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Efficacy of approved Smallpox Vaccines in Human and Canine Cancer Therapy: Adipose - tissue derived Stem Cells (ADSC) take up VACV and serve as a protective vehicle for virus delivery to tumors

Dissertation

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SUMMARY

Cancer is one of the major causes of mortality in developed countries. In 2020, there were more than 19.3 million new cases of tumor malignancies worldwide, with more than 10 million deaths. The high rates of cancer cases and mortality necessitate extensive research and the development of novel cancer treatments and antitumor agents. In most cases, conventional treatment strategies for tumor therapy are based on chemotherapeutic treatment, which is supplemented with radiotherapy and/or surgical resection of solid tumors [1]. The use of chemotherapy for the treatment of cancer has significant side effects, the most dangerous of which is toxicity [2] [3].

Modern methods of treating tumors focus on specific drug delivery to the tumor site, actively targeting the tumor cells, as well as the reduction of side effects. One of the most promising current approaches is based on oncolytic viruses. Antitumor properties of viruses were documented at the beginning of the 20th century when some cancer patients recovered after acute viral infections, particularly influenza [4]. Vaccinia virus (VACV) is a member of the Poxviridae family, has natural antitumor properties, and provides a good basis for generating efficient recombinant oncolytic strains. Furthermore, VACV has never been shown to integrate into the host genome [5]. VACV is likely one of the safest and well-studied viruses due to extensive research being done in molecular biology and pathophysiology to investigate its potential as a vaccine for smallpox eradication programs. It has been administered to over 200 million people worldwide. VACV antitumor therapeutic effectiveness has been established in xenograft models with a variety of tumor types for human and canine cancers. Furthermore, recombinant oncolytic VACVs expressing genes encoding light-emitting proteins are a big improvement in a treatment strategy that combines tumor-specific therapies and diagnostics.

Oncolytic virus treatments are effective in xenograft cancer models in mice, however, the significant improvements found in mice do not always translate to human cancer patients. These therapies should be tested in dogs with spontaneous cancer not only to offer well-translatable information regarding the possible efficiency of viral therapy for human cancers but also to improve the health of our household pets as well. Spontaneous canine tumors are starting to be regarded as an essential model of human cancers that can reproduce the tumor microenvironment and immune response of cancer patients [6]. Just as data obtained in dog experiments can improve cancer therapy for human patients, these findings can also be used to improve treatment protocols in canine patients.

Hundreds of studies and dozens of reviews have been published regarding the antitumor effects of various recombinants of VACV, but information on the anticancer features of initial, genetically-unmodified "naïve" VACV is still limited. In the first studies, we compared different wild-type, non-modified strains of VACV and tested their oncolytic properties on a panel of various cancer cells derived from different organs. In addition, we also tested a protection system based on the "Trojan horse" concept - using a combination of human Adipose tissue-derived Stem Cells (hADSC) and three different wild-type single plaque purified Vaccinia virus strains: W1, L1, and T1. We showed that all tested human cell lines (FaDu, MDA MB 231, HNT-13, HNT-35, and PC-3) are permissive to L0, W0, T0, L1, W1, and L1 infection. Furthermore, we tested the cytotoxicity of VACV in different cancer cell lines (A549, PC-3, MDA-MB 231, FaDu, HNT-13, HNT-25, and HNT-35). All strains lysed the cells, which was most visible at 96 hpi. We also showed that all tested strains could efficiently infect and multiply in hADSC at a high level. In our *in vivo* study, we tested the therapeutic efficacy of the wild-type Vaccinia viruses L1, W1, and T1 alone or in combination with hADSC. Wild-type VACV strains were tested for their oncolytic efficiency in human lung adenocarcinoma (A549) in a xenograft model. Treatment of A549 tumors with different doses of L1 and W1 as well as with a L1/ADSC or W1/ADSC combination led to significant tumor regression compared to the PBS control. Additionally, the treatment with L1 and W1 and the combination of L1/ADSC and W1/ADSC was well tolerated by the animals. In the case of the wild-type Tian Tan strain, results were not obtained due to the high cytotoxicity of this strain. Therefore, it should be attenuated for further studies.

In the second part of the current study, we investigated the oncolytic effect of C1-opt1, W1-opt1, and L3-opt1 strains based on the wild-type Copenhagen, Wyeth, and Lister vaccines with additional expression of turboFP635. Replication and cytotoxicity assays demonstrated that all 3 viruses were able to infect, replicate in and kill canine tumor cell lines STSA-1 and CT1258 in a virus dose- and time- dependent fashion. Cytotoxicity and replication assays were also performed on cultured canine Adipose-derived Mesenchymal Stem Cells (cAdMSC). The results showed that the cells were lysed much slower than the tumor cells. It suggests that these cells can harbour the virus for a long-term period, allowing the virus to spread into the body - and there is enough time to reach the primary tumor or metastases before the cell carrier is destroyed. The viral replication in cAdMSC in our study was lower than in canine cancer cells (STSA-1 and CT1258) at the same MOI. After being studied in cell culture, C1-opt1 and their combination with cAdMSC (C1-opt1/cAdMSC) were used in canine STSA-1

tumor-bearing nude mice. We tested the oncolytic effect of the C1-opt1 virus alone and in combination with cAdMSC in the canine STSA-1 xenograft mouse model. Altogether, our findings have shown that both C1-opt1 and cAdMSC/C1-opt1 significantly reduced tumor size or eliminated the tumor. There was no significant difference between C1-opt1 alone and cAdMSC/C1-opt1. The virus particles were mostly found within the tumor after 24 dpi, some amount of virus particles were found in the lungs of mice injected with a combination of cAdMSC/C1-opt1 but not in the group injected with virus alone (cAdMSC might get stuck in the lungs and cause virus propagation there).

Taken together, this study provided a proof-of-concept that hADSC/cAdMSC can be used as a carrier system for the "Trojan horse" concept. However, it should be confirmed in another experimental model system, such as canine patients. Moreover, these findings suggest that wild-type, non-modified strains of Vaccinia virus isolates can be considered promising candidates for oncolytic virotherapy, especially in combination with mesenchymal stem cells.

ZUSAMMENFASSUNG

Krebs wird zu einer der Hauptursachen für die Sterblichkeit in den Industrieländern. Im Jahr 2020 gab es weltweit mehr als 193 Millionen neue Fälle von tumormalen Erkrankungen mit mehr als 10 Millionen Todesfällen. Folglich erfordern die hohen Krebsfälle- und Mortalitätsraten umfangreiche Forschung und Entwicklung neuartiger Krebsbehandlungen und Antitumormittel. Konventionelle Behandlungsstrategien zur Tumortherapie basieren in den meisten Fällen auf einer chemotherapeutischen Behandlung, die durch Strahlentherapie und/oder chirurgische Resektion solider Tumoren ergänzt wird [1]. Die Verwendung von Chemotherapie zur Behandlung von Krebs hat erhebliche Nebenwirkungen, insbesondere die gefährlichste Intoxikation [2] [3].

Die modernen Methoden zur Behandlung von Tumoren konzentrieren sich auf die spezifische Zielabgabe an die Tumorstelle, die selektive Wirkung auf die Tumorzellen; sowie auf die Neutralisierung der Nebenwirkungen. Einer der vielversprechendsten aktuellen Ansätze basiert auf dem Einsatz onkolytischer Viren. Antitumoreigenschaften von Viren wurden zu Beginn des 20. Jahrhunderts dokumentiert, als sich einige Krebspatienten nach akuten Virusinfektionen - insbesondere Influenza – erholten [4].

Das Vaccinia-Virus (VACV) gehört zur Familie der Poxviridae, hat natürliche Antitumoreigenschaften und bietet eine gute Grundlage für die Erzeugung effizienter rekombinanter onkolytischer Stämme. Darüber hinaus wurde VACV nie in das Wirtsgenom integriert gefunden [5]. VACV ist wahrscheinlich eines der sichersten und am besten untersuchten Viren, da umfangreiche Forschungen in der Molekularbiologie und Pathophysiologie als Impfstoff für Pockenausrottungsprogramme durchgeführt wurden und bei über 200 Millionen Menschen weltweit bereits injiziert wurden. Die therapeutische Wirksamkeit von VACV gegen Tumore wurde in Xenograft-Modellen mit einer Vielzahl von Tumorarten für Krebs sowohl beim Menschen als auch bei Hunden nachgewiesen. Darüber hinaus sind rekombinante onkolytische VACVn mit fluoreszierenden Gene expression eine große Verbesserung in einer Behandlungsstrategie, die tumorspezifische Therapien und Diagnostik kombiniert.

Onkolytische Virusbehandlungen sind in Xenograft-Krebsmodellen bei Mäusen wirksam, jedoch sind die signifikanten Verbesserungen, die bei Mäusen gefunden wurden, nicht immer bei menschlichen Krebspatienten umsetzbar. Diese Therapien sollten bei Hunden mit spontanem Krebs getestet werden, um nicht nur gut übersetzbare Informationen über die

mögliche Wirksamkeit der Virustherapie bei menschlichen Krebserkrankungen zu bieten, sondern auch, um die Gesundheit unserer Haustiere zu verbessern. Hundetumoren, die sich spontan entwickeln, gelten als wesentliches Modell für menschlichen Krebs, das die Tumormikroumgebung und die Immunantwort der Krebspatienten reproduzieren kann [6]. Die Daten aus Hundeexperimenten können so die menschliche Krebstherapie bei Krebspatienten verbessern und diese Erkenntnisse können auch zur Verbesserung der Behandlungsprotokolle bei Hundepatienten verwendet werden.

Hunderte von Studien und Dutzende von Übersichten wurden über die Antitumorwirkungen verschiedener Rekombinanten von VACV veröffentlicht, aber Informationen über die Krebsmedikamente von anfänglichen, genetisch unveränderten " naïve" VACV sind immer noch begrenzt. In unseren ersten Studien verglichen wir verschiedene wildtypische, nicht modifizierte VACV-Stämme und testeten ihre onkolytischen Eigenschaften auf dem Panel verschiedener Krebszellen, die aus verschiedenen Organen stammen. Darüber hinaus haben wir auch ein Schutzsystem getestet, das auf dem "Trojanischen Pferd" -Konzept basiert, wobei eine Kombination aus menschlichen ADSC und 3 verschiedenen Wildtyp-Single-Plaque-gereinigten Vaccinia-Virusstämmen verwendet wurde: W1, L1 und T1. Wir zeigten, dass getestete menschliche Zelllinien (FaDu, MDA-MB231, HNT-13, HNT-35 und PC-3) für L0-, W0-, T0-, L1-, W1-, L1-Infektionen freizügig sind. Darüber hinaus haben wir die Zytotoxizität von VACV in verschiedenen Krebszelllinien (A549, PC-3, MDA-MB231, FaDu, HNT-13, HNT-25 und HNT-35) getestet. Alle Stämme lysierten die Zellen, was bei 96 hpi am sichtbarsten war. Wir haben auch gezeigt, dass alle getesteten Stämme in hADSC auf hohem Niveau effizient infizieren und sich vermehren können. In unserer In-vivo-Studie haben wir die therapeutische Wirksamkeit der Wildtyp-Vaccinia-Viren L1, W1 und T1 allein oder in Kombination mit hADSC getestet. Wildtyp-VACV-Stämme wurden in einem Xenograft-Modell auf ihre onkolytische Wirksamkeit bei A549 (humanes Lungenadenokarzinom) getestet. Die Behandlung von A549-Tumoren mit unterschiedlichen Dosen von L1 und W1 sowie mit L1/ADSC- oder W1/ADSC-Kombination führte im Vergleich zur PBS-Kontrolle zu einer signifikanten Tumorregression. Darüber hinaus wurde die Behandlung mit L1 und W1 und die Kombination von L1/ADSC und W1/ADSC von den Tieren gut vertragen. Im Fall des Wildtyp-Tian-Tan-Stammes wurden die Ergebnisse aufgrund der hohen Zytotoxizität dieses Stammes nicht erzielt. Es sollte für weitere Studien abgeschwächt werden.

Im zweiten Teil der aktuellen Studie untersuchten wir die onkolytische Wirkung von C1-opt1, W1-opt1- und L3-opt1-Stämmen basierend auf den Wildtyp-Impfstoffen Copenhagen, Wyeth

und Lister mit zusätzlicher Expression von turboFP635. Durchgeführte Replikations- und Zytotoxizitätstests zeigten, dass alle 3 Viren in der Lage waren, die Tumorzelllinien STSA-1 und CT1258 von Hunden in Virusdosis- und zeitabhängig zu infizieren, zu replizieren und abzutöten. Zytotoxizitäts- und Replikationstests wurden auch an kultivierten Hunde-AdMSC durchgeführt. Die Ergebnisse zeigten, dass die Zellen viel langsamer lysiert wurden als die Tumorzellen. Es deutet darauf hin, dass diese Zellen das Virus für einen langen Zeitraum halten können, so dass sich das Virus in den Körper ausbreiten kann und genügend Zeit bleibt, um den Tumor oder die Metastasierung zu erreichen, bevor der Zellträger zerstört wird. Die Virusreplikation in cAdMSC unserer Studie war niedriger als in den Hundekrebszellen (STSA-1 und CT1258) bei gleichem MOI. Nach der Untersuchung in Zellkultur wurden C1-opt1 und ihre Kombination mit cAdMSC (C1-opt1/ cAdMSC) bei stSA-1-tumortragenden Nacktmäusen bei Hunden eingesetzt. Wir haben die onkolytische Wirkung des C1-opt1-Virus allein und in Kombination mit cAdMSC im Hunde-StSA-1 Xenograft-Mausmodell getestet. Gänzlich, unsere Ergebnisse sowohl mit C1-opt1 als auch mit cAdMSC/C1-opt1 zeigen signifikante Reduzierung in Tumorgröße und die Eliminierung der Tumoren. Es gab keinen signifikanten Unterschied zwischen C1-opt1 allein und cAdMSC/C1-opt1. Meistens wurden die Viruspartikel nach 24 dpi im Tumor gefunden, eine gewisse Menge an Viruspartikeln wurde in den Lungen von Mäusen gefunden, denen eine Kombination von cAdMSC / C1-opt1 injiziert wurde, aber nicht in der Gruppe, die nur mit Virus injiziert wurde. cAdMSC können in der Lunge haften bleiben und eine Virusvermehrung verursachen.

Zusammengefasst lieferte diese Studie einen Beweis dafür, dass hADSC/cAdMSC als Trägersystem im "Trojanischen Pferd"-Konzept verwendet werden kann. Es sollte jedoch an einem anderen experimentellen Modell, wie z. B. Hundepatienten, bestätigt werden. Darüber hinaus deuten diese Ergebnisse darauf hin, dass wildtypische, nicht modifizierte Stämme des Vaccinia-Virus als vielversprechende Kandidaten für eine onkolytische Virotherapie gelten, insbesondere in Kombination mit mesenchymalen Stammzellen.

CHAPTER 1: INTRODUCTION

1.1. Overview of human and canine cancers

According to the recently published data by GLOBOCAN 2020 database (Global Cancer Incidence, Mortality, and Prevalence), there were an estimated 19.3 million new cases of cancer and nearly 10 million cancer deaths worldwide in 2020. The study analysed the incidence and mortality of 36 types of cancers in 185 different countries and found that the most frequently diagnosed cancer was breast cancer (11.7% of all new cancer cases). Lung cancer (11.4%), colorectal cancer (10%), prostate cancer (7.3%), and stomach cancer (5.6%) were the other most dominant types. It is predicted that by 2040 there will be 28.4 million new cancer cases [1]. This increase is partly due to the longer lifespan of the world population. Some scientists link this phenomenon to the worsening ecological situation and living in the polluted environment, increasing the degree of negative impact on the human body [7]. The dominant factors that trigger cancer development are also a person's lifestyle, including unhealthy habits such as smoking, alcohol, drugs, and low physical activity that leads to obesity, nutritional deficiencies such as lack or low dietary intake of fruits and vegetables [8]. Genetic predisposition to cancer, as well as workplace associated with harmful toxins, do play a significant role in the induction of carcinogenesis.

Cancer is a threat not only to human life but also to the life of domesticated animals such as cats and dogs. For example, nearly 1 in 4 dogs will have neoplasia during their life. Approximately 50% of all dogs older than 10 years will be killed by cancer. Generally, dogs develop cancer with a similar frequency as humans [9]. Cancer development in dogs is of strong resemble that of humans. Several types of canine and human cancer have very similar morphology, mutations, histology, and metastatic distribution. Different types of lymphoid neoplasia, mesenchymal tumors, including soft tissue sarcoma, and epithelial tumors, especially pediatric cancers, have been confirmed to be nearly identical between the two species [10]. Moreover, it has been shown that cancer therapies that work well against human cancers are also safe and effective against corresponding canine tumors. [11]. All the aforementioned facts suggest that dog cancers are a very promising model for the development of new antitumor therapeutic strategies not only for dogs themselves but also applicable of human patients.

1.1.1. Clinical cancer therapy methods today

The most commonly used treatments of cancer are surgery, chemotherapy, and radiation therapy. Monotherapies are used mainly, but combinational treatment is expected to be more successful: surgical excision of the solid tumor followed by radiation therapy and chemotherapy or immunotherapy combination, boosting patient's own immune response against cancer and increasing the sensitivity of cancer cells to medical drugs.

Historically, the first treatment method for tumor patients was surgery, which remained the only method of treatment until the end of the 19th century. The point of surgical operation is the direct removal of tumor tissue. This method is most effective for treating primary tumors at the early stage of the disease, provided that this primary tumor is accessible for surgery. Tumor resection surgery is still used in modern clinical practice today. The survival rate after the surgery is about 30-40% in the following 5 years. To improve therapy efficacy and minimize the risk of the development of metastases, tumor resection surgery is usually combined with radio- or chemotherapy treatments [12].

The discovery of X-rays and radioactivity at the end of the 19th and beginning of the 20th century led to use of radiotherapy for cancer treatment [13]. The basis of the method is to target tumors with ionizing radiation. Radiation kills both healthy and malignant cells. Since the malignant cells grow faster than healthy cells, therefore tumor cells growth is preferentially inhibited. Currently, radiotherapy is used mainly as a preventive, adjuvant, or supportive treatment to reduce metastases and thereby increase the survival rate of patients after cancer [14]. Radiotherapy is the primary treatment option for cancer such as head and neck cancer, prostate cancer, and Hodgkin's lymphoma. Statistical data analysis shows that radiotherapy is being used in 45-50% of cancer treatments. One of the reasons why radiotherapy is so widespread is that it has a good cost/efficacy ratio of therapy [15], [16]. The combination of radiotherapy and surgery remained the primary treatment for tumor disease until the 1960s. However, the efficacy of this approach was still relatively low: the treatment led to a complete recovery in only 33% of cases, in the rest of the cases, metastases would still develop [17].

In the second half of the 20th century, chemotherapy became a fundamentally new method for tumor treatment [18]. The principle of chemotherapy is to destroy the malignant cells with a specific chemical agent. The era of chemotherapy is believed to have begun in the 1940s when there was active development of chemical weapons for mass people destruction, which led to the discovery of the antitumor properties of mustard gas (bis-β-chloroethyl amine derivatives).

The first antitumor drug based on this compound was invented in 1946. Later antitumor properties were found after actinomycin D treatment, originally developed as an antibiotic. In 1964, this drug was approved by FDA as an anticancer agent [19]. In 1948 Farber et al. discovered that aminopterin and methotrexate also have anticancer activity. Later it was found that it was the most effective drug for treating children suffering from leukemia [20].

In the 1960s, combination chemotherapy methods (or "polychemotherapy") were introduced by combining more than one chemotherapeutic medication at the same time to treat cancer. One of the first combined chemotherapy was the VAMP combination (Vincristine, Amethopterin, 6-mercaptopurine, Prednisone), which showed promising results. The use of the VAMP polychemotherapy combination led to reduced tumor size in 60% of cases, and in many patients, complete tumor regression was observed [21]. Other polychemotherapy combinations such as MOMP (Melphalan, Methotrexate, Vincristine, Prednisone) and MOPP (Melphalan, Procarbazine, Vincristine, Prednisone) are widely used due to their high effectiveness against Non-Hodgkin's Lymphoma [22].

During the 1960-1980s, the most extensive chemotherapeutic drugs were cytostatics. According to their name, they are functioning as cell division blockers. Cytostatics are divided into alkylating agents (this agent covalently binds to DNA and prevents cell division), antitumor antibiotics (these agents can act by different mechanisms, for example, acting as DNA intercalators or prevent DNA repair), alkaloids (they are capable of altering the main signaling of cellular pathways, which are responsible for cell cycle and cell division), antimetabolites (these ones interact with DNA and inhibit cell growth and replication) and taxanes (this group of substances changes the cytoskeleton causing a block of cell division) [23], [24]. Mainly two main mechanisms of action of cytostatics can be distinguished: alterations of DNA structure, including abnormal twists that prevent DNA strands from separation during cell division as well as disruptions in DNA repair and disruption of cell division - formation of the cell spindle that divides chromosomes into daughter cells. Each cytostatic has its own mechanism of antitumor action, or cell damage mechanisms, but most of them are unknown. Finally, all cytostatic actions lead to cellular apoptosis [25]. Unfortunately, the use of cytostatics is very limited due to the severe side effects they cause. Cytostatics are not tumor-specific, their toxic effect spreads on all cells of the human body, especially rapidly dividing cells, which is leading to side effects such as suppression of hematopoiesis, hair loss, and digestive problems. Moreover, after some time, cancer cell clones develop resistance to cytostatics, therefore reducing the effectiveness of chemotherapy [26]. Tamoxifen was another

chemotherapeutic agent frequently used against cancer. It was approved for the treatment of patients with breast cancer in 1977 [27]. It was demonstrated that this drug is safe, and it doesn't harm healthy cells. Later Tamoxifen was broadly used and became a standard gold treatment for women with estrogen (ER) positive breast cancer since it blocks the uptake of ER [27], [28].

Another striking historical event in cancer therapy development was the discovery of the Hepatitis B virus vaccine [29], [30]. In 1981 FDA approved the vaccine, which was developed by Maurice Hillman at Merck, based on insights from Baruch Blumberg, who discovered the Hepatitis B virus [31]. It has been shown that chronic Hepatitis B infections are the principal cause of liver cancer [32], [33]. Hence, the Hepatitis B vaccine is considered to be the first cancer antiviral vaccine.

In the 1990s, the concept of tumor cell-specific anticancer therapy was developed, which allowed selective targeting of tumor cells without significant harming of healthy cells. The first targeted drug was *imatinib mesylate*, approved for patients use in 1996 and by the FDA in 2001 [34], [35]. The drug is the inhibitor of a number of tyrosine kinase enzymes, which are specific for different types of myeloid leukemia cells [36]. One of the most common targeted drugs is *rituximab* which is based on a monoclonal antibody to CD20, which is present on the surface of malignant B lymphocytes [37]. By binding to CD20, *rituximab* can induce direct apoptosis, cell-mediated cytotoxicity, or activate complement against CD20 positive lymphoma cells [38]. In clinical practice, *rituximab* is administered either alone or in combination with cytostatic drugs, which significantly increases the effectiveness of the treatment of B cell non-Hodgkin's lymphoma. Intravenously administered *rituximab* was approved in 1997 by the U.S. Food and Drug Administration (FDA) and in 1998 by the European Medicines (EM) [39].

In the process of developing cancer therapy methods, from surgery to targeted therapy, there is a clear tendency to select a unique treatment scheme for each individual cancer patient. Depending on the origin of the tumor, stage of the disease, age, the health status of the patient, and the other factors. Different combinations of existing cancer treatment methods are used, for instance, different doses of chemotherapy drugs, different doses of radiation, and many other treatment parameters [40]. Despite the patient-oriented approach the personated cancer, treatment there is still very little success in anticancer therapy, and many therapies still don't possess sufficient tumor cell specificity.

1.1.2. Cancer treatment methods presently developed

Currently, the search for new effective approaches to cancer treatment does continue. One of the most promising methods today is immunotherapy, using drugs that stimulate the body's immune system to fight against tumor cells. It is believed that the patient immune system has the ability to destroy tumor cells and prevent further development of solid tumors and metastases [41]. The advantage of immunotherapy is the reduction of side effects associated with chemotherapy. The cancer community is very interested in the approach of activation of dendritic cells, which are key components in the development of the immune response. Activation of dendritic cells leads to increased production of proinflammatory cytokines and chemokines, to an acceleration of maturation and an increase in T-killer cells activity. Dendritic cell activation is performed in *ex vivo* and *in vivo*. The first officially licensed dendritic cell-based tumor therapy was the *Sipuleucel-T* (APC8015). A total of 225 patients with inoperable prostate cancer were enrolled in clinical trials, 147 patients were treated with *Sipuleucel-T* injected intravenously 3 times each. The results showed a 3-year survival in 10% cases compared to the placebo group [42].

An approach based on activation of cytotoxic T cells is also discussed as a potentially promising strategy for introduction into clinical practice and slowly being implemented. It is known that the main regulators of T lymphocyte activity are peptides CTLA-4 and PD-1, and their inactivation inhibition leads to a significant increase in T-lymphocyte activity against cancer cells [43]. The first registered antitumor drug with this function was i*pilimumab*, which is an anti-CTLA-4 antibody. *Ipilimumab* has shown the strongest efficacy against prostate cancer, lung and blood cancers [44].

Less common immunotherapy method is the adoptive T cell transfer, such as chimeric antigen receptor T cell therapy (CAR-T) or tumor-infiltrating lymphocyte therapy (TIL), vaccination with tumor antigens (proteins, peptides, nucleic acids), and activation of immunity by organic nanoparticles. There were attempts to combine immunotherapy with various chemotherapy drugs and oncolytic viruses, which can be themselves considered as immunotherapy. Immunotherapeutic methods of cancer treatment are just being started to be applied in clinical trials. In the future, the role of immunotherapy will rapidly grow, and the number of different tumor-specific therapies will increase [45].

Since the development of nanotechnology, particularly, the idea of synthesizing various nanoparticles to package therapeutic agents for cancer treatment, is becoming more and more

popular (OR “is evolving”) [46]. The main application of nanoparticles in the field of cancer research is their use as vectors for the protection and delivery of antitumor agents at the tumor site. The role of antitumor agents, in this case, can be played by various chemotherapeutic agents and their precursors, or nucleic acids with antitumor activity [47].

Nanoparticles are divided into organic (lipid, polymer, virus-like particles) and non-organic, including those based on gold, iron oxide, or silicon. Lipid nanoparticles can form a hollow sphere in which molecules of the antitumor agents can be placed. These nanoparticles are the most widespread because of the simplicity of their preparation. Several drugs based on lipid nanoparticles carrying chemotherapeutic drugs have already been registered: *Doxil, Myocet, DaunoXome* [48], [49].

Polymeric nanoparticles, for example, act as a nucleus, which carries on its surface molecules from the anticancer agent. The polymer and drug molecules are formed into a complex mainly via hydrophobic bonds. Currently, drugs based on polymeric nanoparticles are *Xyotax* and *CT-2106* (PGA-camptothecin) in clinical trials [50].

Virus-like nanoparticles are protein structures resembling wild-type viruses but carrying no genetic information and constructed to improve the specificity of delivery of a chemotherapeutic agent directly to tumor sites. The specificity is achieved through ligands on the surface of virus-like nanoparticles with affinity to tumor cell receptors, for instance, transferrin receptors, which are broadly present on the surface of the malignant cells. Some nanoparticles have unique optical or electromagnetic properties that can be used as "nanoscalpels" to destroy tumor cells from inside. The nanoparticles can be heated up by laser excitation or by alternating the magnetic field [51].

Stem cells have also been used for the delivery of antitumor agents directly to the tumor site. It has been found that mesenchymal and neural stem cells have a tendency to migrate to primary tumors and metastases [52], [53]. The modified stem cells are "loaded" with antitumor agents and injected into the patient's body. In the case of tumors of the nervous system, this approach can be very effective, because brain tumors are often not accessible for surgical intervention, and the presence of the blood-encephalic and cerebrospinal barrier (blood-brain barrier) makes delivery of antitumor agents with the blood flow problematic. The cell-based delivery may be able to overcome these barriers [54], [55], [56].

1.2. Oncolytic viruses

Starting in the middle of the 17th century, there have been reports of spontaneous regression of hematologic cancers (leukemia, lymphoma) in patients who have had been expired of viral infections. In 1904, Dock described remission of myeloid leukemia in a 42-year-old woman infected with influenza [4]. In the case of leukemia, it was not a complete recovery, but rather just a brief period of remission. There was another case of regression of lymphatic leukemia in a 4-year-old boy after infection with chickenpox and the patient didn't get any antitumor treatment prior to the virus infection. Checking his bone marrow after chickenpox infection confirmed that he was in remission. However, after one month his leukemia progressed rapidly again until his death. These cases were the first documented facts, that viruses could possess anticancer properties [57]. Later, the viruses that can rapidly replicate and destroy tumor cells were named "oncolytic viruses" (OVs). Laboratory studies of the anticancer properties of viruses began in the 1950s. Originally only human viruses were considered as therapeutic agents against cancer because the improvement of the therapy methods was primarily aimed at treating cancer in humans [58], [59]. The first virus that was used for such therapy was Hepatitis B virus (family Hepadnaviridae) [60], [61]. Studies of antitumor activity of West Nile virus (family Flaviviridae), published in 1952, had a great influence on the development of anticancer virotherapy, even despite the fact that the antitumor effect of the virus was observed in a relatively small number of cases, most patients who received therapy with West Nile virus suffered from encephalitis and two cases of death from the side effects were recorded [58]. In 1956 the Adenoidal-Pharyngeal-Conjunctival (APC) virus (which is now known as adenovirus, family Adenoviridae) showed more promising results in clinical trials, compared to the Hepatitis B and West Nile viruses. Administration of the APC virus to patients with cervical cancer led to the development of extensive necrosis of tumor tissue within 10 days. The effect was observed in two-thirds of the patients, nevertheless, more than half of the patients in the study had died because of cancer during a few months after virus injection [62]. Attenuated mumps virus (family Paramyxoviridae) was initially used only as an immunostimulatory agent in patients with metastatic melanoma who didn't respond properly to the standard therapy. Live mumps virus was used in Japan in 1974 to treat a wide range of tumor diseases. In clinical trials, the virus worked quite promising: 37 out of 90 patients showed complete regression of the tumor or reduction of the tumor size by more than half and administration of live mumps virus didn't cause the development of remarkable side effects (mostly these were swelling of lymph nodes, which the therapy was stopped after) [63]. Viremia, as one of the side effects of

virotherapy for malignant diseases, is often of concern since cancer patients often have an exposed immune system. The injection of the virus into a patient with weakened immunity can lead to the development of uncontrolled viral infection, and finally to death. There are generally two ways to avoid developing side effects in virotherapy for cancer: using animal viruses that are non-pathogenic to humans or modifying the human virus for reducing pathogenic and enhancing antitumor properties. The development of recombinant DNA technology over the past 30 years has led to the generation of many genetically modified strains that are attenuated through the deletion of certain genes. The very first modified virus was created by Martuza et al. and in 1991 it was demonstrated that the herpes virus with the thymidine kinase (TK) gene deletion is only able to replicate in glioma tumor cells in immunodeficient mice [64]. There are currently seven different virus families of which modified strains are being tested in clinical trials [65].

The interest in using OVs for cancer treatment has increased when in 2015 FDA and EU approved T-VEC, an oncolytic virus based on HSV, for patients with melanoma cancer [66], [67]. However, there are several limitations of oncolytic virotherapy against a variety of cancers. The first obstacle is the targeted delivery of the virus to tumor sites. Usually, the best way to inject the virus is to administer it intravenously, but in most of the current therapy which uses viruses, the virus is injected intratumorally meaning it can be injected only into visible solid tumors, such as melanoma. In today's clinical practice, ultrasonic and MRI-guided injections can facilitate the therapy to a certain extent and help deliver the virus to relatively large internal tumors and/or metastases [68]. The second problem is that the virus enhances either innate or adaptive immune response, and they both could strongly inhibit viral replication, which in turn drastically reduces viral oncolysis. Despite these limitations, oncolytic virus therapy remains a promising new method for the treatment of cancer.

1.2.1. Vaccinia virus: a cowpox virus in oncolytic tumor therapy

Poxviruses (family Poxviridae) are a family of animal viruses characterized by large (up to 300 nm) and complex structures of virions. The genome of Poxviruses, represented by double-stranded DNA, contains 250.000 base pairs; it contains over 300 genes that encode viral proteins. Some of these proteins antagonize host IFN-α, -β, and γ, IL-1β, TNF-α, and several chemokines as well [69]. Poxvirus virion has a brick shape and consists of a nucleoid containing viral DNA and lateral bodies, surrounded by a double lipid membrane coated with

surface proteins. Notably, in addition to viral DNA, poxvirus particles have proteins that are necessary to initiate gene expression and synthesis of viral proteins: DNA and RNA polymerases, early transcription factors. This allows starting replicating cycle of DNA immediately after the virion enters the cell. Therefore, it is important that Vaccinia virus replicates in specific structures located in the cytoplasm of the cell-virus factories and never integrates into the host cell DNA [5].

Vaccinia virus (VACV) has the most extensive and long-term history of use in human disease treatment compared to all other viruses. The smallpox vaccine has been discovered in 1796 and its historical role has resulted in detailed studies of the biology and pathogenesis of VACV. Therefore there is extensive background knowledge, including general, preclinical, and clinical data on VACV and the most critical studies on children [70]. People born after 1980 have not been vaccinated against smallpox, and they have no immunity to the VACV, for instance, it is about 40% of people in the USA. Only a tiny proportion of people were vaccinated after 1980, which usually includes military personnel and sometimes clinical and scientific staff.

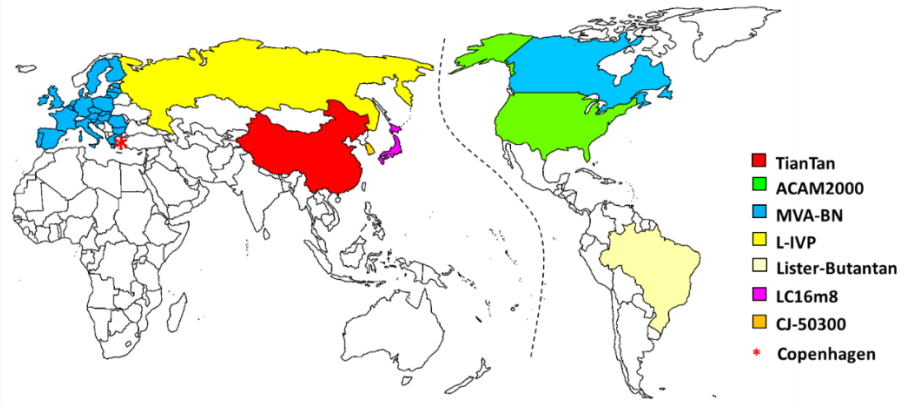
VACV cowpox virus has several unique aspects that make it an excellent candidate for clinical studies. Throughout history, VACV, as a human smallpox vaccine, has been used to vaccinate over 200 million people, including children, without any major adverse effects [71]. In addition, Vaccinia is highly immunogenic and usually induces a strong T-cell response [72], [73]. Another feature of VACV is the fact that the virus has a wide host range and does infect many different human cells as well as cells from other species, such as dogs, cats, hamsters and etc [74]. Therefore, comparative cancer studies with different animals may result in useful applications in human clinical trials. VACV is an excellent vector with a carrying capacity of 25 kbp. It replicates in the cell cytoplasm and doesn't integrate into host DNA, it doesn't need the host DNA replication machine [75], [76]. Moreover, the generation of VACV particles is highly productive and fast; the viral particles are stable in cell lysates and can be stored in frozen or freeze-dried conditions for a long time.

There are two major forms of virions of VACV: intracellular mature virions and extracellular enveloped virions. The intracellular mature virus has a single lipid envelope. When the cell membrane is destroyed, these virions leave the cell and can infect neighbouring cells. The extracellular enveloped virus is a VACV particle, wrapped in an additional membrane formed from membrane vesicles of the Golgi apparatus [77]. It is believed that the enveloped form of VACV provides the systemic spread of the virus through the body [78]. An additional outer

membrane gives the poxvirus particles special antigenic properties and could make them "invisible" to the host's immune system. It has been shown experimentally that antibodies, obtained after immunization with the intracellular uncovered form of the virus don't prevent infection of animals with the enveloped form of the virus [77]. This is happening due to the extra membrane of the extracellular form of the VACV that is host origin and not recognized by the host immune system cells. The relative amounts of the enveloped form of VACV are from 1 to 30% of the total number of virions, depending on the viral strain and the type of infected cells [79]. It has been found that poxviruses are able to penetrate the cytoplasm of most mammalian cell types, but the fact that a virus particle enters the cell doesn't mean that virus will efficiently replicate [80], [81]. The propagation level of the virus in a cell depends not only on the viral strain but also on many biochemical properties of the infected cells. The poxvirus family includes vertebrate pathogens such as chickenpox virus, cowpox virus, mousepox virus, monkeypox virus, variola virus, human smallpox virus, cowpox virus, and insectpox virus. One of the most well-known and well-studied members of the poxvirus family is by far VACV, simply since it was used for the vaccination campaign against variola human smallpox strains, a severe, highly transmissible human disease, which has killed millions of people. The smallpox eradication program in the 1980s has successfully eradicated smallpox worldwide [82], [83].

Since Edward Jenner discovered the smallpox vaccine in the late 18th century, VACV has changed a lot of hosts, propagation methods; thereby, its biological origin is unclear [84] [85]. Probably, all VACV strains could be descended from a single heterogeneous pool of viruses [86]. Vaccinia virus was distributed to many different countries and has received various names according to the country's vaccination program and health institution.

World Distribution of main human Smallpox Vaccines - Vaccinia virus



The most popular Smallpox Vaccines used during smallpox eradication were Lister and TianTan

In one of the comprehensive and interesting papers, the authors, who created POCs (Poxvirus Orthologous Clusters) software, analysed the genome of all 21 poxviruses and concluded that 49 genes are essential for the virus life cycle, and they are strongly conserved for all poxviruses. Part of these genes are responsible for the transcription and replication of virus DNA (25 genes), the other twelve are involved in virion formation and the function of the rest is yet to be determined. All these genes are found in the center sections of the VACV genome [87]. The table with listed conserved genes is presented below:

Completely conserved gene

VV-Cop ORF	Family names of 49 poxvirus conserved genes	Family identification no.	Function*
A1L	Late transcription factor 2 (VLTF-2)	1153	T
A2L	Late transcription factor 3 (VLTF-3)	1228	T
A3L	P4b precursor	1072	M
A5R	RNA polymerase subunit 19 (RPO19)	1225	T
A7L	Early transcription factor—large (VETF-l)	914	T
A9L	Late virion membrane protein (MP), essential	1218	M
A10L	P4a precursor	1750	M
A11R	Unknown	1217	U
A16L	Unknown soluble-myristylated	887	U
A18R	DNA helicase, transcription	896	T
A21L	Unknown	1202	U
A22R	Holliday junction resolvase	1201	R
A23R	Intermediate transcription factor 3—large (VITF-3)	946	T
A24R	RNA polymerase subunit 132 (RPO132)	880	T
A28L	Unknown predicted signal peptide	1099	U
A29L	RNA polymerase subunit 35 (RPO35)	1197	T
A32L	ATPase-DNA packaging protein	1192	M
D1R	mRNA capping enzyme large subunit	1109	T
D4R	Uracil-DNA glycosylase	1130	R
D5R	NTPase, DNA replication	950	R
D6R	Early transcription factor—small (VETF-s), morphogenesis	1027	T, M
D7R	RNA polymerase subunit 18 (RPO18)	1141	T
D10R	Nucleophosphohydrolase-pyrophosphohydrolase downregulator	1143	T
D11L	Nucleophosphohydrolase I (NPH-I), virion	1093	T, M
D12L	mRNA capping enzyme small subunit, VITF	1151	T
D13L	Rifampin resistance MP, morphogenesis	883	M
E1L	Poly(A) polymerase large subunit	939	T
E6R	Unknown	1253	U
E9L	DNA polymerase	910	R
E10R	Redox–EVR-1, morphogenesis	900	M
F9L	Unknown predicted MP	1001	U
F10L	Serine-threonine kinase, morphogenesis	1065	M
G1L	Protease morphogenesis	1275	M
G5R	Unknown	1017	U
G6R	Unknown	1131	U
G9R	Myristylated protein	957	U
H2R	Unknown	1116	U
H3L	Intracellular mature virus morphogenesis viral protein (VP55)	1269	M
H4L	RNA polymerase-associated protein (RAP94)	1695	T
H6R	Topoisomerase type I	908	R
I7L	Virion core protease	1015	M
I8R	RNA helicase, NPH-II	1104	T
J3R	Poly(A) polymerase small subunit VP39	895	T
J5L	Late MP, essential	1777	M
J6R	RNA polymerase subunit 147 (RPO147)	1040	T
L1R	Myrisylated MP virion	1044	M
L3L	Unknown	1285	U
L4R	Core packaging transcription	1283	T, M
L5R	Unknown predicted MP	1511	U

*** T, transcription; M, morphogenesis; U, unknown; R, replication**

Today, many countries still have stockpiles of live Vaccinia virus as human smallpox vaccines to protect their populations against sudden smallpox outbreaks or terrorism from outside. Most of these vaccines are first-generation vaccines, meaning that they were used in the eradication program and were propagated on the skin of live animals, such as caws, rabbits, sheep, calves, and buffaloes, or in chicken eggs [70]. Later these methods were replaced to propagate the virus in cell cultures under sterile conditions, which reduces vaccine contamination levels. These vaccines are known as 2nd generation vaccines. The most well-known Vaccinia virus strain globally is Lister, which was used in Great Britain, Germany, the Netherlands, and other European countries [88]. It is well known that the USA used the New York City Board of Health (NYCBH) strain (or DRYVAX), which originated from England (in 1856) and was broadly used in the USA and West Africa. In 2007 this DRYVAX 1st generation vaccine was replaced with the 2nd generation vaccine - ACAM2000™, clone number 2, derived from parental DRYVAX and propagated in the Vero monkey cell line. Currently, this strain is FDA approved as a smallpox vaccine in the USA and is used for vaccination of military personal and people exposed to smallpox risks infected areas. In the former Soviet Union and India, strain EM-63 (based on NYCBH) was used and later replaced in Russia by Lister - L-IVP (based on the Lister strain). In Europe, various strains of smallpox vaccines were used depending on the country. For instance, in Germany and Austria, the VACV Bern strain was used during the period between the 1950-1960s [71]. Great Britain used the Lister-Elstree strain since the strain was first isolated there [89]. In the Netherlands, the Copenhagen strain was used until 1962 and later replaced with the Lister-Elstree strain [90]. In China, Vaccinia virus strain Tian Tian was used for variola eradication during the period between the 1920s - 1980s [91]. At the same time, in Japan, the Ikeda strain was used and later replaced with the attenuated smallpox vaccine strain named LC16m8 [92]. Ankara strain was used in Turkey [93]. In France, the Paris strain was used for people vaccination [94].

The 3rd generation vaccines based on highly attenuated strains of VACV, such as MVA (Modified Vaccinia Ankara) and LC16m8, currently licensed in Japan for smallpox vaccination, were injected into more than 100.000 infants without noticeable side effects [95]. MVA-BN® (Bavarian Nordic) was approved in Canada and the EU (under the trade names IMVAMUNE® and IMVANEX®, respectively) as smallpox vaccines. MVA doesn't replicate in most mammalian cells except BHK-21 cells. The replication cycle of this strain in most cell lines is blocked at the late stage, therefore no new viral particles can't be formed and released.

This advantage makes MVA an excellent vaccine vector backbone but not useful for oncolytic therapy.

Table 1. List of stockpiles of first, second and third generation smallpox vaccines (WHO manuscript, 2014)

Generation	Strains	Production platform	Description
1st	Lister/Elstree NYCBH/Dryvax Ikeda Dairen 1 Tian Tan Copenhagen Bern	Lymph, animal skin lesion, egg choriolantroic membranes	All strains were used in the global eradication campaign 1960-1970
2nd	Elstree-BN (Lister/Elstree) VVLister/CEP (Lister/Elstree) ACAM2000 Lister/Elstree-RVIM	Embryonic chicken fibroblasts cells Chicken embryo cells Vero cells Rabbit kidney cells	Stockpiled in EU countries Licensed in France and the UK Stockpiled and licensed in the USA Stockpiled in the Netherland
3rd	LC16m8 (Lister/Elistree) MVA (Imvanex/Imvamune)	Primary rabbit kidney cells at 30°C chicken embryo fibroblast cells BHK-21	Licensed in Japan Licensed in Europe and Canada

It is hard to find updated information about the location and number of stockpiles today. Many of the strain descriptions are not accessible or published in different languages.

After many years of using VACV strains as smallpox vaccines, it was suggested to use them for cancer therapy. It was reported that VACV possesses broad tropism to various mammalian cancer cells, which means that VACV could potentially become a potent wide range "weapon" against tumors of various origins. The safety profile of VACV is one of the most deciding criteria for using it in clinical trials since it has already been used safely for global vaccination programs, and there is access to antiviral agents in case of emergency.

VACV oncolytic properties for the first time were described in 1922 when Levaditi et al. discovered that the smallpox vaccine could inhibit the growth of different tumors in rodents [96]. Human clinical trials of VACV started in the 1960s. The first patients who were treated

with wild-type Vaccinia virus were melanoma patients due to the high visibility and accessibility of primary tumors. Belisario et al. (1961) showed that both directly injected and non-injected melanoma lesions shrunk or even completely disappeared after treatment with VACV [97], [98]. Hunter-Craig et al. administered the smallpox vaccine into the sites of lesions to 19 melanoma patients. A complete response to treatment has been observed in 6 cases and 5 patients were without deterioration of health during the period from 2 to 22 months after treatment, the nodules have disappeared. However, these data were in contrast to that of the other scientific groups, where Belisario, Milton, Burdick, and Hawk didn't observe widespread tumor regression, tumor shrinking was found only in the place of vaccination [99]. Roenigk et al. treated patients with stage II or III metastatic malignant melanoma and attempted to focus on the immune response. Eight out of 20 patients showed visible regression of melanoma spots. The authors hypothesized that the Vaccinia virus caused cell cytotoxic immunity against melanoma cells [100]. In 1995, Mastrangelo et al. conducted a Phase I clinical trial. Unmodified VACV was injected into patients with recurrent superficial melanoma. They showed that the presence of neutralizing antibodies against VACV didn't prevent its replication in tumor lesions during at least the first 4 days after injection. In 1978, a case report from the Medical College of Wisconsin attracted the attention of many scientists to oncolytic VACV therapy. A 3-year spontaneous remission after smallpox revaccination was documented in a 78-old-year man with chronic lymphocytic leukemia (CLL) [101].

At that time, oncolytic VACV clinical studies weren't limited to just melanomas. Dryvax vaccine was applied intratumorally to patients with bladder cancer. It resulted in inflammation and attraction of eosinophils, plasma cells, and lymphocytes to the tumor site in three out of four patients. There were no major side effects documented. These 3 patients were in 4-year remission after treatment. This study showed how safe the intratumoral injection of the virus is, resulting in just inflammation of the injected region [102]. In another remarkable work, the authors used a non-modified Dairen I (DI) smallpox vaccine, attenuated by several passages in different species' cells at suboptimal conditions (33℃ incubation temperature). The virus was injected intravenously into two patients with metastatic lung and kidney adenocarcinomas, resulting in significant regression of tumors and also metastatic lesions [103]. Later, the same group conducted another study, where a 67-year-old patient with IgA multiple myeloma was administered with the DI vaccine, which resulted in reduced levels of monoclonal IgA and an increased number of NK cells. All these findings confirm that wild-type VACV have an

antitumor effect in different cancer types and, at the same time, are absolutely safe with zero or close to zero side effects [104].

In the past two decades, more than 650 different VACV based recombinant viruses have been constructed. The most well-studied oncolytic Vaccinia virus up to now, based on the number of publications, is GLV-1h68 (also known as GL-ONC1), first constructed by Zhang et al. in 2007. This novel strain was based on the L-IVP backbone with genetically inactivated genes encoding thymidine kinase (J2R), F14.5, and hemagglutinin (A56R) by insertion of genes encoding GFP (Green Fluorescent Protein), the bacterial β-galactosidase, and β-glucuronidase, respectively [105]. In the first experiments *in vivo*, the recombinant GLV-1h68 virus strain was injected either intratumorally or intravenously into mice bearing implanted different human tumor xenografts. The results showed that the VACV strain replicated in the tumors and significantly reduced their size. Furthermore, it has been shown that deletion of the genes encoding thymidine kinase (TK) and hemagglutinin improved GLV-1h68 tropism [105], [106], [107]. Later, it was demonstrated in Phase I human clinical studies that this approach, in combination with chemo- and radiotherapy, is safe for cancer patients with advanced head and neck cancer as well as for patients with advanced-stage peritoneal carcinomatosis [108], [109]. A Phase Ib&II study is currently ongoing with 64 patients with recurrent or refractory ovarian cancer peritoneal carcinomatosis (NCT02759588). The virus treatment was performed either alone or in combination with chemotherapy/bevacizumab. The safety and efficacy of this study have been presented on the poster section at the 51st Annual Meeting of the Society of Gynecologic Oncology [110].

Another recombinant VACV strain is JX-594 or Pexa-Vec that was constructed on the Wyeth strain base by inserting human GM-CSF and β-galactosidase (lacZ) for more specific cancer cell infection and enhancement of virus immunostimulatory activity and replication capabilities. It helped increase antitumor immunity via the recruitment and activation of cytotoxic T lymphocytes (CTL) and antigen-presenting cells (APC). The JX-594 was first engineered and characterized as an oncolytic agent by Kim in 2006 [111]. Clinical trials of Phase I, II are now completed [112], [113], [114], [115], [116], [117]. It was planned to recruit 600 advanced liver cancer patients into a Phase III human clinical trial of Pexa-Vec in combination with sorafenib. However, for an unknown reason, it was terminated early, after recruiting approximately two-thirds of the planned number of patients. Obtained data didn't demonstrate any significant clinical benefit when compared to the conventional treatment, so

the antitumor efficacy of the Pexa-Vec remains unclear and requires further investigation (NCT02562755) [118].

The recombinant vvDD strain of VACV (also called JX-929 or vvDD-CDSR), based on the Western Reserve (WR) strain, has deletions in both TK (thymidine kinase) and VGF (vaccinia growth factor) genes and carries GFP as a reporter gene. The secreted VGF protein is a viral analog of EGF (epidermal growth factor) protein. It is released from the infected cells during virus infection and stimulates the replication of neighbouring epithelial cells, thereby increasing overall virus yield [119]. In preclinical studies, the antitumor efficacy of the virus was shown against a variety of malignancies, including ovarian cancer, liver metastases, and colon cancer [120], [121]. The results of Phase I clinical trial of vvDD on 17 patients with different solid types of cancer showed that a high dose of vvDD (3×109 PFU) wasn't toxic; the virus was selectively replicating in injected as well as in non-injected tumors. One patient's response to the therapy resulted in his arm-located melanoma tumor shrinking to the point of being safely removed by surgery with negative margins. However, the overall effect of the virus wasn't significantly better than the conventional therapy [122]. Later, in the second clinical study with 11 patients with advanced colorectal or other solid cancers, no toxicity was demonstrated, and a strong Th1-mediated immunity response against the virus and probably the cancer was observed. There were almost no clinical benefits, but it was exceptionally safe, so it could probably be improved by combining the virus with other therapeutic agents such as checkpoint inhibitors [123].

Table 2. Examples of modified oncolytic Vaccinia virus used in clinical trials. Source: www.clinicaltrials.gov

Virus name	Transgene expressed/ combination	Tumor type	Phase	Status of research	Ref.
JX-594	GM-CSF/ anti-PD-1	Renal cell carcinoma	I	Recruiting	NCT03294083
JX-594	GM-CSF/ ipilimumab	Liver cancer	I	Recruiting	NCT02977156

Table 2. Continued

Virus name	Transgene expressed/ combination	Tumor type	Phase	Status of research	Ref.
JX-594	GM-CSF/ cyclophosphamide and avelumab	Solid tumors and soft tissue sarcoma	I&II	Recruiting	NCT02630368
JX-594	GM-CSF/ sorafenib	Hepatocellular carcinoma	II	Completed	NCT02630368
JX-594	GM-CSF	Hepatocellular carcinoma	II	Completed	NCT02630368
JX-594	GM-CSF/BSC	Hepatocellular carcinoma	II	Completed	NCT01387555
T601	FCU1/ 5-Fluorocytosine	Solid tumors	I&II	Recruiting	NCT04226066
TBio-6517	FLT3L, IL-12, αCTLA-4/ Pembrolizumab	Solid tumors	I&II	Recruiting	NCT04301011
GL-ONC1	Ruc-GFP, β-Galactosidase/ β-glucuronidase/ Bevacizumab	Ovarian cancer, peritoneal carcinomatosis cancer of fallopian tube	I&II	Ongoing	NCT02759588
GL-ONC1	Ruc-GFP, β-Galactosidase/ β-glucuronidase	Head and neck cancer	I	Completed	NCT01584284
GL-ONC1	Ruc-GFP, β-Galactosidase β-glucuronidase	Solid tumors	I	Completed	NCT00794131
vvDD	Cytosine adeaminase and somatostatin receptor	Solid tumors	I	Completed	NCT00574977

1.3. Vaccinia virus for canine cancer treatment

Mouse models are extremely useful for the study of cancer development, progression, and therapy response. They also have several disadvantages; mouse tumors are usually not spontaneous but mostly implanted tumors. Mouse as a host is not supporting efficient VACV replication. Mice have a short lifespan and are slightly different from the human tumor microenvironment (TME) and metastases formation [124]. Due to these facts, there may be a poor correlation between preclinical and clinical trials, leading to difficult interpretations and unexpected effects. Therefore, other models for better understanding and modeling of human cancer are needed. The spontaneous canine tumor models would be much more comparable to human tumors. [125], [126]. Canine tumors are usually relatively slow-growing, similar to human cancers [6]. Many mammary canine tumors have similar molecular and histological features, hormonal profiles, as well as clinical manifestations to human breast cancer [127], [128]. Thus, understanding and studying the action of oncolytic viruses in spontaneous canine tumors should be a very valuable benefit in human and veterinary oncology. Several preclinical studies with canine cancer patients have been completed with oncolytic viruses, including VACV [129].

Gentschev et al. showed that GLV-1h68 efficiently infected, replicated in, and killed canine mammary pleomorphic adenoma (ZMTH3) cells *in vitro* as well as inhibited tumor growth in canine tumor xenografts *in vivo* [130]. In another set of experiments, two strains of VACV, non-modified isolate LIVP1.1.1, and GLV-1h68 efficiently eliminated canine soft tissue sarcoma (STSA-1) xenografts. Not attenuated non-modified LIVP1.1.1 strain had higher oncolytic potential than highly attenuated GLV-1h68 [131]. Later the group also evaluated that vaccinia virus strain GLV-1h109 successfully replicated and lysed STSA-1 and canine prostate carcinoma (DT08/40) in murine xenograft models [132]. The GLV-1h109 strain was derived from GLV-1h68 by replacing the lacZ gene (beta-galactosidase) with the anti-VEGF single-chain antibody GLAF-1. GLAF-1 protein (anti-VEGF single-chain antibody) can specifically bind to VEGF, leading to reduced formation of blood vessels. In contrast to direct oncolysis of cancer cells, it has also been shown that GLAF-1 protein expressed in the tumors and inhibited angiogenesis and increased recruitment of innate immune cells to tumor site [133]. LIVP6.1.1, one of the plaque purified clones from the LIVP Vaccinia mix, has also shown strong oncolytic activity in canine tumor cells. The authors confirmed that strain LIVP6.1.1 efficiently eliminated melanoma (CHAS), osteosarcoma (D-17), soft tissue sarcoma (STSA-1), and prostate carcinoma (DT08/40) canine tumor cell lines. It killed more than 82%

of tested cells. The therapeutic effect of LIVP6.1.1 was determined on the canine STSA-1 and DT08/40 subcutaneous xenografts. Significant tumor regression was found in both types of tumor compared to the PBS control [134].

The clinical trial in canine cancer patients with various tumor types treated with LIVP6.1.1 (trade name V-VET1) was done by Genelux Corporation, but the data has not been published up to date [135]. The study's findings were being analysed; however, no harmful effects of viral inoculation have been found (AA Szalay, University of Wuerzburg, Germany, personal confidential communication). Adelfinger et al. tested new recombinant VACV GLV-5b451 expressing the anti-VEGF single-chain antibody GLAF-2 (doesn't have FLAG-Tag unlike GLAF-1) in different canine cancer cell lines and compared it with the parental strain LIVP6.1.1. The mammary adenoma (ZMTH3), prostate carcinoma (CT1258), mammary carcinoma (MTH52c), and soft tissue sarcoma (STSA-1) were permissive and supported high replication of GLV-5b451. The construct showed a stronger oncolytic effect than both parental LIVP6.1.1 strain and GLV-1h109 (construct expressing GLAF-1 protein). Hence, it can be a promising candidate for therapy for cancer dog patients with CSTS [136].

Béguin et al. treated canine mammary tumor explants with highly attenuated strain TG6002 [137]. This strain is based on attenuated by deletions in RR (a subunit of the ribonucleotide reductase) and TK locus of Copenhagen backbone armed with an FCU1 suicide gene. FCU1 gene expresses a chimeric protein that catalyses the conversion of non-toxic 5-fluorocytosine (5-FC) into toxic metabolites 5-fluorouracil (5-FU) and 5-fluorouridine monophosphate. It helps to target chemotherapy directly to the tumor site [138]. The data in this work investigated that TG6002 efficiently replicated and lysed canine fibroblast cancer (A72) *in vitro* and canine mammary carcinoma (REM134) cell lines in the canine xenograft model. Two years later, the same group experimented on seven healthy Beagle dogs. No side effects were reported, and no virus particles were detected in blood or any other fluids or organs of the dogs after intramuscular injection of 5×10^7 PFU/kg of TG6002. The authors claim that the oncolytic potency of TG6002 can now be further tested in canine/human clinical trials [139].

A Finnish scientific group designed oncolytic Vaccinia virus strain vvdd-hCD40L-tdTomato based on the Western Reserve strain with complete deletion of VGF and a partial deletion of TK and inserted lacZ, human CD40L, and tdTomato. CD40L has been found to induce Th1 responses, downregulate the suppressive effect of Treg cells, and be able to directly induce apoptosis in tumor cells in both preclinical and Phase I clinical trials of cancer [140]. Vaccinia

virus vvdd-hCD40L-tdTomato was tested first *in vitro* on the canine osteosarcoma (Abrams and D17) and prostatic carcinoma cells (ACE-1). At lower viral doses, the virus replicated in D17, ACE-1 significantly faster than the control vvdd-tdTomato virus (which is not cytokine-armed), but not in the Abrams cells. Since human CD40L doesn't function in mice, *in vivo* activity of the virus was tested in a subcutaneous xenograft canine ACE-1 tumor model with a new construct, vvdd-luc, expressing luciferase. Vvdd-luc was able to delay tumor growth in the model significantly. To check the action of human Cd40L, the authors infected A549 cells with Ad5/3-hTERT-CD40L strain and then collected the supernatant from the infected cells. Ad5/3-hTERT-CD40L is an adenovirus-based vector carrying human telomerase reverse transcriptase (hTERT) promoter for tumor selectivity and human CD40L for increased efficacy [141]. Then they tested the function of CD40L by incubating canine PBMCs with supernatants from Ad5/3-hTERT-CD40L infected cells, which resulted in increased secretion of IL-8 from PBMCs. This confirmed that human CD40L could activate canine PBMCs and can potentially be used in the treatment of dogs as an immunostimulatory agent [142]. The safety and biodistribution of this strain vvdd-hDC40L-tdTomato were also examined in a Phase I trial in two healthy Beagles. There were only a few moderate side effects in the second canine patient. In both dogs, hematology, different blood tests, and histologic examination of the bone marrow showed no noticeable changes and no sign of toxicity. Finally, a large dose of oncolytic vvdd-hCD40L-tdTomato based on WR strain appears to be safe in dogs [143]. Currently, canine patients with inoperable solid tumors are recruited for the Phase I clinical trial.

1.4. Carrier cells for VACV delivery

It is well established that the first administration of the Vaccinia virus gives a rapid immune response against the virus even if the patients were not vaccinated against smallpox [144]. This inactivation does seriously minimize the introduced dose of VACV as an oncolytic agent. However, naked virus particles can't survive in the blood flow for a long time (usually for a few minutes only). For this reason, to deliver the virus particles to tumors and to protect the virus against inactivation of the host immune system (circulating neutralizing antibodies, complement proteins, circulating blood cells, liver and spleen macrophages) and physical barriers such as tumor extracellular matrix, it was suggested by several groups to hide viral particles in or by carrier vehicles [145]. This way, one can increase the selectivity of the

treatment and may reduce the dosage of the virus without reducing its effectiveness. It would also be beneficial if the carrier cell system itself could be a "factory" and amplify the virus. However, the virus should replicate slower or less efficiently than in tumor cells to prevent the fast lysis of carrier cells. Based on numerous preclinical studies, there are three known types of carrier delivery cells: a) immune cells, b) tumor cells, and c) progenitor cells [146]. It would be perfect if these cells could not only protect the virus from inactivation but specifically enhanced delivery to tumor sites and in addition also have antitumor efficacy on their own. Another clinical advantage would be isolation and culturing/manufacturing methods.

One of the possibilities is to infect tumor cells with the Vaccinia virus and use patient-specific autologous cells as carriers, or culturing and manufacturing of allogeneic cell lines or use cancer cells themselves as carriers. The advantages of using patient's tumor cells are that they are usually easy to obtain and culture and are highly permissive to VACV infection. Guo et al. demonstrated this concept on the immunocompetent mice model using MC38 cancer cells expressing luciferase as a carrier system. The authors tested different combinations with both vvDD-infected and intact cells. In all cases, no implanted cell-specific tumor formation in recipients was detected, proving that these cells are safe for using them as virus carriers. Furthermore, in combination with tacrolimus, mycophenolate mofein, and methylprednisolone sodium succinate, the simultaneous addition inhibited immune response against VACV and significantly improved viral entry and replication in tumors [147].

Another potential cell carrier for the Vaccinia virus, which has already been tested, is CIK (cytokine-induced killer) cells. These cells belong to a specific population of cytotoxic T cells derived from blood after stimulation with interferon-γ, CD3-specific antibodies, and interleukin-2 (IL-2). They are usually easier to obtain than T-cells. The pilot study demonstrated that CIK cells alone homed to the tumor site 72 h after administration into the blood. Thorne et al. in 2006 first showed that it could be supplied for the VACV. The authors took CIK cell cultures and infected them with vvDD. After 48 h there was no virus progeny released. They demonstrated that CIK cells infected with the virus don't change their phenotype and did not influence the surface markers of the cells. At the same time, there were no virus antigens on the surface of the infected cells. In addition, the authors also showed the interaction of both infected and uninfected CIK cells with human ovarian tumor cells *in vitro*. An oncolytic killing effect has been demonstrated in both infected cells due to active viral replication and in CIK cells alone. CIK cell distribution infected with the virus was assessed using luciferase-labeled vvDD virus in human ovarian cancer xenograft bearing mice. Three

days after virus administration, the luminescence from mice treated with infected CIK was higher than in the case of the virus alone. These results confirmed that using CIK cells as carrier vehicles for oncolytic Vaccinia virus to tumor sites was effective [148].

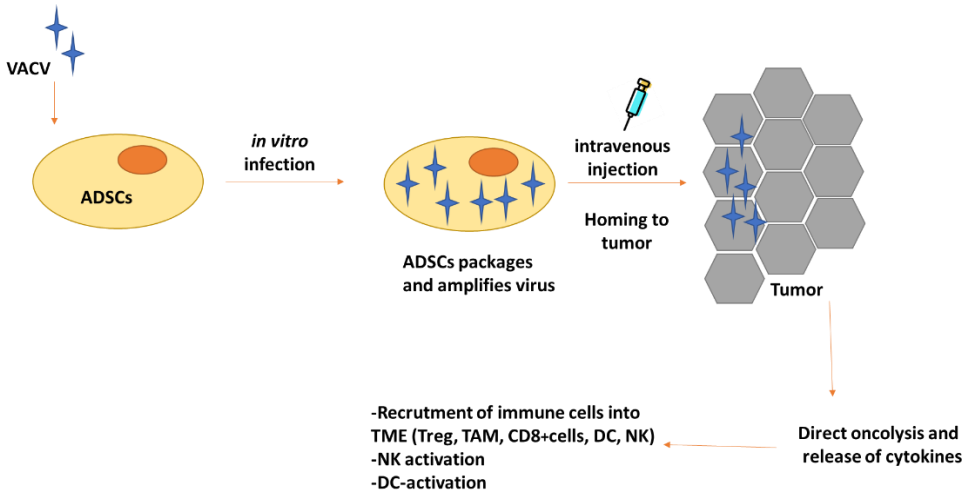
Progenitor cells are also a good candidate for being an effective packaging system. Mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSC/AdMSC), neural stem cells have become promising cellular vehicles for antitumor drug delivery due to their natural ability to home sites of inflammation [149]. MSCs can be derived from various tissues, such as bone marrow, adipose tissue, placenta, umbilical cord, dental pulp, amniotic fluid, and Wharton's jelly [150]. MSCs are very similar to ADSC/AdMSC and can differentiate into a variety of cell types. MSCs and ADSC/AdMSC have the natural homing to the sites of inflammation, damaged tissue, and also to the tumor microenvironment (TME) [149], [151]. In addition, MSCs have immunosuppressive functions, can inhibit proliferation of NK cells, cytokine production, differentiation, and function of DCs, and induce regulatory T cells [152]. An *in vitro* study demonstrated that MSCs don't express HLA-I and costimulatory molecules such as CD86, CD154, CD80, CD40, and CD83 [153]. Instead, MSCs release different cytokines that are responsible for immunosuppression, such as TGF-β, IL-10, IL-6, iNOS (inducible nitric oxide synthase), IDO (indoleamine 2, 3-dioxygenase), and heme oxygenase-1 [154]. It has recently been shown that MSCs suppress the activity of a number of key immune cell types such as DCs, T and B lymphocytes, monocytes, macrophages, and NK cells [155], [156].

MSCs were first isolated in 1970 from bone marrow by Friedenstein et al. [157]. According to ISCT (International Society for Cellular Therapy) recommendation, the stemness of MSCs should be confirmed by the presence of surface markers CD90, CD105, CD73 and absence of CD79α, CD14, CD11b, CD19, HLA-DR and as well as hematopoietic markers CD34 and CD45. MSCs should be able to differentiate into adipocytes, osteoblasts, and chondroblasts under selective conditions in cell cultures. There are two main sources for obtaining MSCs: bone marrow and fat tissue. If the cells are obtained from bone marrow, they are named BM-MSCs; if they are extracted from the fat tissue, they should be called ADSC/AdMSC. These two types of MSCs share a lot of similar features. It is preferable for the clinical application to use ADSC/AdMSC due to their simple harvest from fat tissue with minimally invasive liposuction technology. They can be efficiently expanded in cell cultures. ADSC/AdMSC can be isolated from stromal vascular fraction (SVF) [158]. ADSC/AdMSC are also more stable in cell culture when compared to BM-MSCs [159]. MSCs are well

characterised, safe, and can be used in clinical trials [160]. Most preclinical trials confirmed that MSCs could serve as carriers for oncolytic virus delivery and their usage other benefits to patients [161], [162]. The combination of MSCs and Herpes Simplex Virus (HSV) in immunodeficient mouse models resulted in protected and targeted delivery of the virus with melanoma tumor sites and prolonged the animals' survival. In immunocompetent melanoma-bearing mice, the combination with anti-PD-L1 elicited an increase of $CD8^{+}$T lymphocyte infiltration and as well as significantly prolonged the mouse survival [163]. In the different preclinical studies, the combination of measles virus and with MSCs led to a more selective virus delivery to hepatocellular carcinoma (HCC), which was implanted in the mouse liver; *in vitro*, MSCs could also protect the measles virus in the presence of anti-measles virus antibodies [164]. The efficacy of this concept was also confirmed by Castleton et al. in acute lymphoblastic leukemia tumor xenograft. The authors demonstrated that MSCs loaded with measles virus prolonged the survival of mice and improved the antitumor effect in comparison to treatment with virus alone [165]. No completed human clinical trial data were reported yet.

The only published clinical study used the combination of wild-type Vaccinia virus - ACAM2000 (approved vaccine in the USA) and autologous adipose SVF cells. A Phase I study of the intravenous and intratumoral application of a combination of ACAM2000/SVF has been completed with the participation of 24 patients with solid tumors and 2 patients with AML. The safety of the treatment has been demonstrated, with no severe side effects have been reported. In addition, treatment feasibility, infectivity, and significant response to the therapy in some patients have been shown [166]. This concept may be considered as a promising strategy in enhancing the antitumor efficacies of virotherapy.

In the current work, I focused my attention on the Adipose-derived Stem Cells (ADSC) as a delivery system for VACV. ADSC have strong inherent tropism to the tumor microenvironment where they can regulate the tumor size [167], it also supports virus replication [168], [169], [170], [164], protects virus particles against the immune host neutralization, and also suppresses immune response on the way to the tumor site [171].



A scheme of the “Trojan horse” concept using ADSCs and VACV

CHAPTER 2: AIMS

It has been demonstrated during the last several years that oncolytic viruses, especially the Vaccinia virus, in combination with conventional cancer treatments, can provide a solid basis for therapy design and can be successfully used in clinical trials [172] [173] [174]. It was shown that the Vaccinia virus could effectively replicate in different tumor cells, has a natural ability to home to tumor sites, and can lyse malignant cells - releasing tumor-associated antigens that can trigger the antitumor immune response. Systemic administration of the virus allows treatment of tumors in deep tissue that are otherwise not accessible to treatment. However, intravenously-administered VACV can be rapidly neutralized by the host immune defence system. Therefore, it is necessary to design a protection system for virus delivery to patients.

Some of the spontaneous canine cancers have grown to be relevant tumor models for human cancers. In addition, many characteristics of caner, such as its histological status, hormonal status, molecular genetic alteration, microenvironment, and response/resistance to therapy, are pretty similar between the two species. Therefore, assessing the potency of oncolytic viruses with regard to spontaneous canine cancers should be could be beneficial for human medicine.

The main goal of this work is to test several wild-type strains of the Vaccinia virus to understand whether or not they can be used for a feasible country-origin specific therapy of cancer-based on readily available vaccine stockpiles as wild-type vaccines or as the basis of gene-modified oncolytic virus therapy. For this purpose, we initiated a comparative analysis of oncolytic potency of various polyclonal as well as single clone, plaque purified, wild-type Vaccinia virus strains on a panel different of human cancer cell lines *in vitro*. In addition, the "Trojan horse" concept should also be tested using the wild-type VACV strains in combination with human ADSC *in vivo*. In clinical trials, the therapeutic efficacy of the combination of the wild-type viruses loaded into hADSC was studied separately in the lung adenocarcinoma (A549) mouse xenograft model.

The second aim of this thesis is to test whether the wild-type strains are suitable for the therapy of canine cancers. For monitoring effectiveness, the Vaccinia virus wild-type strains are armed with TurboFP635, a light-emitting reporter gene. We tested whether the C1-opt1, W1-opt1, or L3-opt1 (opt-1 represents a virus where Turbo-FP635 is inserted in the TK locus) can infect and replicate in canine AdMSC - to see if these cells can be used as carriers for the "Trojan horse" concept. For this purpose, we used the canine Soft Tissue Sarcoma (STSA-1) xenograft mouse model. Viral replication as well as viral distribution in organs, blood serum,

and tumors was analysed. The extent of tumor regression after intravenous injection was also analysed. In the future, the combination of VACV and canine AdMSC should be tested in autologous canine cancer patients.

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals and enzymes

Materials	**Manufacturer**
Acetic acid (3%)	Sigma
Alcian blue 8 GS	Carlroth
Alizarin Red S	Carlroth
Crystal violet	Sigma
Carboxymethylcellulose (CMC)	Sigma
Dubelcco's Modified Eagle's Medium (DMEM)	Fisher
DMEM low glucose	Fisher
Dimethylsulfoxide (DMSO)	Sigma
Phosphate Buffered saline (PBS)	Sigma
EDTA	Sigma
EDTA-Trypsin	Fisher
Ethanol (96%)	Chemical dispensing/Sigma
Tryple (TrypLE^{TM})	Fisher
Tris-HCl	Fisher
Fetal Bovine Serum (FBS)	Fisher
Fetal calf serum	Capricorn
Formaldehyde (37%)	Sigma
Isopropanol	VWR
Non-essential amino acid (NEAA)	Fisher
MTT 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazoliubromide thiazolyl blue	Sigma
Penicillin/Streptomycin	Sigma
Sodium Azide	Fisher
Stem cell supplement mTeSR^{TM}1	Stemcell technologies
Optical microscopy (EVOS M5000)	Termo Fisher Scintific
Oil Red O solution	MERCK
5% Human Platelet Extract	Cook Regentec, Stemulate, PL-SP-100

3.1.2. Buffers and solutions

CMC overlay medium	1.5% Carboxymethylcellulose 2% FBS 1% pen/str 1× DMEM medium
Crystal violet solution	1.3g crystal violet 5% ethanol 30% formaldehyde (37%) dd H20
Buffer for FACS	Ice cold PBS 10% FBS 1% sodium azide
DMEM 10%	10% FBS 1% pen/str DMEM
DMEM 2%	2% FBS 1% pen/str DMEM
Freezing-medium for cells	90% FBS 10% DMSO
MTT solution	2.5 mg/ml dimethyl thiazolyl diphenyl tetrazolium salt (MTT) DMEM without phenol red
Tissue homogenization buffer	50 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 7.4) supplemented with Complete Protease Inhibitor Cocktail
10% HCl in isopropanol	10 mL of HCl 90 mL of Distilled Water
3.7% PFA	3.7g PFA in 37C H_20 ad NaOH unit solution clears 10 mL 10×PBS (pH 7.4) ad 100 mL d H_20

3.1.3. Cell lines and media

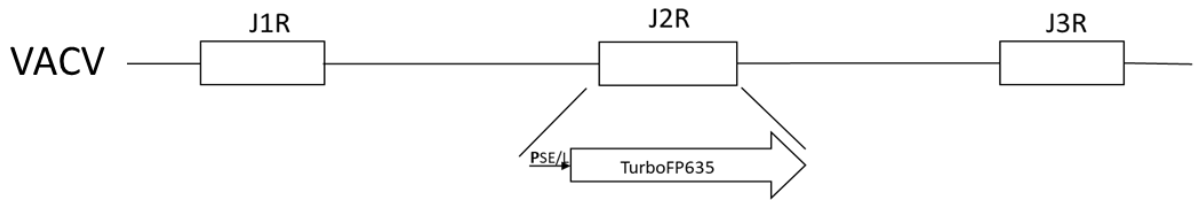
CV-1	Green monkey kidney fibroblast cell line (ATCC) DMEM with 10% FBS
A549	Human lung carcinoma cell line (ATCC) DMEM with 10% FBS, 1% Pen/Strep
PC-3	Human prostate carcinoma cell line (ATCC) DMEM with 10% FBS, 1% Pen/Strep
STSA-1	Canine soft tissue sarcoma cell line (kind gift from Dr. A. MacNeill (University of Colorado, USA) and was derived from a canine patient with a low-grade II soft-tissue sarcoma) [131] MEM with Earle's salt with 10% FBS, 1% Pen/Strep, 2 mM Glutamine, 1 mM Sodium pyruvate, 0.1 mM non-essential amino acids
CT1258	Canine spontaneous prostate carcinoma cells (kind gift from Dr. Nolte School of vet. medicine Hannover) [175] DMEM high glucose with 20% FBS, 1% Pen/Strep
MDA-MB231	Human breast adenocarcinoma (DSMZ) DMEM with 10% FBS, 1% Pen/Strep
FaDu	Human Pharynx carcinoma DMEM with 10% FBS, 1% Pen/Strep
HNT-13	Human head and neck carcinoma (kindly provided by Stem Immune) DMEM with 10% FBS, 1% Pen/Strep
HNT-25	Human head and neck carcinoma (kindly provided by Stem Immune) DMEM with 10% FBS, 1% Pen/Strep
HNT-35	Human head and neck carcinoma (kindly provided by Stem Immune) DMEM with 10% FBS, 1% Pen/Strep
hADSC	Human Adipose derived mesenchymal stem (cells were obtained from adipose tissue from healthy donors) DMEM 5% Human Platelet Extract (mTeSRTM) 1% Pen/Strep
cAdMSC	Canine Adipose derived mesenchymal stem cells (were obtained from adipose tissue from healthy canine donors) DMEM low glucose 10% fetal calf serum 1% Pen/Strep

3.1.4. Vaccinia virus strains

Virus	Description
L0	Parental Lister (LIVP) strain
L1	LIVP1.1.1 is a plaque purified isolate of the non-attenuated LIVP strain
L3-opt1	LIVP 1.1.1. expressed FP635, plaque purified
W0	Parental Wyeth strain
W1	ACAM2000, clone 2, derived from W0, plaque purified
W1-opt1	ACAM2000 expressed FP635, plaque purified
C1	Clone derived from parental Copenhagen strain; plaque purified
C1-opt1	Clone derived from parental Copenhagen strain expressed FP635, plaque purified
T0	Parental Tian-Tan strain
T1	Clone number 5 derived from T0; plaque purified

3.1.5. Recombinant Vaccinia virus constructs

Sucrose gradient purified C1-opt1, W1-opt1 and L3-opt1 Vaccinia viruses were provided by Genelux and StemVac GmbH, Bernried, Germany. TurboFP635 expression cassettes was inserted into thymidine kinase (TK) of Copenhagen (C1), Wyeth (W1) or LIVP (L3) strains and used as reporters to monitor the ability of the virus to infect tumor cells.



Schematic representation of construction of C1-opt1; W1-opt1; and L3-opt1; VACV strains

3.1.6. Kits

LDH Cytotoxicity Assay Kit (CyQUANT™, Invitrogen)

Freezing Medium for hADSC/cAdMSC (synth-a –Freeze, Fischer)

ADSC-GM (SingleQuots media, Lonza)

StemPro™ Adipogenesis Differentiation Kit (Fischer)

StemPro™ Chondrogenesis Differentiation Kit (Fischer)

StemPro™ Osteogenesis Differentiation Kit (Fischer)

3.1.7. Laboratory materials and equipment

Biosafety hood	Camfil HERA safe
Flow cytometry	BD Accuri
Cell culture plates (6-, 24- & 96-well)	Cellstar
Cell culture flasks	Cellstar
Cell Lab Quanta SC Flow Cytometer	Beckman Coulter
Cell scrapers	SPL
Cell culture incubator	New Brunswick Galaxy 170 S
Centrifuge for reaction caps	Eppendorf
Cryo tubes 2 ml	Clearline
Digital caliper	VWR
Falcon (15 ml, 50 ml)	Cellstar
Syringe	Penta
Pomogonizer	MP FastPrep-24
Multichannel pipettor 8 channels	INTEGRA
Multichannel pipettor 9 channels	PEQLAB
Needle 29G	BD
Pipette controller	INTEGRA
Pipettes 10, 20, 200 & 1000 µl	Eppendorf
Pipettes 5, 10 & 25 ml	Sarstedt
Refrigerator	Liebherr
Sample tubes (0.5 ml, 2 ml)	Eppendorf
Scalpel, sterile disposable	Ratiomed
Scales	KERNewj
TC20™ cell counter	Bio-Rad
ELISA-photometer Sunrise™	Tecan, Maennedorf, Switzerland
Tabletop centrifuge	Heraeus biofuge fresco
Titer plate shaker ‘Mini rocker MR-1’	PEQLAB, Erlangen, Germany
Vortex mixer	VWR
Cell centrifuge	VWR
Table microscope	Zeiss
Water bath	GFL

3.1.8. Laboratory animals

All *in vivo* experiments were carried out with Female Athymic Nude-Foxn1^{nu} (6 weeks old) and maintained under approved protocols by animal welfare regulations of the government of Upper Franconia, Germany, and conducted according to the German animal protection guidelines (permit number: 55.2.2.-416 2532-2-344 and 55.2.2.-416 2532-2-610).

These mice have a (dysfunctional) rudimentary thymus which leads to a limited immune response due to the lack of T-cells. Otherwise, B cells and NK cells function to a certain extent [176]. The weakened adaptive immune response enables transplantations and growth of human tumor tissues and cells with no rejection caused by the mouse immune system, which makes this mouse breed an ideal model in immune allogenic studies. Another chromosomal mutation leads to a completely hairless phenotype in nude mice [177].

Phenotype of an athymic nude FoxN1 mouse

3.2. Methods

3.2.1. Cell biological techniques

All cell cultures have been carried out under sterile conditions under laminar flow hood. The equipment and the bench surface were disinfected with 80% ethanol prior to use. The antibiotics penicillin and streptomycin (pen/str) were added to the culture media to avoid bacterial or fungal contamination. All the used materials were sterilized and sprayed with 80% ethanol before entering the hood.

The different cell lines were grown in specific cell culture media, which contain a variety of nutrients, vitamins, salts, and buffer substances. Phenol red was frequently used as a pH indicator in most of media. Media were supplemented with antibiotic-solution (100 U/mL penicillin G, 100 U/mL streptomycin), 10% heat-inactivated Fetal Bovine Serum (FBS), or 20% FBS for STSA-1 cells, or 5% stem pellet (instead of FBS) for hADSC, kept at 4°C in the refrigerator, and warmed up in a 37°C water bath before use. For cAdMSC was used DMEM low glucose was supplemented with 10% Fetal Calf Serum (FCS). All cell lines were grown under standard cell culture conditions at 37°C in a cell incubator in 5% CO_2 and 100% relative humidity inside.

3.2.1.1. Culturing of mammalian cells

CV-1 cells, human cancer cell lines (A549, PC-3, MDA-MB231, FaDu, HNT-13, HNT-25, HNT-35), canine prostate cancer cell lines (CT1258 and STSA-1), human Adipose tissue-derived Stem Cells (ADSC) and canine Adipose - derived Mesenchymal Stem Cells (cAdMSC) were cultured in well plates or flasks, depending on the experimental use. The stock cell cultures were grown cultured in 175T flasks. Cells were grown in a humidified 5% CO_2 incubator at 37°C. At 80-90% confluence, the cells were rinsed with PBS and treated with 5 ml of EDTA-trypsin or with triple (TrypLE™) for the more sensitive cell lines, like hADSC and cAdMSC. After 5-8 min (cells should become detached), the flasks were shaken to completely detach the cells. Then, 10 ml of regular growth medium containing Mg^{2+} and Ca^{2+} was added to stop the EDTA-trypsin reaction. In the case of TrypLE treatment, there is no need for the inactivation step. Cells were resuspended by being pipetted up and down several times to disrupt the cell clumps, followed by cells being counted with an automated cell counter

(TC20™). The cells were then ready for passaging with a designated ratio into suitable plates or flasks.

3.2.1.2. Freezing and thawing of cells

Cells with a confluence of 90-100% were washed with PBS once and trypsinized with EDTA-trypsin or TrypLE (for hADSC, cAdMSC). Then cells were centrifuged for 5 min at 1200 rpm at room temperature (r.t.). The cell pellet was diluted with a freezing medium and was transferred to a cryotube. Tubes were put in the special freezing box, rapidly cooled by 1°C per 1 min at 80°C. Tubes were then stored in liquid nitrogen permanently.

To thaw cells, the frozen tubes were taken from liquid nitrogen tank storage, placed into the water bath at 37°C until most of the liquid was defrosted. The cells were resuspended in 5 ml of fresh growth medium in a 15 ml falcon, then centrifuged 5 min at 1200 rpm and aliquots were transferred to fresh medium into cell culture flasks for further incubation at 37°C.

3.2.1.3. Adipose-derived stem cells: isolation and culture

Canine AdMSC were isolated, cultured, and characterised by the Institute of Veterinary - Anatomy, - Histology and - Embryology, Faculty of Veterinary Medicine, Justus - Liebig University Giessen, Germany.

Human Stromal Vascular Fraction (SVF) was provided to non-cancer donor patients with consent at Clinics in Rancho Mirage and Berkeley Hills, California, USA. Homogenous hADSC (isolated from SVF) were sent to the University of Wuerzburg (for research use only) at passages 2-3. The mesenchymal stem cells were characterized: fibroblast morphology of adherent cells; verification of multilineage differentiation potential into adipogenic, osteogenic, and chondrogenic phenotype after culturing hADSC in specific cell culture media for differentiation (Fisher, Life Technologies), followed by lipid drop (oil red O), bone extracellular matrix (alizarin red) and chondrogenic glycosaminoglycans (alcian blue) staining respectively. The flow cytometry expression profile analysis using monoclonal antibodies (ABs) to human CD44, CD90, CD105, CD34, CD146, CD31, CD14, CD45. All these ABs are presented in **Table 3**.

Table 3. Antibodies

Antibody/Fluorochrome	Company/Cat№/Clone
CD90 mouse IgG1	BD biosciences, 559869, 5E10
CD44 mouse IgG2b	BD biosciences, 555479, G44-26
CD14 mouse IgG2a	BD biosciences, 557742, M5E2
CD45 mouse IgG1	BD biosciences, 555482, HI30
CD105 mouse IgG1	Invitrogen, 12-1057-42, SN6
CD31 mouse IgG1	BD biosciences, 555446, WM59
CD146 mouse IgG1	BD biosciences, 562135, P1H12
CD34 mouse IgG1	BD biosciences, 555824, 581

3.2.1.3.1. Trilineage differentiation potential of human ADSC

Adipogenic differentiation

The hADSC were differentiated into adipocytes when grown in adipogenic induction media (StemPro Adipogenesis Differentiation Kit). Once the adipogenic induction media was added, cell proliferation stopped after 20 days. Then the cells were fixed with 4% paraformaldehyde (PFA) for 30 min, washed and stained with a working solution of 0.5% oil red O for 20 min and then washed with water. Oil droplets were visualised using optical microscopy (EVOS M5000, Thermo Fischer Scientific).

Osteogenic differentiation

When hADSC were grown in osteogenic induction media (StemPro Osteogenesis Differentiation Kit), cells differentiated into the osteogenic lineage and showed mineralization. To confirm osteogenic differentiation, after 21 days, cells were fixed with 70% ethanol, washed twice with water, and stained with 2% Alizarin Red S (pH 4.2) for 30 min. Then, they were washed with PBS, and images were obtained using optical microscopy (EVOS M5000, Thermo Fischer Scientific).

Chondrocyte differentiation

Micromass culture was used for chondrogenic differentiation (StemPro Chondrogenesis Differentiation Kit) by seeding 5-mkl droplets of cell solution on the center of the 96 well plates. A spherical cell pellet was observed after 7 days of culture. After 21 days micromass was washed with PBS and fixed with 4% PFA for 30 min. Then cells were washed again and

stained with 1% Alcain Blue 8GX prepared in 3% acetic acid. The solution was filtered and added to each well and incubated for 2 h. Fixed cells were washed with water. Images were obtained using optical microscopy (EVOS M5000, Thermo Fischer Scientific).

3.2.1.3.2. Immunophenotyping by flow cytometry (FACS analysis)

The flow cytometer is an instrument used to detect and measure the physical and chemical characteristics of a population of cells.

The cells were trypsinized and the pellet was resuspended in PBS. The cells were harvested and washed, then resuspended in a buffer (ice cold PBS, 10% FBS, 1% sodium azide) at a concentration of $1x10^6$ cells/ml. Then the cells were stained in eppendorf tubes with 0.1-10 µg/ml of the primary AB (CD90 (APC), CD34 (APC), CD73 (APC), CD45 (FITC), CD105 (PE), CD44 (PE), CD146 (PE-Cy™), CD31 (PE), CD14 (PE-Cy™)) in the dark for 30 min at 4°C. Then the cells were washed 3 times by centrifuged at 400 g for 5 min and resuspended in 500 µl of ice-cold PBS, 10% FBS, 1% sodium azide solution, and then the cells were kept in the dark on ice or in a fridge at 4°C until analysis. All the anti-human ABs were of mouse origin and obtained from BD Pharmingen, USA. The samples were analysed by BD Accuri Flow Cytometry and C-Flow Plus software.

3.2.1.4. Cell counting

For most *in vitro* experiments, the number of cells per unit volume of a suspension needs to be determined. Cells were counted using automated cell counter TC20™.

3.2.2. Virology methods

3.2.2.1. Infection of cells with Vaccinia virus strains

All the Vaccinia virus strains were stored at -80°C. Before infection, they were thawed and then kept on ice. To prevent the formation of virus aggregates all VACV stocks were vortexed for 30 sec 3 times before infection.

The cell count for each virus infection was determined by the TC20™ cell counter. The required virus amount was calculated using the following formula:

$$\frac{\text{Desired MOI} \times \text{Cell number}}{\text{Virus titer}} = \text{Virus amount}$$

The concentration of each virus strain is defined as Plaque Forming Units (PFU), with infectious virus particles per milliliter. MOI (Multiplicity of Infection) is the ratio of infectious virus particles to cells. For example, MOI of 0.1 means 0.1 virus particle per cell or 1 plaque forming units per 10 cells. The virus amounts needed for each infection experiment were calculated accordingly. The infection medium was prepared by diluting the required volume of virus stock in a medium supplemented with 1% antibiotic solution (10.000 U/ml penicillin G, 10.000 µg/ml streptomycin) and 2% FBS.

3.2.2.2. Determination of the virus titer

Cancer cells were seeded onto the 6 well plates one day before infection. After 24 h in culture, or when cells reached a confluence of 95-100%, cells growth media was supplemented with Vaccinia virus infection medium (supplemented with 2% FBS) containing desired MOIs of virus particles. Cell cultures were incubated for 1 h at 37°C with gentle agitation (every 20 min). After 1 h, the infection medium and virus particles were removed, and the infected cells were cultured further in a fresh, growth (regular) medium.

3.2.2.3. Viral production assay (VPA)

The replication efficiency of VACV strains in various cancer cell lines was compared at the different MOIs. After culturing, cells and supernatants were harvested at different time points post infection (3h, 24h, 48h, 72h and 96h), and viral titers were determined by infecting CV-1 cells. The virus particles were released by 3 cycles of freezing and thawing. Supernatant and cell debris were stored at -80°C for future use.

3.2.2.4. Viral plaque assay

The viral titer in the cell infected samples was determined by standard plaque assay for VACV. The plaque assay is a method to determine the concentration of virus particles in a cell lysate, in suspension, and in tissue homogenates. After virus infection, samples were frozen and

thawed three times, followed by vortexing 3 times for 30 sec. Tenfold dilutions of the samples in DMEM with 2% FBS were prepared. For virus titer determination CV-1 cells was used. These cells were grown in 24-well plates until they reached 95-100% confluence. The CV-1 growth medium was aspirated and 200 µl from the virus containing samples were placed into each well in duplicate. Cells were incubated for 1 h at 37°C, shaken at every 20 min. After this incubation, the CV-1 cells were covered with 1 ml of carboxymethyl cellulose (CMC) medium, which does prevent the virus from spreading further. Cells were further incubated for 40 h at 37°C. During this incubation period, the virus particles replicate in the infected cells, the cells are lysed, and virus particles spread into the neighbouring CV-1 cells. After two days 500 µl of crystal violet (CV) were added to each well and for staining overnight. Plates were rinsed with tap water for the removal of the staining solution. Plaques were visualised in wells; 12 -100 plaques were counted per well. The virus titer was calculated in PFU per milliliter (PFU/ml) using the following formula:

PFU×dilution factor×5= viral titer [PFU/ml]

3.2.2.5. Cytotoxicity assays

3.2.2.5.1. MTT assay

MTT (3(4.5-Dimethyl-2-thiazolyl)-2.5-diphenyl-2H-tetrazolium bromide) assay is a colorimetric assay used to assess the number of viable cells after infection with Vaccinia virus in cell cultures. When cells die, they lose the ability to convert MTT into formazan, thus colour formation serves as a useful and convenient marker for the viable cells' detection. Tumor cells seeded in 96 well-plates, cultured for 24 h and infected in triplicates with the viral constructs with different MOIs (0.01, 0.1, and 1) or mock-infected, with infection medium alone. Following cell culture at different time points (24, 48, 72, and 96 hpi), shortly before performing the assay, the yellow tetrazolium salt MTT was solubilized in PBS at a final concentration of 5 mg/ml, resulting in a light-sensitive yellowish solution. MTT solution (10 µl) was added to infected cells and untreated control cells (100 µl) at a final concentration of 0.45 mg/ml under aseptic conditions in a laminar flow hood. Plates were placed back to the incubator for 2 h. The yellow colour of MTT was changed by metabolically active cells into an intracellular insoluble purple formazan product. After 2 h 110 µl of 10% HCL in isopropanol were added to all wells to dissolve the formazan crystals. An aliquot of 100 µl from each sample

was transferred to a new 96 well-plate and absorbance was measured at 570 nm in a spectrophotometric system from the Tecan, Maennedorf, Switzerland. Decreasing quantities of formazan due to the death of the infected tumor cells over time led to a decrease in absorbance. Uninfected cells are alive and were considered as 100% viable. Cytotoxicity levels were calculated in Microsoft Excel using the following formula:

$$\frac{\textbf{OD (infected cells)}}{\textbf{OD (control cells)}} = \textbf{Percentage of viable cells (\%)}$$

3.2.2.5.2. Lactate dehydrogenase (LDH) cytotoxicity assay

This analysis was performed by a commercial LDH Cytotoxicity Assay Kit (CyQUANT^{TM}, Invitrogen). To determine the release of LDH is a cytosolic enzyme present in many different cell types. LDH is a reliable and sensitive indicator of lactate dehydrogenase released from damaged cells.

Canine AdMSC were seeded into 24 well-plates and infected with C1-, W1-, or L3-opt1 Vaccinia virus strains at MOI 0.5. After 1 h incubation at 37°C, the infection medium was replaced by a fresh growth medium. After 3, 24, 48, 72, and 96 h of incubation, supernatant aliquots were collected from each well and transferred into 96 well-plates. In the case of human ADSC, the cells were seeded into 96 well-plates and infected with different strains of VACV: L0, L1, W0, W1, T0, T1 and C1 at MOI 1. After 48, 72, 96 hpi, and 5 dpi supernatants were harvested and transferred into 96 well-plates. LDH release into the culture supernatants was measured using the Pierce LDH Cytotoxicity Assay Kit, following the manufacturer's instructions. The measurements were performed with the Tecan Sunrise Remote microplate reader (Tecan, Maennedorf, Switzerland) at the wavelength of 490 nm. The results were shown as of LDH as released that of the total LDH content of healthy cells. Data were presented as mean standard deviation (SD), n = 3.

3.2.3. Mouse tumor model experiments

3.2.3.1. Subcutaneous (SC) implantation of tumor cells into mice

To establish canine or human tumor xenograft models (STSA-1 or A549, respectively), approximately 4 x 10^6 of STSA-1 cells or 5 x 10^6 of A549 cells in 100µl PBS were injected subcutaneously into the right hind leg of athymic nude FoxN1 mice, using a 29 G sterile needle and syringe.

The mice were then monitored for swelling of the leg or for any adverse reactions to the tumor cell implant. After the solid tumors did form, their growth was measured and daily monitoring.

The mouse weight was also recorded regularly for the tumor bearing mice during the study. Together with the tumor volume, the net body weight of each mouse was calculated and as sign of the general well-being of mice. The net body weight of the mouse is defined as followed:

$$\frac{\textbf{Body weight [g]} - \textbf{Tumour weight [g]}}{\textbf{1000}} = \textbf{Net body weight [g]}$$

This body weight is later represented as the fractional body weight calculated from the net body weight at any given time over the initial body weight at the starting point of treatment, in this case, day 0. The tumour weight was converted from the tumour volume using the conversion formula: 1 mm^3 = 1 mg.

3.2.3.2. Tumour size measurements

Each tumour is measured 2 times weekly using a digital caliper. The dimensions width (w), length (l), and height (h) of the tumour were noted in mm. The tumor volume was calculated as

$$\frac{\textbf{L (mm)} \times \textbf{W (mm)}^2}{\textbf{2}} = \textbf{Tumour volume (mm)}^3$$

3.2.3.3. Intravenous injection of Vaccinia virus strains alone and a combination of Vaccinia virus infected canine AdMSC or human ADSC in canine or human tumor-bearing mice

For the first experiment after 2 weeks implantation of subcutaneous (SC) STSA-1 cells, a single dose of C1-opt1 alone (2.5×10^6 PFU), cAdMSC alone (2.5×10^5 cells), or a combination of C1-opt1/cAdMSC (2.5×10^6 PFU C1-opt1/2.5×10^5 cAdMSC; MOI of 10) was injected intravenously (i.v.). For the second animal experiment, when the tumor volume (A549 xenograft) reached around 300-500 mm^3, these A549 xenograft mice were randomized into 10 matched groups based on tumor size (n=5 per group): a single dose of 5×10^5 PFU of LIVP 1.1.1 (L1) (a), 5×10^5 PFU of ACAM 2000 (W1) (b) or 5×10^5 PFU of Tian-Tan 1 (T1) (c), 2.5×10^6 PFU of L1 (d), 2.5×10^6 PFU of W1 (e), 2.5×10^6 PFU of T1 (f), or in combination of L1/hADSC (viruses 5×10^5 PFU / ADSC 1×10^5) (g), W1/hADSC (viruses 5×10^5 PFU/ ADSC 1×10^5) (h), T1/ hADSC (viruses 5×10^5 PFU / ADSC 1×10^5) (i) and PBS (100µl). The injection was performed intratumorally. cAdMSC/hADSC and viruses were previously co-incubated for 2 h at 37 ºC in the cell incubator with gentle swirling every 20 min. Tumor volumes were recorded continuously as described above. In addition, mice were monitored for changes in body weight and any signs of toxicity.

3.2.3.4. Determination of Vaccinia virus distribution in tumor tissues, body organs, and in the blood of tumor-bearing mice

To evaluate the viral replication tumors, body organs (spleen, kidney, liver, lungs) and serum of virus-treated animals were sacrificed and the tumors surgically excised at 20 dpi. The harvested tissue was placed in two volumes of homogenization buffer [50 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 7.4)] supplemented with a complete protease inhibitor cocktail. Tumors were homogenized using a MagNA Lyser at a speed of 6.000 rpm for 30 sec (3 times). After three freeze-thaw cycles, supernatants were collected by centrifugation (6.000 rpm, 5 min, 4°C). The viral yield was measured by standard plaque assay using CV-1 cells (please see viral plaque assay methods **3.2.2.4**).

3.2.4. Statistical analysis

All values are presented as the mean ± standard deviation (SD). Statistical analysis in the animal experiments was calculated by one-way ANOVA (GraphPad Prism 5). The difference was statistically significant if the value was less than 0.05 ($p < 0.0001$).

CHAPTER 4: RESULTS

4.1. First aim: Comparison analyses of oncolytic efficacy of wild-type Vaccinia virus strains in human cancers

The oncolytic virus must overcome several natural barriers before it can reach the tumors in patients. When the virus is injected intravenously, most virus particles are immediately cleared by the reticuloendothelial system of the liver and spleen. To overcome these barriers, larger doses of the virus should be used, which increases the cost of treatment and generates additional safety risks. There are also adaptive barriers in the immunocompetent organism, such as the rapid formation of neutralizing antibodies in response to the virus administration, which promotes rapid clearance of the virus from the bloodstream, especially when it needs to be injected several times. In fact, the blood of the cancer patients in the clinical trials usually contains antibodies against the injected virus. This barrier can be partially overcome by injecting the virus directly into the tumors. As an alternative approach, the so-called “Trojan horse” method can be used when the taken host cells are pre-infected with virus particles and reintroduced to the same patient. Viruses “hidden” inside the cell may not be recognized by the host immune system, allowing infected cells to reach the tumors, where the infected cells function as a "factory" for focal reproduction of the virus.

In this part of the thesis, I compared the oncolytic virus efficacy of different wild-type strains and their isolates in cell cultures *in vitro* and also tested the “Trojan horse” concept using human Adipose-derived Stem Cells (hADSC) in the combination of different non-modified Vaccinia virus strains in a mouse model *in vivo*. All information about strains can be found in the materials and methods section (**3.1.4**).

4.1.1. Characterization of the replication of Lister (L0, L1), Wyeth (W0, W1), Copenhagen (C1) and Tian-Tan (T0, T1) strains in different human tumor cell lines and human ADSC (RM0035) under cell culture conditions

4.1.1.1. Analysis of the cell viability of mammalian cancer cell cultures infected with L0, L1, W0, W1, T0, T1 and C1 strains

To analyse and compare the antitumor activity of different smallpox vaccines to infect and lyse cancer cells, a panel of different cancer cell lines was tested. A total of 7 human cancer cell lines derived from 5 different organs were infected with L0, L1, W0, W1, T0, T1 and C1 viruses

at an MOI 0.01 and 1 and cell viability was measured by MTT assay for periods ranging from 24 to 96 hpi. The cancer cell lines used for the viability study include A549 (lung), PC-3 (prostate), MDA-MB231 (breast), FaDu (pharynx), HNT-13, HNT-25, HNT-35 (head and neck).

4.1.1.1.1. Cell viability assay in lung adenocarcinoma cells (A549) in real time, infected with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains

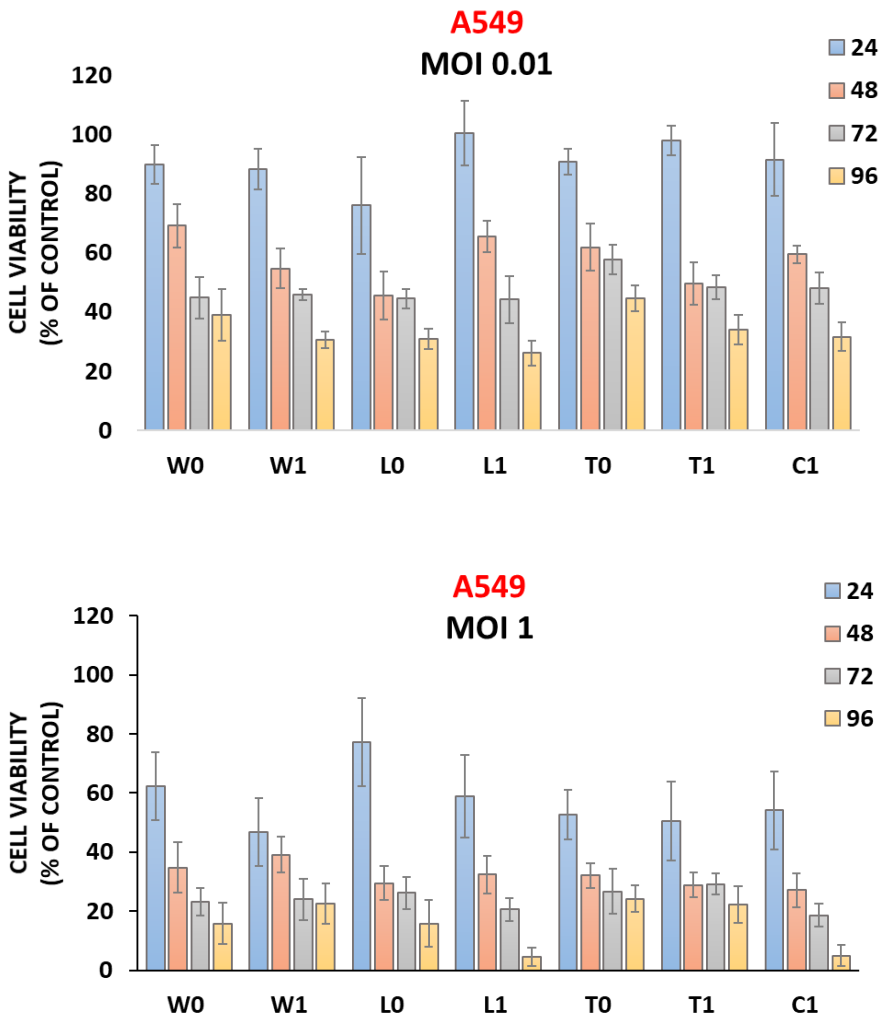


Figure 1.1. Cell viability of A549 cancer cells after infection with L0, L1, W0, W1, T0, T1 and C1 virus strains.

Analysis of cell viability of A549 cells after infection with Vaccinia viruses L0, L1, W0, W1, T0, T1, and C1 was performed by MTT assay, using MOIs of 0.01 and 1, respectively. Viability was monitored over the course of 96 h and was measured in triplicates. Experiments were repeated at least 3 times. Plotted averages are normalized against uninfected controls for each time point considered 100% viable. The average ± SD of 3 independent samples is shown.

The efficiency of cell lysis of all viruses at MOI 1 is shown in **Figure 1.1**. It demonstrates a dose-dependent cancer cell death from all strains tested. At high MOI, cell lysis was very fast with all the tested viral strains. A much stronger cytotoxic effect was observed with an MOI of 1 for L1 and C1 strains. Only 5% ± 3.61 of the cells infected with C1 and 4% ± 3.05 of the L1 were still viable at 96 hpi (**Table 4**).

4.1.1.1.2. Cell viability assay in human prostate cancer cells PC-3 infected with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains

The ability of L0, L1, W0, W1, T0, T1, and C1 to lyse cancer cells was analysed in PC-3 cells.

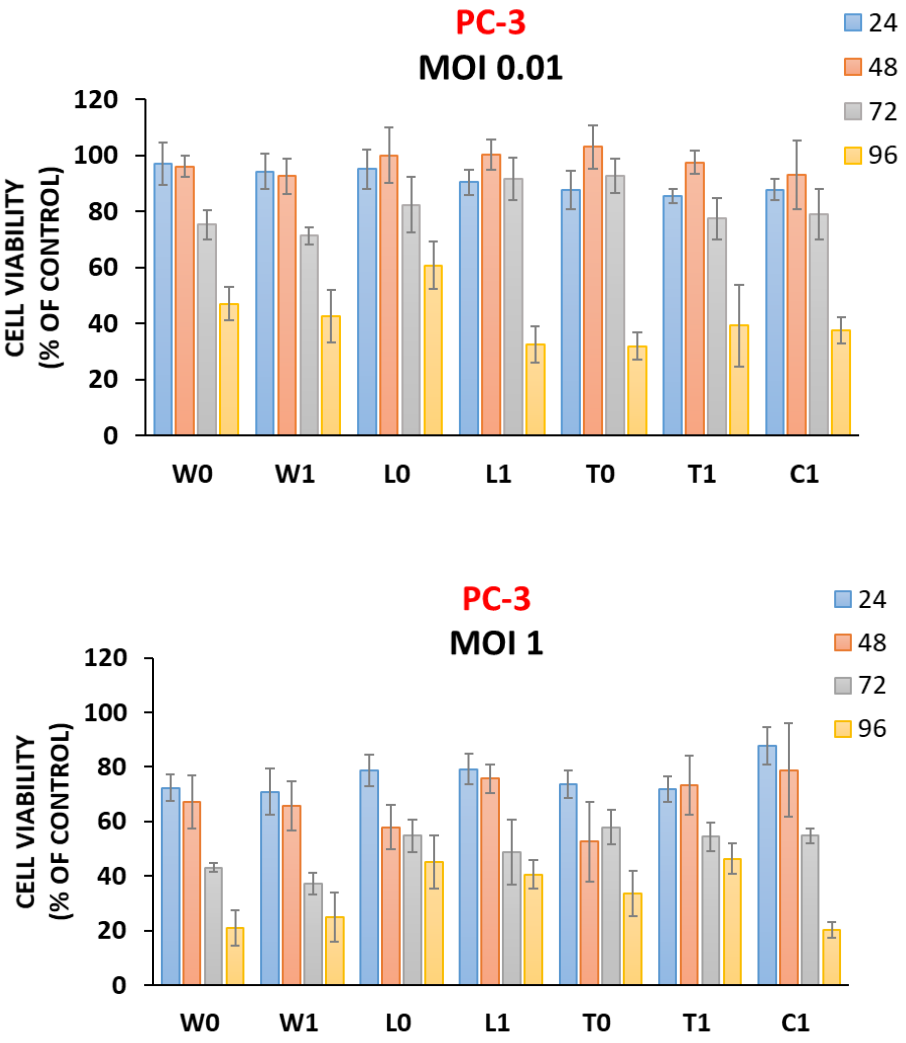


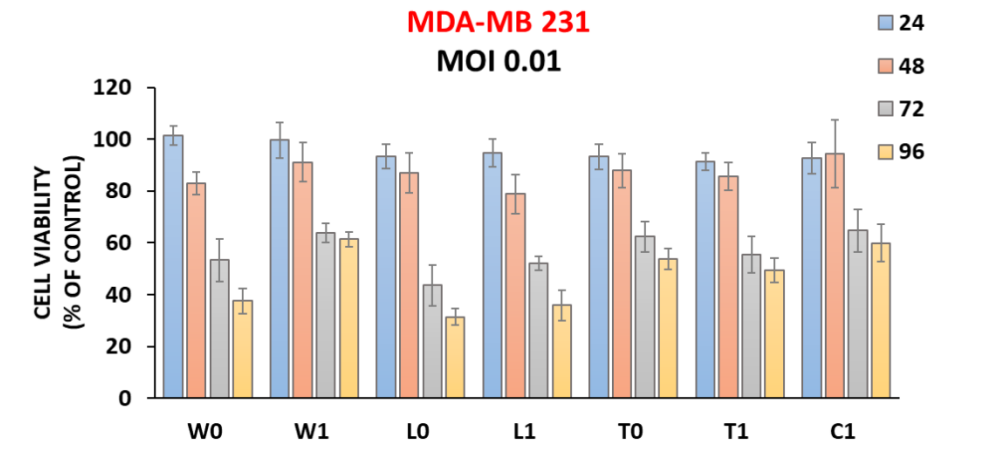
Figure 1.2. Viability of PC-3 cells after infection with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains.

Analysis of cell viability of PC-3 cells after infection with Vaccinia viruses L0, L1, W0, W1, T0, T1, C1, using MOIs of 0.01 and 1, respectively. The relative amounts of viable cells after infection were measured at 24, 48, 72, and 96 hpi by MTT assay. Experiments were performed in triplicates and repeated at least 3 times. Plotted averages are normalized against uninfected controls for each time point considered 100% viable. The average ± SD of 3 independent samples is shown.

As presented in **Figure 1.2**, the Wyeth strains and C1 at MOI of 1 were highly cytotoxic to PC-3 resulting in approximately 80% cytotoxicity over 4 days. Conversely, the Lister and T1 strains lysed human prostate cancer cells with lower efficiency at MOI 1; only around 55% of cells were lysed (**Table 4**).

4.1.1.1.3. Cell viability assay in MDA-MB 231 (breast adenocarcinoma) infected with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains

Here, the cytopathic effects of the L0, L1, W0, W1, T0, T1 and C1 were detected and analysed through infection of the MDA-MB 231 cell line.



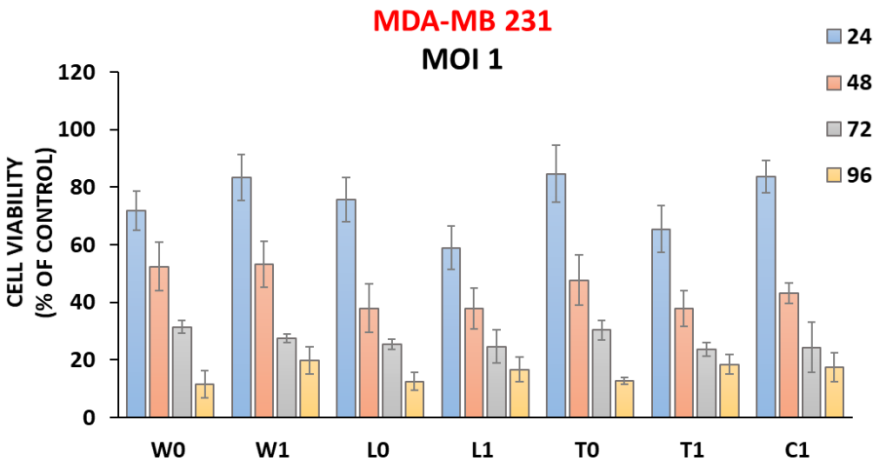


Figure 1.3. Viability assay of MDA-MB231 cells after infection with L0, L1, W0, W1, T0, T1 and C1 virus strains.

The viability of MDA-MB 231 cells infected with L0, L1, W0, W1, T0, T1, and C1 at an MOI of 0.01 or 1 was monitored over 96 h by MTT assay. Experiments were performed in triplicates and repeated at least 3 times. Plotted averages are normalized against uninfected controls for each time point considered 100% viable. The average ± SD of 3 independent samples is shown.

The living cell number decreased during the time, and the cell viability rate was around 10-20% of living MDA-MB 231 cells at an MOI 1 at 96 hpi (**Figure 1.3.**).

All viruses showed efficient cytotoxicity on MDA-MB 231 cancer cells. All viruses lysed the cancer cell lines in time-, MOI- dependent and in a similar manner. A much stronger cytotoxic effect was observed with an MOI of 1. Only 20% to 12% of the cells infected with different viruses survived after 96 h of the infection (**Table 4**).

4.1.1.1.4. Cell viability assay in FaDu (pharynx carcinoma) cells infected with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains

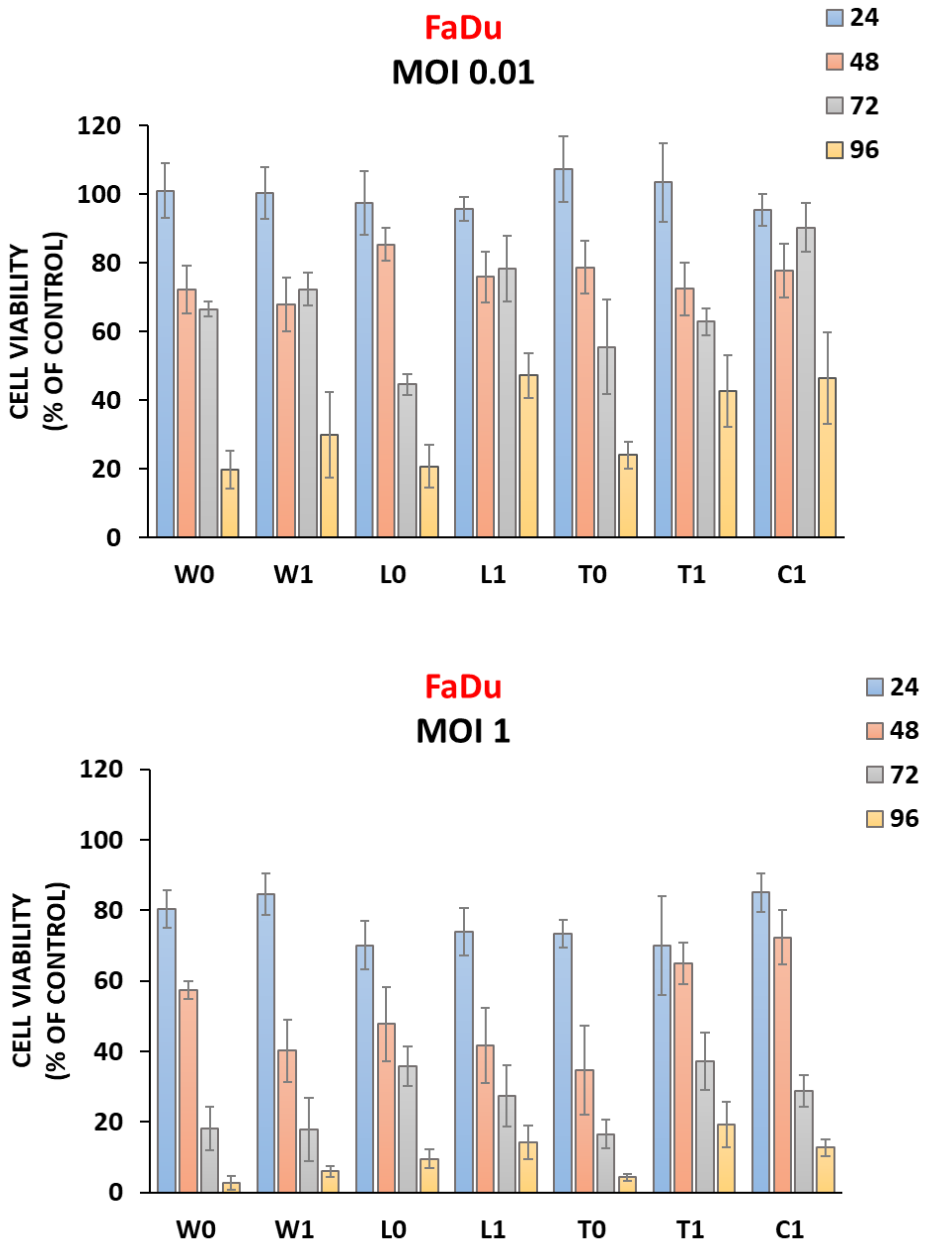
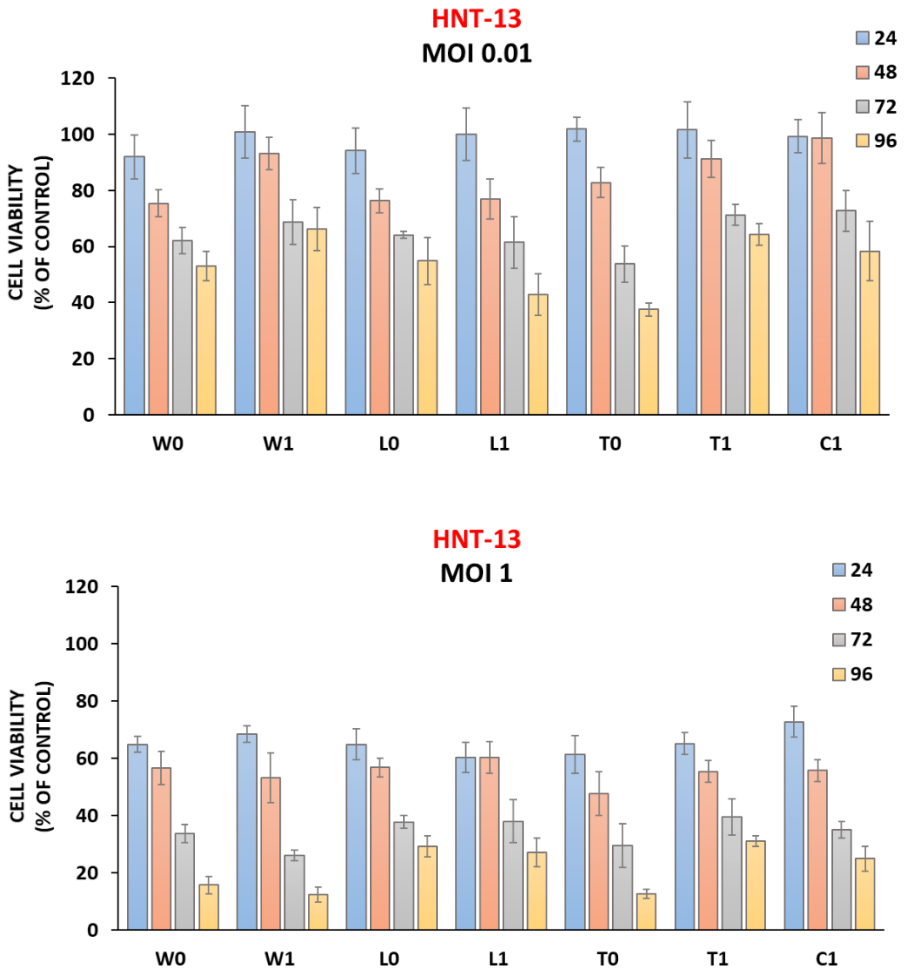


Figure 1.4. Viability of FaDu cells after infection with L0, L1, W0, W1, T0, T1 and C1 virus strains.

The viability of FaDu cells infected with L0, L1, W0, W1, T0, T1 and C1 at an MOI of 0.01 or 1 was monitored over 96 h using an MTT assay. Experiments were performed in triplicates and repeated at least 3 times. Plotted averages are normalized against uninfected controls for each time point that were considered to be 100% viable. The average ± SD of 3 independent samples is shown.

FaDu infected cells showed a dose-dependent response to the infection; cells were lysed more efficiently at higher MOI in comparison with the ones infected with lower MOI (**Figure 1.4**). Dose-dependent cell killing was seen in all strains. More than 90% of the cells died on day 4 at an MOI of 1 by T0, L0, W1, and W0 strains. W0 and T0 showed a higher oncolytic effect than other viruses; only 3% ± 1.91 and 4% ± 1.07 of cells were found viable at 96 hpi, respectively (**Table 4**).

4.1.1.1.5. Cell viability assay in head and neck cancer cell lines (HNT-13, HNT-25 and HNT-35) infected with L0, L1, W0, W1, T0, T1 and C1 Vaccinia virus strains



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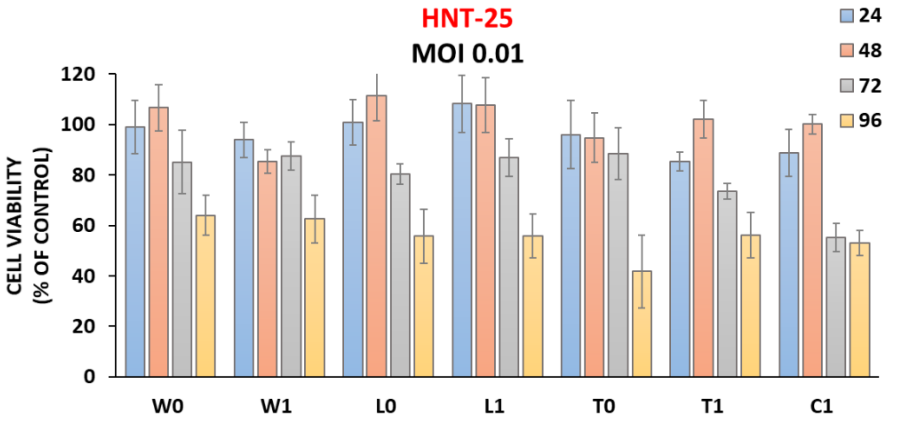
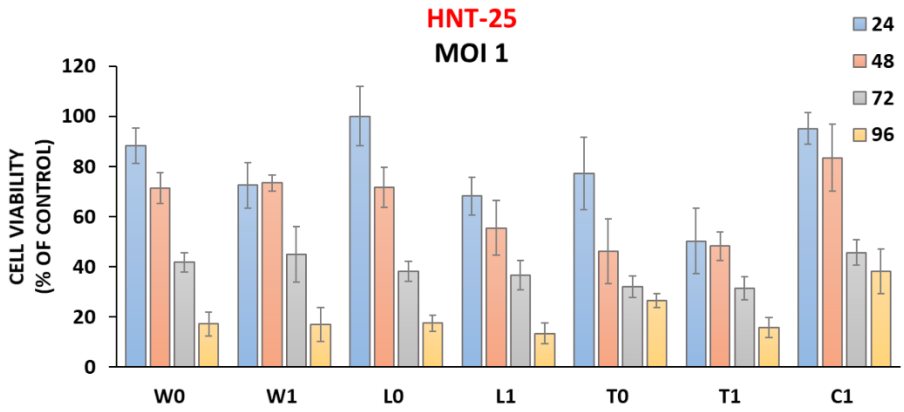
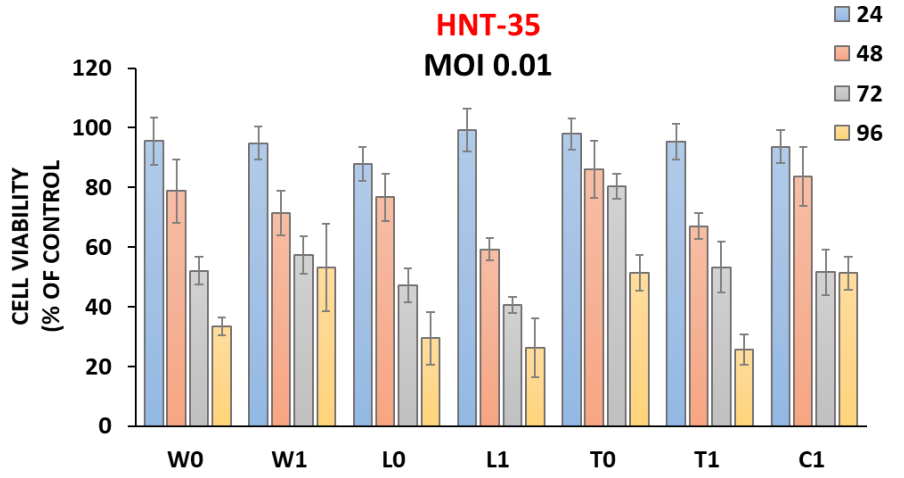






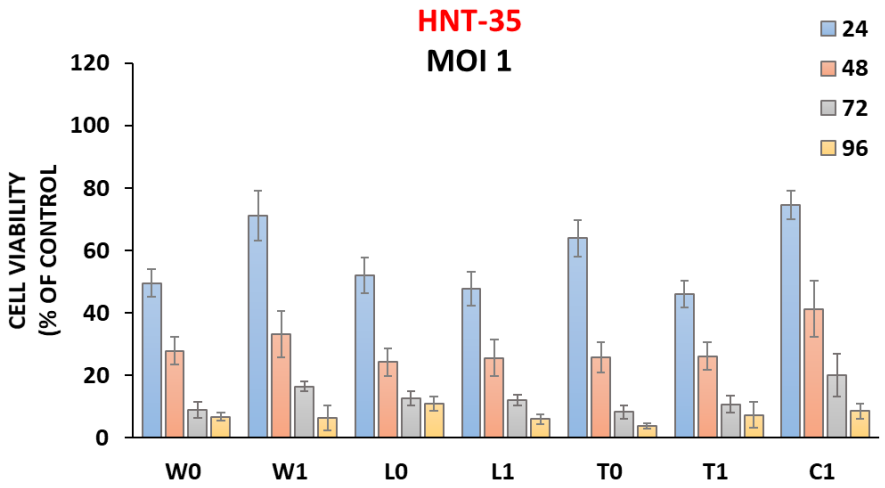


Figure 1.5. Viability of HNT-13, HNT-25 and HNT-35 cells after infection with L0, L1, W0, W1, T0, T1, and C1 virus strains.

Viability of HNT-13, HNT-25, and HNT-35 cells infected with L0, L1, W0, W1, T0, T1, and C1 at an MOI of 0.01 or 1 was monitored over 96 hours using MTT assay. Experiments were made in triplicates and repeated at least 3 times. The viable cells were calculated as a percentage of the mock-infected control cells for each time point, which was considered 100% viable. The average ± SD of 3 independent samples is shown.

After the first 24 hours post-infection with an MOI of 0.01, the cell signal was slightly increased, probably due to virus-mediated activation of cell metabolism. Then, cells were lysed, and the number of living cells decreased down to 38% (HNT-13; T0), 42% (HNT-25; T0), 26% (HNT-35; L1 and T1) at 96 hpi at MOI of 0.01. For all the 3 cell lines, infection with an MOI 1 was more efficient in comparison with the infection with an MOI 0.01. From the beginning, the cell viability decreased constantly, leading to only 13% ± 2.61 (HNT-13; W1 and T0), 13% ± 4.23 (HNT-25, L1), and 4% ± 0.92 (HNT-35, T0) viable cells at 96 hpi. Although, HNT-35 cell line was more sensitive to viral lysis compared to the other tested cell lines, even at lower MOIs, efficient cell killing was noted in all virus strains (**Figure 1.5.**). For all the tested viruses, more than 90% of the cells were lysed at day 4 with an MOI of 1 (**Table 4**).

Taken together, the sensitivity to virus-induced cell killing varied between the cell lines, and the results showed that the cytotoxic effect of different smallpox vaccines was time- and MOI- dependent.

Table 4. Viability of different tumor cell lines infected with different VACV strains (L0, L1, W0, W1, T0, T1, and C1) at 96 hpi at MOI 1 in real time. Cell viability was assessed by MTT assay.

	Cell viability (%)						
Cell lines	**W0**	**W1**	**L0**	**L1**	**T0**	**T1**	**C1**
A549 (Human lung carcinoma)	16±7,00	23±6,73	16±7,89	4±3,05	24±4,55	22±6,16	5±3,61
PC-3 (Human prostate carcinoma)	21±6,36	25±8,90	45±9,81	41±5,35	34±8,31	46±5,59	20±2,95
MDA-MB 231 (Human breast adenocarcinoma	12±4,76	20±4,72	13±3,11	17±4,35	13±1,16	19±3,51	17±5,00
FaDu (Human pharynx carcinoma)	3±1,91	6±1,62	10±2,62	14±4,78	4±1,07	19±6,33	13±2,41
HNT-13 (Human head and neck cancer carcinoma)	16±2,96	13±2,61	29±3,58	27±5,04	13±1,54	31±1,83	25±4,39
HNT-25 (Human head and neck cancer carcinoma)	17±4,72	17±6,76	17±3,32	13±4,23	27±2,77	16±4,02	38±8,95
HNT-35 (Human head and neck cancer carcinoma)	7±1,18	6±4,03	11±2,92	6±1,62	4±0,92	7±4,13	9±2,48

All cell lines showed less than 50% viability after 4 days of infection at an MOI of 1, indicating that all strains had a broad range of target cancer cell tropism (**Table 4**).

4.1.1.2. Replication efficiency of Vaccinia virus strains (L0, L1, W0, W1, T0, T1, and C1) in human cancer cell lines under cell culture conditions

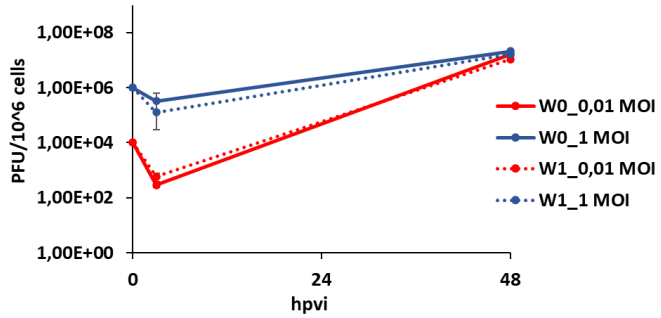
The oncolytic potential of VACV strains is dependent on their ability to infect and replicate in cancer cells efficiently. The efficiency of replication of different smallpox vaccines was analysed in human cancer cell lines. Moreover, the replication kinetics of different VACV strains was compared to each other in the same tumor cell lines.

The viral replication starts after the virus has entered the host cell. The replication rate depends on two parameters: the used viral strain and the target cell line.

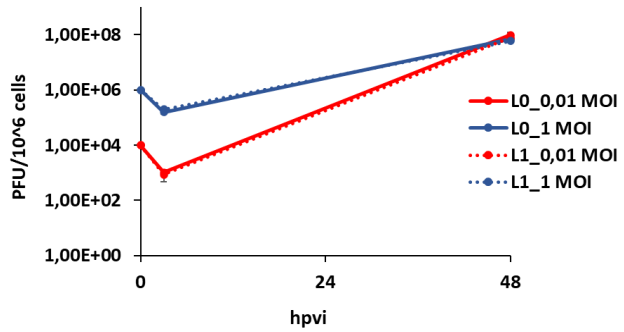
For analysis of the replication efficiency, the mentioned cell lines were infected with viruses at low MOI (0.01) and high MOI (1), followed by the determination of viral titers at selected time points of 3 and 48 hpi. At 3 hpi the virus enters inside the cells, and the viral replication cycle begins. At 48-72 hpi the concentration of the virus reaches the maximum level and remains constant. For easy comparison, the viral titer is always presented as particles per $1x10^6$ cells.

The replicative activity of L0, L1, W0, W1, T0, T1, and C1 viruses to replicate and spread was determined in various human carcinomas derived from 4 different organs. The cancer cell lines used for the replication study include FaDu (pharynx), MDA-MB 231 (breast), HNT-13, HNT-35 (head and neck), and PC-3 (prostate). All these cell lines were efficiently infected by all tested VACV strains, producing viral titers at the range of 6-8 $\log^{10}$ PFU per $1x10^6$ cells.

A: The replication efficiency of W0, W1, L0, L1, T0, T1, C1 strains in FaDu cell line

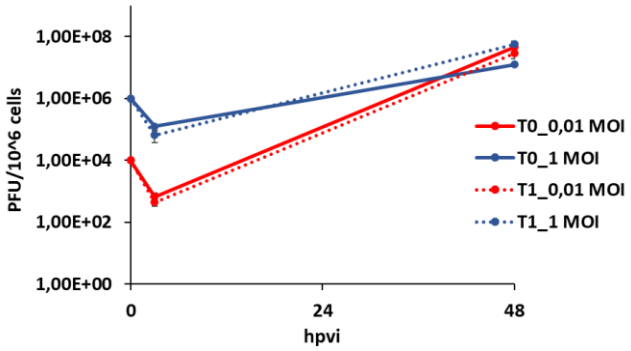


FaDu			
0,01 MOI	0	48	Virus Yield
W0	1.00E+04	1,63E+07	16.3
W1	1.00E+04	1,06E+07	10.6
1 MOI	0	48	Virus Yield
W0	1.00E+06	2.12E+07	21.2
W1	1.00E+06	1.60E+07	16.0

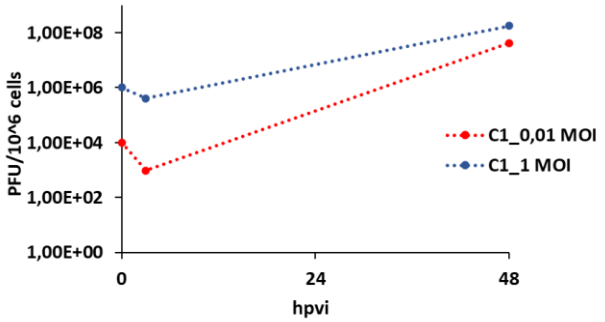


FaDu			
0,01 MOI	0	48	Virus Yield
L0	1.00E+04	9,88E+07	98.8
L1	1.00E+04	7,77E+07	77.7
1 MOI	0	48	Virus Yield
L0	1.00E+06	6,88E+07	68.8
L1	1.00E+06	5,97E+07	59.7

A: Continued

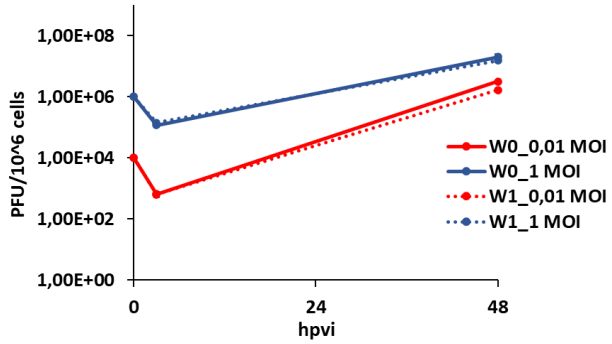


FaDu			
0,01 MOI	0	48	Virus Yield
T0	1.00E+04	4,53E+07	45.3
T1	1.00E+04	2,84E+07	28.4
1 MOI	0	48	Virus Yield
T0	1.00E+06	1,23E+07	12.3
T1	1.00E+06	5,65E+07	56.5

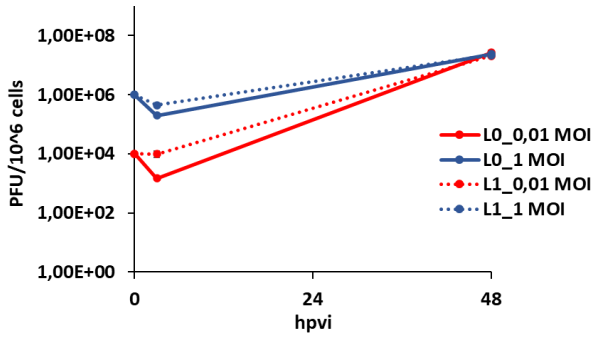


FaDu			
0,01 MOI	0	48	Virus Yield
C1	1.00E+04	4,15E+07	41.5
1 MOI	0	48	Virus Yield
C1	1.00E+06	1,78E+08	177.9

B: The replication efficiency of W0, W1, L0, L1, T0, T1, C1 in MDA-MB 231 cell line

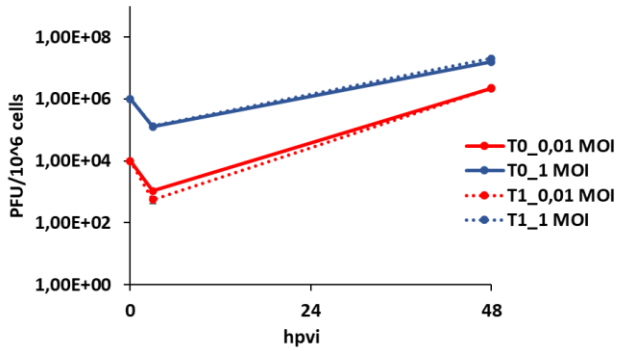


MD-MBA231			
0,01 MOI	0	48	Virus Yield
W0	1.00E+04	3,13E+06	3.1
W1	1.00E+04	1,67E+06	1.7
1 MOI	0	48	Virus Yield
W0	1.00E+06	1,97E+07	19.7
W1	1.00E+06	1,54E+07	15.4

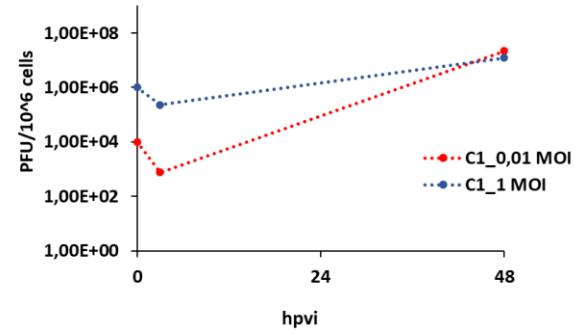


MD-MBA231			
0,01 MOI	0	48	Virus Yield
L0	1.00E+04	2,63E+07	26.3
L1	1.00E+04	2,04E+07	20.4
1 MOI	0	48	Virus Yield
L0	1.00E+06	2,35E+07	23.5
L1	1.00E+06	2,20E+07	22.0

B: Continued

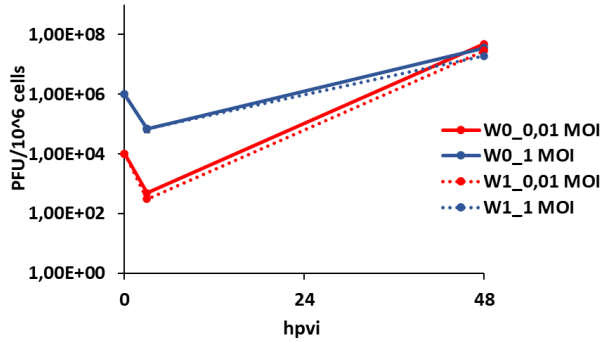


MD-MBA231			
0,01 MOI	0	48	Virus Yield
T0	1.00E+04	2,17E+06	2.17
T1	1.00E+04	2,24E+06	2.24
1 MOI	0	48	Virus Yield
T0	1.00E+06	1,56E+07	15.6
T1	1.00E+06	2,03E+07	20.3

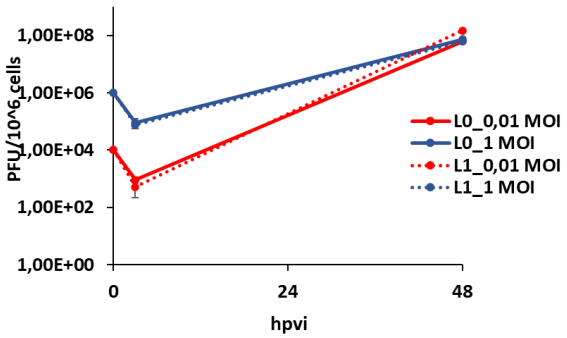


MD-MBA231			
0,01 MOI	0	48	Virus Yield
C1	1.00E+04	2,15E+07	21.5
1 MOI	0	48	Virus Yield
C1	1.00E+06	1,21E+07	12.1

C: The replication efficiency of W0, W1, L0, L1, T0, T1, C1 strains in HNT-13 cell line

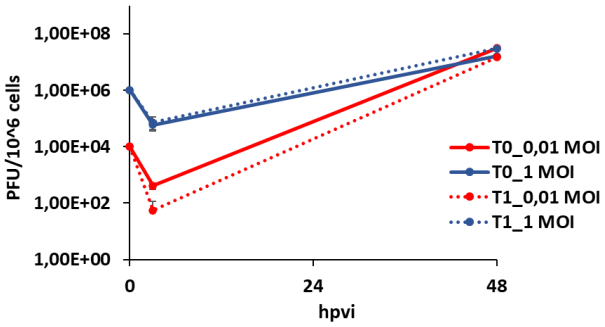


HNT-13			
0,01 MOI	0	48	Virus Yield
W0	1.00E+04	4,82E+07	48.2
W1	1.00E+04	2,85E+07	28.5
1 MOI	0	48	Virus Yield
W0	1.00E+06	3,55E+07	35.5
W1	1.00E+06	1,90E+07	19.0

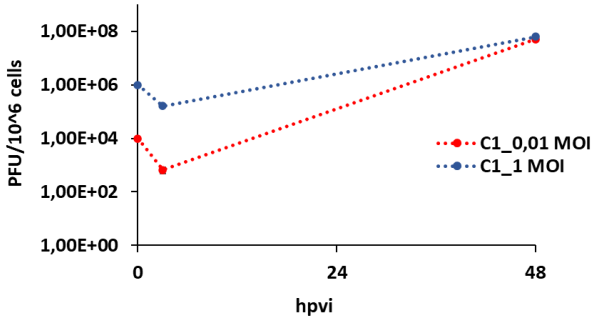


HNT-13			
0,01 MOI	0	48	Virus Yield
L0	1.00E+04	5,31E+07	53.1
L1	1.00E+04	1,47E+08	146.7
1 MOI	0	48	Virus Yield
L0	1.00E+06	7,28E+07	72.8
L1	1.00E+06	6,18E+07	61.8

C: Continued

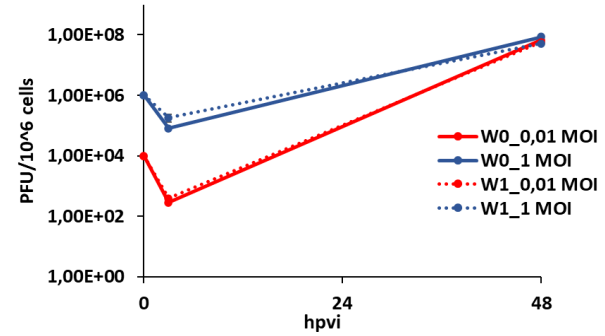


HNT-13			
0,01 MOI	0	48	Virus Yield
T0	1.00E+04	3,12E+07	31.2
T1	1.00E+04	1,44E+07	14.4
1 MOI	0	48	Virus Yield
T0	1.00E+06	1,60E+07	16.0
T1	1.00E+06	3,00E+07	30.0

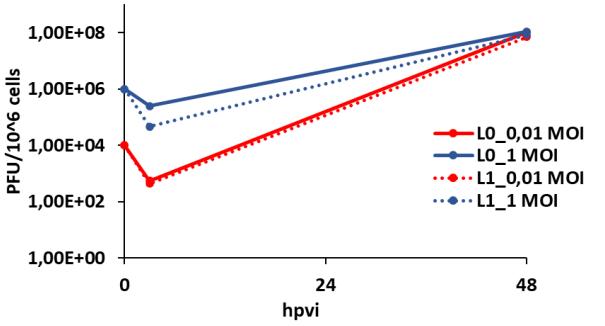


HNT-13			
0,01 MOI	0	48	Virus Yield
C1	1.00E+04	5,10E+07	51.0
1 MOI	0	48	Virus Yield
C1	1.00E+06	6,23E+07	62.3

D: The replication efficiency of W0, W1, L0, L1, T0, T1, C1 strains in PC-3 cell line

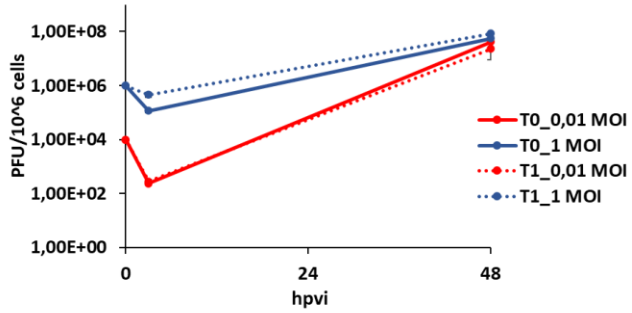


PC-3			
0,01 MOI	0	48	Virus Yield
W0	1.00E+04	6,76E+07	67.6
W1	1.00E+04	5,64E+07	56.4
1 MOI	0	48	Virus Yield
W0	1.00E+06	8,53E+07	85.3
W1	1.00E+06	5,09E+07	50.9

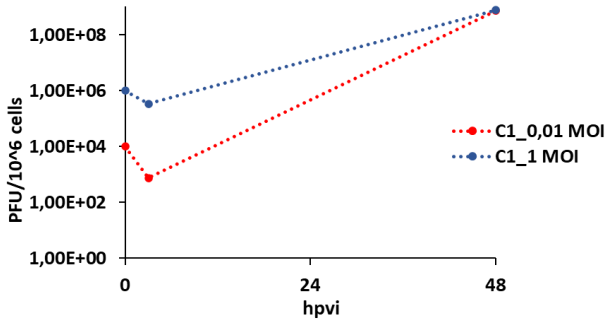


PC-3			
0,01 MOI	0	48	Virus Yield
L0	1.00E+04	1,04E+08	103.6
L1	1.00E+04	6,96E+07	69.6
1 MOI	0	48	Virus Yield
L0	1.00E+06	1,08E+08	108.4
L1	1.00E+06	8,36E+07	83.6

D: Continued

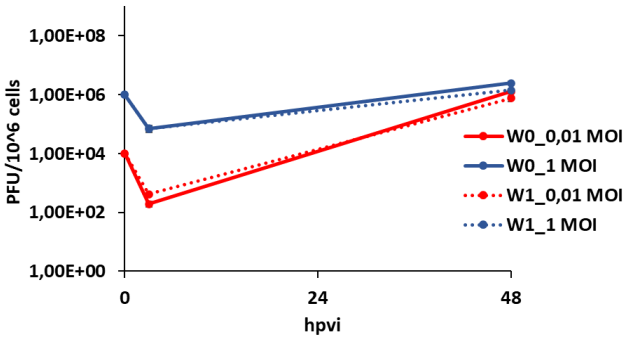


PC-3			
0,01 MOI	0	48	Virus Yield
T0	1.00E+04	4,20E+07	42.0
T1	1.00E+04	2,29E+07	22.9
1 MOI	0	48	Virus Yield
T0	1.00E+06	5,64E+07	56.4
T1	1.00E+06	8,42E+07	84.2

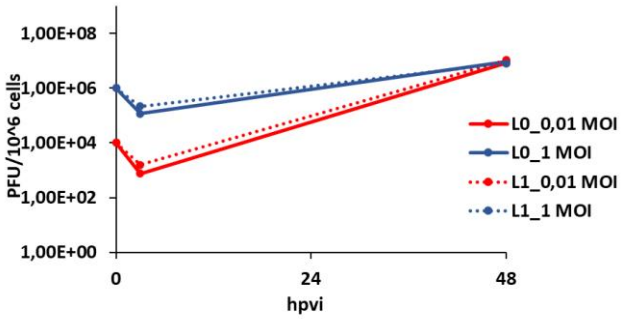


PC-3			
0,01 MOI	0	48	Virus Yield
C1	1.00E+04	7,13E+08	713.3
1 MOI	0	48	Virus Yield
C1	1.00E+06	7,44E+08	744.4

E: The replication efficiency of W0, W1, L0, L1, T0, T1, C1 strains in HNT-35 cell line

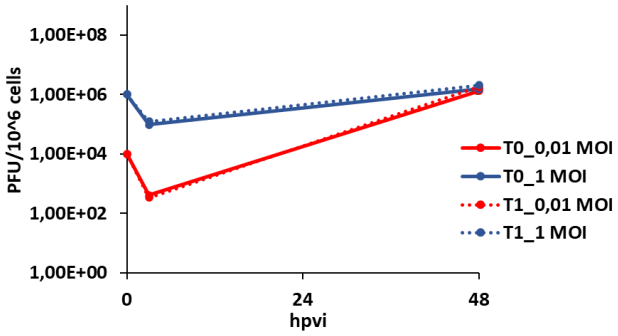


HNT-35			
0,01 MOI	0	48	Virus Yield
W0	1.00E+04	1,28E+06	1.28
W1	1.00E+04	7,51E+05	0.75
1 MOI	0	48	Virus Yield
W0	1.00E+06	2,48E+06	2.48
W1	1.00E+06	1,41E+06	1.41

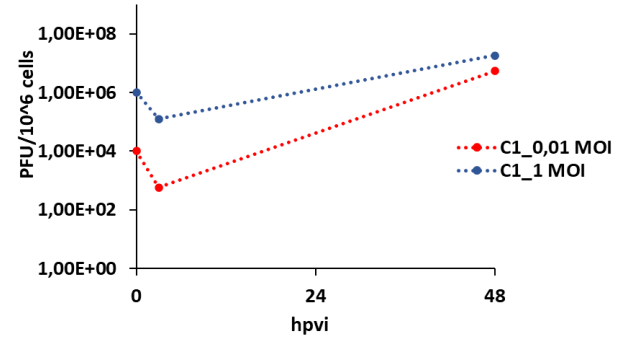


HNT-35			
0,01 MOI	0	48	Virus Yield
L0	1.00E+04	8,20E+06	8.2
L1	1.00E+04	1,05E+07	10.5
1 MOI	0	48	Virus Yield
L0	1.00E+06	9,42E+06	9.4
L1	1.00E+06	7,97E+06	8.0

E: Continued



HNT-35			
0,01 MOI	0	48	Virus Yield
T0	1.00E+04	1,32E+06	1,32
T1	1.00E+04	1,75E+06	1.75
1 MOI	0	48	Virus Yield
T0	1.00E+06	1,50E+06	1.50
T1	1.00E+06	2,05E+06	2.05



HNT-35			
0,01 MOI	0	48	Virus Yield
C1	1.00E+04	5,42E+06	5.4
1 MOI	0	48	Virus Yield
C1	1.00E+06	1,80E+07	18.0

Figure 2. Replication of L0, L1, W0, W1, T0, T1, and C1 in the human carcinoma cell lines: FaDu, MDA-MB 231, HNT-13, PC-3, and HNT-35. The replication efficiency of W0, W1, L0, L1, T0, T1, C1 in FaDu (**A**), MDA-MB 231 (**B**), HNT-13 (**C**), PC-3 (**D**), and HNT-35 (**E**) is shown at different time points (3 and 48 hpi) using MOI 1 and 0.01. Standard plaque assay was performed for all samples to determine the viral titers. The average ± SD of three replicates is shown. Virus yield is the cumulative number of progeny viruses produced by a single cell after 48 hpi [PFU/cell].

We calculated the virus production capacity (how many viral particles could be produced after 48 hpi from a single cell) to monitor and compare the release of new virus particles in different cancer cell lines. The employed formula for the virus yield from the single cell is indicated below:

$$\frac{[\text{PFU}/1\times10^{6}\text{cells}]_{\text{(virus titer at 48 hpi)}}}{10^{6}\text{cells}} = \text{Virus yield [PFU/cell]}$$

FaDu cells can efficiently be infected with all viruses - the non-plaque purified smallpox vaccines as well as their plaque-purified clones (**Figure 2A**). Virus yield from a single FaDu cell was comparable for cells infected with an MOI of 1 and an MOI of 0.01, meaning that the

viral replication efficiency is not MOI-dependent. Among the used viral strains, the most replicative virus was C1, with an MOI of 1. On average, a single FaDu cell produced 177.9 virus particles at MOI 1. For the same strain, the virus yield was 41.5, with an MOI of 0.01. This result indicates that C1 replicates 4 times better with a high MOI (1) than with a lower MOI (0.01). FaDu produced the lowest viral titers at 48 hpi of W1 strain at MOI 0.01 and T0 strain at MOI 1, when compared with other tested strains. The virus yield for W1 was only 10.6 PFU/cell and 12.3 PFU/cell for T0. For L0 and L1 viruses, the progeny was slightly higher with an MOI of 0.01 than with an MOI of 1. Based on the virus replication capacity, the Lister strain has higher replication efficiency in comparison with the Wyeth strains in FaDu cells.

The replication efficiency of different strains was also analysed in the breast adenocarcinoma (MDA-MB 231) cells (**Figure 2B**). In the case of several cell lines, we observed dose-dependent replication efficiency (for Wyeth and Tian Tan strains). If more virus was used to infect cells, more viral particles were generated upon infection. The virus yield from one MDA-MB 231 cell was higher for cells infected with high MOI than with low MOI. Lister strains displayed no MOI-dependent replication. According to the data, the Lister strain replicates significantly better than Wyeth and Tian strains at low MOI. Both Wyeth and Tian Tan strains show low virus yield (around 2-3 PFU/cell) in MDA-MB 231 in comparison with other strains at MOI 0.01.

Taken together, these results indicate that the Lister strain (L0 and L1) and C1 are the most replicative strains in MDA-MB 231 cells at low MOI, and they could be used for further studies. In contrast, the Wyeth (W0, W1) and Tian Tan (T0, T1) strains at low MOI are replicating with low efficiency in MDA-MB 231 cells.

Below, the infection efficiency of the HNT-13 cells, the PC-3 cells, and the HNT-35 cells with the indicated strains is demonstrated.

There was no MOI-dependent replication observed in HNT-13 cells for Wyeth, L0, C1 (**Figure 2C**). The viruses replicate at low MOI and high MOI with comparable efficiency. The highest virus yield was found in L1 infected HNT-13 cells – 147 PFU/cell and the lowest one in T1 infected, 14.4 PFU/cell.

The highest amount of the virus has been produced in PC-3 cells (**Figure 2D**). The prostate carcinoma cells had the most virus progeny at 48 hpi for C1 strain at both MOIs tested; the virus yield was more than 700 viral particles produced by a single cell. It was the highest virus

yield among all the cell lines and strains. Regarding the other viruses, the plots show that correlation wasn't dose-dependent for this strain.

HNT-35 cell line was also permissive for all strains (**Figure 2E**). The virus replication efficiency was low for all strains when compared to other cell lines. There was no dose-dependent fashion in Lister and Tian Tan virus production. The highest virus yield was demonstrated for C1 at MOI 1; the number of virus particles from one cell reached 18 PFU/cell.

Table 5. The summary tables with a number of virus particles per 10^6 cells which produced after 48h at MOI 1 in **A:** FaDu cell line; **B:** MDA-MB 231; **C:** HNT-13; **D:** PC-3; **E:** HNT-35.

A) FaDu

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	2.12E+07
W1	1.00E+06	1.60E+07
L0	1.00E+06	6.88E+07
L1	1.00E+06	5.97E+07
T0	1.00E+06	1.23E+07
T1	1.00E+06	5.65E+07
C1	1.00E+06	1.78E+08

B) MDA-MB 231

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	1.97E+07
W1	1.00E+06	1.54E+07
L0	1.00E+06	2.35E+07
L1	1.00E+06	2.20E+07
T0	1.00E+06	1.56E+07
T1	1.00E+06	2.03E+07
C1	1.00E+06	1.21E+07

C) HNT-13

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	3.55E+07
W1	1.00E+06	1.90E+07
L0	1.00E+06	7.28E+07
L1	1.00E+06	6.18E+07
T0	1.00E+06	1.60E+07
T1	1.00E+06	3.00E+07
C1	1.00E+06	6.23E+07

D) PC-3

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	8.53E+07
W1	1.00E+06	5.09E+07
L0	1.00E+06	1.08E+08
L1	1.00E+06	8.36E+07
T0	1.00E+06	5.64E+07
T1	1.00E+06	8.42E+07
C1	1.00E+06	7.44E+08

Table 5. Continued

E) HNT-35

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	2.48E+06
W1	1.00E+06	1.41E+06
L0	1.00E+06	9.42E+06
L1	1.00E+06	7.97E+06
T0	1.00E+06	1.50E+06
T1	1.00E+06	2.05E+06
C1	1.00E+06	1.80E+07

In summary, it was shown that all virus strains efficiently infect and did replicate in all different human cancer cell lines with a different replication behaviour for each strain and cell tumor type.

4.1.1.3. Characteristic of human ADSC in cell culture

4.1.1.3. Identity of human ADSC (RM0035)

Human ADSC were obtained by culturing stromal vascular fraction (SVF) cells taken of healthy donors. Morphology of cultured hADSC was characterized together with their ability to differentiate into adipocytes, osteoblasts, and chondrocytes, as well as the presence of MSC-specific markers. The hADSC (RM0035) have a phenotype that is a characteristic of MSCs. According to the results, by studying the expression profile of MSC markers via flow cytometry, the hADSC were positive for CD105, CD90, and CD44, as MSC-associated surface proteins (**Figure 3A**). The cells were negative for the hematopoietic marker, CD45, and also for CD34, CD146, CD31, and CD14. (**Figure 3B**). The obtained results confirmed the mesenchymal identity of the isolated cells [178].

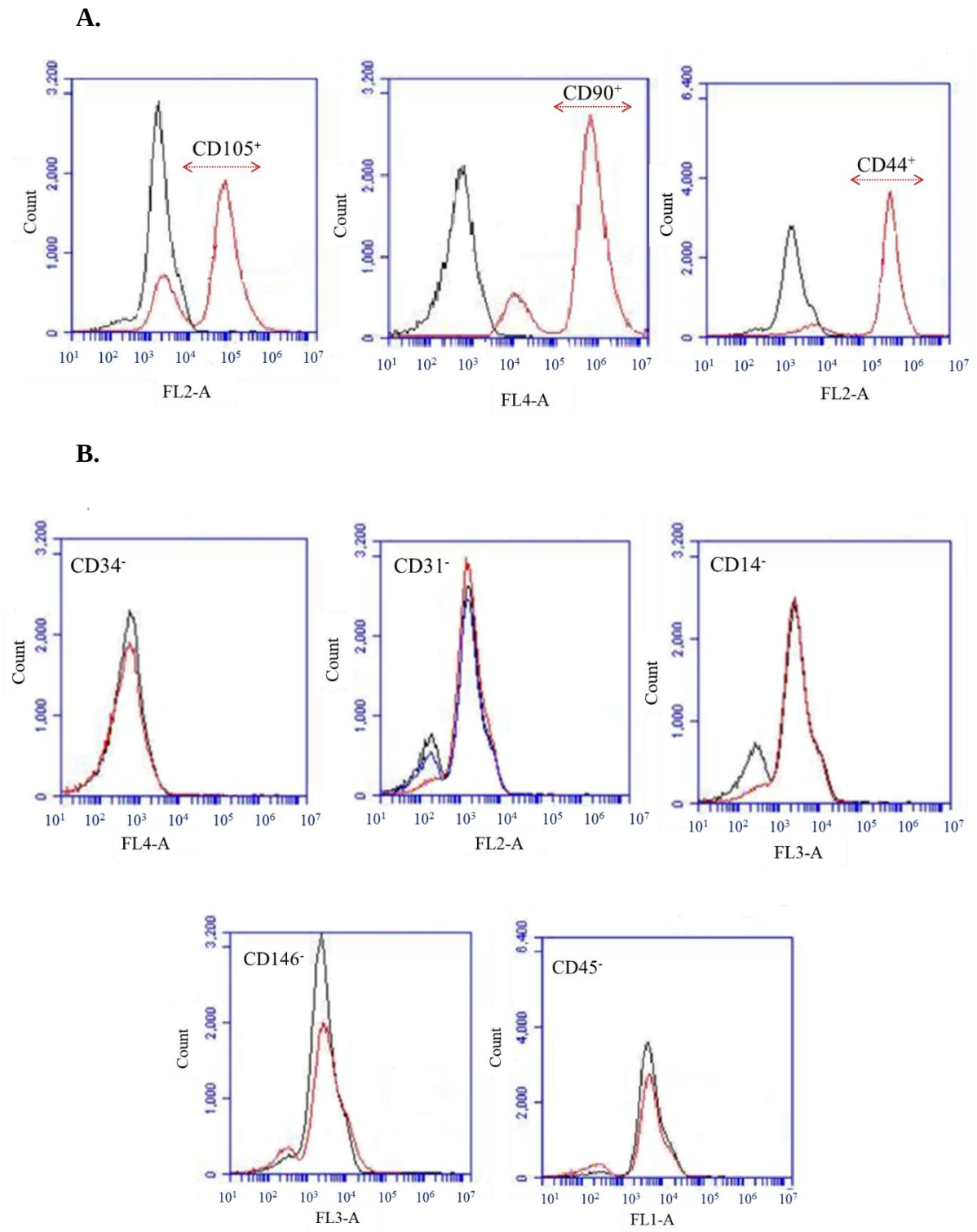


Figure 3. Characterization of the cell surface markers for hADSC by flow cytometry. ($CD105^+$ (PE), $CD90^+$ (APC), $CD44^+$ (PE), $CD34^-$ (APC), $CD31^-$ (PE), $CD14^-$ (PE-Cy^{TM}), $CD146^-$ (PE-Cy^{TM}), $CD45^-$ (FITC). The black histograms represent unstained negative control cells. **A**: positive markers. **B**: negative markers.

ADSC demonstrate fibroblast-like morphology and differentiate into adipocytes (**Figure 4A**), osteocytes (**Figure 4B**) and chondrocytes (**Figure 4C**). After oil red O staining, we monitored the presence of lipid vacuoles. Alizarin red S staining indicated the presence of calcified nodules and Alcian blue staining displayed sulfated glycosaminoglycans and chondrogenesis. These observations indicated the multi-potent identity of the isolated cells.

A: **B:** **C:**

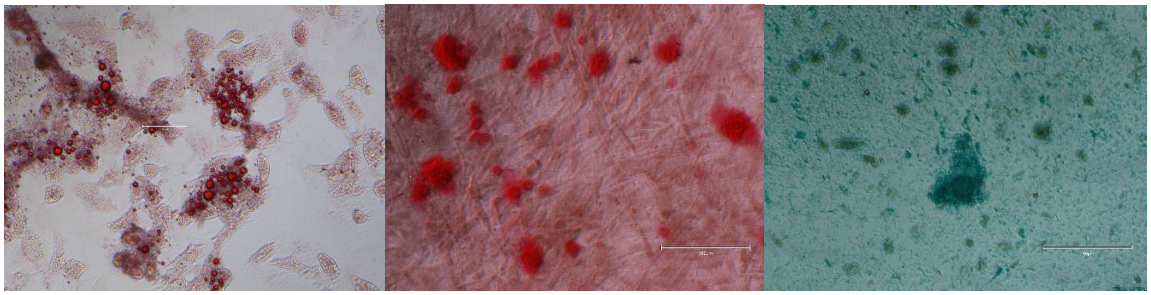


Figure 4. Human ADSC retain multipotent lineage. Representative results of *in vitro* differentiation of hADSC into adipocytes, osteocytes, and chondrocytes post culture in differentiation media. Respective stains used are as indicated. **A**: Adipocytes with oil droplets are visible on day 14 of adipogenic induction; the size and number increased as the differentiation progressed. Oil Red O stained the lipid droplets red on day 20 (magnification 20×). **B**: Day 14 of osteogenic induction showed visible mineral deposits. There was a significant increase in the mineralization on day 21 of osteogenic induction (magnification 20×). **C**: Micromass culture of human ADSC on day 21. Alcian blue staining indicates the synthesis of proteoglycans by differentiated chondrocytes (magnification 20×).

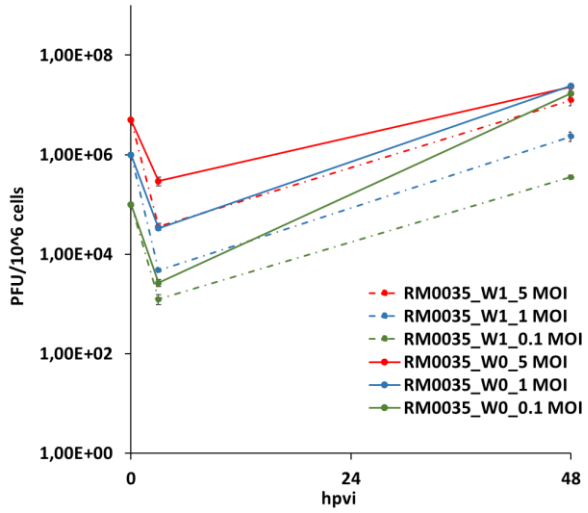
This study shows that human ADSC are capable of forming osteoblasts, chondrocytes, and adipocytes *in vitro*. Having successfully isolated hADSC, we next sought to determine the permissivity of these cells by comparing the cytotoxic activity and replication of wild-type Vaccinia virus strains.

4.1.1.3.2. Vaccinia virus replication in human ADSC followed by oncolysis under cell culture conditions

To determine the ability of the viruses to infect and replicate in hADSC, cells were infected at MOI 5, 1, and 0.1. We found that all tested VACV strains replicated efficiently. However, the replication efficacy was different. For non-purified smallpox vaccines L0, W0, and plaque purified T1 there was no difference between different MOIs conditions, the final amount of

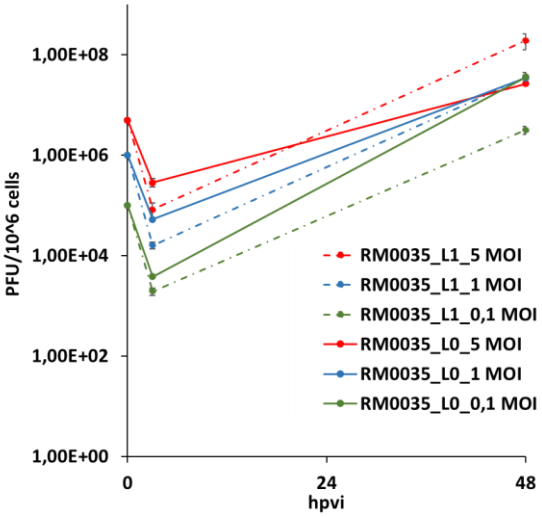
virus particles was the same (after 48 hpi). In comparison to the single-plaque purified strains - L1 and C1 strains, the more virus loaded into the cell, the more viral particles were produced after 48 hpi. There was also an MOI-dependent manner in infection kinetics for plaque purified W1, L1, and C1 compared to their parental strains. In the case of the T1 purified clone, the replication efficiency was the same as the parental strain T0 (**Figure 5**). The virus yield was maximum high for C1 strain (598 PFU/cell at MOI 5 and 423 PFU/cell at MOI 1), as well as in tumor cell line PC-3. The virus yield for L1 at MOI 5 was also high and reached 193 PFU/cell. The lowest titer was found for W1 at low MOI, it produced only 0.4 PFU/cell.

A) Replication of W0, W1 virus strains in RM0035 cells (ADSC)



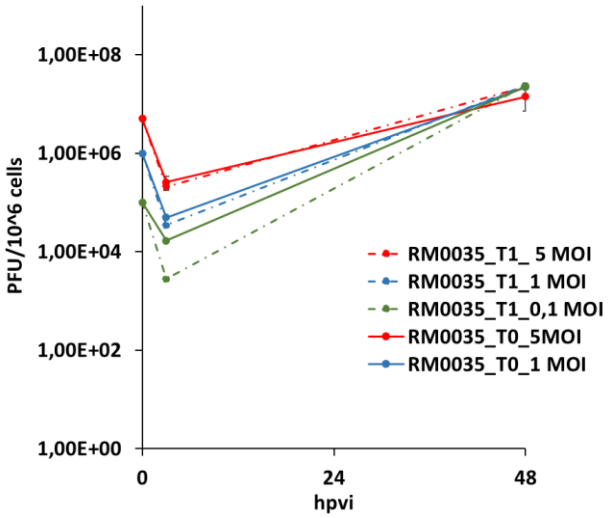
RM0035			
0,1 MOI	0	48	Virus Yield
W0	1.00E+05	1,70E+07	17.0
W1	1.00E+05	3,57E+05	0.4
1 MOI	0	48	Virus Yield
W0	1.00E+06	2,42E+07	24.2
W1	1.00E+06	2,33E+06	2.3
5 MOI	0	48	Virus Yield
W0	5.00E+06	2,33E+07	23.3
W1	5.00E+06	1,25E+07	12.5

B) Replication of L0, L1 virus strains in RM0035 cells (ADSC)



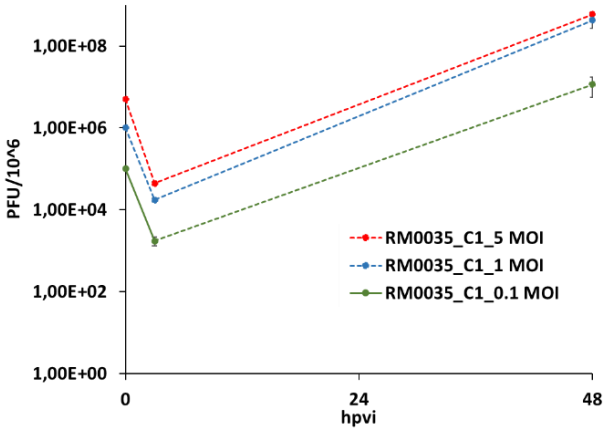
RM0035			
0,1 MOI	0	48	Virus Yield
L0	1.00E+05	3,61E+07	36.1
L1	1.00E+05	3,17E+06	3.2
1 MOI	0	48	Virus Yield
L0	1.00E+06	3,46E+07	34.6
L1	1.00E+06	3,55E+07	35.5
5 MOI	0	48	Virus Yield
L0	5.00E+06	2,63E+07	26.3
L1	5.00E+06	1,93E+08	193.0

C) Replication of T0, T1 virus strains in RM0035 cells (ADSC)



RM0035			
0,1 MOI	0	48	Virus Yield
T0	1.00E+05	2,25E+07	22.5
T1	1.00E+05	2,39E+07	23.9
1 MOI	0	48	Virus Yield
T0	1.00E+06	2,18E+07	21.8
T1	1.00E+06	2,38E+07	23.8
5 MOI	0	48	Virus Yield
T0	5.00E+06	1,42E+07	14.2
T1	5.00E+06	2,23E+07	22.3

D) Replication of C1 virus strain in RM0035 cells (ADSC)



RM0035			
0,1 MOI	0	48	Virus Yield
C1	1.00E+05	1,15E+07	11.5
1 MOI	0	48	Virus Yield
C1	1.00E+06	4,23E+08	423.0
5 MOI	0	48	Virus Yield
C1	5.00E+06	5,98E+08	598.0

Figure 5. Replication of **A)** W0, W1; **B)** L0, L1; **C)** T0, T1, and **D)** C1 in RM0035 cells.

RM0035 cells were seeded onto 6 well-plates and infected with L0, L1, W0, W1, T0, T1 and C1 using MOI 5, 1, and 0.1. The cells were harvested at 3 and 48 hpi. Standard plaque assay was performed for all samples to determine the viral titers. The data showed as the mean ± SD of triplicates. Virus yield is the cumulative number of progeny viruses produced by a single cell after 48 hpi [PFU/cell].

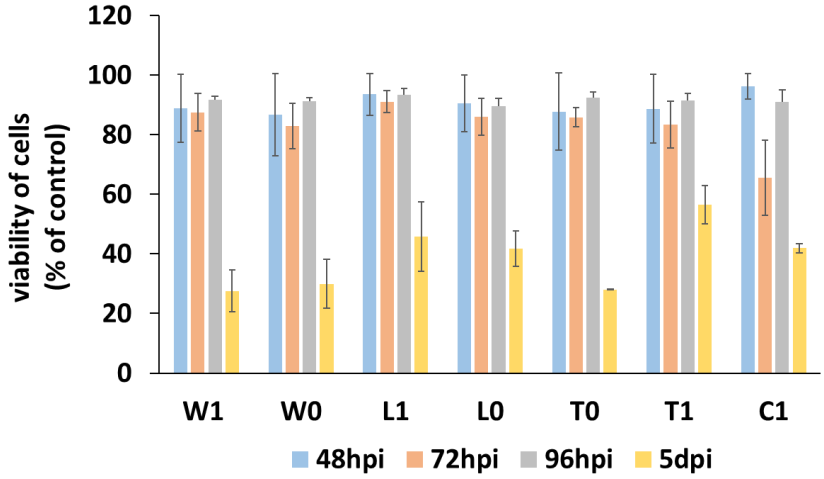
Table 6. The summary tables with a number of virus particles per 10^6 cells which produced after 48 h at MOI 1 in RM0035 (ADSC) cell line.

Virus strains	0h	48h
	[PFU/10^6 cells]	
W0	1.00E+06	2.42E+07
W1	1.00E+06	2.33E+06
L0	1.00E+06	3.46E+07
L1	1.00E+06	3.55E+07
T0	1.00E+06	2.18E+07
T1	1.00E+06	2.38E+07
C1	1.00E+06	4.23E+08

Taken together, all viruses were able to efficiently infect and replicate in human ADSC (RM0035), suggesting that the system can potentially be used for VACV delivery.

4.1.1.3.3. Cytotoxicity assay of ADSC (RM0035) infected with different Vaccinia virus strains

The cell-killing effect of each viral strain was assessed by LDH-cytotoxicity assay after 48, 72, 96 hpi, and 5 dpi.



Viability (%)				
	48hpi	72hpi	96hpi	5dpi
W1	89	88	92	28
W0	87	83	91	30
L1	94	91	93	46
L0	91	86	90	42
T0	88	86	92	28
T1	89	83	91	56
C1	96	66	91	42

Figure 6. Lysis of hADSC (RM0035) after virus infection at an MOI of 1 after 48, 72, 96 hpi, and 5 dpi. The viability of hADSC after infections with L0, L1, W0, W1, T0, T1, C1 viruses was assessed by LDH-cytotoxicity assay. The LDH activity was measured as described in materials and methods. The viability of the cells was calculated as a percentage of the mock-infected control cells for each time point, which was considered to be 100% viable. The

average data and the error bars indicated the ± SD of the mean values of three separate experiments are shown for all virus strains.

Interestingly, only after 5 days post infection a significant amount of hADSC (44%-72%) was lysed (**Figure 6**). The highest level of LDH was observed in cells infected with T0 and Wyeth virus strains at 5 dpi.

It was demonstrated that RM0035 cells are permissive for all tested Vaccinia virus strains. However, VACV displayed a stronger cytotoxic effect in human cancer cell lines compared to human ADSC at the same MOI.

4.1.2. Oncolytic effects of L1, W1, and T1 Vaccinia virus strains and combination with hADSC in human cancer xenografts of tumor-bearing mice

4.1.2.1. Growth inhibition of lung adenocarcinoma (A549) xenografts with the Vaccinia viruses (L1, W1, and T1) and in combination with Vaccinia virus infected hADSC

To confirm the oncolytic effect of this approach *in vivo*, A549 (human lung cancer) tumor-bearing nude mice were treated with W1, L1, and T1 in different doses or with the combination of W1+ADSC, L1+ADSC, and T1+ADSC, or PBS starting 3.5 weeks after tumor inoculation. (See the design of the experiment in **Figure 7**).

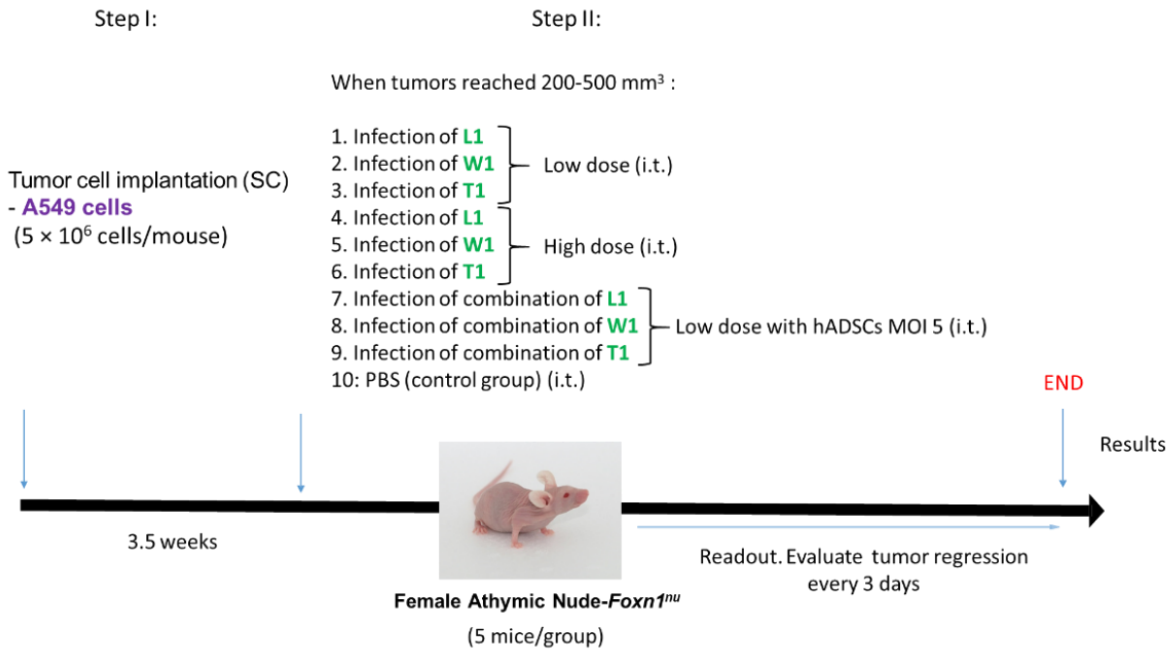


Figure 7. Design of animal experiment. SC - subcutaneous; i.t. - intratumorally; hADSC - human Adipose-derived Stem Cells; A549 cells - human lung adenocarcinoma;

L1 - LIVP 1.1.1; W1 - ACAM2000; T1 - Tian Tan clone number 5; Low dose – 5×10^5 PFU/mouse; High dose - 2.5×10^6 PFU/mouse; MOI 5 means a mix of 2.5×10^6 PFU of virus and 1×10^5 cells of hADSC per mouse.

At 2 weeks from the initial treatment, tumors in the control group had significantly increased in size, while those in the W1, L1, T1 and W1+ADSC, L1+ADSC, T1+ADSC - treated groups had decreased (**Figure 8**). Tian-Tan virus was too toxic for mice causing poxes all around the body, especially in the nasal area, which prevented animals from eating, so the animals had to be sacrificed by day 20. There was no difference between the Lister groups (2.5×10^6 (high dose) = 5×10^5 (low dose) = low dose of virus + ADSC). In the case of Wyeth groups, the combination of W1 (low dose) + ADSC worked slightly better than the low dose of W1 and has oncolytic activity closer to a high dose of W1. At high dose concentration, the Wyeth strain worked slightly better than Lister strain. Generally, L1 and W1 have the same antitumor effect.

Notably, the antitumor effect of W1 and L1 was attributable to the replication of the viruses alone as no therapeutic genes had been introduced into the viruses. These results strongly indicated that wild-type Vaccinia virus strains had a notable antitumor effect as an oncolytic virus.

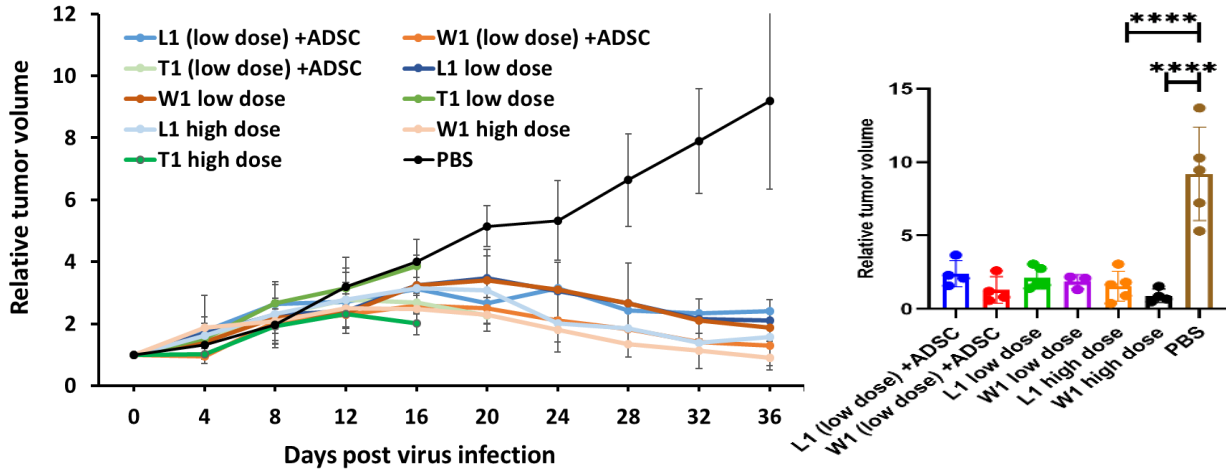


Figure 8. Growth curve of subcutaneous A549 tumor in L1, W1, T1, L1, W1+ADSC, L1+ADSC, T1+ADSC, or PBS-treated mice. Animals of 10 groups (n = 5) were treated with a single injection of 2.5×10^6 (high dose) or 5×10^5 (low dose) PFU of virus or with the combination of 5×10^5 PFU of the virus with hADSC (1×10^5 cells) or PBS only, after three and half week post tumor implantation A549 (human lung adenocarcinoma). Tumor size was measured twice a week. The average data including ± SD are plotted. Significant regression

was noted in xenografts injected with different strains when compared to PBS injected control at 36 days after infection (****$p < 0.0001$).

Besides the determination of the tumor volume, mice were weighed at least twice a week, and taking into account the tumor volume, the net body weight of the mice was calculated. The net body weight was chosen as a parameter to control the general state of health of the animals including viral toxicity, other types of infections, development of metastases. As shown in **Figure 9**, the general state of health of the mice was stable over the whole-time course observed for the mice treated with all viruses except the Tian-Tan strain. The mice in the Tian Tan groups had to be sacrificed due to severe virus-mediated side effects. In the Lister group, there was no mortality until the end of the experiment. More importantly, mice treated with the combination of L1 and W1 with ADSC also exhibited stable body weight, indicating that the well-known safety of the viruses is not compromised when it is combined with ADSC.

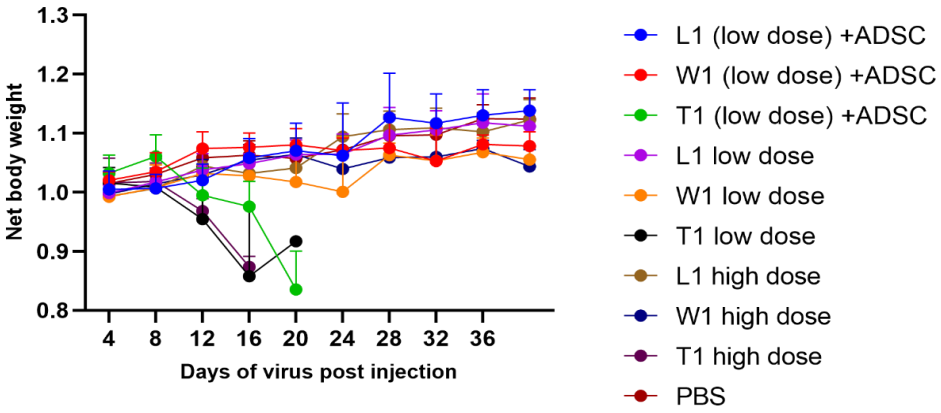


Figure 9. Fractional net body weight of mice with A549 xenograft tumors treated with different Vaccinia virus strains and combination it with human ADSC. The average data including ± SD are plotted.

In this initial study, we could demonstrate that tumor growth of SC implanted A549 xenografts is significantly reduced when treated with oncolytic VACV alone or VACV in combination with human ADSC.

4.1.2.2. Survival of tumor bearing mice after treatment with L1, W1 Vaccinia strains alone, and with L1/ADSC, W1/ADSC in mice with implanted lung adenocarcinoma xenografts

Mice had to be euthanized due to sudden, strong weight loss, in a short time (20%), as signs of suffering or weakness or when the tumor reached a volume more than 4000 mm^3. As shown in

Figure 10, all groups with VACV Tian Tan were sacrificed on day 19 due to their weight loss and severe side effects like pox formation at the tail, nose, pads, and mouth. In the case of Vaccinia Wyeth groups, also only half of the animals survived. In this case, extensive pox formation was detected on the tail, pads, nose, and mouth. In contrast, all mice's body conditions remained excellent in all Lister groups, no adverse clinical signs were seen, and all mice continued to gain weight during the study (**Figure 10**). However, the tumors were eliminated slightly faster in Wyeth groups in comparison to Lister groups.

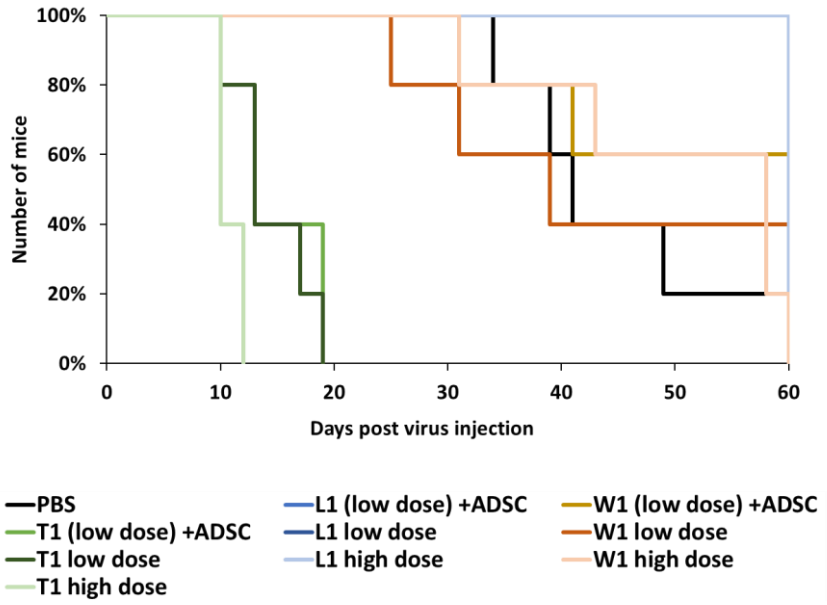


Figure 10. Kaplan-Meier survival curves for A549 tumor-bearing mice treated with L1, W1, T1 or the combination of L1+ADSC, W1+ADSC, T1+ADSC, or PBS (n = 5 per group). The survival of 5 mice was set as 100%.

Overall survival of mice in Lister/Wyeth virus-treated groups was significantly higher than the PBS-treated group.

4.2. Second aim: Characterization of oncolytic virus efficacy of vaccines derived and engineered Vaccinia virus strains: L3- opt1, W1- opt1, and C1- opt1 in canine cancers

In these studies, 3 new recombinant Vaccinia virus strains (VACVs): L3-opt1 (Lister strain derivate), W1-opt1 (Wyeth strain derivate), and C1-opt1 (Copenhagen strain derivate), constructed by Dr. D. Nguyen at StemImmune, USA, amplified by Dr. T. Auth in StemVac GmbH, Bernried, Germany. We analysed the oncolytic effect of these recombinant VACVs on a panel of two different canine cancer cell lines (CT1258, STSA-1) and second, tested the

viruses on the canine Adipose-derived Mesenchymal Stem Cells (cAdMSC). These stem cells were used as a "Trojan horse" to hide and deliver the oncolytic Vaccinia virus strains to tumors in a canine tumor xenograft model in mice.

4.2.1. Infection of canine tumor cell lines and canine AdMSC in cell culture with L3-opt1, W1- opt1, and C1-opt1 Vaccinia virus strains. (Most of the data presented in this part of the work are generated in collaboration and published with I. Petrov et al. (2020) [179]).

4.2.1.1. Replication efficiency of Vaccinia virus strains (L3-, W1-, and C1-opt1) in canine cancer cell lines (CT1258, STSA-1) in cell culture

A virus replication assay was performed to determine the ability of L3-opt1, W1-opt1, and C1-opt1 to efficiently infect canine prostate carcinoma (CT1258) and canine tissue sarcoma (STSA-1) cells in cell culture. All cancer cells were infected with different Vaccinia virus strains at an MOI 0.5.

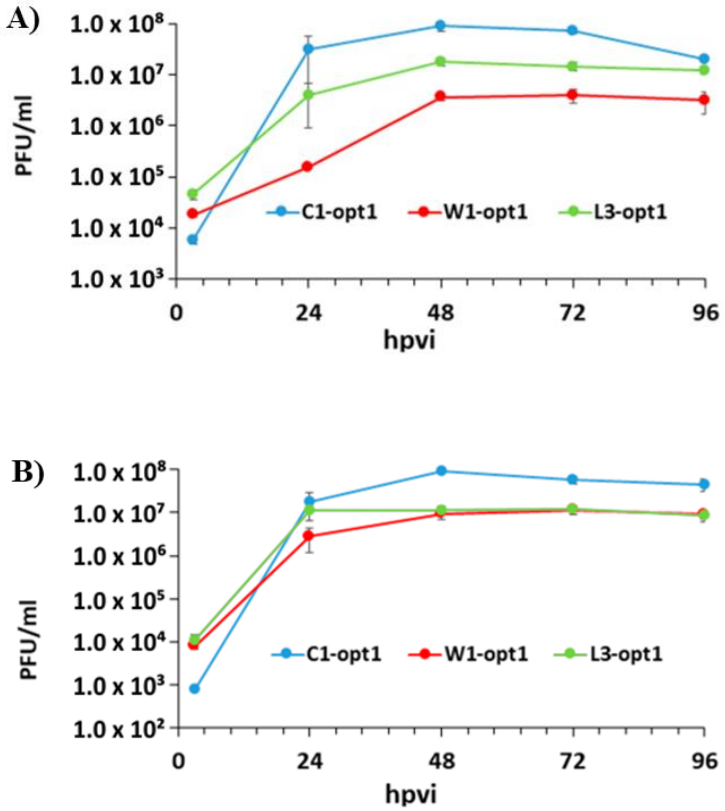


Figure 11. Replication assay of Vaccinia virus strains (L3-opt1, W1-opt1, and C1-opt1) in CT1258 (**A**) and STSA-1 (**B**) cells.

Cells were seeded into 6 well-plates and infected with L3-opt1, W1-opt1, or C1-opt1 at an MOI 0.5. After 24, 48, 72, and 96 hpi cells and supernatants were collected and viral titers determined in a standard plaque assay. Dots represent the mean value and ± SD are indicated for each measurement. Samples were analysed in triplicates.

All recombinant Vaccinia virus strains expressed the turbo red fluorescent protein (FP635) are shown in **Figure 11.** All tested Vaccinia virus strains were infected efficiently and replicated well (>100-fold virus titer increase at 24 hpi to 96 hpi) in both canine cancer cell lines. The maximum titer of 9.18×10^7 PFU/ml was reached at 48 hpi in case of C1-opt1 virus infected with CT1258 cells.

The data were generated in collaboration with Mr. D. Bohn and Dr. I. Gentschev.

4.2.1.2. Replication efficiency of Vaccinia virus strains L3-opt1, W1-opt1, and C1-opt1 in canine Adipose-derived Mesenchymal Stem Cells (cAdMSC) in cell culture

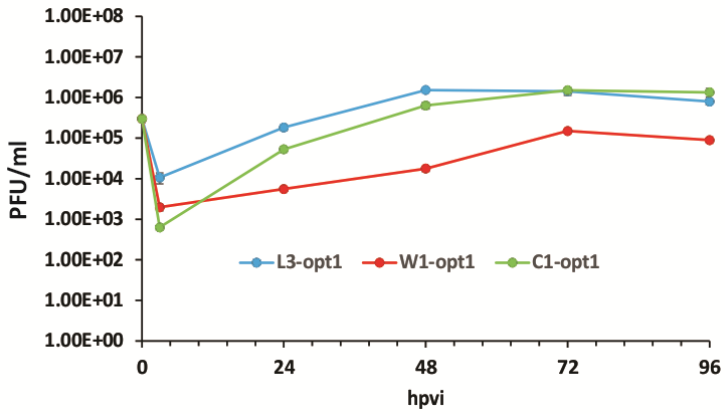


Figure 12. Replication assay of L3-opt1, W1-opt1, and C1-opt1 in canine AdMSC.

Cells were seeded into 6 well-plates and infected either with L3-opt1, W1-opt1, or C1-opt1 at an MOI 0.5. After 24, 48, 72, and 96 hpi cells and supernatants were collected and viral titers determined in a standard plaque assay. Points represent the mean value from three measurements (n=3).

We analysed the permissiveness of cAdMSC to L3-opt1, W1-opt1, and C1-opt1 at different time points **(Figure 12).** The cAdMSC used in this study were obtained from adipose tissue from a healthy canine donor. The highest viral titers were observed with VACV C1-opt1 strain at 72 hpi (1.51×10^6 PFU/mL) and with VACV L3-opt1 strain at 48 hpi (1.52×10^6 PFU/mL).

Results of the viral replication assays demonstrate that cAdMSC are permissive to L3-opt1, W1-opt1, or C1-opt1 and support the replication of different Vaccinia virus strains.

4.2.1.3. Viability of cAdMSC after infection with L3-opt1, W1-opt1, or C1-opt1 Vaccinia virus strains

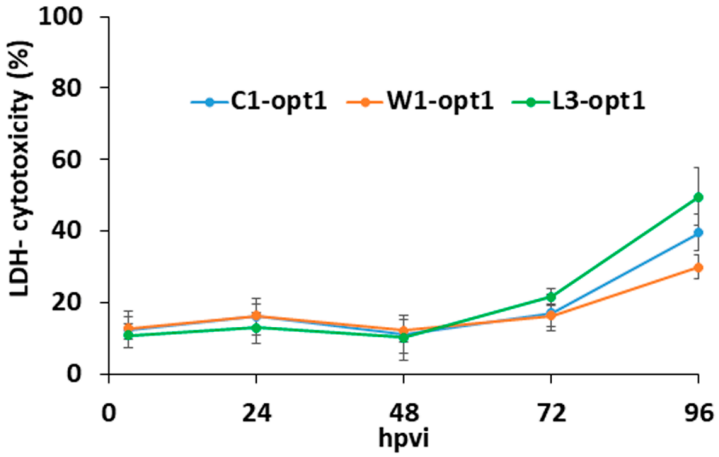


Figure 13. Cell cytotoxicity assay of cAdMSC after infection with L3-opt1, W1-opt1, or C1-opt1 infected at an MOI 0.5.

Each data point was measured in triplicates at 24, 48, 72, and 96 hpi with an LDH cytotoxicity assay. Values were normalized to non-infected cells that were considered 100% viable.

To investigate the ability of L3-opt1, W1-opt1, C1-opt1 to infect and lyse cAdMSC, a Lactate Dehydrogenase (LDH) cytotoxicity assay was performed. This method is based on measuring the activity of cytoplasmic enzymes (LDH) released by damaged cells. The highest level of LDH was observed in cells infected with all 3 viruses at 96 hpi, however, only about 30% to 50% of cells were lysed after 4 days post infection (**Figure 13**).

Taken together, the titers of VACV infected cAdMSC were lower compared to viral titers in infected canine tumor cell lines, meaning that VACV is replicating more efficiently in the canine tumor cells at an MOI 0.5.

4.2.1.4. Analyse of Vaccinia virus infections based on expression of engineered Vaccinia encoded protein FP635

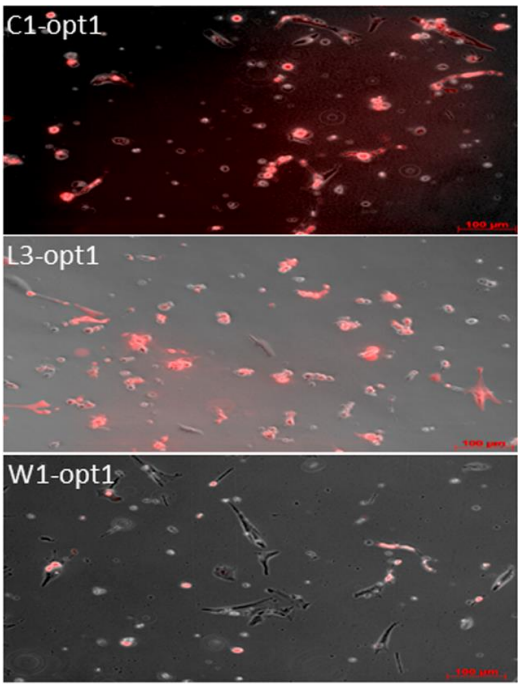


Figure 14. Fluorescence microscopy images of the FP635 protein expression in cAdMSC line infected with VACV L3-opt1, W1-opt1, or C1-opt1 strains after 96 hpi. The FP635 expression is detected as a red signal. (Scale bars 100 μm).

To monitor the Vaccinia virus infection efficiency, the expression of the fluorescent red protein was analysed by fluorescence microscopy. The gene expressing the FP635 was integrated into Vaccinia virus genome (see **3.1.5.** in material and methods section). Once the virus replicates infected cells, the FP635 expression can be detected by fluorescence in red spectra. To monitor the infection of Vaccinia virus strains via turbo FP635 protein expression, cAdMSC were infected with L3-opt1, W1-opt1, or C1-opt1 viruses at an MOI 0.5 and observed under fluorescence microscope at 96 hpi **(Figure 14)**.

The figure was generated and provided by Dr. I. Gentschev.

4.2.2. Effect of C1-opt1 virus strain in combination with cAdMSC in canine Soft Tissue Sarcoma (STSA-1) bearing mouse xenografts

To assess the antitumor efficacy of the VACV/stem cells combination canine tumor-bearing nude mice, which were treated with C1-opt1 alone, with a combination of C1-opt1/cAdMSC or with cAdMSC alone. Please, see the experimental design in **Figure 15** below.

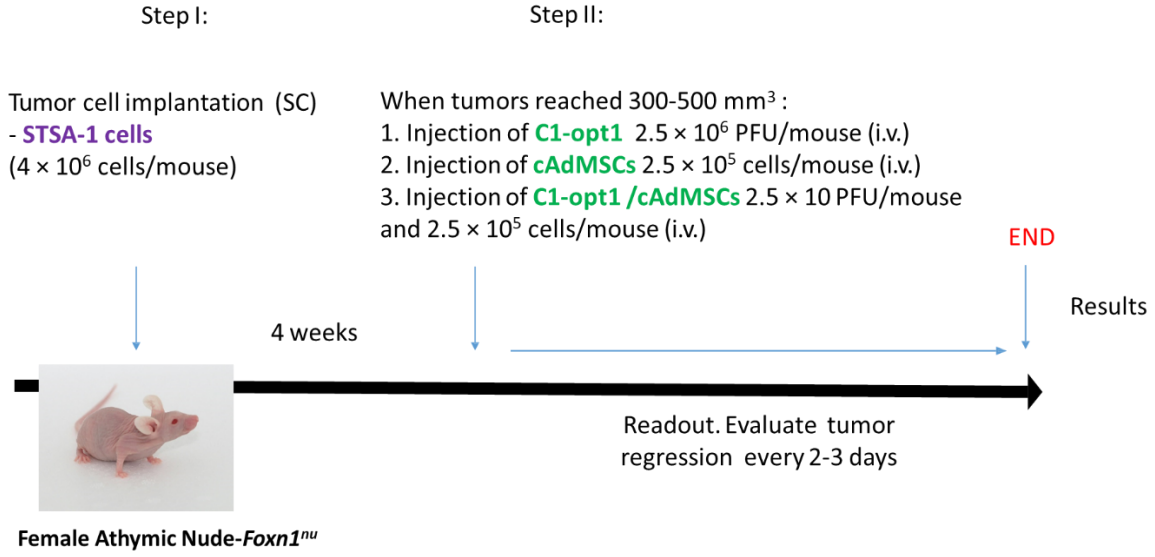


Figure 15. Design of animal experiment. SC – subcutaneous; i.v. – intravenously; cAdMSC - canine Adipose-derived Mesenchymal Stem Cells; STSA-1 - canine Soft Tissue Sarcoma cells.

At first, nude mice with canine tumor were generated. STSA-1 cells ($4x10^6$) were implanted subcutaneous (SC) into the right flank of 7-week-old female nude mice. Four weeks post implantation, the animals were intravenously (i.v.) injected with C1-opt1 alone, C1-opt1/cAdMSC or cAdMSC alone in 100 µl of PBS. The mice treated with C1-opt1 virus alone were compared to ones treated with C1-opt1/cAdMSC and cAdMSC alone. Canine AdMSC alone were used as control. Every treatment consisted of a cohort of 3 mice minimum. Significant differences in tumor volume between C1-opt1, C1-opt1 loaded in cAdMSC and cAdMSC alone, treated mice were found. Tumors treated with C1-opt1 showed no significant differences in tumor volume compared with the ones treated with C1-opt1/cAdMSC (**Figure 16**).

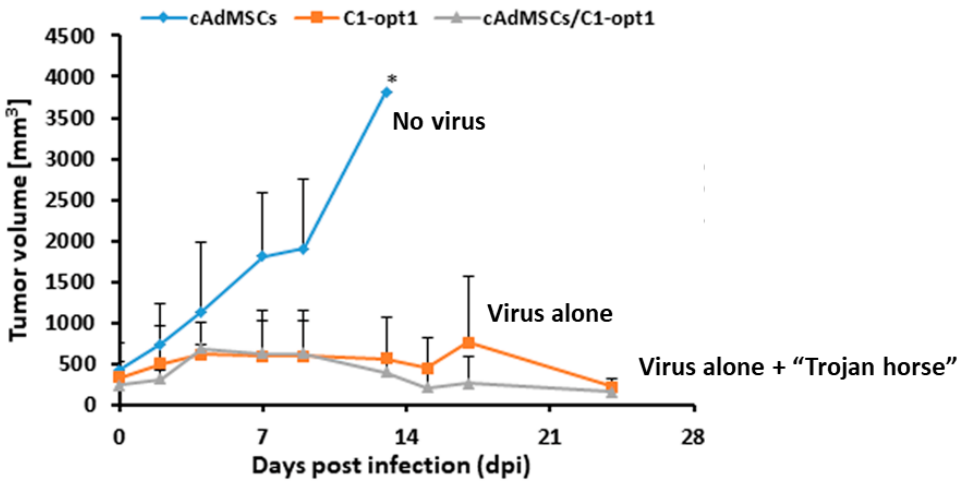


Figure 16. Tumor volume measurements in immunodeficient mice treated with C1-opt1, cAdMSC or C1-opt1/cAdMSC combined. Points represent the mean value from 3 measurements. SD is indicated as bars. Mice cohorts = 3.

Results indicate that the tumor mass is negatively affected by the C1-opt1 virus infection. Indeed, in the C1-opt1/cAdMSC and the C1-opt1 treated mice, the tumor volume is reduced at 28 dpi compared to the cAdMSC treated control mice.

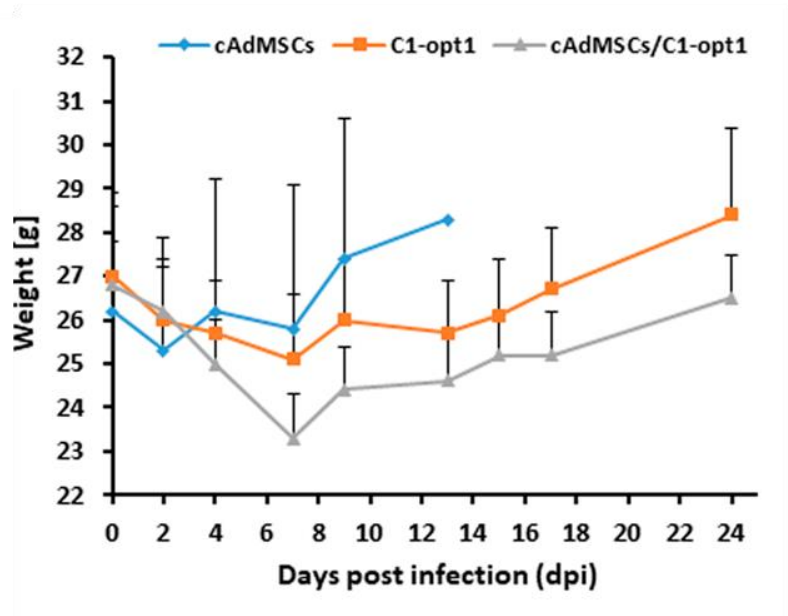


Figure 17. Body weight measurements of mice with STSA-1 xenografts treated with C1-opt1 or C1-opt1/cAdMSC. Net body weight was calculated by subtracting the tumor mass and measurements were plotted as a fraction of the net body weight. Points represent the mean value from three measurements. SD is indicated as bars. Mice cohorts = 3.

The toxicity was determined by body weight measurements and examination of the animals for possible pox formation and overall health status. Mice didn't show any signs of viral toxicity since body weights of all treated groups remained stable through the whole course of the study (**Figure 17**).

Taken together, there were no differences in survival and health status between nude mice treated with the virus (C1-opt1) alone or with the combination of C1-opt1/cAdMSC. Moreover, cAdMSC was safe and didn't cause any sign of toxicity in mice.

Table 7. Biodistribution of Vaccinia virus (C1-opt1) in tumors, other tissues and in the serum after 24 dpi.

PFU/g of organ	**Mice treated with C1-opt 1alone**			**Mice treated with cAdMSC/C1-opt1**		
№ mouse	78	88	89	80	83	86
Tumor	4.80×10^4	1.54×10^5	5.78×10^5	2.50×10^6	3.83×10^5	2.37×10^6
Lung	n.d.	n.d.	n.d.	3.45×10^2	3.45×10^2	n.d.
Liver	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Spleen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Kidney	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Serum	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Mice were treated intravenously with 2.5×10^6 PFU of C1-opt1 virus or a combination of 2.5×10^6 PFU of C1-opt1 and 2.5×10^5 cells of cAdMSC. The data were obtained by standard plaque assays on CV-1 cells using aliquots 50 µL of the homogenized organs (see protocol in methods **3.2.3.4**) and were presented as PFU per gram of organ. Each aliquot was measured in duplicates. N.d.: not detected.

The biodistribution after i.v. injection of C1-opt1 was evaluated in immunodeficient tumor-bearing mice to determine the viral persistence and dissemination from the initial injection site. The viral yield of C1-opt1 in tumors, organ tissues, and serum was evaluated 24 h after a single injection with C1-opt1 or cAdMSC/C1-opt1 into the tail vein of athymic nude mice bearing STSA-1 tumors. After dissection of the tumors, various tissues including spleen, liver, lungs, kidneys and blood serum, the results represented in **Table 7,** indicates that the highest viral titers were identified in tumor tissue of cAdMSC/C1-opt1-treated mice, whereas no viral particles were present in other organs, such as liver, spleen, kidney, or blood. Only a little amount of virus was detected in the lungs of cAdMSC/C1-opt1 group, but it was a trace residual amount.

This result suggested that the viruses have an inherent affinity to the tumor, or in other words, have the “tumor homing" capability.

The animal data were obtained in collaboration with Mr. I. Petrov and Dr. I. Gentschev.

CHAPTER 5: DISCUSSION

Dogs with spontaneous cancer are a very important experimental model for the study of human cancer that provides an excellent platform for the development of new cancer treatment therapies in human and canine cancer patients. Currently, in addition to traditional methods, a number of studies have been initiated to develop new therapeutic approaches to find more effective cancer therapies. The natural oncolytic properties of certain wild-type or genetically modified viruses against various tumor types are examined as a promising method in preclinical and clinical cancer treatments. In November 2005, Oncorin (based on adenovirus) was approved by the Chinese State Food and Drug Administration [180]. Later, another oncolytic virus, T-VEC, an engineered Herpes Simplex virus-1, expressing GM-CSF was approved by FDA and by the European Commission and Australian Health authorities for oncolytic virotherapy [181], [182], [183]. This step triggered increased interest in this novel therapeutic approach for cancer treatment [184]. Vaccinia virus is one of the most studied oncolytic viruses, used in millions of patients as a safe smallpox vaccine, mostly in children.

However, oncolytic virus (OV) therapy of cancer faces numerous challenges that limit its successful application, particularly when injected systemically (intravenously). The limiting factors include the clearance of the virus by the liver and spleen, macrophages, neutralizing interactions in blood, pre-existing antibodies, complement proteins, and coagulation factors. In addition, the physical endothelial barriers to infection and fast clearance by the immune system are added factors [185], [186]. Today, OVs in clinical trials need several doses of injection, and thus, therapeutic effects are dependent on higher doses, which simultaneously will increase viral toxicity and the inflammatory immune response [171].

Therefore, to disguise the virus from the host immune system to be able to decrease the dose of the virus in clinical trials and improve VACV safe delivery to the tumor site, we used ADSC/AdMSC as carrier cells due to their natural tropism for inflammation sites [187]. Mesenchymal stem cells are thought to migrate to sites of inflammation, but it is unknown how they will affect tumor growth. At the same time, ADSC/AdMSC is not tumor-specific, and part of the administered cells can be found in organs other than tumors [188]. For clinical applications, it is also important that these cells can be easily obtained by a minimally invasive procedure (e.g., liposuction) and can be grown in cell cultures following isolation (Good Manufacturing Practice guidelines) [189].

Many papers have been published regarding the antitumor effects of different recombinants of VACV, but information on the anticancer effects of original, genetically unmodified VACV strains or "native" strains is still limited [105], [109], [113], [121].

In the first part of this study, we compared different wild-type, non-modified strains of VACV (approved smallpox vaccines which were used in the human smallpox virus eradication program) and tested their oncolytic properties on the different types of cancer cells from different organ origins. In addition, we also tested the protection system based on the “Trojan horse” concept using a combination of human ADSC and 3 different wild-type vaccines, which were the single plaque purified Vaccinia virus strains W1, L1, and T1.

Although VACV has been broadly investigated, it is critical to select the strain that might be preferred, efficient and safe for routine clinical use in cancer therapy.

In this study, we used the following smallpox Vaccinia strains: L0, W0, T0, L1, W1, L1, and C1. Some of these strains, such as L0, W0, T0, and W1, are approved vaccines that have been used in the international smallpox eradication program, while T1, L1, and C1 have only been used in research studies but they were isolated from approved polyclonal wild-type vaccines, without genetic alterations. The oncolytic features of tested VACV strains (based on Lister, Wyeth, and Copenhagen) had already been confirmed before, except for the Chinese vaccine strain - Tian Tan. However, the efficacy of the wild-type Tian Tan strain with regard to oncolytic therapy and its clinical application has not been reported yet. Therefore, we tested the replication and cytotoxicity of the Tian Tan smallpox vaccine and its isolated clone number 5 *in vitro* and *in vivo*. Clone number 5 or T1 was derived from the Chinese approved Tian Tan smallpox vaccine (T0) using simple plaque-cloning selection, so it is a non-engineered single plaque purified clone. The profile of this strain is extremely similar to the parental Tian Tan strain, and it also remains neurotoxic, suggesting that it might have to be attenuated before human clinical studies [190].

The concept of using wild-type VACV to cure cancer was basically abandoned after extensive efforts in the 1960s and 1970s due to the misunderstanding of virus pathogenicity at that time. Despite the clear oncolytic effect of the live virus, physicians didn’t know the mechanisms of action of viruses and were unable to alter (attenuate) their genomes. They preferred to use chemical compounds since their composition and mechanism of action were much better understood. Therefore, the use of viruses was not as widespread in clinical applications. The viral therapy concept was reborn with the development of genetically-engineered, attenuated

viruses, and the medical and scientific community became interested in using genetically-modified viruses only while ignoring the wild-type vaccine strains. In the case of VACV, we believe that understanding the oncolytic mechanism of recombinant viruses and determining the real effects of genetic alterations requires a deep understanding of the parental VACV, including its "natural" anti-cancer properties. Each oncolytic, engineered/modified virus has the features of the parental wild-type strain. Using parental, wild-type viruses has several advantages, such that their effects have been extensively tested through their use in vaccination programs in humans (mostly children), they are approved for use in humans, stockpiles are available in many countries today, and it is usually easier to get permission for a clinical trial in which one plans to use repurposed agents. The goal of this work was to examine the antitumor properties of different wild-type VACV strains and compare them to each other in the same cell culture and preclinical animal experiments.

Among all the tumor cell lines and different VACV strains tested, we wanted to find the most effective one, but we were not able to decide because the oncolytic properties of the different viruses were changed depending on the cancer cell line used. In the current study, we tested the susceptibility of a panel of various human cancer cell types and human ADSC to the efficacy of infection of different VACV strains. We found that all seven human cell lines tested were efficiently infected by VACV strains. Most of the human cell lines infected by different strains of VACV produced viral titers in the range of 6–7 $\log_{10}$ PFU/10^6. According to our findings, PC-3 cells had the highest infection efficiency with the C1 strain. The virus production per cell was over 700 new viral particles after 48 hpi. The other cancer cells were also infected, but with lower permissivity. Wyeth and Lister Vaccinia strains had the lowest infection efficiency in HNT-35 (head and neck cancer) cells of all the human cell types and viruses tested. It is known that oncolytic Vaccinia virus replicates faster in tumor cells than in normal cells [191], [121]. Moreover, the replication of oncolytic Vaccinia virus in cancer cells is influenced by the type of cancer cell [192]. Our results also revealed that the tested strains can efficiently infect and multiply in human ADSC; the replication efficacy was comparable in several tested cancer cell lines, especially for the C1 strain, where the virus production levels from one ADSC was close to those of PC-3 tumor cells at an MOI of 1 after 48 hpi. Interestingly, canine AdMSC, when compared to human ADSC, are less permissive to VACV. This can be explained by the fact that dogs are less permissive hosts for VACV compared to humans.

Overall, our results have confirmed that there are differences in permissivity of many cancer cells to VACV infection. We also found that there were differences between the parental VACV strains and their purified isolates.

Furthermore, we tested the cytotoxicity of Vaccinia strains L0, W0, T0, L1, W1, L1 in different cancer cell lines (A549, PC-3, MDA-MB231, FaDu, HNT-13, HNT-25, and HNT-35) using the MTT assay. All Vaccinia strains were potent against the entire panel of different malignant cells *in vitro*, which was most visible at 96 hpi. HNT-35 was the most susceptible tumor cell line for all the VACV strains tested and the PC-3 cell line, despite producing the highest number of viral particles after 48 hpi, had the highest killing ability at day 4 after infection. Unfortunately, the MTT approach failed to detect purple formazan crystals in hADSC, both infected with VACV and not infected, probably due to the low metabolic activity of the cells. Therefore, we decided to test hADSC viability under virus infection conditions with the LDH assay. LDH was constantly released from Vaccinia virus-infected hADSC into a culture medium during the progression of virus growth. The viability of hADSC was not significantly reduced (1-4 days) after VACV infection in contrast to the cells of tumor cancer lines tested at the same MOI, which were rapidly killed by the infection. Even though two different approaches to determine cell cytotoxicity were used for tumor cells and hADSC, these findings and microscopic observations of infected cells may suggest that VACV can induce cell killing faster in cancer cells than in hADSC at the same MOI. The significant increase in LDH activity was evident in the infected cell cultures to day 5 post-infection, suggesting that the cells are able to survive longer while infected. These findings may particularly be useful if we want to deliver the VACV inside ADSC to a tumor site.

Dragonov et al. (2019) demonstrated in *ex-vivo* experiments that hADSC (not the same ADSC that were used in the current study) might also act as a virus-amplifying “Trojan horse” in the presence of autologous and allogeneic human PBMCs, and potentially be able to suppress both the innate and adaptive branches of anti-viral immunity [168].

In our study *in vivo*, we also tested the therapeutic efficacy of the different wild-type Vaccinia viruses L1, W1, and T1, alone or in combination with hADSC. We chose these particular vaccines strains because they are single plaque purified clones, and their oncolytic potential was generally similar to their parental licensed smallpox vaccines L0, W0, and T0, respectively. Furthermore, it is better to use a plaque purified clone, for example, for further genetic alterations instead of a parental vaccine strain which consists of a mix of virus clones.

The therapeutic efficacy and safety of the wild-type L1, W1, and T1 strains were tested in A549 human lung adenocarcinoma xenografts. After the tumors formed, the mice were injected i.t. with a single different dose of L1, W1, and T1, the combination of the virus and the cells (L1/ADSC, W1/ADSC, and T1/ADSC) or PBS. Treatment of A549 tumors with different doses of L1 and W1 as well as with L1/ADSC or W1/ADSC combination led to tumor regression. Unfortunately, we could not evaluate tumor growth of T1, and T1/ADSC treated groups due to the high toxicity of this virus strain: all mice had to be sacrificed by 19 dpi. Treatment with L1, W1, or L1/ADSC, W1/ADSC resulted in efficient tumor regression and, in most cases, almost complete elimination of tumors. During the first two weeks after treatment, tumors would rapidly increase in size in all groups, including the PBS control group. During the third week, the tumor sizes started to plateau. After 3 weeks, we observed significant tumor regression in all treated groups compared to the control PBS group. These data follow the tumor growth curves other researchers have observed [172], [193]. This standard three-phase tumor growth curve in mice implanted with A549 cells and treated with different recombinant Vaccinia virus strains such as GLV-1h164, GLV-1h442, or GLV-1h28, GLV-1h68 was also previously described [194]. Systemic injection of LIVP1.1.1 (L1) was previously shown to be able to boost viral replication and eliminate human and murine tumors in focally irradiated tumor xenografts compared to nonirradiated glioma xenografts [195].

Additionally, the treatment with L1 and W1 and the combination of L1/ADSC and W1/ADSC was well tolerated by the animals. There was no significant change in the relative body weight of mice in the Lister groups, and they all survived until the end of the experiment. However, half of the animals in the Wyeth treated group were sacrificed due to extensive body pox formation, particularly in the nasal area. Such animals were frequently unable to eat and started to lose weight. Only half of the animals in the Wyeth treated groups survived until the end of the experiment. In the Lister groups, no side effects such as pox formation were observed. It is worth noting that tumors vanished faster in the Wyeth groups than in the Lister groups. It can be explained by the fact that LIVP.1.1.1. has a naturally disrupted J2R locus of the TK gene, whereas ACAM2000, the approved US smallpox vaccine strain, is a TK-positive strain and therefore might be slightly more virulent. There were no significant differences in tumor volume regression between virus only treated groups and groups treated with a combination of virus and hADSC. These results are in contrast to the study where researchers using the three - dimensional (3D), fluorometric tumor model with parts of TME (used the Matrigel™ Basement Membrane Extract) *in vitro*, demonstrated that human bone marrow stromal cells (hMSC)

enhance the growth of ER-positive breast cancer cells such as MCF-7, T47D, BT474, and ZR-75-1. However, the growth rate of the ER-negative cell line such as MDA-MB 231 was unaffected by hMSC [196].

In this study, we have found that hADSC is safe in nude mice, however, we were unable to confirm that hADSC enhanced the viral replication of the *in vivo* system and protected the virus against the host immunity due to limitations of the model used. Therefore, it would be preferable to use dogs or human patients since mice are not natural hosts of VACV.

The next step could be implementing immunocompetent models to investigate the trafficking of ADSC-loaded VACV in the presence of an intact immune system. It would be essential to measure the serum level of IL-2, IL-4, IL-10, and IFN-γ after virus inoculation because cytokines play a crucial role in the host immune response [197]. It is also possible to improve wild-type strains by inserting an additional therapeutic agent to enhance their oncolytic effect or some genes that can influence ADSC properties for more efficient delivery and escape from the host immune response. This approach might be an excellent supportive mechanism to traditional cancer therapy.

A Phase I clinical study using the wild-type FDA-approved smallpox vaccine ACAM2000 (W1) loaded with cancer patient freshly isolated SVF (stromal vascular fraction) was overseen by Minev et al. in collaboration with the University of Wuerzburg [166]. In this clinical trial, 24 patients with different advanced metastatic and solid tumors and 2 patients with AML (Acute Myelogenous Leukemia) were recruited. Each patient was injected with their own SVF infected for 15-30 min with the ACAM2000 smallpox vaccine (MOI 1) i.v. and i.t. once. No serious side effects or significant increase in cytokine levels were noted for dosages from 1.4×10^6 to 1.8×10^7 PFU. Interestingly, viral DNA in blood disappeared 1 day after injection and returned 1 week later in 8 patients, indicating active viral replication at tumor regions, and the return corresponded with response to the therapy. In addition, several patients showed signs of antitumor effect (especially in combination with checkpoint inhibitors). It would be interesting to run clinical trials using a combination of ACAM2000 (W1) and autologous hADSC based on our findings. The use of SVF might be more feasible than using autologous ADSC, because SVF can be generated in large quantities immediately without cell culturing, thus largely avoiding the possibility of pathogen contamination [198]. However, SVF is a heterogeneous cell population that contains endothelial precursor cells, mesenchymal stem cells, T regulatory cells, fibroblasts, pericytes, M2 macrophages, smooth muscle cells,

preadipocytes. SVF samples may also contain fragments of many other types of cells, depending on how they are generated and purified, and these could be harmful [199]. Therefore, it would be preferable to use a homogeneous cell population because of the known qualities of ADSCs. In addition, it has been shown that both autologous and allogeneic ADSC could be used in the clinic for the same patient [168].

In the second part of this work, we analyzed the oncolytic effect of three new different engineered Vaccinia virus strains, C1-opt1, W1-opt1, and L3-opt1, all expressing turboFP635 as a reporter, and based on the wild-type Copenhagen, Wyeth, and Lister vaccines, respectively. TurboFP635 was inserted in TK-locus; this manipulation did not change the replication activity of the strains. In the current study, C1-opt1, W1-opt1, and L3-opt1 strains were tested for their replication efficiency and oncolytic potential in STSA-1 and CT1258 canine cancer cell lines in cell culture. We observed that all three novels VACV strains effectively infected, replicated in, and lysed STSA-1 and CT1258 canine tumor cell lines *in vitro*. The result was comparable with previous data, where different clones of LIVP (Lister strain) such as LIVP 1.1.1, LIVP 6.1.1, GLV-5b451, GLV-1h109, and also the widely known GLV-1h68 modified strain were tested [131], [132], [136]. The results showed that all tested viruses can efficiently replicate in these tumor cells, albeit with differences between virus strains and cancer cell types. The maximum virus titer was observed at 48 hpi in CT1258 cells infected with C1-opt1, the same result was observed in another STSA-1 cell line, indicating that C1-opt1 Vaccinia virus replicates more efficiently in comparison to W1-opt1 and L3-opt1 strains.

We demonstrated that canine AdMSC used in the study had the proper morphology and differentiation multipotency. Cytotoxicity analysis was performed on cultured cAdMSC infected with C1-opt1, W1-opt1, and L3-opt1 strains at an MOI of 0.5 by LDH assay to determine the percentage of lysed cells. Analyses of data showed that only 15% of the cAdMSC were killed after 2 dpi. It confirmed that these cells could harbor the virus for a long-term period, allowing the virus to spread into the body - and there is enough time for the carrier cell to reach the tumor or metastasis before it is destroyed. The mechanism whereby AdMSC is permissive to the Vaccinia virus is still unknown. Interestingly, in our study, the level of viral replication in cAdMSC was lower (approximately 40–60 fold) than in canine cancer cells (STSA-1 and CT1258) at the same MOI. Therefore, we decided to use a higher MOI for a more effective outcome and designed the animal experiment with an MOI of 10.

We tested the oncolytic effect of the C1-opt1 virus alone and its combination with cAdMSC (C1-opt1/ cAdMSC) in the canine STSA-1 xenograft mouse model. Altogether, our findings showed that both C1-opt1 and cAdMSC/C1-opt1 significantly reduced or completely eliminated tumors in the STSA-1 tumor-bearing mice. The tumor-shrinking was observed after 14 dpi that is consistent with our previous studies [131], [132], [136]. There was no significant difference between C1-opt1 alone and cAdMSC/C1-opt1, however, there was a slight difference at the period between 13 and 21 dpi. This can be explained by the aforementioned advantages of using cAdMSC.

To evaluate the distribution of C1-opt1 *in vivo*, we collected and checked viral titers in different organs, tumors, and blood serum. Data revealed that the virus mainly accumulated in the tumor tissue after i.v. administration (via tail vein) of C1-opt1 alone and combination of cAdMSC/C1-opt1 after 24 dpi, some amount of virus particles were found in the lungs of mice injected with a combination of cAdMSC/C1-opt1, but not in the group which received only virus alone. The cAdMSC might get "stuck" in the lungs and cause local virus propagation.

Here, there are several important observations. First, we showed that cAdMSC are permissive to Vaccinia virus infection and are able to support productive replication. This is a key requirement for the cell carrier: they should be permissive to virus infection and hold the virus in the cell or attached the cell surface for a certain period of time post-infection in order to deliver the virus to the tumor site and support virus progeny production [200]. Second, this is the first demonstration of the potential applicability of cAdMSC to deliver the Vaccinia virus in an experimental model of canine soft tissue sarcomas (STSA-1). We showed that cAdMSC infected with the Vaccinia virus produce a significant increase in mouse "patient" survival in *in vivo* models of canine soft tissue sarcomas in mice compared to the control group. Third, we confirmed that cAdMSC are not toxic to nude mice. An important consideration for the clinical use of adipose stem cells as therapeutic vehicles is the question of their influence on tumor microenvironment and growth. Finally, we found that uninfected canine cAdMSC do not significantly impact the growth of STSA-1 tumor *in vivo*, and furthermore, they did not significantly impact the median survival of the xenograft mouse model compared to virus therapy alone.

I cannot compare our findings with other, similar research because no other studies exist using a combination of ADSC/cAdMSC and VACV - at least none that have been published yet. However, I can compare our data with other oncolytic viruses loaded into MSCs. For example,

Shah et al. showed that a combination of adipose-derived MSC loaded with adenovirus (ICOVIR17) encapsulated in biocompatible synthetic extracellular matrix (sECM) eliminated HA-expressing GBM (Glioblastoma) xenograft tumors and increased mice survival to a significantly greater extent and more rapdily compared with injected ICOVIR17 alone. The authors explained this in terms of the fact that the naked virus is washed out by the cerebrospinal fluid immediately after injection, while the virus loaded into the cells delivered the virus to the tumor [201]. Another study found that a combination of bone marrow mesenchymal stromal cells loaded with adenovirus virus (OAd Ad5/3-TRAIL) first triggered a significant antitumor response and, second, efficiently infected and lysed pancreatic ductal adenocarcinoma (PDA) xenografts [202]. Hakkarainen et al. reported that the combination of MSCs and adenovirus not only prolongs the survival of mice but also increases homing to advanced-stage orthotic breast and lung tumors in comparison to virus alone, which was not detectable in the tested tumor xenografts [203]. Furthermore, when MSCs were administered alone, it was shown that they inhibited tumor growth of Kaposi's sarcomas (KS). Human MSCs were found to suppress the activation of the Akt protein kinase in various primary cell lines, which is an important factor of KS tumor growth and survival [204].

Du et al. studied the homing ability of MSCs to brain metastases. They generated melanoma brain metastasis in both immunocompromised and immunocompetent mice to investigate the therapeutic efficacy of HSV (Herpes Simplex Virus) and its combination with MSCs in melanoma brain metastasis. They showed that only HSV loaded into MSCs can efficiently migrate to the brain metastasis, therefore suppressing tumor growth and prolonging survival of mice [163].

Taken together, it was shown that cADSC could potentially act as “Trojan horses” that are capable of migrating and delivering the Vaccinia virus to tumors. The further therapeutic efficacy of the combination of cAdMSC and the C1-opt1 Vaccinia virus strain should be confirmed in clinical trials with canine cancer patients, as it was done by Minev et al. (2019) with human SVF derived autologous stem cells infected with the smallpox vaccine ACAM2000 [166].

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LIST OF ABBREVIATIONS

◦C	Degrees Celsius
µg	Microgram
µl	Microlitre
aa	Amino acid
AB	Antibodies
ATCC	American Type Culture Collection
BHK-21	Baby hamster kidney cell line
cAdMSC	Canine Adipose-derived Mesenchymal Stem Cells
CO_2	Carbon dioxide
CLL	Chronic lymphocytic leukemia
CMC	Carboxymethylcellulose
DMEM	Dubelcco’s Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dpi or dpvi	Days post virus injection/infection
EtOH	Ethanol
FBS	Fetal Bovine Serum
g	Grams
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PFU	Plaque Forming Units
rpm	Revolutions per minute
TurboFP	Red Fluorescent Protein
h	Hour
hADSC	Human Adipose Derived Stem Cells
hpi	Hours post infection
kbp	Kilobase pairs
LDH	Lactate Dehydrogenase
LIVP	Lister vaccine strain
mg	Milligram
min	Minute
ml	Millilitre

mm	Millimetre
mM	Millimolar
MOI	Multiplicity of Infection
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
MVA	Modified Vaccinia Ankara
ng	Nanogram
nm	Nanometre
OD	Optical density
OV	Oncolytic virus
r.t.	Room temperature
SD	Standard Deviation
SC	Subcutaneous
VACV	Vaccinia Virus
VEGF	Vascular Endothelial Growth Factor
VPA	Viral Production Assay
WHO	World Health Organization
U/ml	Units/millilitre
VPA	Viral Production Assay

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AFFIDAVIT

I hereby confirm that my thesis entitled "**Testing efficacy of approved Smallpox Vaccines in Human and Canine Cancer Therapy: Adipose - tissue derived stem cells (ADSC) take up VACV and serve as a protective vehicle for virus delivery to tumors**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis. Furthermore, I confirm that this has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wurzburg,

Place, Date Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "**Wirksamkeit zugelassener Pockenimpfstoffe in der Krebstherapie bei Menschen und Hunden: Aus gewebe gewonnene Stammzellen (ADSC) nehmen VACV auf und dienen als schützendes Vehikel für den Viruseintrag in Tumore**" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberates, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittle verwendet zu haben. Ich erkläre außedem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelen hat.

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