells is mainly driven by genetic modifications and epigenetic reprogramming [7]. Analogies between human and canine

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DLBCL (cDLBCL) have been identified and the main distinctive feature in both species emerged from tumor transcriptome studies, where the constitutive activation of the NF-κB pathway was demonstrated as a common hallmark [8]. The causes of the aberrant NF-κB activity have not yet been completely resolved in dogs, but canonical NF-κB signalling resulted enhanced in cDLBCL compared to non-canonical pathway, and both cell-extrinsic and cell-intrinsic stimuli were supposed to contribute [9]. Therefore, the elucidation of the mechanisms underlying NF-κB activation may result in a high clinical relevance, possibly identifying specific therapeutic targets.

IL-1R8, also known as SIGIRR or TIR8, belongs to the IL-1 receptor superfamily [10], and is highly conserved among vertebrates [11]. Physiologically, IL-1R8 inhibits NF-κB activation and other transcription factors induced by Toll-like/Interleukin-1 receptor (TIR) signalling through different mechanisms, such as interfering with the TLR/ILR extracellular domain dimerization or blocking the recruitment of TIR domain containing adaptor molecules, eventually blocking TLR and ILR signalling cascades [12-15]. Conversely, IL-1R8 deficiency has been associated to several pathological conditions in mice, including infections, autoimmune diseases, sterile inflammations, and cancers. In particular, IL-1R8 deficiency has been associated to B-cell lymphoma development and tumor progression in murine models of chronic lymphocytic leukaemia [16].

To date, the role of IL-1R8 in cDLBCL is unknown, but we recently hypothesised the induction of proliferation in canine nodal B-cell lymphomas upon triggering of several TLRs. Given the ability of IL-1R8 to tune the activation of TLRs, we sought to gain insight into the potential role of this molecule in the pathogenesis of cDLBCL through an integrated analysis of mRNA and protein expression complemented by a comparison with normal canine lymph nodes. Also, genes known to be involved in the regulatory mechanisms of IL-1R8 and cDLBCL pathogenesis, including *TLR7*, *TLR9*, *MYC*, and *p52* were investigated at the mRNA level. Finally, potential associations with clinico-pathological features were analysed.

2. Materials and Methods

2.1. Animals and samples

Fifty dogs with newly diagnosed and previously untreated multicentric DLBCL of any World Health Organization (WHO) clinical stage were enrolled. These dogs underwent a complete staging work-up (history and physical examination, complete blood cell count, serum biochemistry profile, thoracic radiographs and abdominal ultrasound, cytological evaluation of liver and spleen, and immunophenotype determined by flow cytometry on a lymph node aspirate, peripheral blood and bone marrow aspirate). An enlarged lymph node (LN) was always obtained from each dog for routine histology and immunohistochemistry (CD3, CD20, CD79 and PAX5), while 12 control LNs were obtained from dead dogs undergoing necropsy and not bearing tumors. Gross and light microscopy examination excluded lymph nodal lesions other than reactive hyperplasia. Tissue samples from cDLBCL and control dogs were divided in two portions: one was fixed in formalin for histological and immunohistochemical analyses, and one was stored in RNAlater (Sigma-Aldrich, St. Louis, USA) at -80°C for RNA extraction.

Dogs were treated either with chemotherapy or with chemo-immunotherapy depending on owner's choice. Unvaccinated dogs received a CHOP-based protocol, including L-asparaginase, vincristine, cyclophosphamide, doxorubicin, lomustine and prednisone. Chemo-immunotherapy consisted in a CHOP-based protocol with the addition of APAVAC vaccine, as previously described [17]. At the end of treatment, all dogs underwent end-staging including flow cytometry (FC) on a peripheral LN, peripheral blood and bone marrow, and imaging. Follow-up evaluation consisted of monthly physical examination, peripheral LN size measurement and cytological evaluation during the first year, and every other month thereafter. Relapse was defined as the clinical reappearance and cytological evidence of lymphoma with or without FC

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confirmation in any anatomical site in dogs having experienced complete remission. Time to progression (TTP) was calculated as the interval between initiation of treatment and progressive disease or relapse, whereas lymphoma-specific survival (LSS) was measured as the interval between initiation of treatment and lymphoma-related death.

This study complied with Italian laws on animal experimentation and ethics and approved by the Ethical Committee of the Università degli Studi di Milano (OPBA_61_2018 25/06/2018).

2.2. RNA extraction and reverse transcription

Control nodes were processed using guanidine isothiocyanate method. Briefly, the samples were homogenized in 5 ml of guanidine isothiocyanate 4 M, pH 7 (Sigma-Aldrich, St. Louis, USA) using a rotor-stator system (Ultra Turrax, IKA WERKE, Staufen, Germany). 1.3 mL of lysate was centrifuged for 16 h at 42,000 rpm at 18 °C upon a 1 mL caesium chloride 5.7 M layer (Invitrogen, Paisley, Scotland, UK) by ultracentrifugation (Beckman Instruments, Inc., Palo Alto, CA, USA). Supernatant was discarded and the RNA pellet was dissolved in sterile water and precipitated with absolute ethanol (Carlo Erba S.p.a., Chaussée du Vexin, France) and sodium acetate 3 M, pH 5.4 (Carlo Erba S.p.a., Chaussée du Vexin, France) in dry ice for 2 h. After centrifugation at 12,000 g at 4°C for 30 min, the RNA pellet was washed in 1 mL of ethanol 70% and dissolved in sterile water.

cDLBCL tissues were processed using the TRIzol (Sigma-Aldrich, St. Louis, USA) method following the manufacturer's instructions. RNA concentration was established by measuring absorbance at 260 nm with a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany), while RNA integrity was assessed through 2% agarose gel electrophoresis (GellyPhor LM Agarose, Euroclone S. p. a., Pero-Milano, Italy). Samples were stored at -20°C until use. 2 µg of total RNA from each sample were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions using random primers. The reverse transcription was performed according to the following program: step 1 incubation at 25°C for 10 min, step 2 at 37°C for 2 h and step 3 at 85°C for 5 min.

2.3. Real-Time qRT-PCR

The cDNA obtained from each sample was used as a template for Real Time PCR in an optimized 25 µl reaction volume using Sybr Green chemicals (ThermoFisher Scientific, Waltham, USA), as previously described [18]. Primer pairs for canine *p52, MYC, IL-1R8, TLR7, TLR9* and the housekeeping *CCZ1* genes were designed using the Primer Express Software (Applied Biosystem, Foster City, CA, USA) and purchased from Invitrogen (Carlsbad, CA, USA). Primer sequences are listed in Table S1. Quantitative RT-PCR was performed using the ABIPRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A duplicate no-template control (NTC) was included in each experiment. The analysis of gene expression data was performed using the $2^{-\Delta Ct}$ method (Livak method [19]).

2.4. Immunohistochemistry

cDLBCLs and control lymph node samples were formalin-fixed and paraffin embedded for histopathologic examination and immunohistochemistry to evaluate IL-1R8 expression. Deparaffinization, rehydration and antigen retrieval were performed in a dewaxing buffer solution (pH=9) (Dewax and HIER Buffer H, Thermo Fisher Scientific, Cheshire, UK) at 100 °C for 40 minutes. Samples were cooled and subsequently washed in PBS and 0.005% Tween20 (Agilent, Santa Clara, California, USA). Sections were then stained with the Thermo Scientific Autostainer 480S system (Bio Optica, Milan, Italy). After peroxidase inhibition and non-specific binding blocking, sections were incubated with the primary anti-SIGIRR antibody (Polyclonal Rabbit AHP1784-BioRad, Hercules, California, USA; dilution 1:900) for 1 hour at room temperature. Samples were then incubated with a goat anti-rabbit biotinylated secondary antibody (Vector Lab BA-2000,

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Vector Laboratories, Burlingame, California, USA; dilution 1:200) and with an avidin-biotin peroxidase system (Vectastain® Elite® ABC Kit, Vector Laboratories. Burlingame, California, USA). Immunolabelling was revealed by incubation with 3,3-diaminobenzidine tetra-hydrochloride (DAB; Vector Laboratories) for 3 minutes. After washing in distilled water, the sections were counterstained with Mayer's hematoxylin (Diapath S.p.A, Martinengo, Italy), dehydrated in a graded alcohol series and mounted. Normal lymph nodes of known immunoreactivity were included as positive controls in each immunohistochemical run. Stained tissue sections were analyzed at the optical microscope (Leica, Wetzlar, Germany). A semiquantitative evaluation of IL-1R8 cytoplasmic expression was performed: the final score was obtained as the product of the number of positive cells (score 0 < 1%; 1= 1-10%; 2=11-50%; 3 > 50%) and the intensity of the staining (score 0=absent; 1=slight; 2=moderate; 3=strong).

2.5. Statistical analysis

Statistical analyses were performed in R environment. Continuous variables were tested for normal distribution by conducting a Shapiro-Wilk test. Gene expression results of cDLBCLs and control lymph nodes were compared by means of a Mann-Whitney test. The correlation matrix between target genes was computed using the *ggcorrplot* R package and significant associations were checked using a Spearman correlation test. Benjamini-Hochberg correction was applied to multiple testing. To explore associations between gene expression and clinico-pathological variables (breed, age, sex, weight, stage, substage, bone marrow and peripheral blood infiltration, serum LDH levels, pretreatment with steroids and treatment), Mann-Whitney test was performed for categorical variables, with the exception of 'stage' where a Kruskall-Wallis test was used, while a Spearman correlation test was conducted for continuous variables. Finally, the impact of clinico-pathological variables and gene expression levels on both TTP and LSS was explored by means of univariate and multivariate Cox proportional-hazards model. Dogs lost to follow-up or dead for lymphoma-unrelated causes before progressive disease, as well as those still in complete remission at the end of the study, were censored for TTP analysis. Dogs alive at the end of the study, lost to follow-up or dead due to causes other than lymphoma were censored for LSS analysis [20]. Differences in immunohistochemical expression of IL-1R8 were evaluated by Mann-Whitney test.

3. Results

3.1. Study population

Fifty dogs with newly diagnosed, previously untreated DLBCL were enrolled. Detailed signalment and clinico-pathological data are reported in Table S2. Mixed-breed dogs (n=11, 22%), German shepherd dogs (n=7, 14%), Rottweilers (n=4, 8%) and Golden Retrievers (n=4, 8%) were the most represented breeds. Twenty-six dogs were males (52%), while 24 were females (48%). Median age was 7.5 years (range: 3 – 15 years), while median weight was 28.9 kg (range: 4.5 – 81.3 kg). Thirty-two dogs (64%) had stage V disease, 16 dogs (32%) had stage IV and two dogs (4%) had stage III disease. In addition, 33 dogs (66%) were asymptomatic, while 17 (34%) presented clinical symptoms. Fifteen dogs (30%) presented bone marrow infiltration (i.e. >3% of neoplastic cells) (median: 1.4%; range: 0.2 – 50%) and peripheral blood infiltration (median: 1.0%; range: 0.1 – 55.5%). Twenty-six dogs (52%) showed an increased level of serum LDH. Finally, 14 dogs (28%) had received steroids before lymphoma diagnosis. Median TTP was 171 days, while median LSS was 237 days. TTP was significantly affected by treatment (p=0.02) (Table S3a), while LSS was significantly affected by the percentage of bone marrow infiltration (p=0.03) (Table S3b).

3.2. Gene expression analysis

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We evaluated the expression of *IL-1R8, TLR7, MYC, TLR9,* and *p52* genes in cDLBCL and control lymph nodes through qRT-PCR. Gene expression results are reported in Figure 1a-e and Table S4. *IL-1R8* and *p52* were significantly downregulated in tumors compared to control lymph nodes (p<0.001). Conversely, *TLR7, TLR9* and *MYC* showed higher expressions in cDLBCL compared to controls (p<0.01). Interestingly, a significant correlation between *TLR9* and *p52* (rho=0.49, p<0.001) was retrieved (Figure 1f and Table S5). Conversely, no association between gene expression and any clinico-pathological variable was obtained. Likewise, none of the target genes significantly affected outcome (Table S3a and S3b).

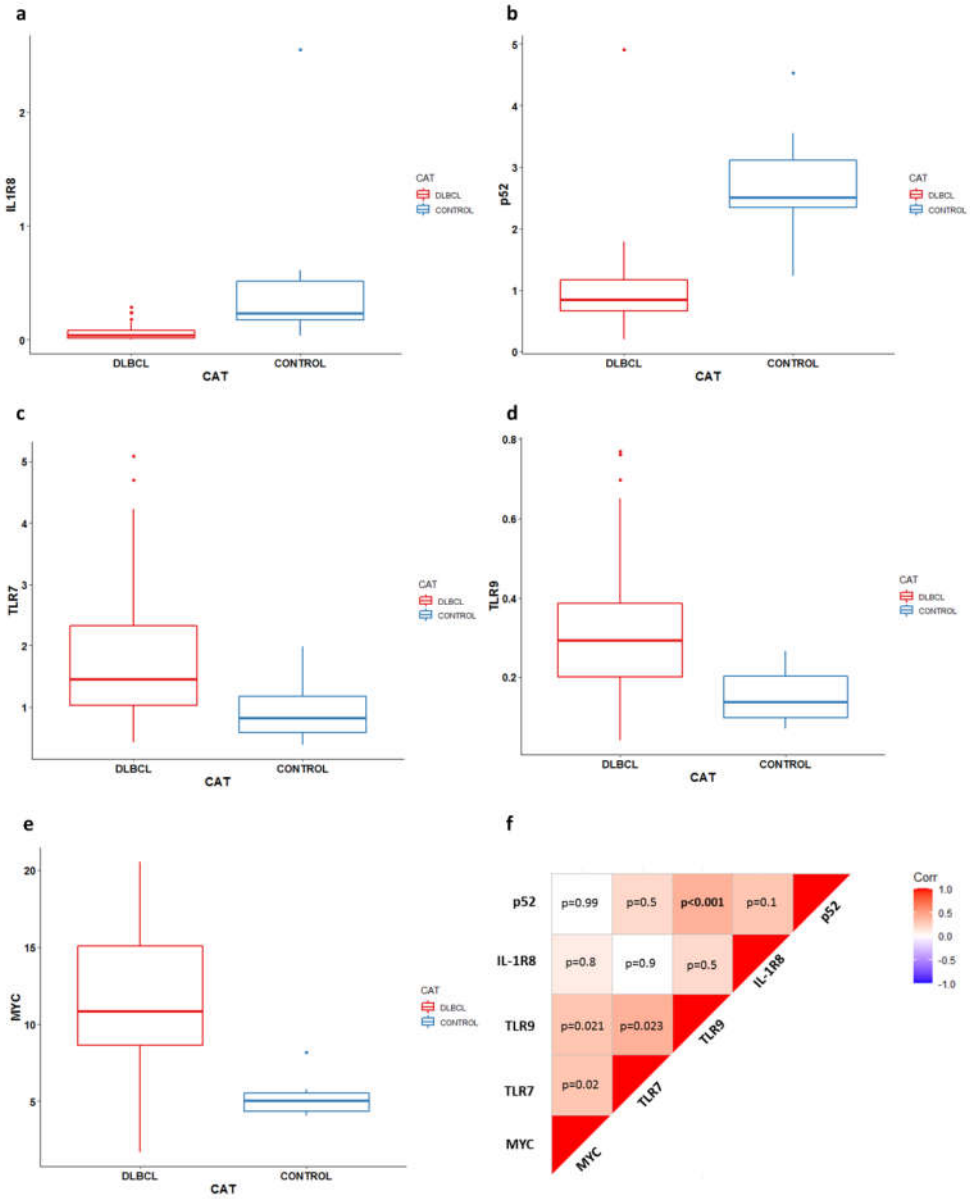

Figure 1. Gene expression results and correlations between target genes. (**a-e**) Real-time PCR results of *IL-1R8* (**a**), *p52* (**b**), *TLR7* (**c**), *TLR9* (**d**), and *MYC* (**e**) in 50 cDLBCLs and 12 control lymph nodes. (**f**) Correlation matrix showing statistical correlations between target genes expression. Correlation coefficients were calculated using the Spearman method.

3.3. Immunohistochemistry

In line with gene expression results, immunohistochemical analysis confirmed a significant down regulation of IL-1R8 protein in cDLBCL compared to control lymph

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nodes (p<0.0001) (Figure 2a). In the latter, B-cells within the germinal centers were diffusely and moderately positive for IL-1R8, while only scattered positive lymphocytes were detected in the remaining regions of the lymph node, including the paracortex (Figure 2b). Therefore, the final score was obtained on selected areas compatible with lymphoid follicles and ranged from 3 to 6 (mean=5.5). The immunohistochemical score ranged from 0 to 1 (mean=0.38) in cDLBCL (Figure 2c).

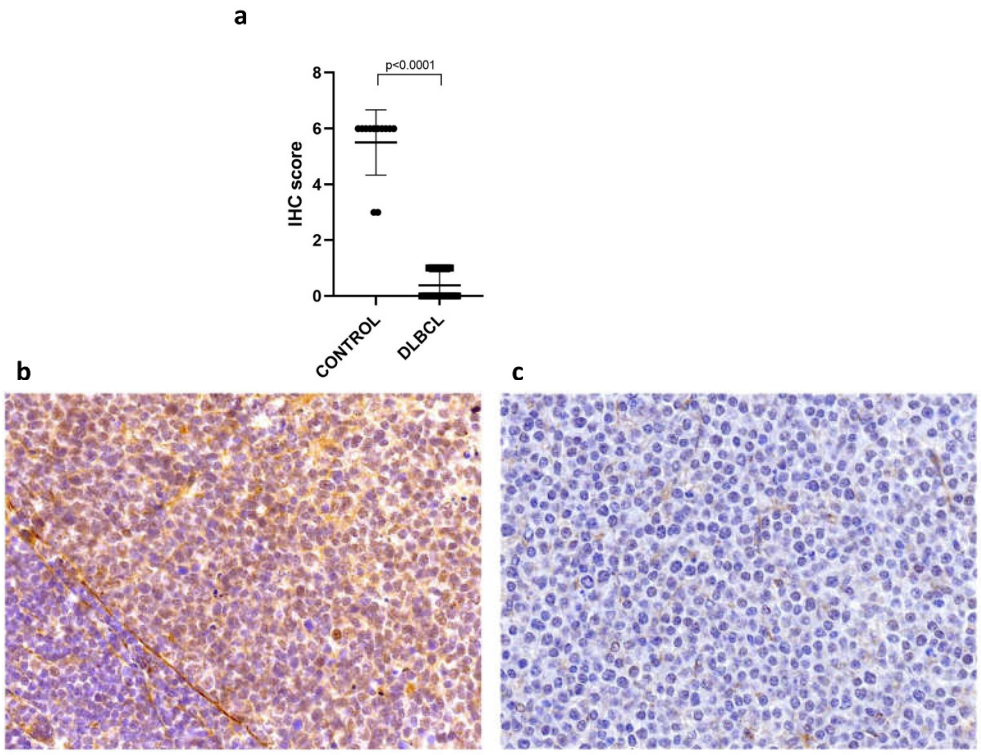

Figure 2. Immunohistochemical expression of IL-1R8 in DLBCLs and control lymph nodes. (**a**) Immunohistochemical semiquantitative score in control lymph nodes and canine DLBCL. (**b**) Diffuse expression of IL-1R8 in germinal center B cells in a control lymph node. Immunohistochemistry for IL-1R8, 400x magnification. (**c**) Lack of IL-1R8 expression in neoplastic lymphocytes in cDLBCL. Immunohistochemistry for IL-1R8, 400x magnification.

4. Discussion

Persistent activation of NF-κB signalling pathway has been identified as a fundamental molecular event required to amplify the survival and proliferation of neoplastic cells both in humans and dogs with DLBCL [21]. However, mechanisms driving aberrant activation are scarcely investigated in dogs and two hypotheses have been proposed so far. The first correlates the presence of *TRAF3* inactivating mutations with NIK stabilization and constitutive NF-κB activity via the alternative pathway [22]. The second hypothesis is more intriguing and associates the activation of NF-κB with the enrichment of the TLR signaling pathway in neoplastic B-cells. Indeed, several TLRs were found upregulated in dogs with DLBCL and, consequently, a pathogenesis mimicking a chronic antigen-like stimulation was supposed [23].

Here, to gain further insights into the mechanisms involved in NF-κB activation in cDLBCL, we first investigated one of the most well-known negative TLRs regulators, named IL-1R8, both at the gene and protein level. As hypothesised, IL-1R8 mRNA expression was significantly downregulated in tumors compared to normal lymph nodes. Likewise, at the protein level, IL-1R8 immunostaining was prevalently detected in normal B-cells within the germinal centers. In contrast, in cDLBCLs the IL-1R8 signal varied from

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absent to very low. In particular, both staining intensity and number of positive cells were significantly lower compared with normal B-cells. These results are in line with previous studies in human tumors. In colon cancer a lower expression of *IL-1R8* was associated to tumor development, and an alternative splicing causing IL-1R8 inactivation was also identified [24,25]. In human DLBCL a reduced *IL-1R8* expression was reported when compared to peripheral blood mononuclear cells and germinal center B-cells, and correlated with a better overall survival, suggesting its role in the pathogenesis of this lymphoma subtype [26]. Finally, deficiency of IL-1R8 predisposed *Fas*^{lpr} mice to severe lymphoproliferation, autoimmune lupus-like disease and TCL1 mice to leukaemia [14,16,26].

In our experiments we obtained similar results, but we did not investigate the mechanisms causing *IL-1R8* silencing. However, we can almost exclude methylation aberrancies and genetic mutations as possible driving events. Indeed, the methylome of these tumors was previously investigated by methyl binding protein sequencing, but no peaks in the promoter regions were identified in the *IL-1R8* locus [23]. Also, whole exome studies did not report genetic mutations affecting this gene in canine B-cell lymphoma [22,27,28]. One last hypothesis refers to genetic or epigenetic alterations in specific genes interacting directly with *IL-1R8* and favouring its gene transcription, such as *SP1*. The role of SP1 in the regulation of *IL-1R8* was recently confirmed in human primary monocytes and neutrophils and its pharmacological inhibition was able to reduce *IL-1R8* expression [29]. No data are available about the regulation of this transcription factor in veterinary medicine and future experiments are required.

Since IL-1R8 physiologically inhibits several TLRs, we investigated here if this was also true in dogs. The inverse correlation between TLRs and IL-1R8 indirectly confirmed the same constitutive mechanisms described in humans and mice, where IL-1R8 negatively modulates the activation of TLR7 and TLR9 in turn leading to inhibition of NF-κB and JNK signalling [14,30]. Therefore, we can speculate that the reduction of IL-1R8 may participate to the mechanisms beyond TLRs up-regulation in cDLBCL. Furthermore, significant higher *TLR7* and *TLR9* mRNA levels were observed in tumours compared to controls, confirming that their activity is not only restricted to normal immune system cells but is also identified in tumor [23]. In this respect, this data provides evidence of clinical relevance, which will be useful in devising therapeutic trials with TLR9 agonists, such as oligodeoxynucleotides containing CpG motifs (CpG-ODN), in dogs with DLBCL. Hypothesizing a similar mechanism to human B-cell lymphomas we can suppose an inhibition of proliferation and induction of apoptosis in TLR9-positive neoplastic cells after treatment.

To further confirm the activation of the NF-κB classical pathway in cDLBCL, we tested *p52* mRNA expression. The NF-κB-subunits involved in canonical NF-κB pathway are principally RelA, p65, and p105/p50, whereas p52 and p100 are always undetectable. Overall, results showed a down-regulation of *p52* in cDLBCL compared to controls, confirming the NF-κB canonical pathway activation.

In order to test other genes known to be involved in DLBCL pathogenesis we analysed *MYC* mRNA level in the same samples [31]. Our results confirmed the up-regulation of this proto-oncogene in tumours, as previously described by array comparative genomic hybridization and whole exome sequencing studies, where amplifications of the chromosome 13 or specific mutations were catalogued [22,23,27,31]. The opposite correlation between *MYC* and *IL-1R8* in cDLBCLs replicate the results obtained in human colon cells where increased *MYC* levels driven by ERK/MAPK signaling pathway were reported in IL-1R8 deficient Apc$^{Min/+}$colonocytes [32]. More specifically, IL-1R8 deficiency is able to stimulate the activation of ERK that in turn stabilizes MYC, thereby preventing its degradation. We couldn't prove it here, but ERK aberrant activity is fundamental in the pathogenesis of canine B-cell lymphoma, as previously demonstrated by Assumpção et al. [33].

In conclusion, similarly to humans, *IL-1R8* has a reduced expression in cDLBCL. The results obtained here reinforce the previously formulated hypothesis in dogs with

DLBCL, suggesting that *TLR7* and *TLR9* up-regulation and concomitant *p52* and *IL-1R8* downregulation in B-cells could lead to an exaggerated and uncontrolled inflammatory-like process, characterized by activated B-cells, eventually evolving into cancer. The tumorigenesis might be further amplified by *MYC* deregulation. Further experiments are needed to clarify the mechanisms causing *IL-1R8* downregulation and stimuli activating TLRs, including a possible aberrant activation driven by virus integrated in the tumor genome.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, **Table S1**: Primer pair list; **Table S2**: Signalement and clinico-pathological variables of 50 dogs with DLBCL included in the study; **Table S3**: Median TTP and LSS and survival analysis of 50 cDLBCL; **Table S4**: *IL-1R8, p52, TLR7, TLR9* and *MYC* expression in cDLBCLs and control lymph nodes; **Table S5**: Spearman correlation results of *IL-1R8, p52, TLR7, TLR9* and *MYC* expression in cDLBCLs.

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Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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