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NEMO Binding Domain peptide inhibits constitutive NF-κB activity and reduces tumor burden in a canine model of relapsed, refractory Diffuse Large B-Cell Lymphoma

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Abstract

Purpose—Activated B-Cell Diffuse Large B-Cell Lymphoma (ABC-DLBCL) is an aggressive, poorly chemoresponsive lymphoid malignancy characterized by constitutive canonical NF-κB activity that promotes lymphomagenesis and chemotherapy resistance via over-expression of anti-apoptotic NF-κB target genes. Inhibition of the canonical NF-κB pathway may therefore have therapeutic relevance in ABC-DLBCL. Here we set out to determine whether dogs with spontaneous DLBCL have comparative aberrant constitutive NF-κB activity and to determine the therapeutic relevance of NF-κB inhibition in dogs with relapsed, resistant DLBCL.

Experimental Design—Canonical NF-κB activity was evaluated by electrophoretic mobility shift assays and immunoblot analyses, and NF-κB target gene expression was measured by qRT-PCR. Primary malignant canine B lymphocytes were treated with the selective IKK complex inhibitor Nemo Binding Domain (NBD) peptide, and evaluated for NF-κB activity and apoptosis. NBD peptide was administered intra-nodally to dogs with relapsed B-cell lymphoma and NF-κB target gene expression and tumor burden were evaluated pre and post treatment.

Results—Constitutive canonical NF-κB activity and increased NF-κB target gene expression was detected in primary DLBCL tissue. NBD peptide inhibited this activity and induced apoptosis of primary malignant B cells *in vitro*. Intra-tumoral injections of NBD peptide to dogs with relapsed DLBCL inhibited NF-κB target gene expression and reduced tumor burden.

Conclusions—This work shows that dogs with spontaneous DLBCL represent a clinically relevant, spontaneous, large animal model for human ABC-DLBCL and demonstrates the therapeutic relevance of NF-κB inhibition in the treatment of ABC-DLBCL. These results have important translational relevance for ABC-DLBCL treatment in human patients.

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Conflict of Interest Statement: Dr. May holds a patent for the NBD peptide and Dr. Baldwin is the founder of TheraLogics and therefore has commercial interests. All other authors declare no competing financial interests.

Keywords

Diffuse Large B-Cell Lymphoma; NF-κB; canine; animal model; pre-clinical

Introduction

Diffuse Large B-Cell Lymphoma (DLBCL) is the most common adult lymphoid malignancy with approximately 30,000 new cases diagnosed each year in the United States (1, 2). Gene expression profiling has revealed the presence of at least three subtypes of DLBCL, Activated B-Cell (ABC-DLBCL), Germinal Center B-Cell (GBC-DLBCL) and Primary Mediastinal B-Cell Lymphoma (PMBL), that have distinct molecular signatures and different clinical outcomes (1, 3). ABC-DLBCL is the most aggressive subtype and is less responsive to conventional multi-agent chemotherapy than GCB-DLBCL and PMBL. Even with the addition of Rituximab, the overall survival for patients with ABC-DLBCL is 47% (4).

ABC-DLBCL is characterized by the presence of constitutive canonical NF-κB activity that drives expression of NF-κB target genes which promote lymphocyte proliferation (e.g. Cyclin D1 and D2) and inhibit apoptosis (e.g. Bcl-2, Bcl-xl, A1, c-FLIP, and XIAP) (5, 6). Aberrant canonical NF-κB activity contributes to lymphomagenesis and chemoresistance by promoting malignant cell survival and proliferation (7, 8). In health, NF-κB activation is highly regulated. NF-κB dimers are held inactive in the cytoplasm through their association with inhibitory IκB family members (9). Engagement of cell surface receptors by many different infectious and inflammatory stimuli leads to phosphorylation and activation of the IKK complex, which phosphorylates IκBα and targets it for ubiquitination and proteosomal degradation. This allows NF-κB dimers to translocate to the nucleus where they bind to target gene promoters and enhancers and initiate transcription (8). Somatic activating mutations in genes encoding regulatory proteins upstream of the IKK complex have been identified in patients with ABC-DLBCL and lead to constitutive phosphorylation and activation of the IKK complex (5, 10, 11). Constitutive, canonical NF-κB activity is essential for ABC-DLBCL cell survival (3, 7) and inhibition of this pathway with small molecule inhibitors of IKK, IκBα super-repressors, proteosome inhibitors, or inhibitors upstream of the IKK complex promotes cell cycle arrest, chemotherapeutic sensitivity and apoptosis in ABC-DLBCL cell lines (3, 7, 12). Taken together, these findings suggest that targeted inhibition of aberrant NF-κB activity represents a promising therapeutic strategy for patients with primary or relapsed ABC-DLBCL.

The high-quality draft genome sequence of the dog has revealed its close phylogenetic relationship with man, emphasizing the potential benefit of canine models in identifying disease genes and evaluating response to novel therapies (13–16). In particular, the dog is being increasingly recognized as a clinically relevant, spontaneous large animal model for human Non Hodgkin's Lymphoma (NHL) (17–19). NHL is the most common, spontaneous, hematopoietic malignancy in dogs, with an annual incidence of 30/100,000 (20). DLBCL is the most common subtype of canine NHL and shares similar biologic, behavioral, molecular and genetic characteristics with DLBCL in humans (17, 20–22). Dogs with DLBCL are treated with the same cytotoxic agents used in human DLBCL patients that inhibit cell division and induce apoptosis. However, as in human patients, clinical remission is not maintained and 85–90% of canine patients relapse with lethal, drug-resistant lymphoma within 6 to 9 months of initial diagnosis and treatment (19, 23).

NEMO Binding Domain (NBD) peptide is a selective inhibitor of the IKK complex that consists of 11 amino acids located at the carboxy terminus of the catalytic IKKβ subunit that

binds to the scaffold protein NF-κB Essential Modulator (NEMO) (24). The core of six amino acids in the NBD is also present in IKKα and facilitates its association with NEMO (24–26). NBD peptide inhibits the interaction of both IKKα and IKKβ with NEMO and prevents assembly of the IKK complex (25). Fusion of NBD peptide to protein transduction domains, such as that within the drosophila antennapedia protein enables it to enter cells and effectively inhibit canonical NF-κB activation in response to TNFα, lipopolysaccharide and Toll-Like Receptor ligation (24, 25, 27). Systemic administration of NBD peptide results in potent anti-inflammatory effects in animal models of inflammation (24, 28–32). NBD peptide also inhibits constitutive NF-κB activity in tumor cell lines, including pancreatic carcinoma, squamous cell carcinoma, melanoma, cutaneous T cell lymphoma and mantel cell lymphoma, and sensitizes these cells to TRAIL or TNFα-induced apoptosis (33–37). Furthermore, NBD peptide inhibits NF-κB activation that occurs in response to DNA damage induced by cytotoxic chemotherapeutics (38). Taken together, these findings suggest that targeted inhibition of canonical NF-κB activity using NBD peptide may represent an effective therapeutic strategy in patients with ABC-DLBCL.

Before evaluating the therapeutic effects of NBD peptide in human patients with ABC-DLBCL, we performed studies to evaluate its ability to inhibit constitutive NF-κB activity in dogs with spontaneous B-cell lymphoma. We show that constitutive canonical NF-κB activity occurs in the malignant lymph nodes of dogs with DLBCL and that this is associated with over-expression of anti-apoptotic NF-κB target genes. We demonstrate that NBD peptide inhibits constitutive NF-κB activity and induces apoptosis of canine malignant B cells *in vitro*. Furthermore, we show that administration of NBD peptide to dogs with relapsed B-cell lymphoma inhibits the expression of NF-κB target genes and leads to a reduction in tumor burden. These findings provide proof of concept that NBD peptide can inhibit constitutive NF-κB activity in a clinically relevant spontaneous canine model of DLBCL and begin to pave the way for phased clinical trials in humans with ABC-DLBCL.

Materials and methods

Cells and Reagents

Canine lymphocytes were cultured in complete RPMI media containing 10% FBS. The human GCB-DLBCL cell line SUDHL-6 was maintained in complete RPMI media, and the human ABC-DLBCL cell line OCI-Ly10 was maintained in complete Iscove's modified essential medium (IMDM) containing 20% FBS. Cell lines were obtained from Dr. Anne Novak (Mayo Clinic Cancer Center, Rochester, Mn). Malignant lymph node samples were collected from dogs with either primary or relapsed DLBCL following approval from the University of Pennsylvania's Institutional Animal Care and Use Committee. The histopathological diagnosis of DLBCL and the cytological diagnosis of large B cell lymphoma were made by board certified veterinary pathologists (M.G., and A.D., see acknowledgements) and by a board certified clinical pathologist (R.P.) respectively. NBD peptide was synthesized and purified by Dr. James I. Elliott (at the Howard Hughes Medical Institute Biopolymer-Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT) using standard tertbutoxycarbonyl (t-Boc) chemistry, cleavage with hydrofluoric acid, and purification by reversed-phase HPLC (26). TNFα was purchased from Sigma-Aldrich (Saint Louis, MO).

Western Blot Analyses

Cells were lysed in 50 mM Tris-HCL containing 1% NP-40, 150 mM NaCl, 2.5 mM EDTA, 5% glycerol, a 1:50 dilution of Protease Inhibitor Cocktail and 1:50 dilutions of Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich). Protein concentrations were determined by micro BCA assay (Thermo Scientific, Rockford, IL). Proteins were separated by SDS-

PAGE (10%) and transferred to PVDF membrane. Membranes were probed with polyclonal rabbit anti-human antibodies against phospho-IKKα/β (16A6), pan IKKβ (L570), phospho-IκBα (14D4) andβ-actin (4967) (Cell Signaling, Danvers, MA) or rabbit anti-human pan IκBα (C-21) (SantaCruz Biotechnology, Santa Cruz). An HRP-conjugated donkey anti-rabbit IgG was used as the secondary detection antibody (Amersham, Piscataway, NJ). Blots were developed using ECL Plus (Amersham, Piscataway, NJ) or SuperSignal West Femto (Thermo Scientific, Rockford, IL).

Electrophoretic Mobility Shift Assay (EMSA)

Whole cell extracts of malignant canine lymphocytes were prepared as previously described (26). EMSAs were performed using 5μg protein extract and a palindromic NF-κB binding sequence probe (Santa Cruz Biotechnology) as previously described (26). EMSA supershift assays were performed using 10μg of protein extract and polyclonal rabbit anti-human p65 (sc-109X), p50 (sc-114X) and c-Rel (sc-70X) antibodies or control rabbit IgG (sc-2027) (Santa Cruz Biotechnology).

Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using random hexamers and Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad. CA, USA). Transcript sequences for canine A1, c-FLIP, Cyclin D1 and IκBα were obtained from the NCBI (http://www.ncbi.nih.gov/Genbank) and were analyzed for secondary DNA structure using M-Fold (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi). Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/) with the maximum self-complementarity score set at 5 and the maximum 3 self-complementarity score set to 0 to minimize primer-dimer formation (Table 1). Primer sequences for canine Bcl-2, XIAP and β-actin have been previously described (39). Quantitative RT-PCR was performed using SYBR Green (Fermentas, Glen Burnie, Maryland). Samples were run in triplicate using standard conditions on an ABI 7500 sequence detector (Applied Biosystems, Carlsbad, CA) and data were analyzed using β-actin as an endogenous control. Dissociation curves were performed after each experiment to confirm the specificity of product amplification.

Immunohistochemistry

Immunohistochemical analysis was performed on formalin fixed, paraffin-embedded tissue sections of lymph nodes from healthy dogs and dogs with DLBCL. Sections were deparaffinized in xylene, rehydrated and boiled in sodium citrate buffer to unmask antigens. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Immunohistochemistry was performed using a rabbit anti-human p65 antibody (C22B4) (Cell Signaling, Danvers, MA) or rabbit isotype control (sc-2027) (Santa Cruz Biotechnology) and the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Sections were developed using 3′,3′ diaminobinzidine (DAB) and counterstained with hematoxylin. Slides were viewed using a Nikon E600 infinity corrected upright microscope. Bright field images were acquired using a Nikon Digital Sight DS-Fi1 color camera using NIS-Element BR3.0 for image analysis.

In Vitro NBD Peptide Treatment of Malignant B Lymphocytes

Freshly isolated or cryopreserved malignant lymphocytes from lymph nodes of dogs with DLBCL were seeded at 4×10^6 cells/ml and treated with 100μm of either NBD or mutant peptide in DMSO. After 12h, whole cell extracts were evaluated by immunoblot for canonical NF-κB activity as described above, and for apoptosis by flow cytometry. The human ABC- and GCB-DLBCL cell lines OCI-Ly10 and SUDHL-6, were seeded at 0.5 ×

10^6 cells/ml and treated with 0, 25μM or 50μM NBD peptide. After 24 hours, cell death was evaluated by Trypan Blue.

Flow Cytometry

Detection of apoptotic cell death was performed using the PE Annexin-V Apoptosis Detection Kit (BD Biosciences, San Jose, CA). Cells were acquired on a FACSCalibur cytometer (BD Biosciences) and analyzed using TreeStar FlowJo software. Immunophenotyping of primary lymphoma cells was performed using the following antibodies: FITC conjugated rat anti-canine CD3 (Serotec, Raleigh, NC), APC conjugated mouse anti-human CD79a (BD Pharmingen, San Diego, CA) and mouse anti-canine CD21-like molecule (Serotec, Raleigh, NC). A FITC-labeled goat anti-mouse IgG secondary antibody was used to detect anti-CD21 antibody. For CD79a staining, cells were fixed with 1% paraformaldehyde and then permeabilized with 0.1% saponin prior to staining.

Pilot Trial

All *in vivo* studies were performed following approval from the University of Pennsylvania's Institutional Animal Care and Use Committee and the University of Pennsylvania School of Veterinary Medicine Clinical Review Board. Client-owned dogs were enrolled with the following inclusion criteria: 1) cytological confirmation of relapsed large B-cell lymphoma, 2) stage III-V, substage a, with at least 1 set of bilateral, enlarged, measurable, malignant lymph nodes, 3) life expectancy of greater than 1 month, 4) no concurrent systemic disease, 5) adequate hematologic, renal and hepatic function and 6) the presence of constitutive NF-κB activity within malignant lymph nodes as determined by EMSA. All dogs enrolled in the pilot trial had been previously treated with a range of rescue chemotherapeutic agents including L-asparaginase, cyclophosphamide, vincristine, prednisone, lomustine, doxorubicin, mechlorethamine, procarbazine, dacarbazine and total body irradiation. Signed informed consent was obtained from all owners before entry into the study. Baseline evaluation included a complete medical history, full clinical examination, complete blood count (CBC), chemistry screen (CS) and urinalysis, malignant lymph node cytology and flow cytometric immunophenotyping using a basic panel of antibodies including CD3, CD21 and CD79a. Eligible dogs received either 5 or 10mg NBD peptide/100g malignant lymph node mass. Node mass was calculated based on two dimension caliper measurements which were averaged and considered the diameter of the lymph node sphere. Volume was determined based on $V=2/3\pi r^3$. Based on our previous assessments, $1cm^3$ of malignant lymph node mass is equivalent to ~379mg. Peptide was injected directly into one malignant node and the same node was biopsied 24 hours later. qRT-PCR for NF-κB target gene expression was performed on node tissue taken 24 hours post NBD peptide treatment and was normalized to values obtained pre NBD peptide. No control dogs were included in this pilot study. Following the post treatment biopsy, patients received rescue chemotherapy as determined by their primary attending oncologist. All dogs received a follow up full clinical examination plus CBC, CS and urinalysis one-week post NBD peptide injection. Mass of the NBD treated lymph node and the contra-lateral, malignant, untreated lymph node was calculated before, and one week post NBD peptide administration.

Results

Constitutive canonical NF-κB activity occurs in dogs with DLBCL

To determine whether constitutive NF-κB activity is present in dogs with spontaneous, histologically confirmed DLBCL, EMSA was performed on biopsy samples taken from the peripheral lymph nodes of 13 dogs with DLBCL at the time of diagnosis and prior to chemotherapy. Constitutive NF-κB activity was detected in all 13 samples. In contrast, NF-

κB activity was not detected in normal lymph nodes from healthy dogs (Fig. 1A). To determine whether constitutive NF-κB activity was associated with the canonical pathway, malignant lymph node tissue from 11 dogs with newly diagnosed, histologically confirmed DLBCL was analyzed by EMSA supershift using antibodies directed against p50, p65 and c-Rel. In 7 dogs, p50, p65 and c-Rel were all present in the active NF-κB complexes (Fig. 1B). In 2 dogs only p50 and p65 were present, and in 2 dogs only p50 was identified (data not shown). To further confirm the presence of constitutive canonical NF-κB activity, formalin-fixed, paraffin-embedded lymph node tissues from 6 dogs with DLBCL were evaluated for the presence of nuclear p65 by immunohistochemistry (Fig. 1C). All tissue sections had abundant cytoplasmic and nuclear-localized p65 whereas 4 healthy canine lymph node sections had minimal p65 staining.

To determine whether the detected canonical NF-κB activity was associated with constitutive IKK activity, the phosphorylation status of the IKK complex and the inhibitory IκBα protein were evaluated by immunoblot (Fig. 1D). p-IKKβ and p-IκBα were detected in 8 out of 9 lymph node biopsy samples taken from dogs with histologically confirmed, untreated DLBCL with constitutively active NF-κB as determined by EMSA. In contrast, p-IKKβ and p-IκBα were only detected at very low levels in normal lymph nodes taken from healthy dogs (Fig. 1D). Taken together, these findings demonstrate that constitutive canonical NF-κB activity occurs in the malignant tissue of dogs with DLBCL, and that in the majority of dogs this activity is associated with IKK activation and downstream IκBα phosphorylation. These data parallel findings in human ABC-DLBCL, and suggest that dogs with DLBCL represent a clinically relevant, spontaneous large animal model in which to evaluate the therapeutic effects of NF-κB inhibition in the treatment of ABC-DLBCL.

Anti-apoptotic NF-κB target genes are up-regulated in canine DLBCL

To determine whether NF-κB target genes are up-regulated in canine DLBCL, malignant lymph node tissue from 14 dogs with histologically confirmed DLBCL and constitutive NF-κB activity was evaluated by qRT-PCR for Bcl-2, c-FLIP and XIAP gene expression (Fig. 2). Tissue samples were obtained at the time of diagnosis and prior to chemotherapy. Using healthy canine lymph node tissue as a normalizing control, statistically significant increases in Bcl-2 gene expression were identified in 7 dogs. In addition, c-FLIP and XIAP gene expression was significantly increased in 6 and 7 dogs, respectively. These results suggest that constitutive canonical NF-κB activity in canine DLBCL promotes the over-expression of anti-apoptotic NF-κB target genes. This finding parallels that in human patients with ABC-DLBCL and indicates that constitutive canonical NF-κB activity in the dog may contribute to lymphomagenesis and chemoresistance.

NBD Peptide inhibits constitutive NF-κB activity and promotes apoptosis in DLBCL cells *in vitro*

To determine whether NBD peptide can inhibit constitutive phosphorylation of IKK and prevent downstream IκBα phosphorylation, cells from malignant lymph nodes of dogs with DLBCL were treated *in vitro* with either 100μM NBD peptide or 100μM mutant peptide for 12 hours and then analyzed for the presence of p-IKKβ and p-IκBα by immunoblot (Fig. 3A and B). Cells treated with NBD peptide showed a marked reduction of p-IKKβ compared to untreated cells, and those treated with either mutant peptide or DMSO vehicle control. NBD peptide treatment also resulted in complete inhibition of IκBα phosphorylation (Fig. 3B). In contrast, treatment with mutant peptide or DMSO (vehicle control) did not inhibit IκBα phosphorylation.

To determine the effects of NF-κB inhibition on DLBCL cell apoptosis, malignant lymphocytes harvested from canine DLBCL tumors were treated *in vitro* with NBD or

mutant peptide and analyzed by flow cytometry (Fig. 3C). After 12 hours incubation with NBD peptide, more than 50% of DLBCL cells stained positive for Annexin-V while only 15% of cells treated with mutant peptide appeared apoptotic. Comparable results were identified in malignant cells taken from the lymph nodes of 3 dogs with DLBCL. In contrast, when PBMCs isolated from healthy dogs were treated with either 100μM NBD or mutant peptide for 12 hours, less than 5% of PBMCs stained positive for Annexin-V (Fig. 3D). Collectively, these data show that NBD peptide inhibits constitutive NF-κB activity in canine DLBCL cells, leading to selective, rapid apoptosis of malignant cells.

To confirm a selective cytotoxic effect of NBD peptide on human ABC-DLBCL cells, OCI-Ly10 cells and the GCB-DLBCL cell line SUDHL-6 were treated with NBD peptide for 24 hours and the percentage increase in cell death compared to untreated cells was calculated. At 24 hours, NBD peptide induced a dose dependent increase in cell death of the ABC-DLBCL cell line, OCI-Ly10. In contrast, NBD peptide did not induce cell death in the GCB-DLBCL cell line at either concentration used (Fig. 3E).

In vivo administration of NBD peptide inhibits NF-κB target gene expression and leads to a reduction in tumor burden in dogs with relapsed, chemoresistant large B-cell lymphoma

To determine whether NBD peptide can inhibit NF-κB target gene expression in malignant lymphoid tissue *in vivo*, privately owned dogs with relapsed, chemoresistant large B-cell lymphoma were enrolled to a small pilot study (Fig. 4). Eligibility criteria included the presence of histologically or cytologically confirmed large B-cell lymphoma and constitutive NF-κB activity in malignant lymph nodes as determined by EMSA. Response to NBD peptide was determined by evaluating NF-κB target gene expression pre and 24 hours post NBD peptide administration. Clinical response to NBD peptide was determined by comparing the mass of the injected lymph node before NBD peptide and 1 week after administration of peptide plus rescue chemotherapy. The mass of the contra-lateral malignant lymph node that was *not* injected with NBD peptide but was exposed to systemic rescue chemotherapy was also determined at both time points and the change in lymph node mass was calculated for both nodes and compared. Six eligible dogs were enrolled to the study, three dogs received 5mg NBD peptide/100g of node tissue and three received 10mg NBD peptide/100g node tissue via a single intra-nodal injection. At the time of enrollment, qRT-PCR analysis of lymph node tissue revealed that all dogs over-expressed at least 2 out of 6 NF-κB target genes that promote cellular proliferation and inhibit apoptosis when compared to normal lymph node tissue (Fig. 5A left panel). 24 hours post NBD peptide administration 1 out of 3 dogs receiving 5mg NBD peptide/100g nodal tissue showed a reduction in the expression of 4/6 NF-κB target genes within the malignant node when compared to pre-NBD treatment gene expression values. At the higher dose of NBD peptide all three dogs showed a reduction in the expression of at least 3/6 NF-κB target genes including Bcl-2, Cyclin D1 and IκBα when compared to pre-NBD treatment values. (Fig. 5A right panel).

Response to NBD peptide was determined by comparing the change in mass of the NBD peptide treated lymph node with change in mass of the contra-lateral, malignant, non-injected lymph node. Results were available for 4 of the 6 treated dogs (Fig. 5B). Rescue chemotherapy protocols administered to each dog after NBD peptide treatment varied amongst dogs and were determined by the referring oncologist. In 3 out of 4 dogs, the NBD peptide treated lymph node showed between a 1.5 and 6 fold reduction in mass when compared to the contra-lateral, malignant lymph node that did not receive NBD peptide. Two dogs (dogs 7 and 8) were treated on a compassionate basis and given intranodal injections of 10mg NBD/100g tumor into 8 peripheral enlarged lymph nodes, resulting in cumulative NBD doses of 0.33 and 0.8 mg/kg body weight respectively. This amendment to the original protocol was approved by the University of Pennsylvania's Institutional Animal

Care and Use Committee. At enrollment, each dog over-expressed at least 3 NF-κB target genes (Fig. 5A left panel). 24 hours after NBD peptide administration, reductions in NF-κB target gene expression were observed in 3/6 and 6/6 evaluated genes, respectively (Fig. 5A right panel). In these two dogs, a reduction in lymph node mass was observed in 3/8 and 4/8 NBD treated lymph nodes (Fig. 5C). Despite NBD peptide and systemic chemotherapy, the remaining lymph nodes continued to increase in size. In both dogs, the lymph nodes that showed a reduction in mass following NBD peptide treatment were the smallest nodes at the time of treatment.

Discussion

Aberrant, constitutive, canonical NF-κB activity is recognized as a key contributor to oncogenesis and chemotherapy resistance in many different cancers and as such represents a logical target for inhibition in cancer therapy. While nearly 800 inhibitors of NF-κB have been identified, (40) their ability to inhibit constitutive NF-κB activity in spontaneous cancers *in vivo* and the therapeutic relevance of such inhibition in patients with cancer is unknown.

In this study we first set out to determine whether dogs with DLBCL represent an appropriate, spontaneous, large animal model in which to evaluate the therapeutic effects of NF-κB inhibition. DLBCL in the dog is often characterized by total effacement of lymph node architecture with large malignant B-cells that have a high mitotic index and multiple, prominent nucleoli (41). This malignancy in dogs is frequently associated with over-expression of c-myc that occurs as a result of chromosomal translocations that place the oncogene under control of the IgH promoter (17). While these histocytological and cytogenetic changes are also found in ABC-DLBCL in humans, it is unknown whether canine DLBCL is characterized by constitutive canonical NF-κB activity. Here we show that dogs with histologically confirmed DLBCL have constitutive canonical NF-κB signaling in malignant lymphoid tissue at the time of diagnosis. In most cases evaluated, p50/p65 and p50/c-Rel heterodimers were present in the activated complex. Only 2 biopsy samples had evidence of active p50 in the apparent absence of active p65 or c-Rel suggesting the presence of p50 homodimers or complexes with RelB in these patients. These differences may be attributed to the specific mechanism(s) responsible for constitutive NF-κB activity in each tumor sample, as the identity and relative abundance of active homo-and heterodimers depends on the nature of the activating signal or pathway aberrancy (42, 43). Furthermore, as in human ABC-DLBCL patients, constitutive activity was associated with phosphorylation of IKKβ and IκBα in all but one dog evaluated, indicating that in the majority of cases signals leading to constitutive activity occur upstream of the IKK complex (7, 10). These findings confirm on a molecular level that human ABC-DLBCL and canine DLBCL have similar aberrant NF-κB signaling and that the dog may serve as a clinically relevant large animal model in which to study the therapeutic effects of NF-κB pathway inhibition in ABC-DLBCL.

Although constitutive NF-κB activity was present in the malignant lymph nodes of all dogs with untreated DLBCL, the NF-κB target genes Bcl-2, c-FLIP and XIAP were over-expressed in only half of the biopsy samples. These findings serve to underline the multiple layers of complexity involved in the regulation of NF-κB target gene expression. Firstly, the particular combination of activated NF-κB family members influences the target gene expression profile, in part because these family members have different binding affinities for different gene promoters. While NF-κB activation originates upstream of IKK in these dogs, the specific activation triggers have not been investigated and may differ between dogs, resulting in different NF-κB family member usage and different target gene expression signatures. However, in this small dataset, we did not find any correlation between the

expression of NF-κB target genes and activation of specific NF-κB family members. Secondly, other signaling pathways that activate additional transcription factors such as AP-1 and ETS may be concurrently active in these tumors. These transcription factors together with co-factors that modify NF-κB activity influence gene activation and repression, leading to variable gene expression profiles (44, 45). Taken together these points highlight the complex and multifaceted nature of NF-κB regulated gene expression. A much larger sample set of histologically confirmed, DLBCL biopsies with complete gene expression profiling together with a thorough evaluation of activated NF-κB family members will be needed to identify specific NF-κB gene expression signatures.

In the second part of this study we show that NBD peptide can inhibit constitutive IKKβ and IκBα phosphorylation in DLBCL biopsy samples which leads to increased apoptosis of malignant lymphocytes. Furthermore, in a small clinical pilot study NBD peptide showed a dose-dependent inhibition of NF-κB target gene expression in dogs with relapsed, refractory large B-cell lymphoma. In 2 dogs receiving the lower NBD peptide dose (dogs 1 and 3), all NF-κB target genes increased up to 4-fold 24 hours post NBD peptide suggesting that constitutive NF-κB activity in these dogs may be exerting a transcriptional repressive effect. No correlation was identified between 24-hour gene expression profile results and change in lymph node mass 7 days later.

In 3 out of 4 dogs, intra-nodal administration of NBD peptide led to a marked reduction in tumor mass when compared to the contra-lateral, malignant lymph node that did not receive NBD peptide, although it is unclear whether NBD peptide alone, or acting synergistically with rescue chemotherapy exerted this effect. In dogs that received multi-node injections of NBD peptide, the apparent effects on tumor mass were variable. While most nodes continued to increase in size despite NBD treatment and rescue chemotherapy, several nodes in both dogs showed a marked reduction in tumor mass. In both cases, responding nodes were smaller than non-responsive nodes at the time of treatment. These results raise the possibility that the NBD peptide dose and its ability to diffuse within malignant nodes may influence its ability to exert a measurable therapeutic effect.

Given the central role of the IKK complex in canonical NF-κB activation, IKK inhibitors are being investigated as novel therapeutic agents in both solid and hematological malignancies that depend upon constitutive NF-κB activity for their survival. IKKβ inhibitors have been developed to specifically block canonical NF-κB signaling; however, in the presence of selective IKKβ inhibition, compensatory IKKα activity can drive NEMO-dependent canonical NF-κB activity in human DLBCL cells (46). This finding suggests that inhibitors of both IKKα and IKKβ activity (such as NBD peptide) will provide more complete inhibition and have greater therapeutic efficacy than IKKβ inhibitors alone. Furthermore, agents such as NBD peptide that specifically target the scaffolding molecule NEMO are less likely than kinase inhibitors to exhibit off-target effects on other intracellular signaling pathways (27).

Previous studies evaluating the safety and efficacy of NBD peptide as an anti-inflammatory agent *in vivo* have shown that both short and long term administration of NBD peptide to piglets and rodents at doses ranging from 0.75–20 mg/kg is well tolerated (24, 28–31). In this small pilot study, NBD peptide did not cause any systemic toxicity and there were no significant changes in serum chemistries or hematological profiles over the 10 day assessment period that could be attributable to NBD peptide administration. However, the dose of NBD peptide administered on a mg/kg body weight basis was very low. However, 2 dogs treated with the higher dose of NBD peptide developed submandibular abscesses within one week of NBD injection into pre-scapular lymph nodes. In both cases, the commensal skin bacteria *Staphalococcus aureus* was cultured and successfully treated with

drainage and broad-spectrum antibiotics. In these cases it is possible that commensal skin bacteria introduced with NBD peptide were able to colonize the necrotic node and that inhibition of TLR signaling by NBD peptide may have contributed to this process.

In this pilot study, NBD peptide was administered intra-nodally rather than systemically to first determine whether it could inhibit constitutive NF-κB activity in malignant lymphocytes *in vivo*. Based on the favorable *in vitro* and *in vivo* results we are now actively recruiting dogs with ABC-DLBCL to a phase I dose escalation clinical trial in which NBD peptide will be administered systemically via the intravenous route. While the amount of peptide necessary to achieve complete inhibition of NF-κB activity in a dog or human are likely to be high, it is unknown whether complete inhibition is required to see a therapeutic effect when used in combination with chemotherapy.

Taken together, the results reported here provide the first *in vivo* evidence that NBD peptide can inhibit anti-apoptotic target gene expression driven by constitutive NF-κB activity and that this inhibition, either alone or in combination with cytotoxic therapy, can lead to improved clinical responses in dogs with ABC-like DLBCL. Given our reported findings that demonstrate the dog is a highly relevant, spontaneous clinical model for ABC-DLBCL in humans, the therapeutic effects of NF-κB inhibition seen in the canine cancer patient are likely to hold high translational relevance to human patients with ABC-DLBCL.

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Statement of Translational Relevance

Activated B-Cell Diffuse Large B-Cell Lymphoma (ABC-DLBCL) is an aggressive, poorly chemoresponsive lymphoid malignancy characterized by constitutive canonical NF-κB activity that promotes lymphomagenesis and chemotherapy resistance via over-expression of anti-apoptotic NF-κB target genes. We have investigated NF-κB as a therapeutic target using the dog as a clinically relevant, spontaneous, animal model of DLBCL. We show that as in human ABC-DLBCL, malignant lymphocytes from dogs with DLBCL have constitutive canonical NF-κB activity and over-express anti-apoptotic genes. We demonstrate that the selective IKK inhibitor, NBD peptide inhibits NF-κB activity *in vitro* and leads to apoptosis of malignant B cells. We show *in vivo* that intra-tumoral injections of NBD peptide inhibit NF-κB gene expression and reduce tumor burden in dogs with DLBCL. These studies demonstrate the therapeutic relevance of NF-κB inhibition *in vivo* in a canine DLBCL model and have important translational relevance for the treatment of human ABC-DLBCL.

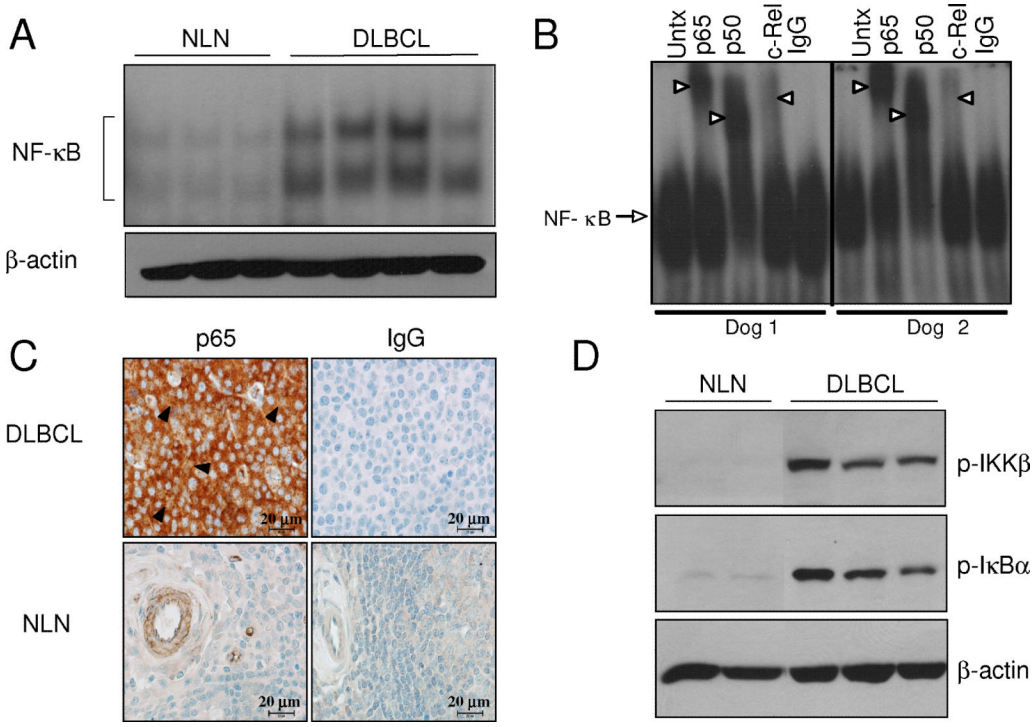

Figure 1. Constitutive canonical NF-κB activity in dogs with spontaneous DLBCL

(A) Whole cell protein extracts of normal lymph nodes (NLN) taken from 3 healthy dogs and lymph nodes taken from 4 dogs with primary, untreated DLBCL were evaluated for NF-κB DNA binding activity by EMSA. Results shown are representative of 13 dogs with DLBCL. (B) Whole cell protein extracts of lymph nodes taken from 2 dogs with primary, untreated DLBCL were subject to EMSA supershift analysis with antibodies against p65, p50, c-Rel, or control rabbit IgG. Results shown are representative of 7 dogs with DLBCL. (C) Immunohistochemical staining for p65 was performed on lymph node sections from healthy dogs and dogs with DLBCL. Rabbit IgG was used as an isotype control. Arrowheads indicate nuclear p65 staining. Results shown are representative of 6 dogs with DLBCL and 4 healthy dogs (NLN). (D) Whole cell protein extracts of NLN and lymph nodes taken from 3 dogs with primary, untreated DLBCL were evaluated for the presence of p-IKKβ and p-IKBα by Western blot. Results shown are representative of 8 dogs with DLBCL. β-actin was used as an endogenous loading control.

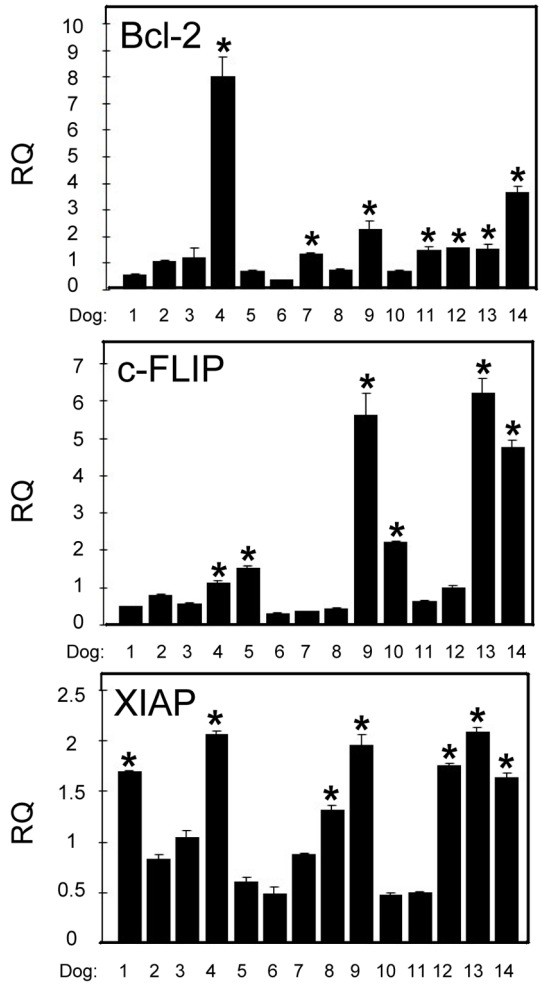

Figure 2. Expression of anti-apoptotic NF-κB target genes in canine DLBCL

RNA was isolated from lymph node biopsies of dogs with DLBCL at the time of diagnosis and prior to chemotherapy. qRT-PCR was performed for Bcl-2, c-FLIP and XIAP. Values are expressed as relative quantification using β-actin as an endogenous control. RNA isolated from biopsy samples of 3 healthy canine lymph nodes were used as a normalizing control. All data shown represent means +/− SEMs of reactions performed in triplicate. Statistical analysis was performed using a two-tailed Student's 't' test where * $p<0.05$.

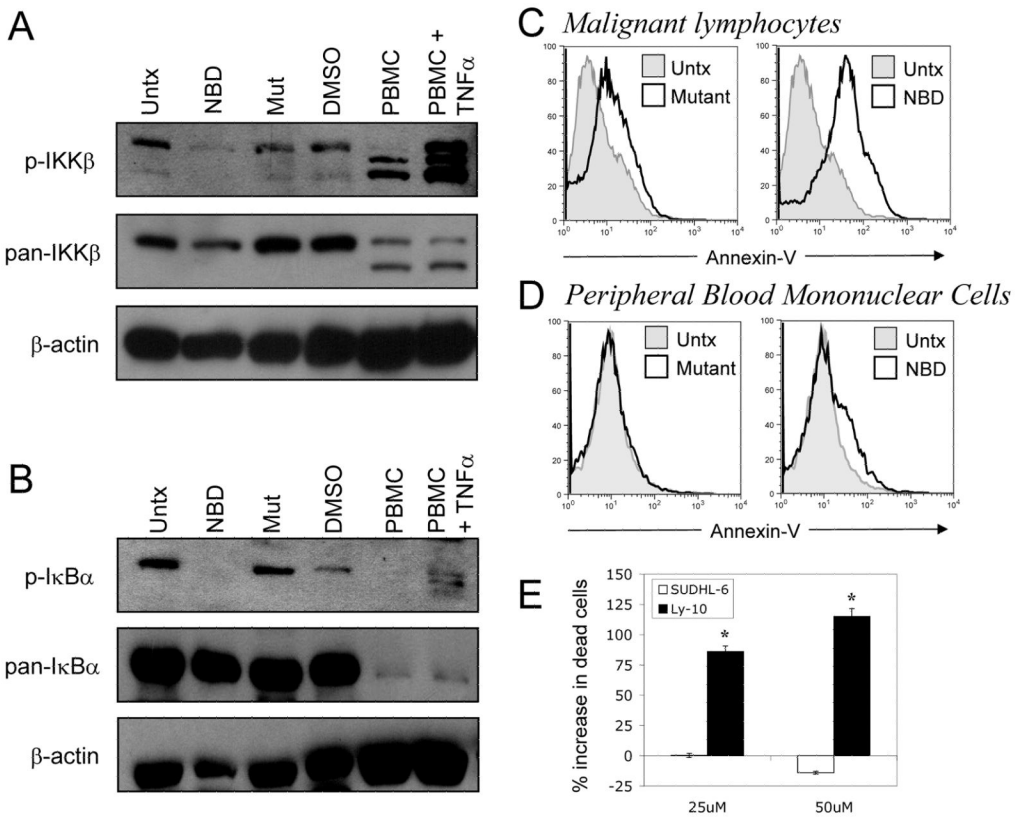

Figure 3. NBD Peptide Inhibits Constitutive NF-κB Activity in Canine Primary DLBCL *in vitro*

Primary canine DLBCL cells were treated with either 100μM NBD peptide or mutant peptide or with DMSO (vehicle control) for 12 hours. 25 μg of whole cell extract was immunoblotted using antibodies against (A) p-IKKβ and pan-IKKβ and (B) p-IκBα and pan-IκBα. β-actin was used as an endogenous loading control. Results are representative of 3 individual dogs. Canine PBMCs with or without TNF-α were included as positive and negative controls for p-IKKβ and p-IκBα. (C) Primary canine DLBCL cells were either untreated (grey histogram) or treated (open histograms) with either 100μM NBD peptide or mutant peptide for 12 hours and then analyzed by flow cytometry for Annexin-V. Results are representative of 3 individual dogs. (D) Healthy canine PBMCs were treated with 100μM NBD peptide or mutant peptide for 12 hours and then analyzed by flow cytometry for Annexin-V. Histograms are gated on CD21+ lymphocytes. (E) OCI-Ly10 and SUDHL-6 cells were treated with 0, 25μM or 50μM NBD peptide for 24 hours. Cell death was quantified by Trypan Blue. Data represent the percent increase in cell death in treated wells compared to untreated cells. Cell counts were performed in triplicate, and data shown represents means +/− SEMs. Statistical analysis was performed using a two-tailed Student's 't' test. *$p<0.05$. Results are representative of 3 separate experiments.

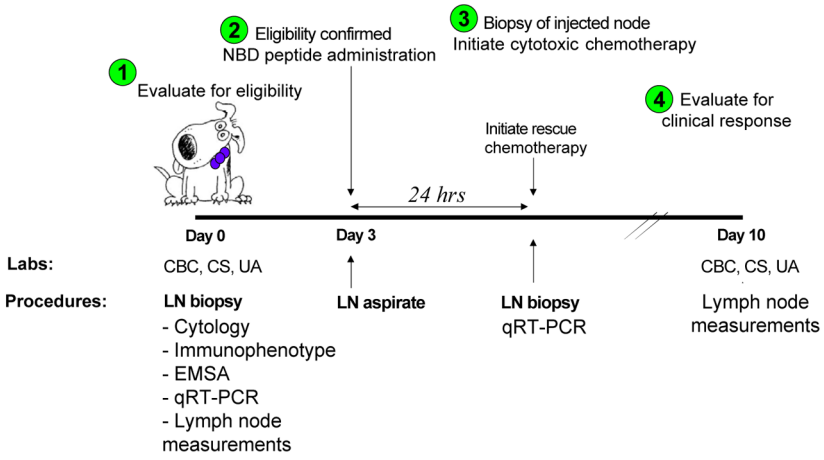

Figure 4.
Schematic of Pilot Clinical Trial.

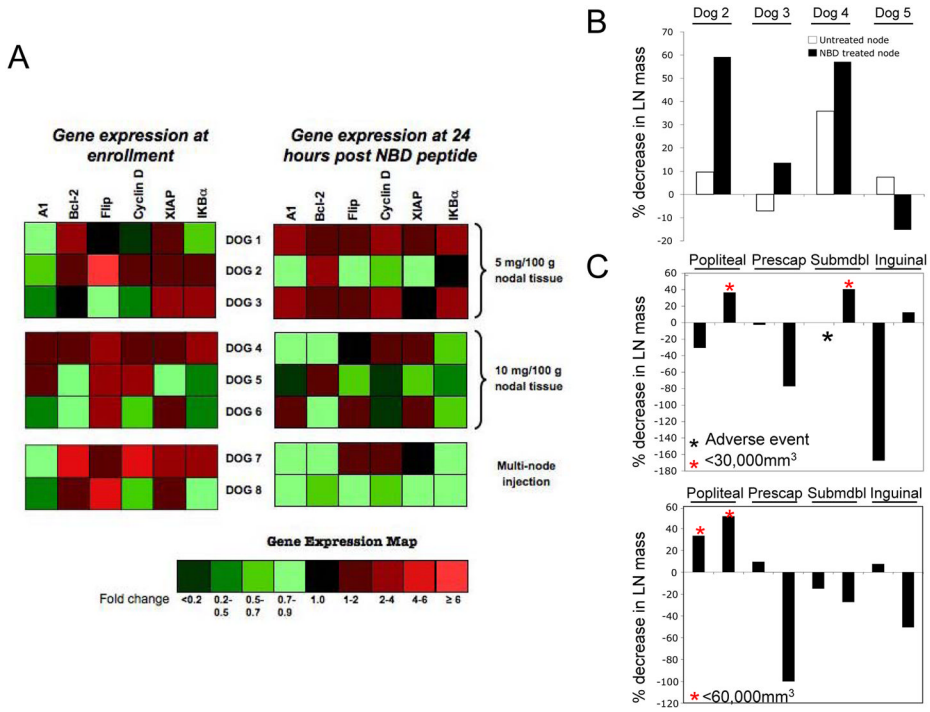

Figure 5. ***In vivo*** **administration of NBD peptide inhibits NF-κB target gene expression in malignant lymph nodes of dogs with relapsed/resistant large B-Cell lymphoma**

(A; left panel) qRT-PCR of malignant lymph nodes of dogs with relapsed large B-Cell lymphoma at enrollment. Values are expressed as relative quantification using β-actin as an endogenous control. RNA isolated from biopsy samples of 3 healthy canine lymph nodes were used as a normalizing control. (A; right panel) qRT-PCR of malignant lymph nodes 24 hours post NBD peptide injection. RNA isolated from biopsy samples of the same dogs prior to NBD peptide treatment was used as a normalizing control. All data shown represents means +/− SEMs of reactions performed in triplicate. (B) Comparison of percent decrease in mass between NBD injected lymph node and contra-lateral, malignant, non-injected lymph node of 4 study dogs. Measurements were taken immediately prior to and one week post NBD peptide administration. (C) Percent decrease in mass of NBD injected lymph nodes from 2 dogs that received multi-nodal injections of NBD peptide.

Table 1

Primer sequences for NF-κB target gene expression analyses.

Gene	RT-PCR Primers		Accession #
A1	Sense:	5′ - TCA ATC AGG TGA TGG AGA AGG - 3′	XM_545888
	Antisense:	5′ - TGC TCG AGG AGT TTC TTG GT - 3′	
Actin	Sense:	5′ - TCC CTG GAG AAG AGC TAC GA - 3′	AF021873
	Antisense:	5′ - CTT CTG CAT CCT GTC AGC AA - 3′	
Bcl-2	Sense:	5′ - TGG ATG ACT GAG TAG CTG AA - 3′	NM_001002949
	Antisense:	5′ - GGC CTA CTG ACT TCA CTT AT - 3′	
c-FLIP	Sense:	5′ - TCC AGG AAT CAG GAC CAT TT - 3′	XM_545592
	Antisense:	5′ - GAT TCC TAG GGG CTT GCT CT - 3′	
Cyclin D1	Sense:	5′ - CAT CTA CAC TGA CAA CTC CAT CC - 3′	NM_001005757
	Antisense:	5′ - CAG GTT CCA CTT CAG TTT GTT C - 3′	
IkB alpha	Sense:	5′ - CCT ACG TCC AGC CAT CAT TT - 3′	XM_537413
	Antisense:	5′ - CAG TTC CTC CTT GGG GTT TG - 3′	
XIAP	Sense:	5′ - ACT ATG TAT CAC TTG AGG CTC TGG TTT C - 3′	AY603038
	Antisense:	5′ - AGT CTG GCT TGA TTC ATC TTG TGT ATG - 3′	