University of Veterinary Medicine Budapest
Department of Pathology

A Review of Oncolytic Virotherapy as a Developing Approach to Canine Cancer Treatment

By Maria Aoun

Supervisor:
Dr. Anna Szilasi
Assistant professor

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List of Abbreviations

Ad5: human adenovirus type 5
ALT: alanine transaminase
anti-VEGF: anti-vascular endothelial growth factor
AST: aspartate transaminase
ATP: adenosine triphosphate
AxCA-cp53: adenoviral vector that expresses wild-type canine p53
AxCA-LacZ: adenoviral vector that expresses β-galactosidase of *Escherichia coli*
CAR: chimeric antigen receptor
CAV1: canine adenovirus type 1
CAV2: canine adenovirus type 2
CD40: cluster of differentiation 40
CHSS and CHSA: solitary histiocytic sarcoma of synovial origin
CIK: cytokine-induced killer cells
CM-MC: cutaneous mast cell lines
CMHM: disseminated histiocytic sarcoma
CPA: cyclophosphamide
CPE: cytopathic effect
CRAds: conditionally replicating adenoviruses
CRT: calrectulin
CTL: cytotoxic T lymphocyte
DAMP: damage-associated molecular patterns
DC: dendritic cell
dsDNA/RNA: double stranded DNA/RNA
ECM: extracellular matrix
E. coli: *Escherichia coli*
EGFR: epidermal growth factor receptor
ER: endoplasmic reticulum
GM-CSF: granulocyte macrophage colony stimulating factor
HCC: hepatocellular carcinoma
HCV: hepatitis C virus
HE: haematoxylin and eosin
HMGB1: high mobility group box 1
Hpi: hours post infection
ICD: immunogenic cell death
IFN- γ: interferon- γ
IHC: immunohistochemistry
IL-18: interleukin-18
IP-10: gamma interferon induced protein-10
iv: intravenous
LICAP: Leeds Institute of Cancer and Pathology
MAPK: mitogen-activated protein kinase
M-CSF: macrophage colony stimulating factor
MCP: monocyte chemoattractant protein
MCT: mast cell tumour
MDSCs: myeloid-derived suppressor cells
MGT: mammary gland tumour
MHT-2: solitary histiocytic sarcoma of unknown origin
MOI: multiplicity of infection
MSCs: mesenchymal stem cells
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIH-III: NIH-bg-nu-xid gene
NK: natural killer cell

OCCAV: osteocalcin promoter-controlled canine adenovirus
PARP: anti-poly ADP ribose polymerase
PAMP: pathogen-associated molecular patterns
PBS: phosphate buffered saline
PFU: plaque forming units
PI: propidium idodide
PKR: dsRNA activated protein kinase
PS: phosphatidylserine
RAGE: receptors for the advanced glycation of end products
Ruc-GFP: Renilla luciferase and Green Fluorescent Protein
STS: soft tissue sarcoma
TAA: tumour associated antigens
TIL: tumour infiltrating lymphocytes
TLR4: toll-like receptor 4
TNF-α: tumour necrosis factor-α
TME: tumour microenvironment
Treg: regulatory T cell
VACV: vaccinia virus
VCOG-CTCAE: Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events
VIMC: visceral mast cell lines
WHO: World Health Organisation
Z-VMD-FMK: N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone

1 Introduction

Cancer is among the most diagnosed diseases in dogs and more often than not it's the top fatal disease. This comes as a result of unresolved tumours after extensive treatment, or their reoccurrence. The study of oncolytic virotherapy as an alternative treatment for canine cancers therefore acts as a novel strategy that aims to overcome the limitations the oncological society faces. The conventional antitumour methods such as surgery, chemotherapy, and radiation, to name a few, have shown to be quite restrictive in case of advanced stages of cancer. Innovative researchers have thus turned to oncolytic viruses as a possible solution. These viruses preferentially infect, replicate in and kill neoplastic cells, and by doing so release virions that will further destroy the remaining tumours without harming the normal tissue.

Cancer treatments tend to be invasive in most cases, however oncolytic virotherapy takes a different approach. It provides higher cancer specificity and a better safety margin due to the tumour selective property of the viruses. Oncolytic viruses integrate the body's immune system, and proceed to activate and redirect the innate and adaptive functional responses towards the tumour causing immunogenic cell death. A critical point in the success of this procedure is the balance between the antiviral and antitumour responses as well as the tumour and host interaction. The effectiveness of the antitumour activity therefore depends on the location and site of administration, the cell phenotype, the permissiveness to virus infection, and the tumour homing ability. The following will be elaborated further on in the review.

Oncolytic viruses can be classified into two categories. First are the viruses that naturally replicate in neoplastic cells and are non-pathogenic such as reoviruses and the myxoma virus, and second are those that are genetically manipulated for use as vaccine vectors such as the vaccinia and poliovirus.

Human medicine has successfully used virotherapy strategies on several accounts. A recent study done in the Leeds Institute of Cancer and Pathology (LICAP) described the use of clinical grade oncolytic orthoreovirus in patients with hepatocellular carcinomas (HCC). The virus elicited innate immune activation, within primary human liver tissue, in the absence of cytotoxicity and independently of viral genome replication. As well as achieving therapy in preclinical models of HCC, through the activation of innate degranulating immune cells, the Reo-induced cytokine responses efficiently suppressed hepatitis C virus (HCV) replication both *in vitro* and *in vivo* (Samson, et al., 2018).

Due to the very strong similarities between human and canine cancers, I chose to investigate the development of this technique in veterinary practices. The most studied viruses to date are the adenovirus, the reovirus, and the vaccinia virus, tested mostly in solid tumours such as osteosarcomas, mammary gland tumours, soft tissue sarcomas, and mast cell tumours (Sanchez, et al. 2018). The review will be concentrating on the development of these viruses and the clinical trials associated with them, and will discuss in depth the scope and capacity of this treatment.

Keeping in mind that as many new trials do, oncolytic virotherapy has its obstacles. There has been concerns regarding toxicity, optimization of delivery of the virus to the target tissue, efficacy of spread through the tumour and biosafety in regards to transmission risk of agents to the pets and personnel treating the pets. Several ongoing clinical trials have shown to be very promising and with the approval of two viruses in human cancer treatments, its application in veterinary medicine is at arm's reach.

2 *Literature Review*

2.1 Natural and Genetically Engineered Oncolytic Viruses

2.1.1 Adenoviruses

Adenoviruses are non-enveloped, double stranded DNA (dsDNA) viruses with an icosahedral symmetry of 80-110 nm. They are widely used in oncolytic virotherapy in humans and due to their ability to infect a broad range of cell types/species they are currently being studied in canine cancer therapy. The most commonly tested adenoviruses are the human adenovirus type 5 (Ad5) and the canine adenovirus type 2 (CAV2) from the *Mastadenovirus* genus of the *Adenoviridae* family. Due to their nature, they fall under the category of genetically manipulated viruses with mutations and deletions in genes required for replication in normal, but not cancer cells. These viruses are restricted to cancer cells at the replication level hence the name, conditionally replicating adenoviruses (CRAds). The insertion of additional foreign sequences can provide further selectivity for cancer cells and transcription with cell specific promoters, leading to safer conditions (Chiocca and Rabkin, 2014).

Ad5 is a prime example of this enhancement, where vectors expressing different genetic and molecular factors associated with cancer, such as p53 and CD40 ligand, have successfully treated canine osteosarcoma xenografts and canine malignant melanoma patients respectively (Gentschev, et al., 2014). The p53 protein is a tumour suppressor gene that induces the transcription factor after DNA damage, recent studies have found that it is also the most commonly mutated gene in tumour malignancies. Once inactivated, p53 impairs the cell cycle and apoptotic pathways leading to tumour proliferation. Therefore, it's been demonstrated that an overexpression of p53 could act as an antitumour mechanism inhibiting proliferation. An extended study of a constructed adenovirus vector inserted with the canine p53 gene (AxCA-cp53) was performed to evaluate the antineoplastic effect of an intratumoural injection in canine osteosarcoma xenografts formed in nude mice. Two canine osteosarcoma cell lines, POS and CHOS, with missense point mutations at codons 162 (CGC to CAC, Arg to His) and 150 (TAT to AAT, Try to Asn) were used. Twenty-one mice were irradiated with x-ray two days before transplantation and subcutaneously injected with the above-mentioned cell lines. Once the tumour masses reached a weight of approximately 80 mg, the group harbouring xenografts of POS cells were divided into three groups and injected with AxCA-cp53 (n=5), an adenoviral vector that expresses β-galactosidase of *Escherichia coli (E. coli)* (AxCA-LacZ) (n=5), or phosphate buffered saline (PBS) (n=5).

Similarly, the group harbouring xenografts of CHOS cells were divided and injected with the aforesaid vectors (n=4). The results showed gradual increase in tumour size, during the 27-day observation period, of POS xenografts injected with PBS mock solution or AxCA-LacZ, whereas the AxCA-cp53 injection inhibited tumour growth showing reduced weight over time (Kanaya, et al., 2011).

Comparably, the adenovector CD40L coding for a molecule that activates dendritic cells (DC) and helper T lymphocytes was used on nineteen patients with canine malignant melanoma to evaluate its efficacy parallel to immunotherapy and cytoreductive surgery. The cases ranged from World Health Organization (WHO) stage I-IV, each either oral, cutaneous, or conjunctival. One to six intratumoural injections were administered every 7 days and followed by either surgery or immunotherapy. The post treatment analysis revealed tumour tissue infiltrated with T and B lymphocytes suggesting immune stimulation, in addition to five complete mass resolutions, eight partial resolutions and four stable disease statuses (Westberg, et al. 2013).

CAV2 based vectors, an osteocalcin promoter-controlled CAV (OCCAV) and pRb-responsive, RGD-modified, and hyaluronidase-armed CAV (ICOCAV17), are also being tested centred around the mechanism of action of osteocalcin promoters and hyaluronidase respectively. The osteocalcin promoter has shown to be active in canine osteosarcoma cells, but not in other canine non-neoplastic cells. A synergistic relationship is therefore formed where the virus is native to the target tissue cells and serves as an advantage to the replication pathways of CAV2 in the dog (Smith, et al., 2006). The cytopathic effects of CAV2 were examined in canine osteosarcoma cells using a crystal violet staining-based assay, where the stain binds to proteins and DNA. To determine cell viability after administering OCCAV, crystal violet generally stains all cells, but dead cells detach (Feoktistova, et al., 2016). The differences in proliferation and cellular growth inhibition are observed accordingly. The remaining stained cells show the degree of survival after chemotherapeutic application.

Hyaluronidase increases the dissociation rate of the extracellular matrix, which enhances the viral distribution following intratumoural administration. A series of clinical cases tackled the subject by examining the efficacy of ICOCAV17 on a sweat gland adenoma, two canine osteosarcomas, a cutaneous mast cell tumour, multiple subcutaneous and hepatic nodules and a subcutaneous fibrosarcoma. Four of the six patients showed viral interaction, out of which one lead to complete tumour regression. The 15-year old spayed Catalan Sheep Dog female with a right leg subcutaneous fibrosarcoma at the distal ulna underwent a complete recovery after a 6-point inoculation within the tumour and surgical removal after 15 days. Despite having been previously treated by radiotherapy and surgery in vain,

immunohistochemical analysis post excision showed regions with and without viral replication in the subcutaneous tumour tissue shown in figure 1, after the intratumoural injection (Laborda, et al., 2014).

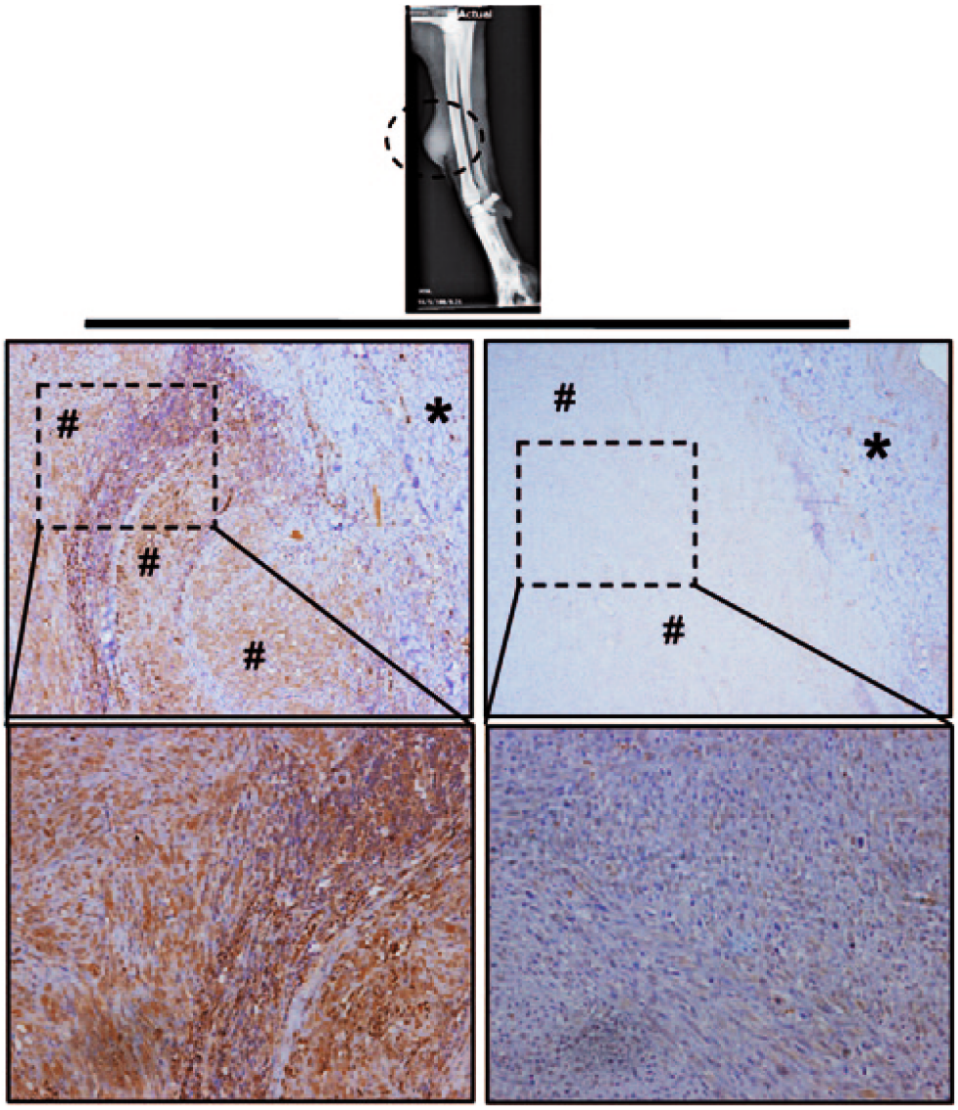

Figure 1: Radiograph revealed a relapsing fibrosarcoma at the distal ulna of the right forelimb. Tumour sections were stained with anti-adenovirus antibody.
Left panel: virus was detected (brown precipitate) in subcutaneous tumor section.
Right panel: tumor section staining did not result in virus detection. (*) skin, (#) tumor tissue.
Upper panel: original magnification x40.
Lower panel: original magnification x200 (Laborda, et al., 2014).

Although some of the patients showed progressive decrease in tumour size in the first few days of trials as well as absence of any signs of systemic toxicity, the advanced stage of tumour spread lead to a poor prognosis and quality of life. Taking into consideration the well-being of the patients, euthanasia was advised.

2.1.2 Poxviruses

Poxviruses, from the *Poxviridae* family, are dsDNA viruses with a complex brick-like or oval shaped symmetry. Their mature virions can be enveloped or non-enveloped, therefore their release from cells occurs either through budding or through exocytosis/cell lysis respectively. They enter the cell via fusion and predominantly multiply in the cytoplasm, therefore are unable to integrate their viral genome into the host's. In dogs, the research on their oncolytic properties mainly involves the *Orthopoxvirus* genus, where preclinical and clinical trials with the vaccinia virus (VACV) strain have revealed promising results. The vaccinia virus was the first widely used vaccine worldwide, eradicating smallpox, and therefore much is known about its safety profile (Patil, et al., 2012). Four VACVs derived from the Lister strain were evaluated in canine cancer cells.

First, GLV-1h68, also known as GL-ONC1, was developed. It carries the gene sequences encoding for a fusion protein of Renilla luciferase and Green Fluorescent Protein (Ruc-GFP), LacZ gene and β-glucuronidase into the thymidine kinase and haemagglutinin loci of the viral genome. Following the construction of this virus, GLV-1h68 was proposed for the treatment of canine mammary gland adenoma and carcinoma, and has been tested successfully in xenograft models. A single iv. (intravenous) dose in nude mice xenografts induced significant tumour regression of canine adenomas, in addition to strong inflammatory and oncolytic responses in canine carcinomas. So, via viral replication-dependent apoptosis and its enhanced expression of monocyte chemoattractant protein-1, -2, and -5 (MCP), macrophage colony stimulating factor (M-CSF), and interferon gamma-induced protein-10 (IP-10) and interleukin-18 (IL-18), GLV-1h68 increased the innate immunity mediated response of dendritic cells, neutrophils and macrophages. The tumour growth inhibition, consequently, confirmed infection, replication capability and tumour specificity. It has also been suggested that the opportunity to localise GLV-1h68 viruses through optical imaging increases the probability of detecting metastases, especially at the early stages. Equally important is GLV-1h109, derived from GLV-1h68, containing the GLAF-1 gene replacing the LacZ gene, which encodes for single chain anti-vascular endothelial growth factor (anti-VEGF) antibody. The above mentioned demonstrated strong

antitumour effects in canine soft tissue sarcoma (STS) and prostate carcinoma xenograft models, notably through the expression of GLAF-1 regulating tumour angiogenesis (Gentschev, et al., 2014). These models displayed an increased accumulation of neutrophils, myeloid derived suppressor cells, and macrophages, as well as a reduction in blood vessel density in STS. Despite the improved tumour specific replication compared to GLV-1h68, a mild level of toxicity was discovered raising the safety concerns involved in the use of oncolytic virotherapy.

The subsequent strain was isolated from a wild type stock of Lister strain and was evaluated both *in vitro* and *in vivo* in a murine model of canine STS. LIVP1.1.1 showed successful infection and destruction of canine STS cells *in vitro*, and more specifically it revealed better replication efficiency in xenograft models when compared to GLV-1h68. Three independent experiments were performed using the STSA-1 cell line derived from the tumour of a seven-year old, male, neutered golden retriever dog. The patient had presented with a firm, painful, erythematous mass on the left forelimb, and the sample was surgically excised from the deep digital flexor and the flexor carpi muscles. Three groups of female nude mice were injected with a single dose (10^7 plaque forming units [pfu]) of LIVP1.1.1, GLV-1h68, or PBS intravenously with the aim of examining the oncolytic effects of the virus on tumour size, tumour volume and survival expectancy. As shown in figure 2A, the treatment led to a significant difference in tumour growth size between PBS controls and virus-treated mice. More importantly, the data indicates that LIVP1.1.1 displays a greater oncolytic potential in comparison to GLV-1h68 against canine STS xenografts. Figure 2B represents the results derived from the second independent experiment, where the outcome of virotherapy was evaluated based on initial tumour volume. It was hypothesised that a larger (600 to 1000 mm^3) initial tumour size at the time of virus delivery would lead to a more responsive treatment than smaller STSA-1 tumours. In this experimental scenario, the average starting volume before injection was 40% larger than the previous LIVP1.1.1 group. A single iv. injection into mice bearing canine STSA-1 xenografts led to near complete tumour regression over a 42-day period with no signs of toxicity. The final experiment analysed the long-term survival of LIVP1.1.1-treated mice compared to PBS-treated mice. The mice of the control group were euthanised at days 17 and 24 due to the development of tumours with volumes greater than 3000 mm^3. In the virus-treated group, however, two animals were found dead at days 39 and 58 post infection with no pathological changes observed. The remaining three animals of this group were euthanised due to weight loss at days 48 and 98, and tumour volume greater than 3000 mm^3 at day 105. The reason for different oncolytic effects of LIVP1.1.1 and GLV-1h68 in STSA-1 xenografts was explained through the

comparison of the virus colonisation and distribution patterns at early and late points in time after viral administration. The highest viral titers were identified in the primary tumours 7 days post infection with no significant differences between the LIVP1.1.1 and GLV-1h68 injected groups. Simultaneously, plaque forming units were found in some organs of the mice injected with LIVP1.1.1, but not with GLV-1h68. At 35 days post infection, GLV-1h68 virus particles were detected in the liver, lungs, and spleen, however, 10^4–10^5-fold more plaque forming units of GLV-1h68 were found in tumour tissue in comparison to healthy tissue at the same point in time. Both viruses, thus demonstrate an enhanced tumour specific replication in STSA-1 xenograft in mice (Gentschev, et al., 2012).

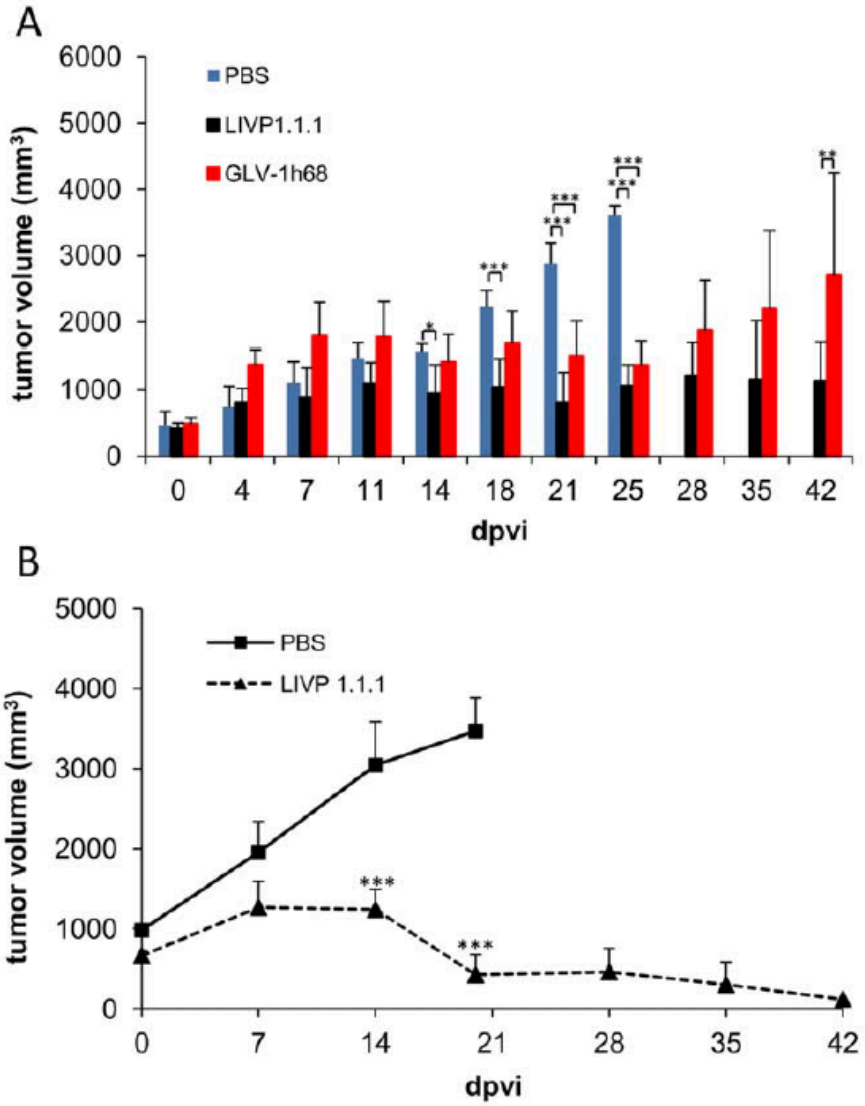

Figure 2: Growth of canine STS in mock and virus-treated mice. **A** Three groups of STSA-1 tumour-bearing nude mice treated with either LIVP1.1.1, GLV-1h68, or PBS (mock control). Tumour size was measured twice a week. Two-way analysis of variance (ANOVA) with Bonferroni post-test was used for comparison. **B** Two groups of STSA-1 (>600mm^3) tumour-bearing nude mice treated with either LIVP1.1.1 or PBS (Gentschev, et al., 2012).

With regards to anti-tumour mechanisms, the host immune response and the tumour vascularisation in the early stages of virus infection were studied. Flow cytometry was used to analyse the presence of host immune cells at 7 days post infection. There were increased levels of CD11b/c$^+$, MHCHII$^+$, CD19$^+$, CD45$^+$, and LY6G high$^+$ in suspensions derived from virus-infected tumours compared to that of uninfected controls. In addition, about 1% of the MHCHII$^+$, the CD45$^+$, and the LY6G high$^+$ cells were GFP positive in the GLV-1h68 infected tumours, indicating that either these immune cells were infected with the VACV or they phagocytised virus infected tumour cells. Turning to the impact of viral tumour colonisation on the tumour vasculature, CD31$^+$ was analysed in tissue sections of the tumours by fluorescence microscopy. The data revealed that there were no significant differences in the vascular density between the LIVP1.1.1, GLV-1h68, and PBS groups. The fluorescence intensity of the vessels of both virus-infected tumours were however significantly higher in comparison to the PBS-injected control tumours. This suggests that virus colonisation led to an upregulation of the CD31$^+$ protein resulting in endothelial activation and migration of immune cells to the tumour site. To conclude, these experiments highlight the different mechanisms through which the oncolytic VACV destroys tumours by either direct oncolysis of tumour cells, destruction of tumour vasculature, induction of the host's immune response, or a combination (Gentschev, et al., 2012).

The most recent VACV strain was also isolated from a wild type stock of Lister strain; however, it is less virulent than its predecessors and bears no genetic manipulation, making it a native virus. LIVP6.1.1 demonstrates a preference for replication at the tumour site leading to increased tumour specificity. *In vitro* analysis of its oncolytic potential against soft tissue sarcoma STSA-1 and prostate carcinoma DT08/40, canine cancer cell lines was performed, where GLV-1h68 was used as a control in order to compare the efficiency of both viral strains. The maximum viral titres for LIVP6.1.1 ($5.34x10^6$ pfu/well) and GLV-1h68 ($2.98x10^6$ pfu/well) were identified at 48 hours post infection (hpi) in STSA-1 cells and at 96 hpi in DT08/40 cells with $8.24x10^6$ pfu/well and $1.53x10^6$ pfu/well, respectively. The numbers helped establish that the replication efficiency is dependent on tumour types and infection time (Gentschev, et al., 2013). *In vivo* experiments in mice xenografted with the same cells showed close to 50% decrease in tumour growth without signs of toxicity after treatment with an intravenous dose of LIVP6.1.1. A flow cytometric analysis of the host immune cells additionally demonstrated an increase in immune cell infiltrates such as Gr-1^{high} CD11b$^+$ myeloid derived suppressor cells (MDSCs), granulocytes, Gr-1^{intermediate} CD11b$^+$ MDSCs monocytes, macrophages, MHCII$^+$, and CD45$^+$ cells was detected in STS tumours (Sanchez, et al., 2018). CD11b$^+$ granulocytes were used as cell markers for

monitoring the viral infection on a systemic level. A parallel flow cytometric analysis was performed in the peripheral blood. The data showed no significant differences in the Gr-1^{high} CD11b^{+} MDSCs population in the peripheral blood between the virus and PBS-treated STSA-1 xenografted mice. The results suggest that this is likely due to direct oncolysis and tumour localised immune stimulation. In other words, it is probable that the changes in granulocytic MDSCs were not systemic, but may be due to a change in recruitment and/or persistence within the virus treated tumours (Gentschev, et al. 2013).

2.1.3 Reoviruses

Reoviruses, belonging to the *Orthoreovirus* genus within the *Reoviridae* family, are non-enveloped, naturally occurring, segmented dsRNA with a double-layered capsid. Their segment re-assortment properties make them genetically diverse, and their common surface antigens often lead to serological cross reactions. Due to their ability to infect the majority of mammalian species, reovirus neutralising antibodies are detected in a high percentage of healthy dogs. As the infection is usually asymptomatic, the reovirus poses no harm to its host (Gentschev, et al., 2013). The reovirus serotype 3 Dearing strain, Reolysin®, is the most commonly studied oncolytic strain in veterinary oncology. Phosphorylation of dsRNA activated protein kinase (PKR) has been determined as one of the major components inhibiting the translation of viral genes and viral replication in untransformed cells. Due to the complexity of their mechanism of action, the exact dynamics determining cell susceptibility to reoviruses are yet to be discovered.

One of the founding studies on reoviruses in veterinary oncology involved the use of four canine mast cell tumour (MCT) cell lines; VIMC (visceral mast cells) and CoMS derived from visceral MCT, and CM-MC (cutaneous mast cells) and HRMC originating from cutaneous MCT. The *in vitro* experiment consisted of infecting and mock-infecting the cell lines with reovirus at a multiplicity of infection (MOI) of 70 pfu per cell. The cells were then stained with 0.25% trypan blue and their viability was counted with a haemocytometer at 72 hpi (Hwang, et al. 2013). The trypan blue dye exclusion test is based on the principle that live cells have membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not and therefore allow the dye to penetrate through. When examined, the viability of the cells is dependent on the colour of their cytoplasm, clear meaning viable and blue meaning nonviable (Strober, 2001). The results revealed 100% cell death induced in VIMC, CoMS, and CM-MC cell lines, in addition to over 80% in HRMC cell lines. Upon morphological evaluation shown in figure 3, virus induced cytopathic effects (CPE) were also observed at 72 hpi.

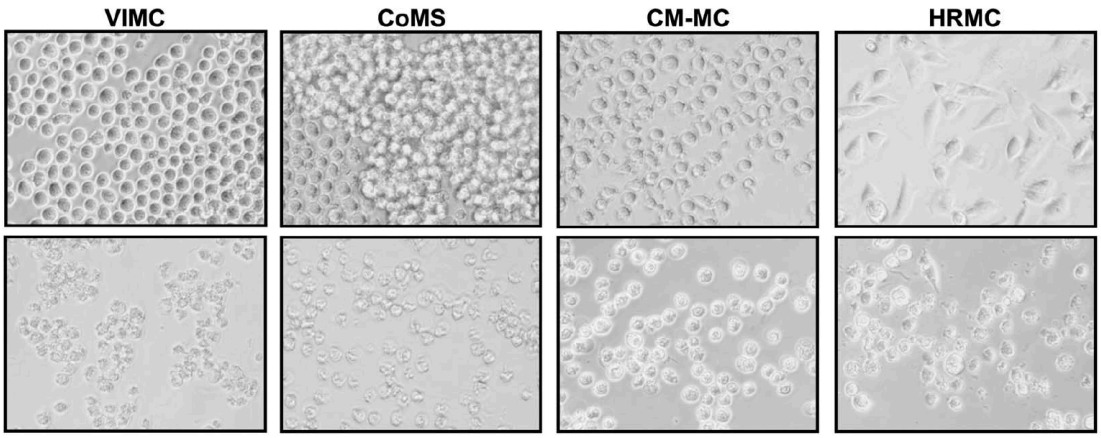

Figure 3: Photomicrographs of mock infected (upper panel) and reovirus infected (lower panel) MCT cells. CPE observed in reovirus infected cells. (Hwang, et al. 2013)

The verification of cell death was subsequently assessed in MCT cell lines using propidium iodide (PI) flow cytometric assay. PI staining is centred around the principle that apoptotic cells are characterised by DNA fragmentation and loss of nuclear DNA content. The fluorochrome, having the ability to bind and label cellular DNA material, is thus able to distinguish viable from non-viable cells (Riccardi, and Nicoletti, 2006). An increase in the proportion of hypodiploid subG1 cells indicated that the number of apoptotic cells increased from 48 to 72 hpi, and reaffirmed the results derived from the cell viability screening with trypan blue.

Similarly to the experiment performed on MCT cell lines, reovirus was able to induce cell death in canine mammary gland tumour (MGT) cell lines. Previous studies indicated that reovirus induces apoptosis by means of caspase 3 activation in the caspase-dependent apoptosis pathway. This suggestion was assessed using the Annexin V/PI assay for early detection of apoptosis. The appearance of phosphatidylserine (PS) residues, normally hidden within the plasma membrane, on the surface of the cell is an indication of the initiation of apoptosis. In flow cytometry, Annexin V has a strong Ca^{2+}-dependent affinity for PS and therefore binds to it when apoptotic cells are detected (Abcam). PI then stains the DNA fragments accordingly. In both CHMp-13a and CHMp-5b cell lines, the percentage of early apoptotic cells increased over time after infection with reovirus compared to the mock-infected cells. In order to prove that the apoptosis of reovirus-infected cells occurred by means of caspase 3 activation, a Western blot analysis was carried out using anti-caspase 3 and anti-poly ADP ribose polymerase (PARP) antibodies. Cleavage of caspase 3 and PARP, using actin as a control, was shown in both cell lines at 48 hpi. More importantly, both cell

lines were pre-treated with N-benzyloxycabonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) before infection with reovirus. Z-VAD-FMK is an irreversible caspase 3 inhibitor, it acts on the effector caspase to prevent the cleavage of PARP in cell undergoing apoptosis (Keoni and Brown, 2015). At 72 hpi cell viability was evaluated by trypan blue dye exclusion test and the cytotoxicity of reovirus infection was completely inhibited by Z-VAD-FMK. For further confirmation on the specific involvement of the caspase 3 pathway, Western blotting showed a decrease in cleaved PARP in CHMp-13 a when pre-treated with Z-VAD-FMK (Igase, et al., 2016). The most recent study on canine histiocytic sarcoma cell lines revealed results consistent with the above-mentioned outcomes before and after treatment with Z-VAD-FMK. At 48 hpi all cell lines except for MHT-2, a solitary histiocytic sarcoma of unknown origin, exhibited significantly increased percentages of early apoptotic cells compared to the mock-infected ones. CHSS, a solitary histiocytic sarcoma of synovial origin, demonstrated a predomination in late apoptotic cells indicating that cell death began earlier than in the other cell lines. After pre-treatment with Z-VAD-FMK, cell death was over 90% inhibited in CMHM, a disseminated histiocytic sarcoma, and MHT-2, however in CHSA, a solitary histiocytic sarcoma of synovial origin, and CHSS approximately 50% of the cell death was inhibited. It is therefore strongly suggested that reovirus infection induces apoptosis in canine tumour cell lines via the caspase signalling pathway (Igase, et al. 2019). With regards to the assessment of reovirus's therapeutic potential *in vivo*, VIMC and CoMS MCT unilateral subcutaneous xenograft models were established in nude mice treated with a single intratumoural injection. All the mice demonstrated significant regression in tumour mass by day 4 post treatment with VIMC and by day 6 post treatment with CoMS. Specifically, the generation and hematogenous dissemination of progeny virus was evaluated. Bilateral cutaneous VIMC xenograft models were created and treated with a single unilateral intratumoural reovirus injection. As anticipated, significant tumour regression was observed not only in the treated limb, but also in the non-treated contralateral mass, which suggests a substantial production of reovirus and release into the blood circulation (Hwang, et al. 2013).

Further studies on the *in vivo* oncolytic potential of the reovirus Dearing strain were performed on canine MGT cell lines. The experiments consisted of CHMp-5b subcutaneous xenograft mice models treated with a single intratumoural injection of either reovirus or UV-inactivated reovirus. At 21 days post injection, tumour growth was significantly reduced in reovirus injected mice compared to UV-inactivated reovirus treated mice as demonstrated in figure 4A. As seen in figure 4B, haematoxylin and eosin (HE) stained histopathological samples from the xenografted models showed extensive necrotic lesions within the reovirus-

treated tumours in contrast to the UV-inactivated reovirus-treated and the untreated tumours. The presence of reovirus proteins was also detected after immunohistochemical staining of the tumours using anti-reovirus antibodies. As a result, it was clear that reovirus infected the tumour cells and limited the tumour growth (Igase, et al., 2016).

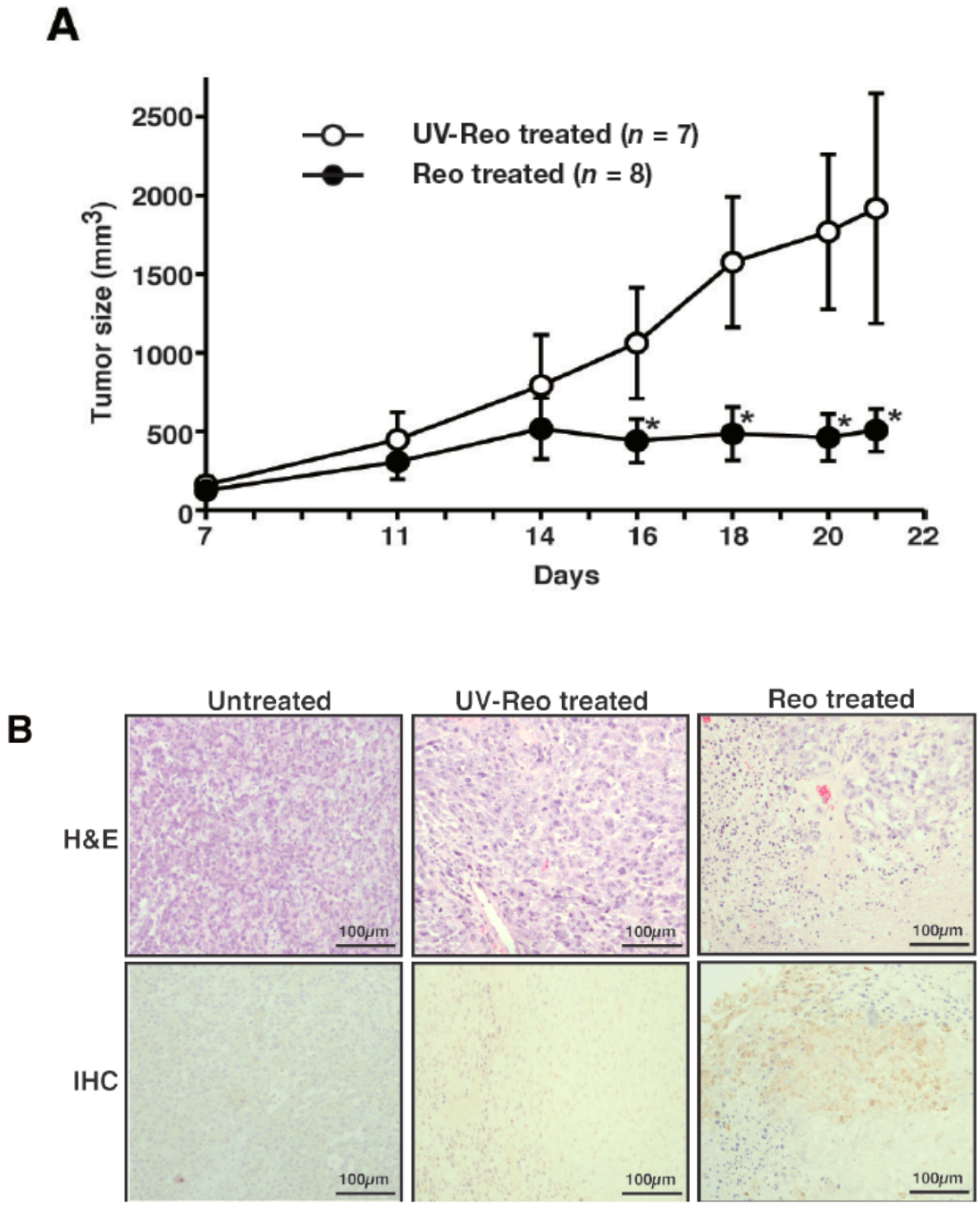

Figure 4: A CHMp-5b (1.0 x 10^7 cells) implanted subcutaneously in mice at day 1. At day 14 the tumours were treated with 1.0 x 10^8 pfus of reovirus or UV-inactivated reovirus as a control. **B** HE and IHC results at 21 days post infection (Igase, et al., 2016).

In relation to the susceptibility of canine histiocytic sarcoma cell lines to the Dearing strain, its oncolytic potential was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The assay is based on the conversion of the water soluble MTT compound into an insoluble formazan product. This pathway only applies to viable cells with active metabolisms, therefore non-viable cells lose this ability and show no signal. The measured absorbance of colour formation at 590 nm is proportional to the number of viable cells. Four canine histiocytic sarcoma cell lines were mock-infected or infected with reovirus (CHSA, CHSS, CMHM and MHT-2). At 72 hpi, the cell growth of all canine histiocytic sarcoma cell lines was suppressed at a seemingly dose-dependent manner. More than 70% inhibition of cell proliferation was seen in CHSA, CHSS, and CMHM at a MOI of 10, whereas MHT-2 demonstrated only 30% inhibition at the same titre (Igase, et al. 2019). Following this experiment, the *in vivo* efficacy of reovirus treatment was tested in subcutaneous xenograft models of severely immunodeficient mice using CHssv1, a tumour line derived from CHSS cell line. The mice were treated intratumourally with a single injection of PBS or reovirus. The initial tumour volume was over 200 mm^3 however, following the single injection, tumour growth was suppressed completely compared to the PBS-treated mice. In spite of the promising results, black tail syndrome, a possible side effect to reovirus exposed mice, was observed in four out of the eight injected mice. Unfortunately, this side effect was also pointed out in the previous study involving MCT in xenografted models and therefore should be anticipated in any further experiments comprising reovirus (Hwang, et al. 2013). Following the notable decrease in tumour mass post treatment, immunohistochemical (IHC) staining of reoviral proteins was applied to confirm the growth suppression. The histopathological samples revealed successful infection of reovirus in the reovirus-injected tumours, which indicates reovirus's ability to infection and suppress the proliferation of canine histiocytic sarcoma cells.

2.2 Mechanism of Action

An oncolytic virus destroys tumours through several possible mechanisms including; direct viral lysis of tumour cells, destruction of tumour vasculature, induction of host antitumour immunity, or through the combination of these mechanisms.

2.2.1 Direct Viral Lysis of Tumour Cells

Direct virus-mediated cytotoxicity leads to a mixture of cell death types, classified based on morphological and ultrastructural changes, such as apoptosis, necrosis, pyroptosis,

and autophagic cell death, depending on the particular virus in use. Previously, all types of cell death, with the exception of apoptosis, were considered to be inflammatory and immunogenic. Recent studies then led to a new concept that categorizes apoptotic cells into "immunogenic cell death (ICD)", and "non-immunogenic cell death (NICD)". Today, apoptotic cell death caused by some oncolytic viruses is classified under ICD. Following the cytotoxic actions of oncolytic viruses, the destruction of cancer and endothelial cells releases and presents damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) as signal 0 and tumour-associated antigens (TAAs) as signal 1 to dendritic cells for antitumour and antiviral immune responses (Barlett, et al. 2013). During the early stages of apoptosis, surface exposed calreticulin (CRT) and secreted adenosine triphosphate (ATP) act as major DAMPs. These stimulate the recruitment of DCs into the tumour bed, the uptake and processing of tumour antigens, and the optimal antigen presentation to T cells. The process of translocation of CRT from the endoplasmic reticulum (ER) lumen to the outer leaflet of the plasma membrane is initiated by ER stress. This occurs prior to the membrane exposure of PS, discussed earlier, as a marker of apoptosis (Wang, et al., 2018). The activation of ER stress is indispensable to the immunogenic character of neoplastic cell death due to its role in coordinating the signalling pathways responsible for trafficking DAMPs. With regards to autophagic cell death, ATP secretion from tumour cells also triggers an immune response as a consequence of stress and nutrient deprivation. During autophagy, cytoplasmic content is sequestered into double-membraned organelles through a series of events; formation of autophagosomes, lysosomal fusion, and cargo digestion which recycle the blocks of cell into energy for metabolic and anabolic reactions. The synergy between autophagy and antitumour effects was studied and exhibited that autophagy-deficient tumours showed reduced ATP secretion and tumour-infiltrating lymphocytes (TILs) in response to chemotherapy, which suggests that autophagy suppression is utilised by malignant cells to evade immunosurveillance. ATP therefore undoubtedly generates a strong location signal for DCs and macrophages upon being liberated from dying tumour cells. Equally important is the role of high mobility group box 1 (HMGB1) released from dying cells undergoing necrosis. Upon its release, HMGB1 triggers a strong inflammatory response by activating DCs and stimulating an optimal presentation of TAAs to T cells as it binds to toll-like receptor 4 (TLR4). It additionally promotes the maturation and migration of DCs through the binding of receptors for the advanced glycation of end products (RAGE) and the activation of mitogen-activated protein kinase (MAPK) (Wang, et al. 2018). As demonstrated in figure 5, ICD stimulates local antitumour activity through the attraction of

immune effector cells into the tumour microenvironment (TME), leading to tumour suppression.

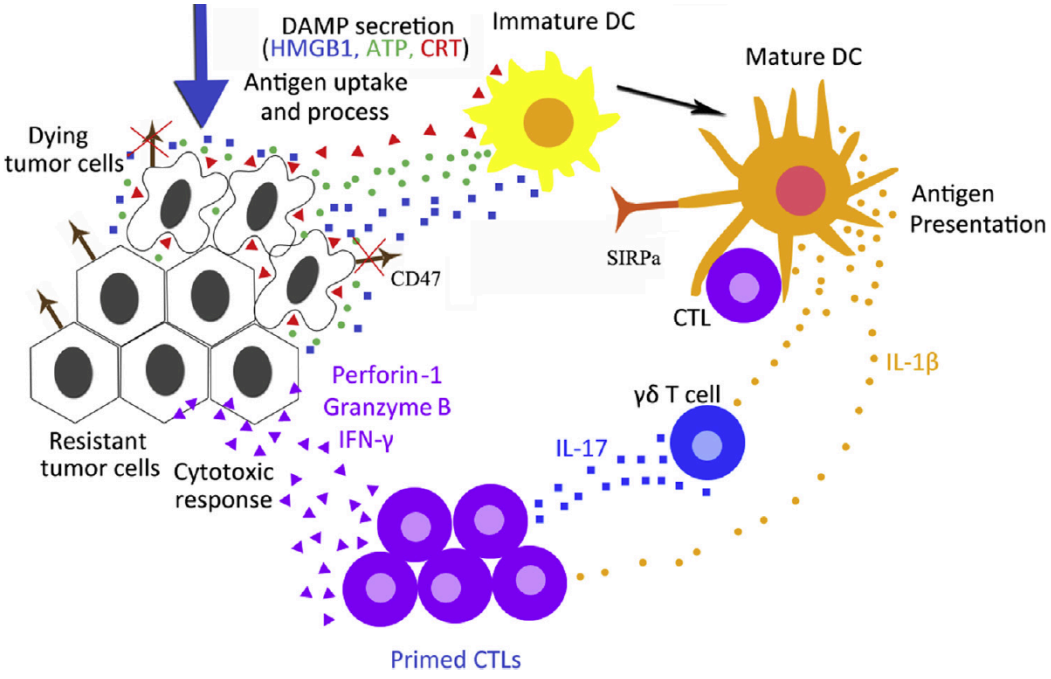

Figure 5: Mechanism of ICD.
In response to oncolytic viruses, tumour cells expose CRT, secrete ATP, and release HMGB1. These DAMPs stimulate the recruitment of DCs, as well as their maturation, and process tumour antigens for presentation to T cells. Cross-priming CD8+ CTLs is triggered by mature DCs and γδ T cells in an IL-1β and IL-17 dependent manner. Primed CTLs elicit direct cytotoxic response and kill remaining tumour cells through IFN-γ, perforin-1 and granzyme B (Wang, et al. 2018).

2.2.2 Destruction of Tumour Vasculature

Oncolytic viruses naturally prevent neoangiogenesis either by direct infection and destruction of tumour vasculature or by vascular normalisation in tumour tissue. Mainly, the tumour-associated endothelium offers a key mechanism for enhanced viral uptake into tumours, creating an access into systemic gene delivery. VEGF is a fundamental regulator of tumour angiogenesis; therefore, anti-VEGF strategies have been developed for the treatment of different cancers (Gentschev, et al. 2014). Aside from being structurally different from normal vessels, the tumour vasculature is antigenically distinct and antigens such as E-selectin, VEGF receptor 2, and integrins, to name a few, have been distinctly overexpressed on the endothelial cells (Russell, et al. 2014). Tumour-associated endothelial cells often display a pro-inflammatory activation of cytokines emanating from the tumour, which promotes faster endothelial proliferation and results in the up-regulated levels of cell

surface markers. The following include tissue necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Bachtarzi, et al., 2011).

As discussed earlier, the therapeutic potential of oncolytic viruses armed with anti-VEGF antibodies was analysed using the GLV-1h109 vaccinia virus strain studied in two different xenograft models based on canine STSA-1 cells and canine prostate carcinoma DT08/40 cells. Following results showing a 9–16 times higher expression of VEGF, as well as scAb GLAF-1 expression in STSA-1 xenografts compared to DT08/40 xenografts, the effects on tumour vasculature and microenvironment were tested in STSA-1 xenograft models only. The anti-VEGF effects were analysed by fluorescence microscopy based on $CD31^+$ vascular networks in tissue sections of GLV-1h109, GLV-1h68 and PBS-treated STSA-1 tumours. The data revealed significant decrease in vascular density of GLV-1h109 infected tumours in comparison to that of GLV-1h68 and PBS injected control tumours. Fluorescence intensity of the CD31 signal was then measured in immunohistologically stained sections of STSA-1 tumours, and the results revealed vessel-related pixels of GLV-1h109 virus infected tumours were significantly decreased compared to that of GLV-1h68, and PBS-injected control tumours. Although this may demonstrate an up-regulation of CD31 protein post virus colonisation in GLV-1h68 infected tumours only, the notable decrease in the fluorescence intensity of GLV-1h109 infected tumours may be justified by the reduction in vascular density following the virus treatment. As a result, the virus colonisation in combination with scAb GLAF-1 led to local inhibition of angiogenesis in the GLV-1h109 infected tumours (Patil, et al. 2012).

2.2.3 Induction of Host Antitumour Immunity

The targeted tumour infection by oncolytic viruses has revealed a potential to create “inflammatory storms” that stimulate the innate and adaptive immune responses against the tumour. The mechanisms underlying successful cancer immunotherapy were shown to link the innate and adaptive responses, and the importance of their initiation came to play a significant role in aiming towards the host’s immune system as a whole. Following careful observation throughout clinical trials, several antitumour immune mediators were selected for their clinical benefits. Cytotoxic T lymphocytes (CTL), tumour-infiltrating lymphocytes (TIL), and chimeric antigen receptors (CAR) modified T cells play a major role in MHC Class I restricted tumour cell lysis leading to enhanced tumour infiltration. Second in line are the natural killer (NK) cells that act on antigen non-specific tumour cell lysis and induce cytotoxicity, in correspondence with DCs that cross present the tumour antigens to CTLs

and activate the NK cells. In addition, regulatory T cells (Tregs) maintain the peripheral tolerance however, their tendency to induce immunosuppression has raised concerns on their benefits. Finally, inflammatory cytokines polarize immune reactions and promote antitumour effector functions, as shown in granulocyte macrophage colony stimulating factor (GM-CSF) inclusion in anticancer vaccine strategies (Melcher, et al., 2011).

Location and site of oncolytic virus administration is an important determinant of the characteristics that will follow the initial host response. This was demonstrated in the intravenous and intraarterial administration of adenovirus, which led to its rapid recognition and elimination by the circulating complement and antibodies of the humoral defence system. The limitation to oncolytic virus replication is however not only seen with intravascular administration, but also with intratumoural administration. As a result, new models were developed where the by-pass of such responses was achieved with the use of immunomodulating drugs. Cyclophosphamide (CPA) is an example of such drugs, it increases replication and inhibits tumour growth by suppressing innate immunity, antibody responses, depleting Tregs, and enhancing the antitumour activity of CTL. Through pharmacological agents, the interference of antiviral responses is applied in a transient manner usually directly before or at the time of administration of an oncolytic virus. An initial surge of replication will consequently lead to tumour cell lysis, and as the pharmacologic effects diminish the debris of oncolytic viruses and tumour antigens become more evident and better recognized by the antiviral host response. Accordingly, a secondary long-term vaccination effect becomes responsible for effective tumour immunity (Chiocca and Rabkin, 2014). The following is detailed in a schematic diagram shown in figure 6. Unfortunately, the quantification of those responses relies on several factors that haven't been thoroughly investigated yet, but will be the focus of future research.

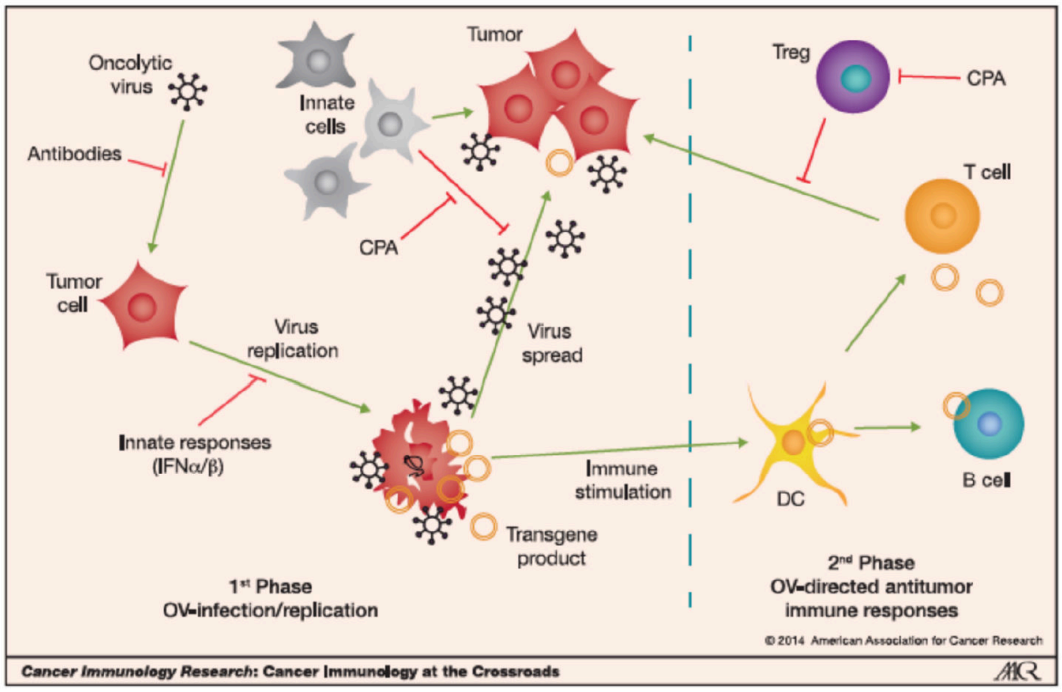

Figure 6: Oncolytic mediated effects in a tumour. **1st Phase** Oncolytic virus delivered for tumour cell infection. Followed by replication, ICD, and spread throughout the tumour. When an armed oncolytic virus is used, transgenes are expressed. **2nd Phase** ICD and inflammatory responses recruit DCs where they take-up TAAs and induce an adaptive immune response (Chiocca and Rabkin, 2014).

2.3 Efficacy of Oncolytic Potential

2.3.1 Selective Targeting of Tumour Tissues

One of the main concerns widespread clinical use of oncolytic virotherapy faces is the assurance that normal tissue cells will not be harmed. Certain viruses such as reovirus and vesicular stomatitis virus have natural tumour tropism, which grade a better safety margin. Better understanding of the molecular events involved in virus-cell interactions recently allowed for the development of genetically modified viruses that target selected molecules or signalling pathways such as p16, p21, p53, IFN, VEGFR. Adenovirus is a prime example of such modifications. The viral protein encoded by the E1 region of the wild type virus binds and inactivates p53, allowing the replication of adenovirus in normal cells. This protein acts as a tumour suppressor by regulating cell division and keeping cells from growing and proliferating uncontrollably. Because tumour cells lacking functional p53 proteins are unable to suppress replication of mutant adenoviruses, E1 gene-deleted adenoviruses have diminished ability to replicate in normal cells and preserve replication in

neoplastic cells. The p53 protein is mutated in several canine cancers, including osteosarcoma, mammary gland tumours, and gastric carcinomas, thus adenoviruses can specifically be targeted to canine cancers by taking advantage of the defective p53 pathway. Similarly, VACV mutants with deletions in the thymidine kinase gene and/or the vaccinia growth factor gene have shown great potential against canine cancers. The mutants grow selectively in cancer cells with high levels of cellular thymidine kinase, and constitutively activate epidermal growth factor receptor (EGFR) pathway. Increased serum levels of thymidine kinase were observed in lymphomas, leukaemia, and hemangiosarcoma tumours of canine origin, in addition to higher expression of EGFR pathway in mammary gland, and glioma tumours, making them ideal candidates for VACV oncolytic therapy (Patil, et al., 2012). Alternatively, a popular strategy to overcome non-selective infection is the use of transductional targeting with conditionally replicative viruses. In a study done to enhance the benefits of conditionally replicating canine adenovirus while restricting its replication in canine osteosarcoma cells, an osteocalcin promoter was used. In healthy dogs, osteocalcin is known to be restricted to osteoblasts, but due to its high activity in osteosarcoma cells it acts as a crucial tumour-specific promoter. Therefore, it falls under the category of type II CRADs, which control the expression of vital adenovirus genes regulating adenovirus replication (Hemminki, et al., 2003).

2.3.2 Spread Throughout Tumour Tissue

Another challenge facing successful virotherapy is the relatively poor penetration of the oncolytic virus throughout solid tumour masses. Unfortunately, slow viral spread can be limiting and often determines the outcome of therapy. Over the years, it was suggested that the slow spread might be related to the relatively large size of oncolytic viruses, such as vaccinia virus and adenovirus being approximately 200 nm and 90 nm, respectively. This was based on the fact that the structural components of the tumour extracellular matrix (ECM), such as collagens and proteoglycans, had shown to hinder the distribution of large therapeutic molecules and viruses. Therefore, protease and hyaluronidase mediated digestion of tumour ECM was used to improve intratumoural spread and efficacy to reach the desired anticancer effects (Patil, et al. 2012).

2.3.3 Optimizing Replication in Immunocompetent Hosts

It is often argued that immune responses against viruses limit ongoing viral replication in immunocompetent dogs. That is to say, a high level of pre-existing immunity to parental viruses in canine populations might limit the use of oncolytic viruses for cancer therapy. In this case, carrier cell-based therapy provided promising results to escape pre-existing immunity. Under these circumstances several cell types such as immune cells, stem cells, and tumour cells were successfully utilised as carriers of oncolytic viruses to tumour sites acting as a "Trojan horse" delivery vehicle. As a result, specific delivery to tumours while escaping the established antiviral immunity increased the effective local viral dose and enhanced the oncolytic effects. Domestic dogs are often vaccinated with CAV2 to protect them from the more serious CAV1 (canine adenovirus type 1), therefore those patients present with high titres of anti-Ad5 antibodies. The previously mentioned OCCAV conditionally replicating canine adenovirus functions as an ideal carrier vehicle for evading pre-existing neutralising antibodies against adenovirus, resulting in successful systemic antitumoural activity (Patil, et al., 2012). Likewise, a study was done on oncolytic VACV, containing deletions in thymidine kinase and viral growth factor genes, involving the combination of cytokine-induced killer (CIK) cells and NK T cell like populations. This approach uses CIK cells infected with the virus prior to their systemic delivery, so the virus is able to utilise the ability of CIK cells to traffic to tumour targets and achieve more effective delivery than cell-free viruses. The murine models were immunised with a western reserve strain of vaccinia 14 days before the implantation of tumours and approximately 24 hours before the application of therapy. 72 hours after intravenous injection of the CIK-luciferase labelled cells there were no significant differences in the levels of CIK cells present in the tumours between the immunised and naïve mice. Thus, demonstrating that infected CIK cells are capable of reaching the tumour regardless of the level of antiviral immunity (Thorne, et al., 2010).

2.4 Safety and Toxicity

As any novel therapy under progressive development; there has been concerns raised on the side effects of oncolytic therapy, however most clinical trials have shown little to no adverse effects as a direct result of the virotherapy. Following the evaluation of efficacy of ICOCAV17 in six canine clinical cases, any unwanted effects were a result of poor prognosis or deteriorated physical condition of the patients prior to the start of the treatment. A 14-year old male Husky that had received surgery 2 years prior to admission to remove a sweat gland

adenoma was later diagnosed with infiltrative sweat gland adenoma. Biochemistry and haematological analysis revealed no signs of systemic toxicity throughout the course of the treatment, yet, general poor body condition and hindlimb weakness were observed. For at least 6 months the tumour progressively decreased in size, unfortunately 11 months post-treatment progressive disease similar to that at the start of the therapy was observed. The second case involved a 7-year old male Ibizan Warren Hound with a large humeral mass on the right proximal humerus compatible with osteosarcoma. The owner reported the lesions became evident 1 month prior to admission, although there was no compromise in physical activity until a week before the visit. Radiography detected multiple lung metastases and a humerus fracture. Biochemistry and haematology results remained within normal values throughout the treatment, and no signs of systemic toxicity were noted. On the 10^{th} day of administration signs of self-trauma were evident on the site, and due to poor prognosis, the dog was euthanised (Laborda, et al. 2014). In the same way, a recent study aimed to establish the safety profile of reovirus in dogs with spontaneously occurring tumours, most of which had been previously treated with conventional chemotherapy methods, in order to determine a recommended dosing regimen. Nineteen dogs with various tumours, mainly in advanced stages, were treated with reovirus serotype 3 Dearing strain ranging from $1x10^8$ to $5x10^9$ $TCID_{50}$ given as intratumoural injections or intravenous infusions daily for up to 5 consecutive days in one of multiple treatment cycles. The characteristics of the dogs studied included, a 5-year old Maltese diagnosed with a grade II cutaneous MCT metastasised to the submandibular lymph node, a 12-year old Dalmatian presented with a grade III inflammatory mammary carcinoma metastasised to the abdominal lymph nodes, an 11-year old Shih Tzu with oesophageal cancer, and a 9-year old Beagle with a grade II hemangiopericytoma, to list a few. The adverse effects were graded according to the Veterinary Cooperative Oncology Group – Common Terminology Criteria for Adverse Events (VCOG-CTCAE) guidelines. Following the therapy, no grade III and above adverse effects were observed, the most common effects were grade I and II, fever and diarrhoea. Bleeding (4/19) and pain (2/19) of the injected tumour and vomiting (2/19) were infrequently observed. The side effects were observed within a few days post administration, but resolved within a couple of days following supportive medical treatment. In addition, no haematological unfavourable effects were seen (Hwang, et al., 2018). Another study intended to determine the efficacy and safety of systemic injections of an upgraded canine version of celyvir (dCelyvir), using canine mesenchymal stem cells (MSCs) and ICOCAV17, a canine oncolytic adenovirus previously mentioned. Twenty-seven canine patients of different dog breeds with progressive cancers despite standard chemotherapeutic

or surgical treatment were enrolled. Some of these patients included, a 5-year old Golden Retriever diagnosed with a grade II rhabdomyosarcoma, a 6-year old Boxer with a grade III mast cell tumour, a 2-year old Border collie presenting with a grade II chondroblastic osteosarcoma, and a 1-year old Rottweiler diagnosed with a pituitary adenoma. The results demonstrated the dCelyvir treatment was well tolerated, and 73% of the patients had a good quality of life. Those presented with bad quality of life were due to their osteosarcomas or STS that affected the limbs causing restricted mobility and pain. Adverse effect related to dCelyvir therapy were graded based on the VCOG-CTCAE criteria. Among the twenty-seven patients, clinical side effects were only documented in four of them, two of which showed grade I/II dermatologic alterations, and one grade I/II diarrhoea, however they were all concurrently treated with corticosteroids. The fourth patient suffered from grade I/II orchitis. None of these clinical events were classified as severe or detrimental to the patients' quality of life. Regarding biochemical blood analysis, serum alanine transaminase (ALT) levels increased to grade I/II in ten patients, but only one showed concomitantly high aspartate transaminase (AST) levels, additionally occasional decrease in phosphorus concentration was detected in eight dogs. There were no significant haematological changes, only two patients exhibited a reduced number of neutrophil granulocytes, one of which subsequently recovered. Therefore, dCelyvir treatment was deemed safe and well tolerated among the studied canine patients (Cejalvo, et al., 2018).

3 Conclusion

Canine tumours have challenged the veterinary profession for years and the complex nature of the disease makes the road to its full understanding demanding. Standard chemotherapeutic and surgical methods have paved a start to overcoming the losses, however there has been no overall substantial improvement in the post-treatment results, and often the outcomes demonstrated adverse effects and poor quality of life. The disappointing results come as no surprise knowing the ability of cancers to find different ways to evade signalling pathways and gain a growth advantage in the host, making it unlikely that pharmacological strategies targeting single molecules will significantly impact the long-term progression of malignancies. Oncolytic virotherapy is an expanding field involving replication competent viruses eliminating tumours through direct viral lysis of neoplastic cells, destruction of tumour associated endothelial cells and vasculature, and stimulation of the innate and adaptive immune responses without causing damage to surrounding healthy tissue cells. Several past and ongoing clinical trials studying those viruses have shown promising therapeutic results, and the direct cytotoxicity and immune-stimulatory effects on the tumour portrays an interesting approach to cancer therapy. Aside from their tumour tropism properties, they have re-ignited the possibility of training the immune system to recognise tumour development and potentially control or eradicate it. Researchers have already established the main mechanisms of action behind a standard oncolytic virus, but the extent of their potential is still unclear despite all the work dedicated to their understanding over the past and present. Although many kinds of viruses have been proposed as candidates, this review discussed the three most commonly evaluated, adenovirus, vaccinia virus, and reovirus. The main challenges facing oncolytic virotherapy are selective targeting of the tumour tissue, spreading of the virus throughout the tumour, optimising the viral replication, and enhancing the antitumour effects of the viruses. Given the above points, an ideal oncolytic virus should demonstrate the following characteristics; efficient, safe and complete destruction of tumour tissue, selective replication in canine cancer cells, resistance against pre-existing immunity in canine populations, eliciting strong immune responses, and efficient clearance from the body preventing latent or recurrent infections. As well as, lack of integration of viral genome into the canine genome, easily engineered to express antitumour agents, large recombinant gene carrying capacity, easy to monitor with respect to successful tumour colonisation, and finally cost effective and economical for widespread use (Patil, et al., 2012). The encouraging nature of medicine is its ability to learn from previous strategies and build upon them by incorporating new approaches. Although there

many benefits associated with the use of oncolytic viruses as a treatment therapy, it is important to point out the restrictions associated with them. Consequently, it seems that the route of combination therapies will lead modern veterinary medicine one step closer to its goal. The immune system makes a crucial contribution to antitumour effects in conventional chemotherapeutic methods as well as in oncolytic virotherapy. Looking forward, branching research on the synergy between chemotherapeutic agents and oncolytic virotherapy revealed a different perspective to overcoming the challenge, and has successfully found a potential solution.

4 Summary

Oncolytic virotherapy is a novel approach against canine cancers emerging as an improvement from standard chemotherapeutic therapies. Its popularity stems from the virus's ability to infect, replicate, and kill neoplastic cells without harming normal tissue cells, thus decreasing the chances of post treatment adverse effects. The preferred viruses of choice are either naturally oncolytic, such as reovirus, or genetically modified to target certain molecules, genes or signalling pathways, such as adenovirus and poxviruses. Several strains were derived from the above-mentioned viruses, and evaluated in comparison to different strains within their virus genera and non-related strains from separate virus genera. Therefore, this review aimed to discuss the past and current experiments performed *in vitro* and *in vivo* on cell lines retrieved from selected cancer types, on murine xenograft models, and on canine patients enrolled in clinical trials. According to researchers, the most common tumour types diagnosed in dogs are mammary gland tumours, osteosarcomas, soft tissue sarcomas, prostate carcinomas, melanomas, and finally mast cell tumours. The studies, discussed throughout the review, analysed the therapeutic potential of oncolytic viruses on the most frequently observed tumour types. The mechanism of action through which the viruses infect and destroy the cancer cells was also elaborated, focusing on the direct cytotoxicity and lysis of the tumour cells, the targeted destruction of the tumour vasculature, and the induced enhancement of the host's innate and adaptive immune systems. Due to the broad and partially uncovered potential of oncolytic viruses, several queries were raised concerning the possibility of reaching full efficacy. The current obstacles are the assurance of selective targeting of cancer cells, the efficient spread throughout the tumour mass, and the evasion of pre-existing immunity. Similarly, to most medical advancements, the safety and toxicity profile poses a great concern to the veterinarians and pet owners. Fortunately, the majority of the clinical trials confirmed no adverse effects were presented as a direct result of oncolytic virotherapy.

5 Bibliography

Annexin V Detection Protocol for Apoptosis. [Website] URL: https://www.abcam.com/protocols/annexin-v-detection-protocol-for-apoptosis Accessed: October 14, 2019

Bachtarzi H., Stevenson M., Subr V., Ulbrich K., Seymour LW., Fisher KD., 2011: Targeting Adenovirus Gene Delivery to Activated Tumour-Associated Vasculature Via Endothelial Selectins. *J Control Release*, 150. 2. 196–203 p.

Bartlett D., Liu Z., Sathaiah M., Ravindranathan R., Guo Z., He Y., Guo ZS., 2013: Oncolytic Viruses as Therapeutic Cancer Vaccines. *Molecular Cancer*, 12, 103.

Cejalvo T., Perisé-Barrios A., del Portillo I., Laborda E., Rodriguez-Milla M., Cubillo I., Vázquez F., Sardón D., Ramirez M., Alemany R., del Castillo N., García-Castro J., 2018: Remission of Spontaneous Canine Tumours After Systemic Cellular Viroimmunotherapy. *Cancer Res*, 78. 17. 489–4901 p.

Chiocca, EA., Rabkin SD., 2014: Oncolytic Viruses and Their Application to Cancer Immunotherapy. *Cancer Immunol Res*, 2. 4. 295–300 p.

Feoktistova M., Geserick P., Leverkus M., 2016: Crystal Violet Assay for Determining Viability of Cultured Cells. *Cold Spring Harb Protoc*.

Gentschev I., Adelfinger M., Josupeit R., Rudolph S., Ehrig K., Donat U., Weibel S., Chen N., Yu Y., Zhang Q., Heisig M., Thamm D., Stritzker J., MacNeill A., Szalay A., 2012: Preclinical Evaluation of Oncolytic Vaccinia Virus for Therapy of Canine Soft Tissue Sarcoma. *PLoS ONE,* 7. 5.

Gentschev I., Patil S., Adelfinger M., Weibel S., Geissinger U., Frentzen A., Chen N., Yu Y., Zhang Q., Ogilvie G., Szalay A., 2013: Characterization and Evaluation of a New Oncolytic Vaccinia Virus Strain LIVP6.1.1. *Bioengineered,* 4. 2. 84–89 p.

Gentschev I., Patil S., Petrov I., Cappello J., Adelfinger M., Szalay A., 2014: Oncolytic Virotherapy of Canine and Feline Cancer. *Viruses,*6. 2122–2137 p.

Hemminki A., Kanerva A., Kremer E., Bauerschmitz G., Smith B., Liu B., Wang M., Desmond R., Keriel A., Barnett B., Baker H., Siegal G., Curiel D., 2003: A Canine Conditionally Replicating Adenovirus for Evaluating Oncolytic Virotherapy in a Syngenic Animal Model. *Molecular Therapy,* 7. 2.

Hwang C.C., Umeki S., Igase M., Coffey M., Noguchi S., Okuda M., Mizuno T., 2014: The Effects of Oncolytic Reovirus in Canine Lymphoma Cell Lines. *Veterinary and Comparative Oncology,* 14. S1. 61–73 p.

Hwang C.C., Igase M., Sakurai M., Haraguchi T., Tani K., Itamoto K., Shimokawa T., Nakaichi M., Nemoto Y., Noguchi S., Coffey M., Okuda M., Mizuno T., 2018: Oncolytic Reovirus Therapy: Pilot Study in Dogs with Spontaneously Occurring Tumours. *Veterinary and Comparative Oncology,* 16. 229–238 p.

Hwang C.C., Umeki S., Kubo M., Hayashi T., Shimoda H., Mochizuki M., Maeda K., Baba K., Hiraoka H., Coffey M., Okuda M., Mizuno T., 2013: Oncolytic Reovirus in Canine Mast Cell Tumor. *PLoS ONE,* 8. 9.

Igase M., Hwang C.C., Kambayashi S., Kubo M., Coffey M., Miyama TS., Baba K., Okuda M., Noguchi S., Mizuno T., 2016: Oncolytic Reovirus Synergizes With Chemotherapeutic Agents to Promote Cell Death in Canine Mammary Gland Tumor. *Cancer J Vet Research*, 80. 1. 21–31 p.

Igase M, Shousu K., Fujiki N., Sakurai M., Bonkobara M., Hwang C.C., Coffey M., Noguchi S., Nemoto Y., Mizuno T., 2019: Anti-Tumour Activity of Oncolytic Reovirus Against Canine Histiocytic Sarcoma Cells. *Veterinary Comparative Oncology,* 17. 184–193 p.

Keoni C., Brown T., 2015: Inhibition of Apoptosis and Efficacy of Pan Caspase Inhibitor, Q-VD-OPh, in Models of Human Disease. *Journal of Cell Death*, 8. 1–7 p.

Kanaya N., Yazawa M., Goto-Koshino Y., Mochizuki M., Nishimura R., Ohno K., Sasaki N., Tsujimoto H., 2011: Anti-Tumor Effect of Adenoviral Vector-Mediated p53 Gene Transfer on the Growth of Canine Osteosarcoma Xenografts in Nude Mice. *J Vet Med Sci*, 73. 7. 877–883 p.

Laborda E., Puig-Saus C., Rodriguez-García A., Moreno R., Cascalló M., Pastor J., Alemany R., 2014: A pRb-Responsive, RGD-modified, and Hyaluronidase-Armed Canine Oncolytic Adenovirus for Application in Veterinary Oncology. *Molecular Therapy*, 22. 5. 986–998 p.

Melcher A., Parato K., Rooney C., Bell J., 2011: Thunder and Lightning: Immunotherapy and Oncolytic Viruses Collide. *Molecular Therapy,*19. 6. 1008–1016 p.

Patil S., Gentschev I., Nolte I., Ogilvie G., Szalay A., 2012: Oncolytic Virotherapy in Veterinary Medicine: Current Status and Future Prospects for Canine Patients. *Journal of Translational Medicine*, 10. 3.

Patil S., Gentschev I., Adelfinger M., Donat U., Hess M., Weibel S., Nolte I., Frentzen A., Szalay A., 2012: Virotherapy of Canine Tumors with Oncolytic Vaccinia Virus GLV-1h109 Expressing an Anti-VEGF Single-Chain Antibody. *PLoS ONE*, 7. 10.

Riccardi C., Nicoletti I., 2006: Analysis of Apoptosis by Propidium Iodide Staining and Flow Cytometry. *Nat Protoc*, 1. 3. 1458–1461.

Russell S., Peng K-W., Bell J., 2014: Oncolytic Virotherapy. *Nat Biotechnol*, 30. 7. 658–670 p.

Samson A., Bentham MJ., Scott K., Nuovo G., Bloy A., Appleton E., Adair RA., Dave R., Peckham-Cooper A., Toogood G., Nagamori S., Coffey M., Vile R., Harrington K., Selby P., Errington-Mais F., Melcher A., Griffin S., 2018: Oncolytic Reovirus as a Combined Antiviral and Anti-Tumour Agent Agent for the Treatment of Liver Cancer. *Gut*, 67. 3. 562–573 p.

Sanchez D., Cesarman-Maus G., Amador-Molina A., Lizano M., 2018: Oncolytic Viruses for Canine Cancer Treatment. *Cancers*, 10. 404.

Smith B., Curiel D., Ternovoi V., Borovjagin A., Baker H., Cox N., Siegal G., 2006: Administration of a Conditionally Replicative Oncolytic Canine Adenovirus in Normal Dogs. *Cancer Biotherapy & Radiopharmaceuticals,* 21. 6.

Strober W., 2001: Trypan Blue Exclusion Test of Cell Viability. *Curr Protoc Immunol*, Appendix 3. Appendix 3B.

Thorne S., Liang W., Sampath P., Schmidt T., Sikorski R., Bilhack A., Contaq C., 2010: Targeting Localized Immune Suppression Within the Tumour Throughout Repeat Cycles of Immune Cell-oncolytic Virus Combination Therapy. *Molecular Therapy*, 18. 9. 1698–1705 p.

Wang Y., Fletcher R., Yu J., Zhang L., 2018: Immunogenic Effects of Chemotherapy-Induced Tumour Cell Death. *Genes & Diseases*, 5. 194–203 p.

Westberg S., Sadeghi A., Svensson E., Segall T., Dimopoulou M., Korsgen O., Hemminki A., Loskog AS., Tötterman TH., von Euler H., 2013: Treatment Efficacy and Immune Stimulation by AdCD40L Gene Therapy of Spontaneous Canine Malignant Melanoma. *J Immunotherapy*, 36. 6. 350–358 p.

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