Master's Thesis of Jun-Hyeong Park

Anticancer activity of IRAK-4 inhibitors against canine lymphoid malignancies

개 림프구성 악성 종양에 있어서 IRAK-4 억제제의 항암 효과

2022년 8월

서울대학교 대학원

수의과대학 임상수의학 수의내과학 전공

박 준 형

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이 논문을 수의학 석사 학위논문으로 제출함

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박준형의 석사 학위논문을 인준함

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위 원 장 _____ (인)

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Abstract

Anticancer activity of IRAK-4 inhibitors against canine lymphoid malignancies

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The interleukin-1 receptor-related kinase 4 (IRAK4), downstream of myd88, plays an essential role in hyperactive TLR signaling seen in some B-cell lymphomas. In particular, efficient IRAK4 inhibitors of activated B-cell subtype of human diffuse large B-Cell lymphoma (DLBCL) are being developed. However, the anticancer effect of IRAK-4 inhibitors in veterinary medicine has not been elucidated. It is therefore explored in this study involving the GL-1 and CL-1 canine lymphoma cell lines in vitro.

MyD88 expression was analysed using polymerase chain reaction. GL-1 and CL-1 cells were subjected to concentration- and time-dependent treatment with an IRAK-4 inhibitor and assessed for viability, TLR signalling association, and apoptosis using a cell counting Kit-8 assay, Western blotting, and flow cytometry.

The GL-1 and CL-1 cells exhibited enhanced MyD88 expression, however, canine peripheral blood mononuclear cells (cPBMCs) did not. The IRAK-4 inhibitor reduced cell viability in a dose- and time-dependent manner, significantly reduced the phosphorylation of molecules associated with TLR signalling at IC50 such as IRAK1, IRAK4, NF-κB and STAT3, and induced apoptosis in GL-1 and CL-1 cells.

The anticancer effect of the IRAK-4 inhibitor on canine lymphoma cells is mediated by apoptosis via downregulation of TLR signalling.

Keywords: Apoptosis, canine lymphoma, cell viability, IRAK-4 inhibitor, MyD88, TLR signalling.

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1. Introduction

1.1. Study Background

Canine lymphoma is generally comparable to non-Hodgkin lymphoma in humans. Multi-agent chemotherapeutic protocols such as cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (CHOP), cyclophosphamide, vincristine, and prednisone (COP) are generally adopted as standard therapeutic regimens in veterinary medicine.[1] Although COP and CHOP are initially effective in treating canine lymphoma, the success rate of treatment, remission, and survival in the long run are poor, and numerous side effects are also common.[2] Canine lymphomas have recently been classified into subtypes similar to human diffuse large B-cell (DLBCL) and activated B-cell (ABC) based on gene expression profiling.[3] Although several studies in humans targeting IRAK-4 inhibitors as therapeutic agents in treating lymphomas are ongoing, similar studies in canines are lacking and are warranted.

Toll-like receptors (TLRs) play a crucial role in the proliferation and survival of immune cells by recognising antigens that activate inflammatory responses through TLR signaling.[4, 5] Dysregulation of TLR signalling is a causative mechanism associated with the development of DLBCL. Accordingly, several studies have focused on the inhibition of TLR signalling as a therapeutic option to suppress tumour growth in humans.[6]

ABC DLBCL tumours harbor highly recurrent oncogenic mutations, such as MyD88-L265P, that promote cell survival by spontaneously aggregating a protein complex involving IRAK1 and IRAK4.[7] Interleukin-1 receptor associated kinase 4 (IRAK-4) inhibitors may suppress the TLR signalling pathway by downregulating the adapter protein MyD88. Consequently, several studies have utilized its therapeutic potential as an anticancer agent for patients with lymphoma. In a phase I trial involving patients with relapsed or refractory non-Hodgkin's lymphoma, CA-4948, an IRAK-4 inhibitor, caused a dose-dependent reduction in tumour burden.[8, 9]

1.2. Purpose of Research

In the present study, canine B- and T-cell lymphoma cell lines were used to evaluate the anticancer effect of IRAK-4 inhibitors in canine lymphoid malignancies in vitro. The expression of MyD88 in canine normal cells was compared and the effects of IRAK-4 inhibitors on cell viability and apoptosis were evaluated. Subsequently, the expression of TLR signalling molecules such as IRAKs, nuclear factor-kappa B (NF-κB), and signal transducer and activator of transcription 3 (STAT3) were evaluated to elucidate the therapeutic mechanism of the drug and to verify the anticancer effect of IRAK-4 inhibitors in canine tumour cells. This study was to determine the effect of IRAK-4 inhibitor on MyD88-dependent canine lymphoma.

2. Material and Methods

2.1. Cell lines & cell culture

The well-characterized canine lymphoid B-cell line GL-1 and the canine T-cell lymphoma cell line CL-1 were obtained from Prof. Yuko Goto-Koshino (Tokyo University, Japan) and validated elsewhere.[10-12] Canine DLBCL cell line CLBL-1 was obtained from Prof. Barbara C Rütgen (University of Veterinary Medicine Vienna, Austria).[13] All cell lines were validated for canine origin by karyotyping. Cells were tested for mycoplasma and were negative. The GL-1 and CLBL-1 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Daegu, Korea) supplemented with 10 % foetal bovine serum (FBS, Gibco®, Paisley, Scotland) and 1 % penicillin-streptomycin (Gibco®). The CL-1 cell line was maintained in DMEM supplemented with 20 % FBS and 1 % penicillin-streptomycin. The cell lines were cultured at 37 °C in 5 % CO2 and 100 % humidity. The media were changed every other day, and cells were sub-cultured at 80 % confluence.

2.2. Isolation of canine peripheral blood mononuclear cells

Blood samples from healthy dogs were obtained from KPC (Gwangju-si, Gyeonggi-do, Republic of Korea; Institutional Animal Care and Use Committee (IACUC) approval no: P203078). cPBMCs from healthy beagle dogs were isolated using Ficoll-Paque™ PREMIUM (Cytiva Life Sciences™, Marlborough, MA, USA) in accordance with the manufacturer’s instructions. Blood samples diluted with an equivalent volume of Dulbecco's phosphate buffered saline (DPBS; WelGENE Inc., Daegu, Korea) were layered onto the Ficoll-Paque medium and centrifuged at 400 × g for 30 min at 20 °C with low brake. After centrifugation, the isolated and aspirated mononuclear cell layer was treated with red blood cell (RBC) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) incubated at 20 °C for 5 min, washed with DPBS, centrifuged at 450 × g for 10 min, and resuspended in

Roswell Park Memorial Institute (RPMI)-1640 (Gibco®, Paisley, Scotland) medium supplemented with 10 % FBS and 1 % penicillin-streptomycin.

2.3. IRAK-4 inhibitors

The IRAK-4 inhibitors FM501 (6-(5-fluoropyridin-3-yl)-N-((1r,4r)-4-morpholinocyclohexyl)-9H-pyrimido[4,5-b]indol-4-amine) and FM504 were provided by Future Medicine Co., Ltd. (Seongnam-si, Gyeonggi-do, Republic of Korea). FM504 is a derivative of FM501 obtained by the addition of MsOH and therefore corresponds to FM501-MsOH. The inhibitors were stored in powder form at 4 °C. For the experiments, FM501 and FM504 were solubilized in dimethyl sulfoxide (DMSO, WelGENE Inc., Daegu, Korea) and primary distilled water, respectively, to obtain the desired concentrations in solution form.

2.4. cDNA synthesis & Reverse transcription polymerase chain reaction

RNA was extracted from cPBMCs isolated from healthy beagle dogs, B cells derived from cPBMCs, GL-1, and CL-1 cells using easy-BLUE™ Total RNA extraction kit (iNtRON Biotechnology, Seoul, Republic of Korea) as per the manufacturer's instructions. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a TAKARA PCR Thermal Cycler (TaKaRa Biomedical, Siga, Japan). cDNA was synthesised from quantified RNA samples using cDNA master mix from CELLSCRIPT™ (Madison, WI, USA) in accordance with the manufacturer's instructions under the following conditions: 25 °C for 10 min, 42 °C for 50 min, and 85 °C for 5 min. As a negative control for PCR reaction, same volume of DPBS was added instead of the RNA sample.

The target DNA was amplified using primers designed specifically for canine GAPDH and MyD88 (Canine GAPDH, forward 5′-GGAGAAAGCTGCCAAATATG-3′, reverse 5′-ACCAGGAAATGAGCTTGACA-3′; Canine MyD88, forward 5′-GATGGGCTTCGAGTACCTGG-3′, reverse 5′-GAGACGACGTGTTGGTGGAA-3′) using Primer3Plus (https://primer3plus.com).

PCR samples were processed using Genomics 5X PCR premix (GenomicsONE, Seoul, Republic of Korea) and subjected to the following PCR conditions: initial denaturation at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The amplified samples (10 μL/lane) were resolved using 1.5 % agarose gel in 0.5 % Tris-Acetate-EDTA buffer with DNA Loading dye (5 μL/mL) and electrophoresed at 100 V using Mupid-2plus (Advance Co. Ltd., Tokyo, Japan). The gel was photographed and the levels of canine MyD88 and GAPDH were quantified using GelGraph (Scinomics Co. Ltd., Daejeon, Republic of Korea). This experiment was performed in triplicate independently.

2.5. Quantitative real-time PCR

MyD88 and GAPDH mRNA expression levels were analyzed by AMPIGENE qPCR Green Mix (Enzo Life Sciences, Inc., New York, USA) and QuantStudio 1 (Invitrogen, Carlsbad, CA, USA). Gene expression levels were determined by normalization against GAPDH, using the $2^{-\Delta\Delta CT}$ method [calculating the fold expression compared with the average ΔCq of cPBMC; ΔCq = Cq (MyD88) - Cq (GAPDH)].[14]

2.6. Cell proliferation and viability test

GL-1 cells were seeded at a density of 104 cells/well in 96-well plates and inoculated with IRAK-4 inhibitor (FM504) at concentration of 0, 1.25, 2.5, 5, 10, and 20 μM respectively, at 0, 24, 48, and 72 h after treatment. To reduce the range of drug concentrations, GL-1 cells were treated with IRAK-4 inhibitor at various concentrations diluted 2-fold starting from 1 μM for 48 h. CL-1 and cPBMCs cells seeded at a density of 104 cells/well in 96-well plates were treated with IRAK-4 inhibitor (FM504) at different concentrations (0, 0.625, 1.25, 2.5, 5, 10, and 20 μM) and times (0, 24, 48, and 72 h). Viability tests were conducted using the D-Plus™ CCK cell viability assay kit (Dongin LS, Seoul, Republic of Korea) as per the manufacturer's instructions. An Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) was used to measure absorbance at 450 nm. The

50 % inhibitory concentration (IC50) value of IRAK-4 inhibitor on GL-1 and CL-1 was calculated using linear regression method with the Prism software.

For proliferation test, GL-1 and Cl-1 cells seeded at a density 104 cells/well in 48-well plates were treated with IRAK-4 inhibitor (FM501). After incubation for different time point (0, 24, 48, 72 h), cells were trypsinized, washed with PBS, and viable cells were scored using Cell Counter (Countess II, Invitrogen). via trypan blue exclusion.

2.7. Annexin V/PI staining

GL-1 and CL-1 cells were seeded at a density of 5 × 105 cells/well in 6-well plates and treated with different concentrations of the IRAK-4 inhibitor (FM501; 0.25, 0.5, 1.25, and 2.5 μM) for 48 h. After incubation, the cells were stained using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) as per the manufacturer's instructions. The collected cells were washed with fresh PBS and resuspended in 1X Annexin V binding buffer. Annexin V and PI were added to 105 cells and incubated for 15 min at 20 °C in darkness. Samples were analysed after less than an hour using FACS Aria II (BD Biosciences).

2.8. Western blotting

GL-1 and CL-1 cells were seeded at a density of 5 × 105 cells/well in 6-well plates and treated with IRAK-4 inhibitor (FM501; GL-1 cells, 250 and 500 nM; CL-1 cells, 1.25 and 2.5 μM) for 48 h. After incubation, proteins were extracted from the treated cells using PRO-PREP™ protein extraction solution (iNtRON Biotechnology) according to the manufacturer's instructions. The extracted protein samples were electrophoresed (10 μg/lane) in 12% Tris-glycine sodium dodecyl sulfate gels, and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5 % skim milk in 1X Tris-buffered saline with Tween 20 (TBST) at 20 °C for 1 h, incubated with primary antibodies at 4 °C overnight, washed thrice with 1X TBST for 10 min, incubated with the secondary antibody (1:5000) at 20 °C for 1 h, and washed four times with 1X TBST for 15 min. ECL

solution (Dongin LS) was used as the detection reagent. Immunoreactive bands were detected with ImageQuant Las 4000 Mini (Cytiva Life Sciences™). The following primary antibodies at a dilution of 1:1000 unless otherwise mentioned were used for Western blotting: anti-Caspase-3 (E8, mouse monoclonal, Santa Cruz, Paso Robles, CA, USA), anti-Caspase-7 (C12, mouse monoclonal, Santa Cruz) anti-IRAK1 (mouse monoclonal, Santa Cruz), anti-pIRAK1 (phospho-Thr387, rabbit polyclonal, Biorbyt, Cambridge, UK), anti-IRAK-4 (1:200, mouse monoclonal, Dendritics, Dardilly, France), anti-pIRAK-4 (pT345, mouse monoclonal, Abnova, Taipei, Taiwan), anti-p65 (rabbit monoclonal, Invitrogen), anti-pp65 (pSer536, rabbit monoclonal, Invitrogen), anti-STAT3 (rabbit polyclonal, LS Bio), anti-pSTAT3 (rabbit polyclonal, LS Bio), and anti-βActin (C4, mouse monoclonal, Santa Cruz). The following secondary antibodies were used for western blotting: goat anti-mouse horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA) and goat anti-rabbit horseradish peroxidase (Enzo Life sciences, Farmingdale, NY, USA)

2.9. Statistical analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad Software version 5 Inc., La Jolla, CA, USA). Data were analysed using one-way analysis of variance and the Kruskal-Wallis and Dunn's multiple comparison tests. Results are presented as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Enhanced expression of MyD88 in canine GL-1 and CL-1 cells

To determine whether MyD88 was overexpressed in canine lymphoid cell lines, RNA expression levels were compared using RT-PCR and qRT-PCR. cPBMCs isolated from the healthy beagle dogs were used as controls. In result, MyD88 expression was higher in canine GL-1 and CL-1 cells than in cPBMCs (Figure 1A). The expression level of MyD88 in GL-1 and Cl-1 was increased by 3.5-fold and 4.9-fold, respectively, compared to cPBMC (Figure 1B).

3.2. IRAK-4 inhibitor decreased cell viability in GL-1 and CL-1 cells but not in cPBMCs

The effect of IRAK-4 inhibitor at different concentrations and exposure durations on cell viability in lymphoid malignancy was assessed in GL-1 and CL-1 cells lines. FM504 was used as IRAK-4 inhibitor in CCK assay because DMSO dissolving FM501 inhibited uniform CCK measurement in cancer cells. IRAK-4 inhibitors significantly reduced the viability of GL-1 and CL-1 cells in a dose-dependent manner. Viability after exposed to IRAK-4 inhibitors at 20, 10, and 5 μM for 24 h (20 μM; $p<0.0001$, 10 μM; $p<0.01$, 5 μM; $p<0.01$) caused a greater reduction in GL-1 cells viability compared to control; the reduction was more pronounced after 48 h (20 μM; $p<0.0001$, 10 μM; $p<0.01$, 5 μM; $p<0.001$, 2.5 μM; $p<0.05$) and 72 h (20 μM; $p<0.001$, 10 μM; $p<0.001$, 5 μM; $p<0.001$, 2.5 μM; $p<0.05$) of treatment (Figure 2A). Thus, a longer incubation time with the inhibitor led to a correspondingly lower survival rate of cells. To confirm the IC50 of IRAK-4 inhibitor, the concentration range was further narrowed from that used in the above experiment to treat GL-1 cells for 48 h (Supplementary Figure 1). The IRAK-4 inhibitor at 1000 ($p<0.0001$), 500 ($p<0.001$), and 250 nM ($p<0.01$) caused

more than 50 % decrease in viability in GL-1 cells. The IRAK-4 inhibitor still significantly reduced the viability of CL-1 cells in a dose-dependent manner, although its effect was not as prominent as in GL-1 cells, which grew more pronounced with longer post-treatment time. Treatment with the IRAK-4 inhibitor at 20, 10, 5, and 2.5 μM for 24 (20 μM; $p<0.0001$, 10 μM; $p<0.001$, 5 μM; $p<0.05$, 2.5 μM; $p<0.05$) and 48 h (20 μM; $p<0.0001$, 10 μM; $p<0.0001$, 5 μM; $p<0.01$, 2.5 μM; $p<0.05$), and 20, 10, and 5 μM for 72 h (20 μM; $p<0.0001$, 10 μM; $p<0.001$, 5 μM; $p<0.01$) reduced the viability of CL-1 cells compared to those of the control (Figure 2B). The IC50 values obtained by CCK-8 was 180.1 nM and 2.36 μM for GL-1 and CL-1, respectively. These results indicate that the IRAK-4 inhibitor inhibits the viability of GL-1 and CL-1 cells in a dose- and time-dependent manner.

The effect of the IRAK-4 inhibitor on cell viability in normal cells was also analysed in the cPBMCs of healthy beagle dogs. Unlike GL-1 and CL-1 cells, cPBMCs treated with IRAK-4 inhibitors at different concentrations and for different durations did not exhibit significant changes in cell viability compared to that of the control. Thus, IRAK-4 inhibitor did not influence the viability of cPBMCs (Figure 2C).

3.3. IRAK-4 inhibitor decreased cell proliferation in GL-1 and CL-1 cells

In addition to cell viability test, actual cell proliferation results were obtained when cell numbers were used as an indicator of cell proliferation (Figure 3A and 3B). Two concentrations of IRAK-4 inhibitor (FM501) were selected by referring to IC50 specific to each cell line (GL-1 cells: 250 and 500 nM; CL-1 cells: 1.25 and 2.5 μM), and incubated for 48 h. Similar to the viability test, both cell lines showed a tendency to decrease in cell number when compared to the control group when IRAK-4 inhibitor was added.

3.4. IRAK-4 inhibitor promotes apoptosis in GL-1 and CL-1 cells

The effect of the IRAK-4 inhibitor (FM501) at different concentrations for 48 h on apoptosis in lymphoid malignancy was assessed in GL-1 and CL-1 cells using Annexin V/PI staining and flow cytometry. These results indicated that apoptosis is the major cell death mechanism involved in the process and the apoptotic ratio corresponding to the drug concentration was measured accordingly (500 nM; $p<0.01$) (Figure 4A, 4B). An increase in the IRAK-4 inhibitor concentration caused an increase in apoptotic ratio in both GL-1 and CL-1 cells (1.25 μM; $p<0.0001$, 2.5 μM; $p<0.0001$) (Figure 4C, 4D). Additionally, to determine whether apoptosis was promoted, caspase activity was indirectly detected using western blot (Figure 4E). In both lymphoma cell lines, procaspase 3 and procaspase 7 were decreased in a dose-dependent manner by IRAK-4 inhibitor. These results indicate that the ability of IRAK-4 inhibitor may promote apoptosis in GL-1 and CL-1 cells in a dose-dependent manner.

3.5. IRAK-4 inhibitor inhibits TLR signaling in GL-1 and CL-1 cells

To investigate the anti-cancer mechanism of the IRAK-4 inhibitors, the protein levels of molecules associated with TLR signalling were measured using Western blot assay. Two concentrations of IRAK-4 inhibitor (FM501) were selected by referring to IC50 specific to each cell line, added to each cell (GL-1 cells: 250 and 500 nM; CL-1 cells: 1.25 and 2.5 μM), and incubated for 48 h. The extracted proteins were subjected to Western blotting with primary antibodies against total IRAK-1, p-IRAK-1, total IRAK-4, p-IRAK-4, total NF-κB p65, p-NF-κB p65, total STAT3, and p-STAT3. Total STAT3, and phosphorylated forms of IRAK-1, IRAK-4, NF-κB p65, and STAT3 were decreased in IRAK-4 inhibitor-treated GL-1 and CL-1 cells compared to those in the control and vehicle-treated cells (Figure 5A, 5B). Phosphorylated and total STAT3 in GL-1 cells treated with 500 nM had weaker band signals than those treated with 250 nM IRAK-4 inhibitor

(Figure 5A). Consistent with the results obtained in GL-1 cells, a dose-dependent decrease in the band signals of phosphorylated and total STAT3 was observed in CL-1 cells treated with 1.25 and 2.5 μM of IRAK-4 inhibitors (Figure 5B). These results indicate that IRAK-4 inhibitors can potentially downregulated molecules associated with TLR signalling in GL-1 and CL-1 cells.

4. Discussion

In the present study, canine lymphoma cell lines GL-1 and CL-1 were treated with the IRAK-4 inhibitors, to analyse their in vitro effects and explore their therapeutic efficacy in treating lymphoid malignancies in dogs. IRAK-4 inhibitor treatment caused a dose- and time-dependent decrease in the proliferation of GL-1 and CL-1 cells. Furthermore, treatment of GL-1 and CL-1 cells with the IRAK-4 inhibitor at a concentration corresponding to the respective IC50, significantly reduced the protein levels of TLR signaling-associated molecules, including phosphorylated IRAK-4. As predicted, downregulation of IRAK-4 affected downstream TLR signalling, resulting in decreased expression of NF-κB and STAT3, which plays a major role in the survival and proliferation of lymphoma cells.

The decreased phosphorylation of NF-κB and STAT3 induced by the IRAK-4 inhibitor may be responsible for the inhibition of cellular proliferation in GL-1 and CL-1 cells. This result is consistent with reports that IRAK-4 inhibitors inhibit the viability of ABC DLBCL cell lines due to NF-κB inhibition associated with the JAK-STAT3 pathway.[15, 16] Although these factors are related to the apoptosis of tumour cells in humans, their role in veterinary medicine is yet to be elucidated. Therefore, the present study aimed to determine the mechanism by which IRAK-4 inhibitors induce apoptosis in canine lymphoma cell lines.

In the present study, the mechanism underlying IRAK-4 inhibitor-induced cell death was identified using Annexin V staining. The IRAK-4 inhibitor was found to increase the apoptotic rate in a concentration-dependent manner. However, the IRAK-4 inhibitor had little effect on cPBMCs isolated from healthy beagle dogs. This suggests that IRAK-4 inhibitors inhibit cellular proliferation in a tumour-specific manner, which attests to its immense therapeutic potential in selective tumour treatment without affecting normal cells.

The results of the present study confirmed that MyD88 expression was higher in canine lymphoid cell lines, GL-1 and CL-1 than in normal cells. MyD88 overexpression was also confirmed in the canine DLBCL cell line CLBL-1 (Figure 1A). Unlike human ABC DLBCL, relatively little is known about the genetic basis

of canine lymphoma, and only a small number of mutations of genes (e.g., TRAF3) are known.[17] Therefore, it is considered that an overall analysis of the MyD88 mutation and MyD88 overexpression in canine lymphatic disease is necessary as further study.

The correlation between canine MyD88 expression and IRAK-4 inhibitor treatment had limitations that were not considered in this study, but has been investigated extensively.[8] IRAK-4 plays a pivotal role in MyD88 signalling, because it is the starting point that mediates the activation and interaction of downstream signals.[18-23] Overexpression of MyD88 caused by oncogenic mutation (e.g. MyD88 L265P) leads to excessive TLR signaling and NF-κB expression, which leads to producing more pro-inflammatory cytokines such as IL-6, IL-10, and IFNβ.[24, 25] These cytokines further activate JAK-STAT3 signalling, contributing to the survival of tumour cells through autocrine effect.[26] Considering this mechanism, the action of IRAK-4 inhibitors is linked to the regulation of TLR signaling by suppressing STAT3 and NF-κB expression in MyD88-dependent lymphoma.[27-29] Our current findings align with those of previous studies in that we confirm the therapeutic potential of IRAK-4 inhibitors as novel drugs to treat canine lymphoma since they selectively reduce the viability of tumour cells that overexpress Myd88 without affecting normal cPBMCs.

In the present study, we elucidated the in vitro effect of IRAK-4 inhibitors in canine lymphoid malignancies and explored its potential as a novel therapeutic agent to treat these malignancies. However, the present study has a few limitations. First, it was conducted on canine lymphoma cell lines GL-1 and CL-1 and did not include canine DLBCL cell lines and lymphoma malignancy cells with normal MyD88 expression. For further evaluation on IRAK-4 inhibitor, comparing with anticancer drugs used for canine lymphoma (e.g. doxorubicin, lomustine) or evaluating the effect of combination therapy are required. Additional studies on in vivo experiments and clinical trials in dogs with lymphoid malignancies could also potentially support the results of this study and its successful implementation in translational medicine.

In conclusion, this study demonstrated that IRAK-4 inhibitors exhibit a selective inhibitory effect on canine lymphoid malignant cells in vitro, but not normal cPBMCs. It also elucidated the associated TLR signalling mechanism. This

is the first study to our knowledge to analyse the effect of an IRAK-4 inhibitor on canine lymphoid malignant cell lines. IRAK-4 inhibitors may be able to overcome and complement the limitations of conventional anticancer therapy protocols, indicating that this inhibitor has potential clinical implications as a treatment for canine lymphoid malignancies.

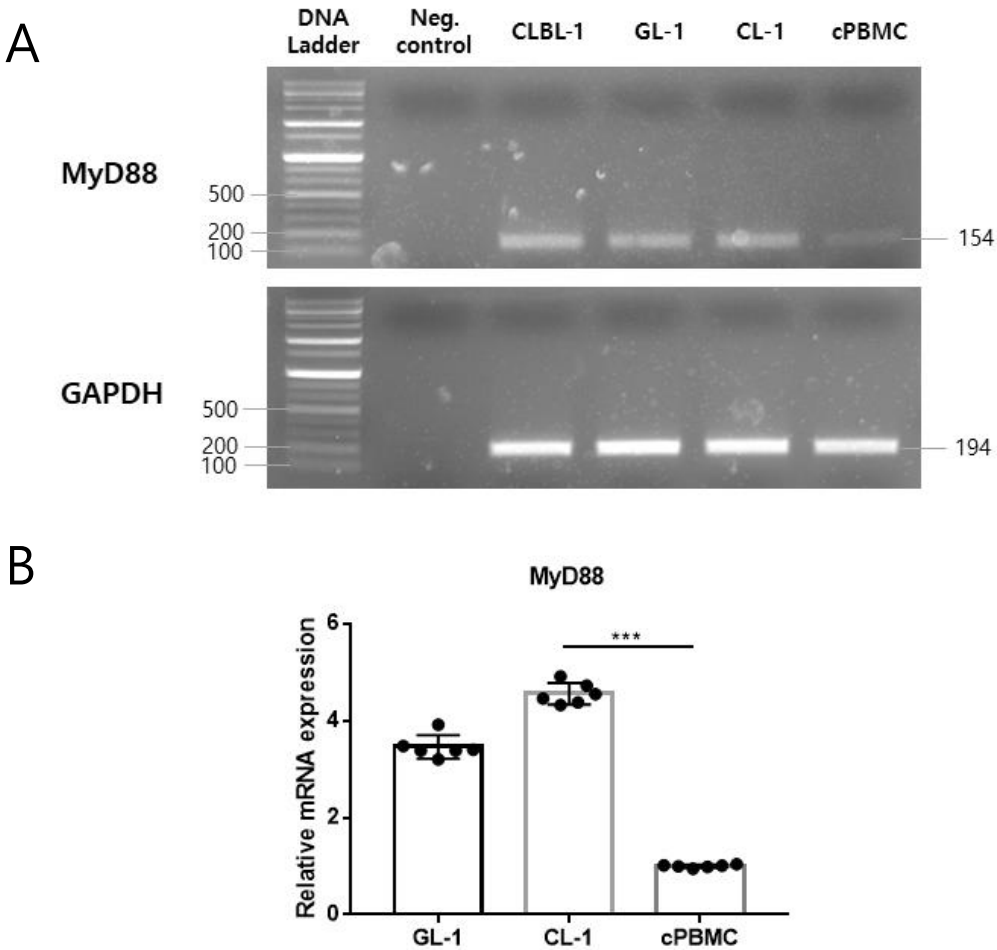
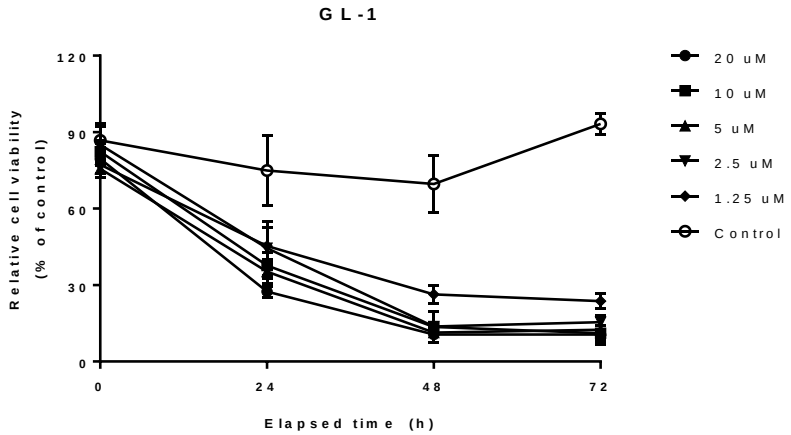


Figure 1. MyD88 expression level compared at the RNA level in GL-1, CL-1, CLBL-1 and normal canine peripheral blood mononuclear cells from healthy beagle dogs. A: MyD88 expression in RNA level in GL-1, CL-1, CLBL-1 and normal canine peripheral blood mononuclear cells (cPBMC) by RT-PCR. B: MyD88 expression in RNA level in GL-1, CL-1 and normal cPBMC by qRT-PCR using the 2^(-ΔΔCT)method. Values are expressed as mean ± standard deviation. *p <0.05, ** p < 0.01, *** p < 0.001, **** p <0.0001; ns, not significant.

A


GL-1
Relative cell viability
(% of control)
120
90
60
30
0
0
24
48
72
Elapsed time (h)
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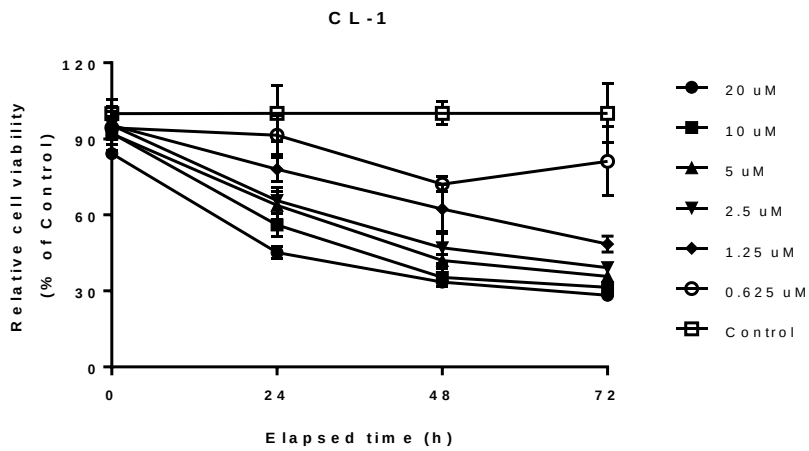
10 uM
5 uM
2.5 uM
1.25 uM
Control

B


CL-1
Relative cell viability
(% of Control)
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90
60
30
0
0
24
48
72
Elapsed time (h)
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5 uM
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1.25 uM
0.625 uM
Control

C

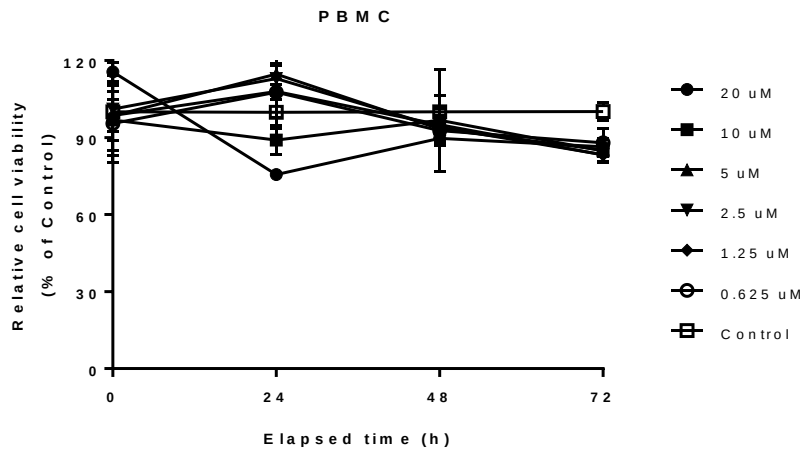
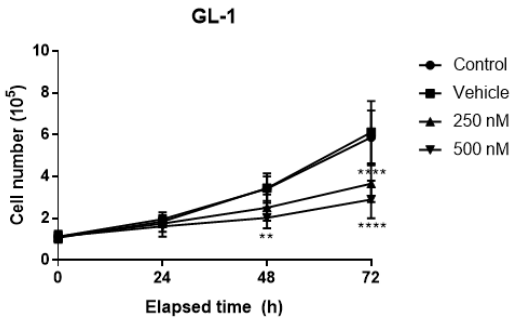

PBMC
Relative cell viability
(% of Control)
120
90
60
30
0
0
24
48
72
Elapsed time (h)
20 uM
10 uM
5 uM
2.5 uM
1.25 uM
0.625 uM
Control

Figure 2. IRAK-4 inhibitor suppressed cell viability of GL-1, CL-1 cell lines in a dose-dependent manner, but not in canine peripheral blood mononuclear cells. A: Viability assay of GL-1 cell line treated with different concentrations of IRAK-4 inhibitor (FM504) for different incubation time conditions. B: Viability assay of CL-1 cell line treated with different concentrations of IRAK-4 inhibitor (FM504) for different incubation time conditions. C: Viability assay of canine peripheral blood mononuclear cells (cPBMCs) from healthy beagle dogs treated with different concentrations of IRAK-4 inhibitor (FM504) for different incubation time conditions. Values are expressed as mean ± standard deviation. *p <0.05, ** p < 0.01, *** p < 0.001, **** p <0.0001; ns, not significant

A



B

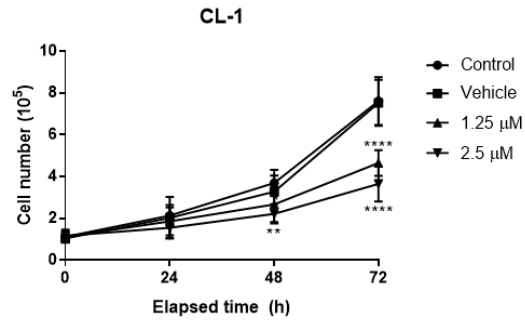
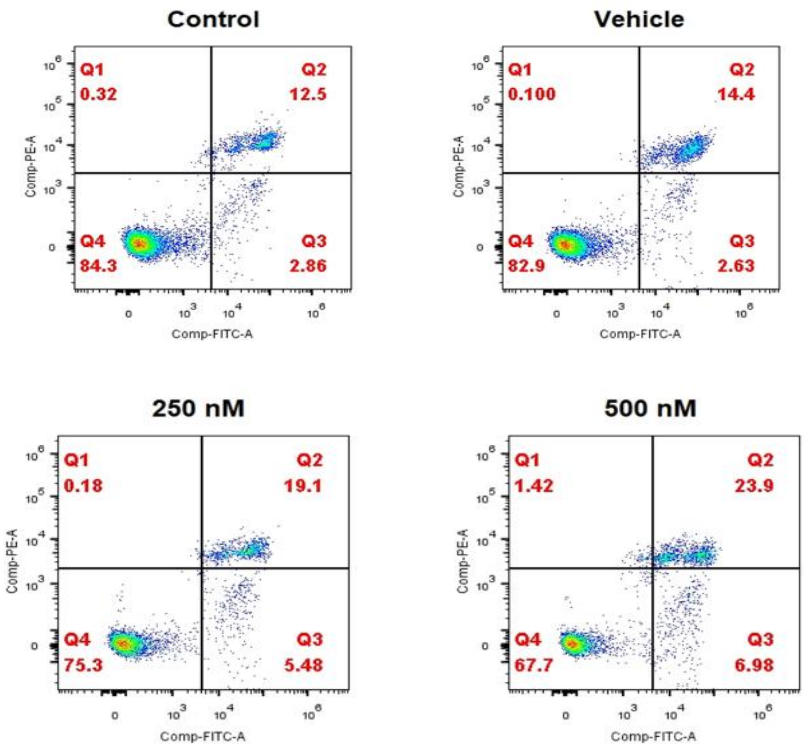
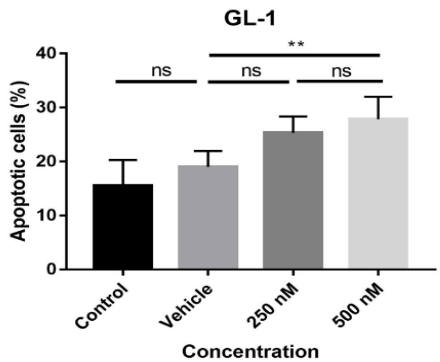


Figure 3. IRAK-4 inhibitor suppressed cell proliferation of Gl-1, CL-1 cell lines. A: Cell count result of GL-1 cell line treated with two different concentrations of IRAK-4 inhibitor ((FM501; 0, vehicle, 250, and 500 nM) for different incubation time conditions. B: Cell count result of CL-1 cell line treated with two different concentrations of IRAK-4 inhibitor (FM501; 0, vehicle, 1.25, and 2.5 μM) for different incubation time conditions. Values are expressed as mean ± standard deviation. *p <0.05, ** p < 0.01, *** p < 0.001, **** p <0.0001; ns, not significant.

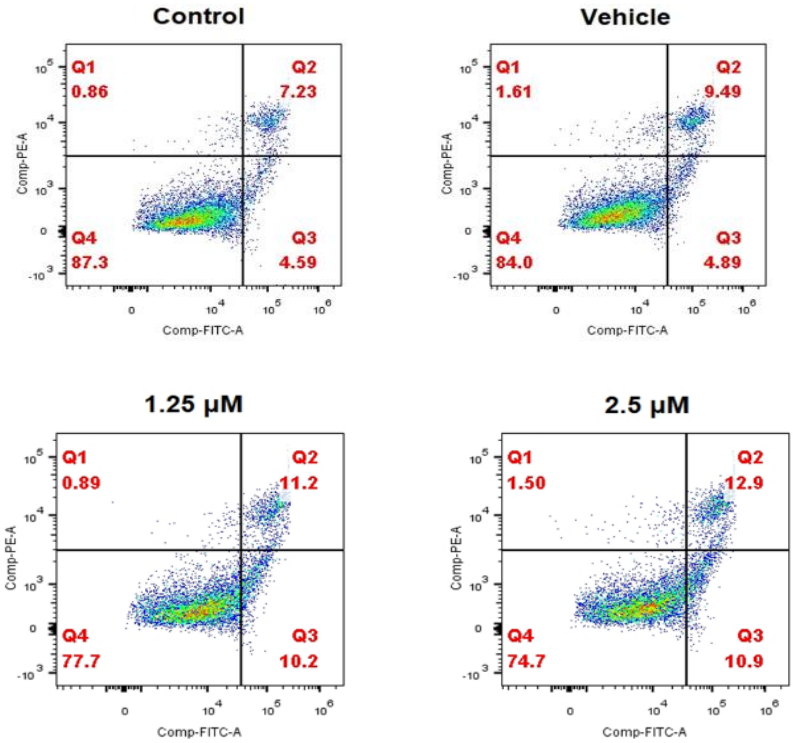
A


Control
Q1
0.32
Q2
12.5
Q4
84.3
Q3
2.86
Vehicle
Q1
0.100
Q2
14.4
Q4
82.9
Q3
2.63
250 nM
Q1
0.18
Q2
19.1
Q4
75.3
Q3
5.48
500 nM
Q1
1.42
Q2
23.9
Q4
67.7
Q3
6.98
Comp-PE-A
Comp-FITC-A

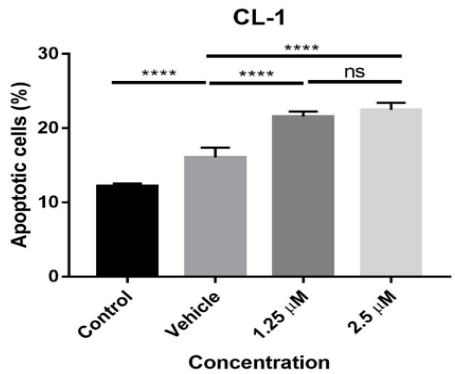
B


GL-1
**
ns
ns
ns
Apoptotic cells (%)
40
30
20
10
0
Control
Vehicle
250 nM
500 nM
Concentration

C


Control
Q1
0.86
Q2
7.23
Q4
87.3
Q3
4.59
Vehicle
Q1
1.61
Q2
9.49
Q4
84.0
Q3
4.89
1.25 μM
Q1
0.89
Q2
11.2
Q4
77.7
Q3
10.2
2.5 μM
Q1
1.50
Q2
12.9
Q4
74.7
Q3
10.9
Comp-PE-A
Comp-FITC-A

D


CL-1
Apoptotic cells (%)
30
20
10
0
****
****
****
ns
Control
Vehicle
1.25 μM
2.5 μM
Concentration

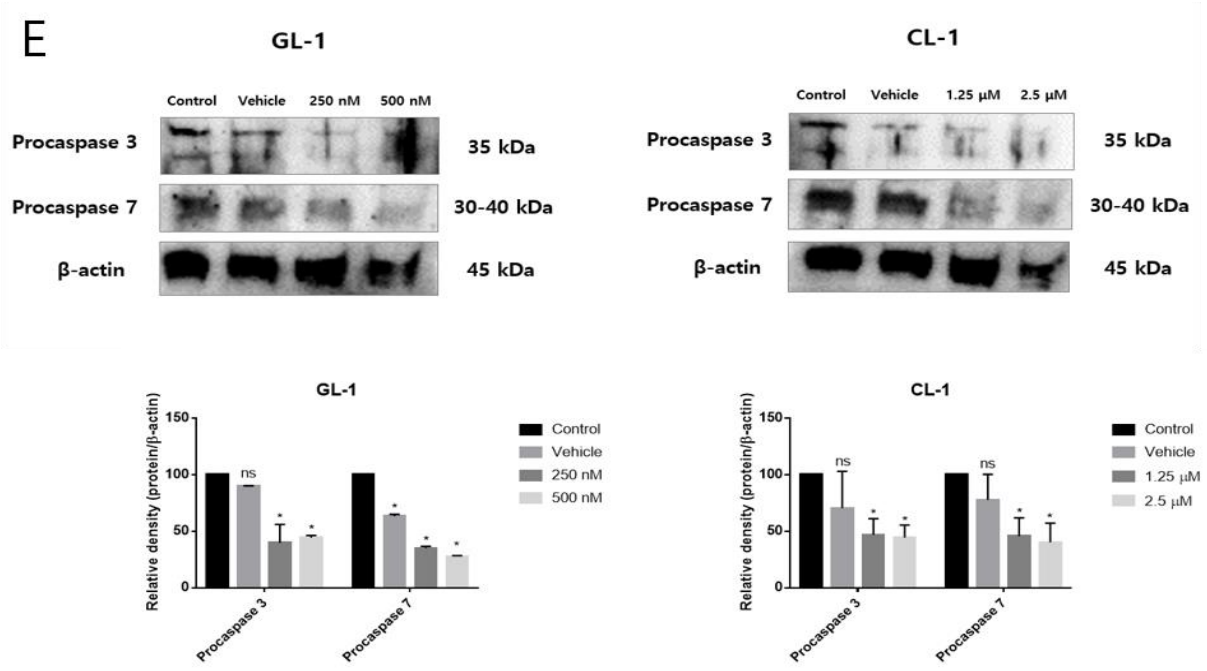


Figure 4. IRAK-4 inhibitor promoted apoptosis of GL-1, CL-1 cell lines. A: The apoptotic ratio was determined using flow cytometry. GL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 250, and 500 nM) for 48 h. B: Quantification of data shown in A. C: The apoptotic ratio was determined using flow cytometry. CL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 1.25, and 2.5 μM) for 48 h. D: Quantification of data shown in A. Percentage of apoptosis was detected using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining. Early apoptotic cells stained as annexin V+/PI−. Late apoptotic cells were stained as annexin V+/PI+. E. Western blot on apoptotic activity. GL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 250, 500 nM), and CL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 1.25, 2.5 μM) for 48 h. Beta-actin was used as a loading control. The calculated band density was normalized using beta-actin (below). Values are expressed as mean ± standard deviation. * $p < 0.05$; ns, not significant.

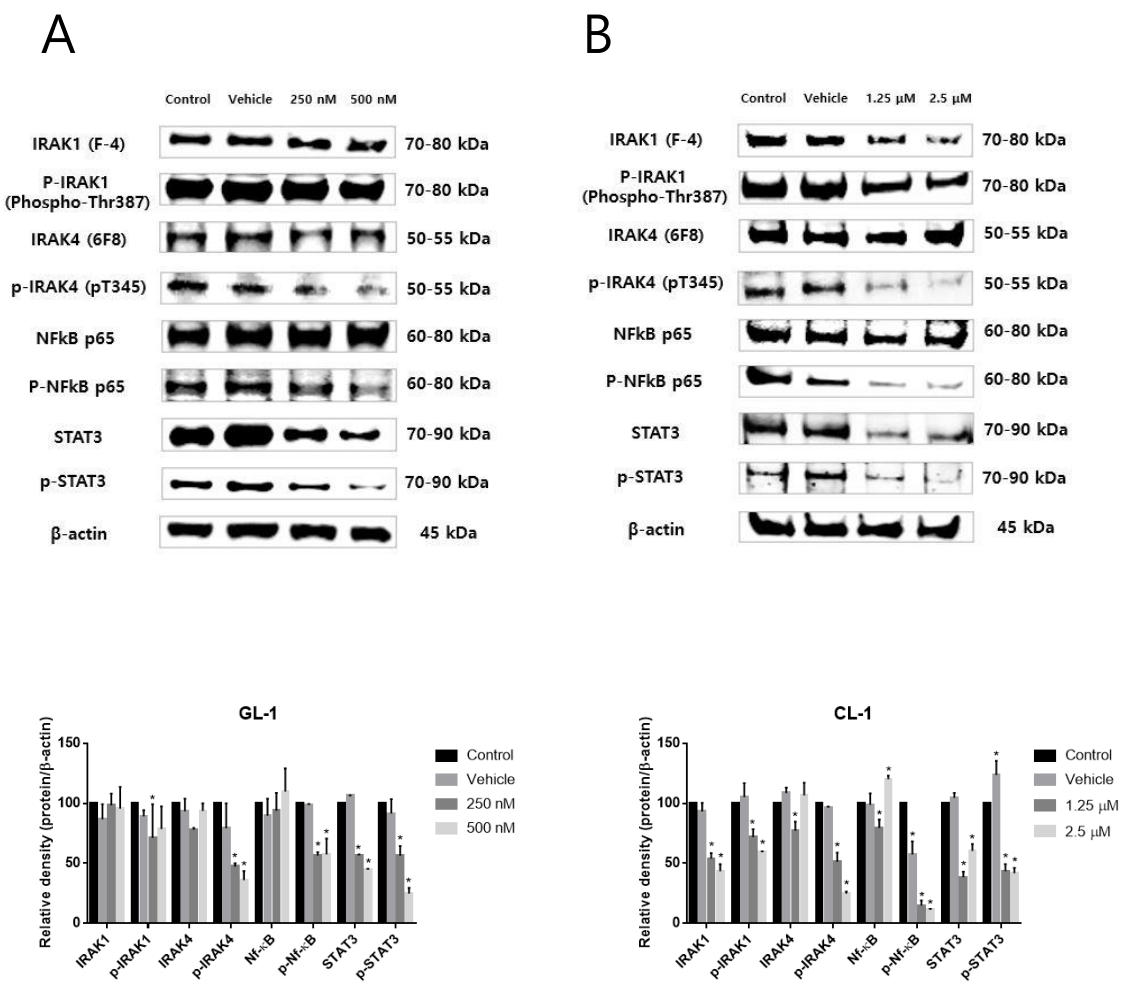
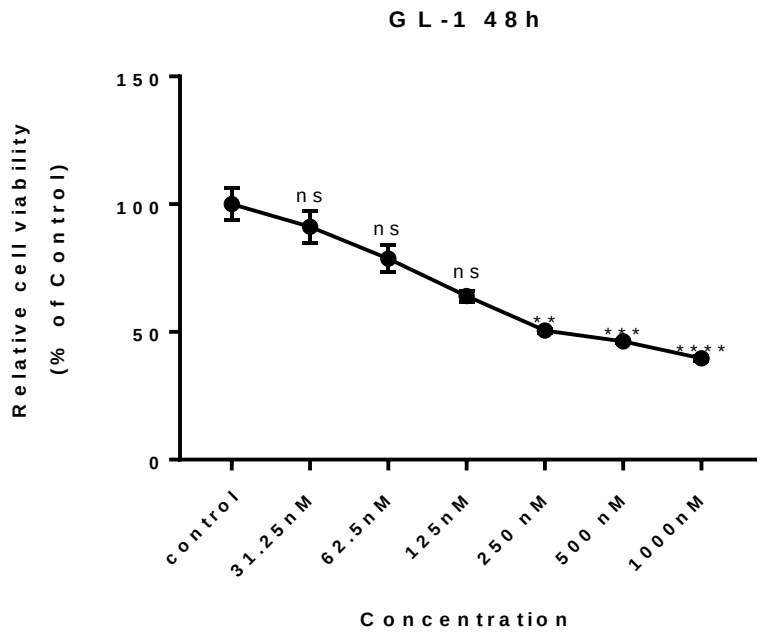


Figure 5. IRAK-4 inhibitor suppressed TLR signaling in GL-1, CL-1 cell lines in a dose-dependent manner. A. GL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 250, 500 nM) for 48 h. B. CL-1 cell line was treated with IRAK-4 inhibitor (FM501; 0, vehicle, 1.25, 2.5 μM) for 48 h. The expression of TLR signaling molecules was measured by western blotting. Beta-actin was used as a loading control. The calculated band density was normalized using beta-actin (below). Values are expressed as mean ± standard deviation. * $p < 0.05$; ns, not significant.



Supplementary figure 1. IC50 of IRAK-4 inhibitor against GL-1. Viability assay of GL-1 cell line treated with different concentrations of IRAK-4 inhibitor (FM504). Results are shown as mean ± standard deviation (n=6). * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001; ns, not significant.

5. References

1. Burton JH, Garrett-Mayer E, Thamm D. Evaluation of a 15-week chop protocol for the treatment of canine multicentric lymphoma. Vet Comp Oncol 2013;11(4):306-315.
2. Wolf-Ringwall Aea. Prospective evaluation of flow cytometric characteristics, histopathologic diagnosis and clinical outcome in dogs with naive B-cell lymphoma treated with a 19-week CHOP protocol. Vet Comp Oncol 2020;18(3): 342-352.
3. Richards KL, Motsinger-Reif AA, Chen H-W et al. Gene profiling of canine B-cell lymphoma reveals germinal center and postgerminal center subtypes with different survival times, modeling human DLBCL. Cancer Res 2013;73(16):5029-5039.
4. Kawai T, Akira S. TLR signaling. Seminars in immunology 2007;19(1):24-32.
5. O'neill L. The toll/interleukin-1 receptor domain: A molecular switch for inflammation and host defence. Biochem Soc Trans 2000;28(5): 557-563.
6. Küppers R. IRAK4 inhibition to shut down TLR signaling in autoimmunity and MYD88-dependent lymphomas. J Exp Med 2015;212(13): 2184.
7. Ngo, Vu N., et al. Oncogenically active MYD88 mutations in human lymphoma. Nature, 2011, 470.7332: 115-119.
8. Rosenthal AC, Tun HW, Younes A, Nowakowski GS, et al. Phase 1 study of CA-4948, a novel inhibitor of interleukin-1 receptor-associated kinase 4 (IRAK4) in patients (pts) with r/r non-hodgkin lymphoma. J Clin Oncol 2019;37(15_suppl): e19055.
9. Booher RN, Nowakowski GS, Patel K, et al. Preclinical activity of IRAK4 kinase inhibitor CA-4948 alone or in combination with targeted therapies and preliminary phase 1 clinical results in non-hodgkin lymphoma. Blood 2018;132(Supplement 1): 4168-4168.
10. Nakaichi M, Taura Y, Kanki M, et al. Establishment and characterization of a new canine B-cell leukemia cell line. J Vet Med Sci 1996;58(5):469-

471.

11. Momoi Y, Okai Y, Watari T, Goitsuka R, Tsujimoto H, Hasegawa A. Establishment and characterization of a canine T-lymphoblastoid cell line derived from malignant lymphoma. Vet Immunol Immunopathol 1997;59(1-2): 11-20.
12. An JH, Song WJ, Li Q, Bhang DH, Youn HY. 3D-culture models as drug-testing platforms in canine lymphoma and their cross talk with lymph node-derived stromal cells. J Vet Sci 2021;22(3): e25
13. Rütgen BC, Hammer SE, Gerner W, et al. Establishment and characterization of a novel canine B-cell line derived from a spontaneously occurring diffuse large cell lymphoma. Leuk Res 2010;34(7):932-938.
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] Method. Methods 2001;25(4):402-408.
15. Kelly PN, Romero DL, Yang Y, et al. Selective interleukin-1 receptor–associated kinase 4 inhibitors for the treatment of autoimmune disorders and lymphoid malignancy J Exp Med 2015;212(13):2189-2201.
16. Roschewski M, Staudt LM, Wilson WH: Diffuse large B-cell lymphoma-treatment approaches in the molecular era. Nat Rev Clin Oncol 2014;11(1):12-23.
17. Bushell, Kevin R., et al. Genetic inactivation of TRAF3 in canine and human B-cell lymphoma. Blood 2015; 125(6): 999-1005.
18. Flannery S, Bowie AGJBp. The interleukin-1 receptor-associated kinases: Critical regulators of innate immune signalling. Biochem pharmacol 2010;80(12): 1981-1991.
19. Kawagoe T, Sato S, Matsushita K, et al. Sequential control of toll-like receptor–dependent responses by IRAK1 and IRAK2. Nature 2008;9(6): 684.
20. Ferrao R, Zhou H, Shan Y, Liu Q, et al. IRAK4 dimerization and trans-autophosphorylation are induced by Myddosome assembly. Mol cell 2014;55(6): 891-903.
21. Qian Y, Commane M, Ninomiya-Tsuji J, Matsumoto K, Li X. Irak-

mediated translocation of traf6 and tab2 in the interleukin-1-induced activation of NF-κB. J Biol Chem 2001;276(45): 41661-41667.

22. Xiang W, Chao ZY, Feng DY. Role of toll-like receptor/myd88 signaling in neurodegenerative diseases. Rev Neurosci 2015;26(4): 407-414.
23. Takaesu G, Ninomiya-Tsuji J, Kishida S, Li X, Stark GR, Matsumoto K. Interleukin-1 (IL-1) receptor-associated kinase leads to activation of TAK1 by inducing TAB2 translocation in the IL-1 signaling pathway. Mol Cell Biol 2001;21(7): 2475-2484.
24. Seelig DM, Ito D, Forster CL, et al. Constitutive activation of alternative nuclear factor kappa B pathway in canine diffuse large B-cell lymphoma contributes to tumor cell survival and is a target of new adjuvant therapies. Leuk Lymphoma 2017;58(7):1702-1710.
25. Jeelall YS, rikawa K.Oncogenic myd88 mutation drives toll pathway to lymphoma. Immunol Cell Biol 2011;89(6): 659.
26. Ding BB, Yu JJ, Yu RY-L, et al. Constitutively activated stat3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. 2008;111(3): 1515-1523.
27. Wang X, Crowe PJ, Goldstein D, Yang JL. STAT3 inhibition, a novel approach to enhancing targeted therapy in human cancers. Int J Oncol 2012;41(4):1181-1191.
28. Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003;101(4): 1535-1542.
29. Bavi P, Uddin S, Bu R et al. The biological and clinical impact of inhibition of NF-κB-initiated apoptosis in diffuse large B cell lymphoma (dlbcl). J Pathol 2011;224(3): 355-366.

국 문 초 록

개 림프구성 악성 종양에 있어서 IRAK-4 억제제의 항암 효과

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박 준 형

Interleukin-1 receptor-related kinase 4 (IRAK4)는 MyD88의 하위 신호로써, 일부 B 세포 림프종에서 보이는 TLR 신호 전달의 과발현에서 매우 주요한 역할을 한다. 특히, 사람에서 미만성 거대 B세포 림프종 (DLBCL) 중 활성화 B 세포 아형에 대한 효과적인 IRAK4 억제제가 개발되고 있다. 하지만 수의학에서 IRAK4 억제제의 항암 효과는 아직 입증되지 않았다. 그러므로 이번 연구에서는 개의 림프종 세포주인 GL-1, CL-1에 대하여 시험관 내 실험을 통해 설명하려 한다. 중합효소 연쇄 반응을 통해 MyD88의 발현을 확인하였다. GL-1 및 CL-1 세포는 IRAK4 억제제 처치 후 농도 및 시간에 따른 세포 계수 Kit-8 분석, 웨스턴 블랏 및 유세포분석을 통해 세포 생존력, TLR 신호 전달과의 연관성 및 세포 사멸 정도를 평가하였다.

GL-1과 CL-1 세포는 MyD88의 상승 발현을 보였지만, 개 말초혈액 단핵세포(cPBMC)는 그렇지 않았다. IRAK-4 억제제는 용량 및 시간 의존적인 방식으로 세포 생존력을 감소시켰고, IC50에서 IRAK1, IRAK4, NF-βB, STAT3와 같은 TLR 신호 전달 물질들의 인산화를 크게 감소시키며, GL-1과 CL-1 세포의 세포 자멸을 유도했다.

결론적으로 IRAK-4 억제제의 개 림프종 세포에 대한 항암 효과는 TLR 신호 전달의 하향 조절을 통한 세포 자멸에 의해 매개된다.

주요어: 세포 자멸, 개 림프종, 세포 생존력, IRAK4 억제제, MyD88, TLR 신호 전달.

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