Oncolytic virotherapy - analysis, design, models

Bhatt, Darshak

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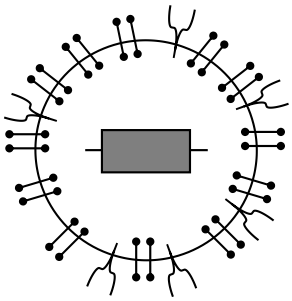
CHAPTER 1

Introduction and outline of the thesis



Preface and scope

Viruses are notorious due to their infectious nature and are infamous for causing a wide range of diseases, from the common cold and flu to more severe illnesses like HIV, COVID-19, and Ebola. Therefore, the idea of using viruses to treat cancer may at first seem to be irrational. Despite the generally negative connotations surrounding the word 'virus', scientific research to date has shown that certain viruses can antagonize cancer and could function as an effective cancer therapy. Therefore, clinical use of such oncolytic (cancer-killing) viruses requires regulatory approval validated through a demonstration of its safety and efficacy comparable to or better than conventional treatment.

The scope of this thesis is to analyze, model, and develop strategies for the improvement of the safety and therapeutic efficacy of oncolytic virotherapy with a focus on Semliki Forest virus, an alphavirus.

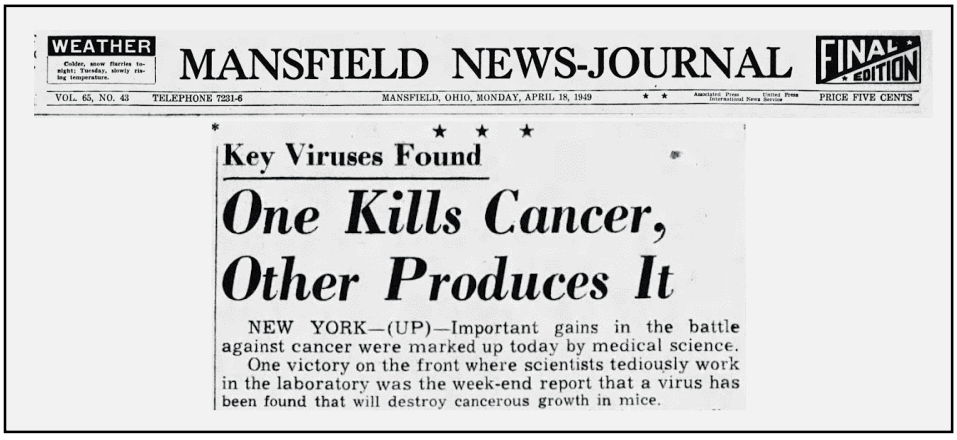
This introduction chapter starts with a summary of historic observations supporting the use of viruses for treating cancer. In the subsequent sections, we provide a brief background of the topics covered in the thesis. First, Semliki Forest virus, an alphavirus we propose for safe oncolytic virotherapy is introduced. Next, we describe the importance of investigating the impact of tumor extracellular vesicles in modulating the tumor microenvironment and thus therapy response to oncolytic viruses. Finally, we introduce how cellular or systemic resistance mechanisms may undermine the efficacy of oncolytic virotherapy in an ecological and evolutionary context.

We end with an outline of all the chapters present in the thesis.

The search for ideal oncolytic viruses (1890-1990s)

From chance observations to a scientific discipline

In 1896, a 42-year-old woman with 'myelogenous leukemia' went into remission after a presumed influenza infection. The patient had a significantly enlarged liver and spleen, which shrank to nearly normal size, and an abnormally elevated leukocyte count, which dropped more than 70-fold after the infection. Unfortunately, the remission was short and her leukemia progressed until death. This case study along with others published since the mid-1800s, were some of the earliest documented cases where tumor regressions have coincided with natural infections caused by bacteria or viruses.[1] Surprisingly, this observation was made 37 years before we learned that influenza was a virus infection.



WEATHER Colder, snow flurries tonight; Tuesday, slowly rising temperature.

MANSFIELD NEWS-JOURNAL

FINAL EDITION

VOL. 65, NO. 43 TELEPHONE 7231-6 MANSFIELD, OHIO, MONDAY, APRIL 18, 1949 PRICE FIVE CENTS

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Key Viruses Found

One Kills Cancer, Other Produces It

NEW YORK—(UP)—Important gains in the battle against cancer were marked up today by medical science.

One victory on the front where scientists tediously work in the laboratory was the week-end report that a virus has been found that will destroy cancerous growth in mice.

Figure 1: A newspaper report on research about oncolytic and oncogenic viruses as novel discoveries of 1949.[2]

The use of viruses for cancer therapy was suggested in the early 20th century.[3–5] Dr. Alice E. Moore's pioneering research in 1949 established viruses as oncolytic agents in mice and identified key factors influencing their effectiveness, such as virus infectivity, tumor specificity, and route of delivery (Figure 1).[6,7] Soon, other studies confirmed tumor destruction in chickens and rabbits using different viruses.[8,9] While these insights were valuable, challenges emerged. Mainly, oncolytic viruses varied considerably in their ability to destroy tumors, making the

response specific to the virus used. Some viruses showed broad oncolytic capacity across different tumors, but they were highly neurotropic and unsafe for clinical use.[7] To address this, Moore and colleagues explored 'directed evolution' techniques to alter virus features for specific functions.[6,7] Through serial passages in tumor tissue, viruses could evolve to be highly oncolytic or lose oncolytic potential, however retained neurotropic potential. Furthermore, immune sera with virus-neutralizing antibodies could protect animals but reduced oncolytic potential. This work emphasized the importance of virus-tumor combinations and antiviral immune responses in cancer virotherapy efficacy.

Oncolytic virotherapy for humans and safety

In 1952, Dr. Chester M. Southam with Alice Moore conducted early clinical trials using different viruses for cancer treatment.[10] These viruses were chosen based on criteria such as showing oncolytic potential in animal studies, expected proliferation in humans, and low virulence to ensure safety. Although virotherapy resulted in transient tumor regression in some patients, substantial therapeutic effects were lacking. However, there were indications that virotherapy led to more virus infection in tumors than in surrounding tissue, and transient tumor damage coincided with viral infection. This and other clinical trials revealed significant obstacles in developing viruses for clinical use, including the development of antiviral immunity, partial tumor eradication, and differences in sensitivity to oncolytic virotherapy among patients.[11] Furthermore, the possibility of toxicity in immune-compromised individuals added to the complexity of the challenge.

To address safety concerns, researchers sought viruses with diminished pathogenic potential. It was hypothesized that non-human animal viruses might exhibit oncolytic activity even in hosts naturally resistant to viral infection. This hypothesis was supported by Moore's observations using Russian encephalitis virus, a human pathogen, to treat murine cancer. However, the trade-off between controlling virulence and expecting complete tumor eradication remained a challenge.[11] Nonetheless, certain exceptions were found. Vesicular stomatitis virus (VSV) of cattle origin was selectively destructive to human tumor cells with defects in the interferon pathway, and it showed limited human pathogenicity. Similarly, New Castle disease virus (NDV) of avian origin demonstrated safety for human

applications and continued to be used for cancer treatment, with some follow-up studies reporting cancer remission lasting at least 10 years.[12] Despite challenges, these examples offered promising options for cancer oncolytic virotherapy.

By the 1970s, evidence emerged that manipulation of the immune system could lead to tumor regression. Transplanting melanoma antigens or using blood products (antitumor antibodies) from regressed patients showed promising results.[13–15] Immune stimulation in cancer patients by injecting BCG vaccine into melanoma nodules resulted in tumor regression, even in non-injected nodules.[15] Similarly, virus-based rabies and mumps vaccines exhibited immunogenicity and led to tumor regression in some patients, validating the possibility of using viruses as agents for cancer immunotherapy.[11,15,16] Subsequent research thus aimed to develop virus-based cancer vaccines and immunogenic viruses to activate potent anti-cancer immune responses in patients.

Beyond oncolysis; oncolytic virotherapy and immune stimulation

Immune responses induced by various oncolytic viruses can help to eradicate cancer cells by recruiting immune cells to the tumor site, activating them to attack cancer cells, and promoting the production of cytokines that enhance the immune response (Figure 2).[17] When oncolytic viruses infect cancer cells, they trigger a series of events that activate the immune system. The virus-infected cancer cells produce signals called danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) that are recognized by immune cells, such as dendritic cells and macrophages. These immune cells then engulf the infected and dying cancer cells or their remnants and present viral or cancer antigens on their surface to T cells. T cells, in turn, recognize and attack the infected cancer cells, leading to their destruction. The activated T cells can also migrate to other areas of the body to seek out and destroy additional cancer cells that may have metastasized. Oncolytic viruses can also simultaneously stimulate the production of cytokines, which are signaling molecules that regulate the immune response. Cytokines can activate immune cells and help to further recruit them to the tumor site, leading to an enhanced anti-tumor response.

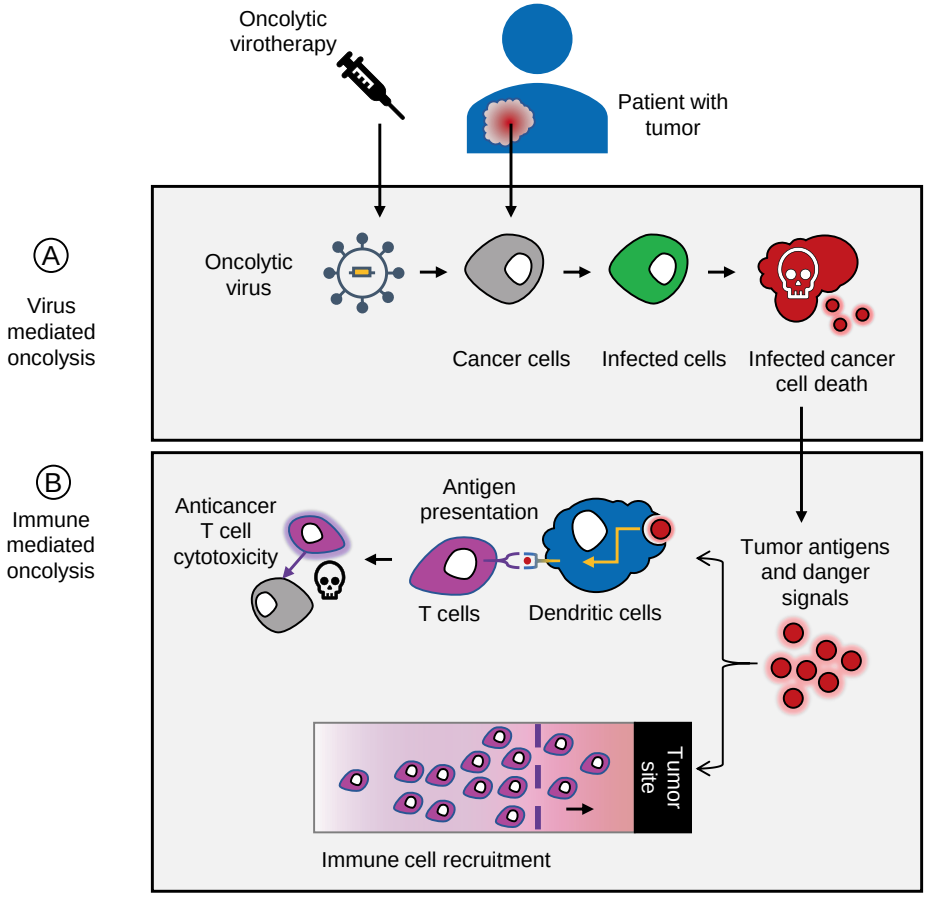

Figure 2: Dual mode of action of oncolytic virotherapy. (A) First, infection by an oncolytic virus leads to cancer cell death and tumor destruction. (B) Second, infected cells release a wide variety of danger signals including tumor antigens that stimulate the recruitment and activation of antitumoral immunity.

How far have we come with oncolytic viruses? (1990-2020s)

Genetic engineering of viruses for therapy

Before we move on, we have to realize that scientific advancements in the field of genetic engineering paralleled progress in oncolytic viruses. Understanding genetic information storage in nucleic acids[18] and its flow to encode functional proteins[19] paved the way for reading and editing cellular genomes.[20–22] Molecular tools enabled precise 'cut and paste' of DNA/RNA sequences[23], while polymerase

chain reaction[24] simplified DNA mutagenesis. These developments led to the emergence of biotechnology and synthetic biology, facilitating the editing of biological systems for therapeutic purposes.[22,25] The 21st century saw a surge in applications, including recombinant proteins, synthetic chromosomes, CRISPR-Cas genetic editing, and chemical synthesis of long DNA sequences.[22,26,27]

Importantly, the rapid developments in the field of biotechnology and synthetic biology aided the development of safer and more effective oncolytic viruses.[17,28,29] A prime illustration of this progress is exemplified by the development and approval of Imlygic or Talimogene laherparepvec (T-VEC), an oncolytic virus for treating melanoma.[30,31] T-VEC was developed using a patient-derived herpes virus demonstrating low pathology. The virus was further genetically modified to be safe and effective for cancer therapy. Safety was ensured by 'deleting' virulent genes that help the virus to infect healthy cells, thus leading to selective replication in cancer cells. Efficacy was improved by 'deleting' viral genes that block immune responses and 'replacing' them with immunogenic genes to boost anti-tumor immunity.

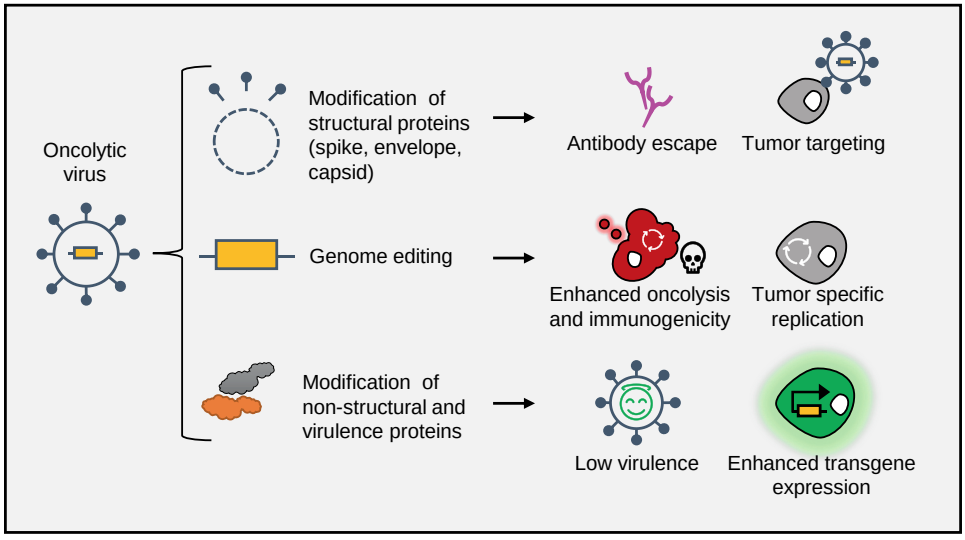

Figure 3: Engineering oncolytic viruses to improve safety and efficacy. Modifications of viral structural proteins can enable escape from antiviral antibodies and enhance tumor infection, or allow precise cancer tissue targeting and reduce toxicity towards healthy tissue. Genome editing techniques fine-tune the virus for improved cancer cell recognition and destruction. Changes in non-structural proteins, such as virus replicase, can boost viral replication and transgene expression within cancer cells. Deletion of virulence factors can mitigate harm to normal tissues.

The current landscape of oncolytic viruses, their design, and clinical use

The approval of T-VEC in 2015 by the US, Europe, Australia, and Switzerland, has renewed the hopes of developing oncolytic viruses for clinical use. Several virus candidates have been similarly modified genetically to improve safety and efficacy (Figure 3).[32] I will not discuss this in detail now as Chapter 2 of the thesis is dedicated to reviewing the clinical efforts in assessing the safety and efficacy of virotherapy for cancer treatment.[29]

Designing safe and effective oncolytic virotherapy based on Semliki Forest virus

So far, it is clear that oncolytic viruses are promising anticancer therapeutics due to their direct tumor-killing potential in combination with their ability to activate anticancer immune responses. To bring this promise to reality requires that the viruses are safe and effective in their profile. As mentioned earlier, advances in biological engineering have provided the molecular tools to make this possible. With the above considerations in mind, we aimed to develop a safe and immunogenic oncolytic virus by genetically engineering Semliki Forest virus (SFV).

Semliki Forest virus

SFV is a positive-strand RNA virus, belonging to the *Alphavirus* genus in the Togaviridae family. It was first isolated in the Semliki Forest region of Uganda and has since been extensively studied as a model virus to elucidate host-virus interactions. The virus has the potential to infect a broad range of mammalian hosts, however is non-pathogenic in natural settings and does not cause fatal symptoms in humans.[33] The only reported fatal case in humans occurred in a laboratory setting leading to fatal human encephalitis, possibly due to an unusual load of virus, or preexisting comorbidities related to immunodeficiency.[34]

The RNA genome of SFV encodes for various viral proteins, simply categorized into structural proteins: the capsid protein and the envelope proteins; and non-structural proteins 1 to 4 (Figure 4).[35] The capsid protein makes up the icosahedral

structure of the virus and packages the RNA forming the nucleocapsid. The envelope proteins are organized in a lipid layer through which they associate with the capsid proteins. The non-structural proteins 1 to 4 function as a replicase complex involved in the rapid expression and replication of the RNA in host cells. Thus, the SFV genome is a replicon, meaning self-replicating as the viral RNA encodes so-called non-structural proteins responsible for RNA replication and translation.

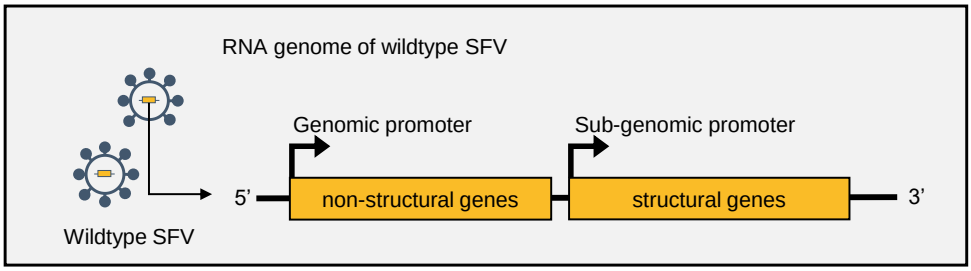

Figure 4: RNA genome of wild-type SFV. The RNA genome encodes non-structural and structural genes under the regulation of genomic and sub-genomic promoters respectively.

Upon infection of a cell, wild-type SFV induces cell death and production of viral progeny (Figure 5). Such virus-mediated lysis is triggered by a chain of events that starts when SFV particles are endocytosed by cells. The electron micrographs of virus infection clearly illustrate these events of the SFV life cycle as described by the lab of Ari Helenius (Figure 5A).[36] After attaching to a cell surface, SFV is trapped into clathrin-coated pits at the membrane, then is internalized by endocytosis in coated vesicles, and sequestered into intracellular vacuoles and lysosomes. Low pH of post-fusion vacuoles triggers the penetration of the nucleocapsid into the cytoplasm followed by the uncoating of viral RNA (Figure 5B). Further processing of viral RNA in the cytoplasm and interaction with the cells' translational machinery leads to a direct expression of the non-structural proteins encoded on the positive-stranded genome under the regulation of the genomic promoter. The non-structural proteins form the replicase protein complex and initiate the replication of the viral genome. The structural proteins are next translated under the regulation of the sub-genomic promoter. At the end of a complex process involving the packaging of the viral genome and assembly of the virus particles, the progeny SFV particles are released from the host cells. SFV

infection triggers cellular-stress responses in the host cells by activating ER stress due to a substantial amount of viral protein expression, inducing a wide range of innate antiviral responses through exposure to viral danger signals (single- or double-stranded RNA and proteins), and inducing loss of plasma membrane integrity through viral budding. These features of SFV, i.e. the high infection rate and lysis of infected cells combined with the observation that SFV infects a variety of cancer cells formed the basis for considering SFV for oncolytic virotherapy.

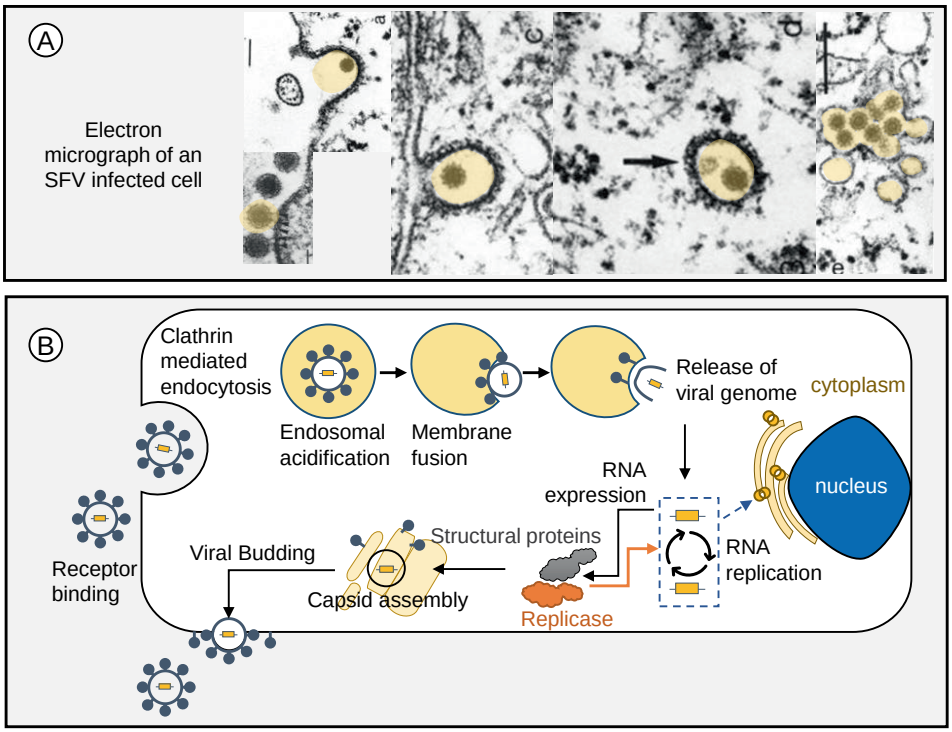

Figure 5: Infection cycle of wild-type SFV. (A) Electron micrograph of an SFV-infected cell, with steps of virus entry and processing highlighted in yellow. Adapted from the work of Ari Helenius et al.[36] (B) Diagrammatic representation of the infection cycle of SFV in target cells starting with entry, followed by uncoating and release of viral genome in the cytoplasm, and expression of viral genes, leading to virus assembly and extracellular release.

Engineering of recombinant replicon particles for safe and effective virotherapy

Liljeström and Garoff developed an expression vector based on the SFV replicon and together with colleagues optimized it to further improve safety.[37,38] Based on

this technology we produced recombinant SFV (rSFV) replicon particles with the aim of developing safe and effective oncolytic virotherapy.

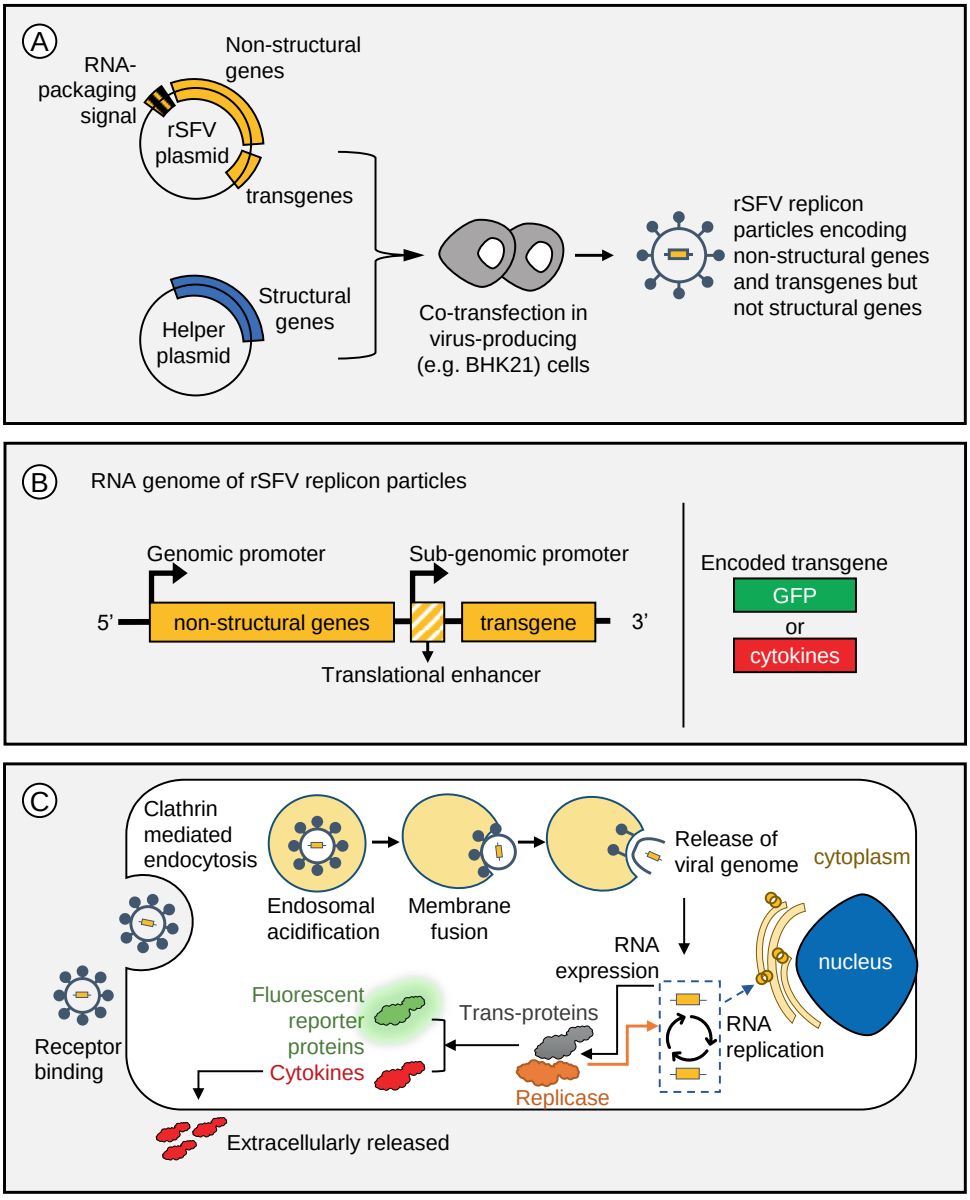

Figure 6: Design and characteristics of rSFV replicon particles. (A) Production of rSFV replicon particles capable of a single round of infection using a helper plasmid system. (B, left) RNA genome of rSFV replicon particles that encode a transgene instead of structural proteins, thus allowing for a single round of infection. (B, right) The encoded transgenes for this thesis are fluorescent reporters or immunogenic cytokines. (C) Diagrammatic

representation of a single round of infection with rSFV replicon particles, where viral genome expression leads to expression of fluorescent reporter proteins or cytokines.

In this vector system, the structural genes, and the non-structural genes are encoded on separate plasmids. Since the helper plasmid(s) that encode the structural proteins do not contain a 'packaging signal', it is ensured that rSFV replicon particles thus produced only contain the non-structural genes encoding for the virus replicase. Thus, as the structural proteins cannot be produced, there is no virus progeny, and infection with these rSFV replicon particles results in a single round of infection (Figure 6A).[38,39] To boost the therapeutic efficacy of rSFV replicon particles, we encoded immunogenic genes under the regulation of a translational enhancer derived from the viral capsid gene. The combination of the single-round infection design and the incorporation of immunogenic genes in the rSFV replicon particles ensured the potential of SFV for improved safety and efficacy as an oncolytic virus (Figure 6B-C).

Smerdou and Liljeström have shown earlier that rSFV particles are safe and unlikely to lead switch back to an infectious wild-type virus.[38] Smerdou and colleagues have also shown that by encoding immunogenic cytokines in rSFV replicon particles, there is an improvement in antitumoral immune responses resulting in better treatment efficacy in animal models.[40–43] Our lab has used rSFV replicon particles as a vaccine platform 'Vvax001' to encode antigens of the envelope proteins from human papillomavirus.[39,44] Through extensive preclinical studies leading up to a recently conducted phase 1 study,[45] our group has shown that rSFV replicon particles are safe and successfully induce strong antitumoral immune responses in receiving patients. A phase 2 study is currently being conducted to demonstrate clinical efficacy.

Understanding cancer to optimize therapeutic outcomes

Cell-centric and tissue-centric perspectives on cancer

Cancer has been described as any one of a large number of diseases characterized by the development of abnormal cells dividing uncontrollably with the ability to infiltrate and destroy normal body tissue. Due to its multifaceted and diverse

nature, understanding the process of cancer development is critical to developing and tailoring therapeutics according to patient requirements for optimal benefits. Researchers have therefore comprehensively evaluated the characteristics of cancer as a disease through interdisciplinary approaches ranging from molecular to systems biology. As a result of this, the cancer research community has brought forth two distinct perspectives for the development of cancer therapeutics: (1) a genetic, cell-centric perspective, and (2) a tissue-centric viewpoint.

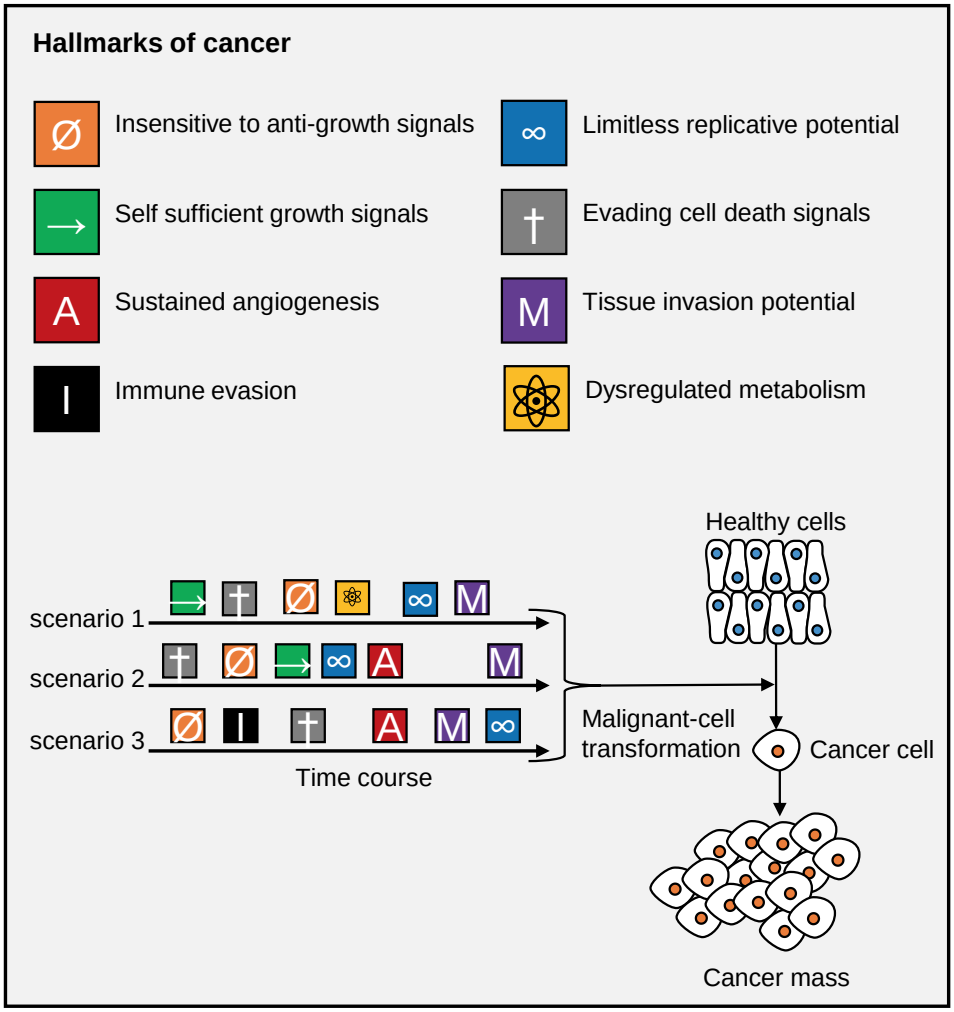

Figure 7: Cell-centric, genetic perspective of carcinogenesis based on hallmarks of cancer. Normal cells transform into malignant cells upon accumulation of mutations in genes regulating the characteristic hallmarks of cancer. Figure adapted from the work of Douglas Hanahan.[46]

From a cell-centric, genetic perspective, the hallmarks of cancer have served as a simplified and comprehensive guide to the fundamental processes that lead normal human cells toward malignancy (Figure 7). [46] This framework explains how somatic mutations acquired by a normal cell can cause carcinogenesis. The hallmarks encapsulate crucial traits resulting from genetic alterations of healthy cells such as uncontrolled proliferation, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, invasion and metastasis, deregulation of cellular energetics, and immune evasion. These characteristics collectively define the intricate nature and adaptability of a cancer cell and have helped researchers focus on the development of therapeutic drugs that target and interfere with these hallmarks at a molecular level.

On the other hand, the tissue-centric perspective offers an alternative interpretation. It proposes that cancer is a tissue-based disease, consisting of a diverse ecosystem of cancer cells, stromal cells, immune cells, and blood vessels within the tumor microenvironment, with evolutionary implications (Figure 8).[47–49] This theory underscores that all cells proliferate and move by default and that cancer arises due to disruptions in the interactions between cells and their surrounding extracellular matrix, as well as among cells themselves. Unlike the defined hallmarks, this theory emphasizes the significance of cellular interactions and the tissue context in driving carcinogenesis. In this view, cancer is seen as a departure from normal developmental processes rather than a mere acquisition of specific cellular traits. The tissue organization field theory highlights that comprehending disturbances in tissue organization and cellular interactions is essential for unraveling the origins and behavior of cancer. This perspective recognizes the dynamic interplay between cells and their microenvironment as pivotal in cancer progression and is vital for therapeutic approaches such as immunotherapy; which modulates the tumor microenvironment to enhance the patient's immune system against cancer.

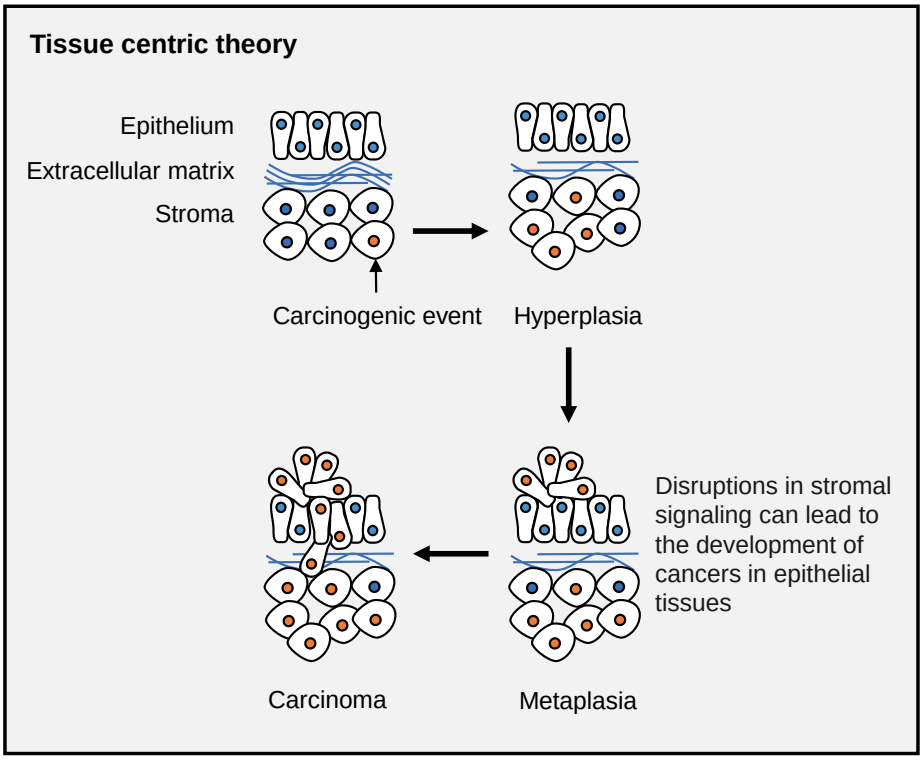

Figure 8: Tissue-centric perspective of carcinogenesis. Cancer arises due to the disruption of cellular interactions at the tissue level causing a dysregulated tissue organization and ultimately cancer formation. Figure adapted from the work of Soto and Sonnenschein.[47]

Role of tumor derived extracellular vesicles in regulating cancer progression

Cancer can be described as a dynamic disease resulting from complex and dynamic interactions between malignant and stromal cells in the tumor microenvironment. The cellular interaction between these components influences cancer development and spread and can be regulated by extracellular vesicle-mediated communication. Extracellular vesicles (EVs) are small lipid-bilayer structures released by cells, once considered insignificant but now recognized for their crucial biological functions.[50] EVs are a heterogeneous population of vesicles with endosomal and plasma-membrane origins (Figure 9A).

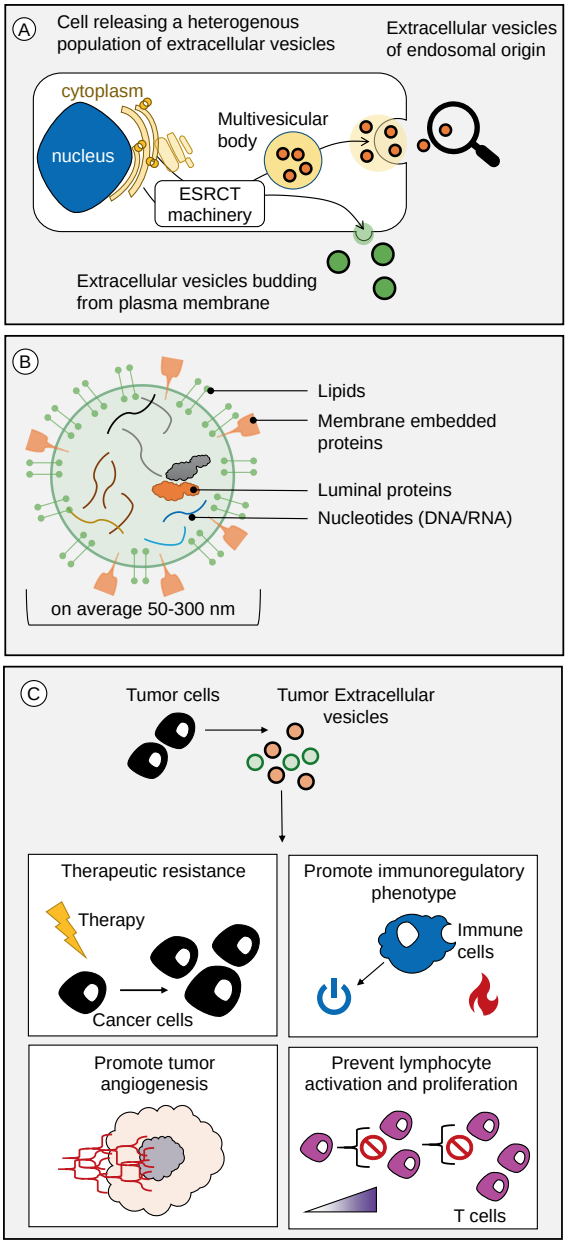

Figure 9: Features of tumor extracellular vesicles. (A) Extracellular vesicles released by cells originate from endosomes (from multivesicular bodies) or the plasma membrane of cells regulated by the endosomal sorting complex required for transport (ESCRT machinery). (B) Biochemical composition of extracellular vesicles consisting of structural and functional proteins, lipids, and nucleotides. (C) Pro-tumoral role of tumor extracellular vesicles. Figure adapted from the work of Clotilde Théry et al. and others.[50,51]

EVs can interact with target cells, triggering specific reactions and influencing their function. EVs can deliver bioactive materials, including proteins, lipids, and genetic material, to recipient cells, affecting their behavior (Figure 9B). Tumor-derived EVs have been reported to impact cancer progression and therapy response by promoting angiogenesis, creating an immunosuppressive microenvironment, and inducing therapy resistance in tumors (Figure 9C). These mechanisms have been reviewed in detail in chapter 4. While the role of tumor EVs has been primarily studied in the context of checkpoint immunotherapy and conventional chemo- or radiotherapy, tumor EVs can likely exploit similar mechanisms to influence the efficacy of oncolytic virotherapy. Conversely, oncolytic virotherapy can also influence tumor EVs. During viral infection, tumor cells produce different EVs, some of which may

contain viral components or viral RNA.[52] These virus-modified EVs can impact neighboring tumor cells, potentially enhancing or inhibiting viral replication or promoting antitumor immune responses. Furthermore, virotherapy-induced cell death and the release of viral particles can trigger the production of apoptotic bodies and exosomes, which may contain viral antigens and stimulate antitumor immune responses.[53] Therefore, understanding the complex interplay between tumor EVs and oncolytic virotherapy can prove useful in optimizing its efficacy.

Why consider ecology and evolution to study therapeutic outcomes of oncolytic virotherapy?

Tumor ecology and evolution

The tumor microenvironment exists as a dynamic and intricate ecosystem composed of cancer cells, normal cells, immune cells, blood vessels, and other elements engaging in diverse interactions continuously. This complexity often shapes the response to therapeutic interventions including oncolytic viruses.[48,54–56] Therefore, by studying the tumor microenvironment within an ecological and evolutionary framework, we can better evaluate these interactions and how they influence tumor growth and treatment outcomes.[54,57–59] For example, through an ecological framework, researchers can gain insights into the context of external factors driving the emergence of distinct cell populations within tumors, and how these variations might impact the efficacy of oncolytic virotherapy. Furthermore, evolutionary principles allow us to explore the adaptive strategies employed by cancer cells within the dynamic tumor microenvironment, shedding light on their ability to evade therapeutic interventions and adapt over time. This eco-evolutionary perspective combining insights from both an ecological and an evolutionary perspective, is not new in cancer research[60] and has helped in understanding the process of carcinogenesis and therapeutic resistance.[60–62] Therefore, we aim to study the interaction between cancer cells and their microenvironment by leveraging existing eco-evolutionary considerations to improve therapy response to oncolytic viruses.

To further advance our understanding of the eco-evolutionary processes behind cancer cells evading oncolytic virotherapy, we want to study it in a spatiotemporal

setting. This would allow us to explore the following dynamics of tumor and immune response to virotherapy:

- Evolution within the Body: Cancer cells evolve within the microenvironment of the body, subject to selection pressures including those inflicted by therapeutic intervention. This can lead to the emergence of diverse cell populations with varying genetic traits, contributing to intra-tumor heterogeneity or even the selection of infection-resistant clones, thus affecting treatment responses to oncolytic viruses.

- Tumor as an Ecosystem: Tumors can be viewed as ecosystems comprising various cell types, blood vessels, and extracellular matrix. Cancer cells can communicate with these elements to alter immune responses, influence tumor growth, and invasion, and thereby also influence therapeutic responses. Accordingly, cancer cells or even immune cells may manipulate these interactions to create a pro-tumoral environment while hampering the activity of oncolytic viruses.

- Evolutionary Arms Race: Cancer cells and the immune system engage in an evolutionary arms race.[63] Cancer cells evolve mechanisms to evade immune surveillance, while the immune system evolves to recognize and target cancer cells. Moreover, cancer cells may also evolve mechanisms to evade virotherapy. This dynamic interaction shapes the progression and outcomes of cancer.

- Trade-offs and Constraints: Evolutionary trade-offs, where certain adaptations come at the cost of others, are observed in cancer progression. For instance, cancer cells may evolve resistance to chemotherapy agents, but this might compromise other cellular functions and/or make them susceptible to other therapeutics.[64–66]

Thus, the adaptation of tumors to selective pressures and its exploitation of the dynamic tumor microenvironment may contribute to its evasion of oncolytic virotherapy. By embracing spatial dynamics, we can harness a comprehensive understanding of the tumor's adaptability in an ecological context. This can potentially inform the development of innovative oncolytic virotherapy strategies that anticipate and counteract tumor tactics, thus optimizing treatment outcomes.

Computational modeling to study spatiotemporal dynamics of oncolytic virotherapy

Therapeutic outcomes of oncolytic virotherapy result from a complex spatiotemporal interplay between the virus, target tumor cells, and the immune response. Predicting outcomes through intuitive reasoning and verbal arguments therefore may lead to an incomplete understanding of these dynamics. While acknowledging the value of traditional in vitro and in vivo laboratory experiments, an in silico computational approach brings remarkable advantages in solving this problem.[67] Computational modeling offers researchers a powerful tool to expedite the exploration of diverse scenarios without the logistical constraints often associated with traditional laboratory experiments. It enables rapid experimentation, bypassing ethical considerations and requirements for the collection and processing of biomaterial. Furthermore, the utilization of high-performance computing and workflow parallelization significantly accelerates the process, allowing for the evaluation of numerous scenarios within a fraction of the time required for in vivo or in vitro studies. Unlike laboratory work, where numerous uncontrollable variables may subtly fluctuate from day to day, computational models allow researchers to maintain consistent conditions, varying only the parameters intentionally introduced. This efficiency in assessing the outcomes of individual experiments empowers researchers to conduct a multitude of trials, facilitating the exploration of a broader spectrum of assumptions and the systematic variation of confounding factors to a far greater extent, ultimately advancing our understanding of the spatiotemporal dynamics of oncolytic virotherapy.

A relevant approach to modeling the spatiotemporal dynamics of oncolytic virotherapy involves utilizing agent-based modeling, where a virtual simulation is created to represent cellular interactions within a tumor microenvironment. Individual agents in this virtual environment represent cells, each following predefined rules integrated into the model. Specifically, it enables one to define cells with distinct characteristics like cell type, proliferation rates, and activation levels, and study their interaction within a two- or three-dimensional spatial grid simulating a tumor tissue (Figure 10A-B). A good example of this is the work by Berg et al., where they modeled a virtual tumor environment, consisting of cancer cells, stromal cells, and infected cells interacting dynamically and influencing

therapeutic outcomes through their distinct characteristics.[68] In principle, an agent-based modeling approach grants precise control over the behaviors and rules governing the actions of each cell type within the model. For example, cancer cells can undergo replication, mutate into resistant forms, or undergo cell death. Immune cells can recognize and attack against cancer cells, or they may become exhausted. Additionally, viruses can infect cancer cells or be neutralized by immune cells. Upon initiating a simulation, these cells sense and react according to their programmed behaviors in the virtual model environment, mirroring the spatial characteristics of the tumor. During and after simulations, data on various parameters are collected, including cell growth, death dynamics, virus-mediated killing, immune responses, etc. Multiple simulations explore diverse scenarios, and results are analyzed to identify insights that inform optimal treatment outcomes (Figure 10C).

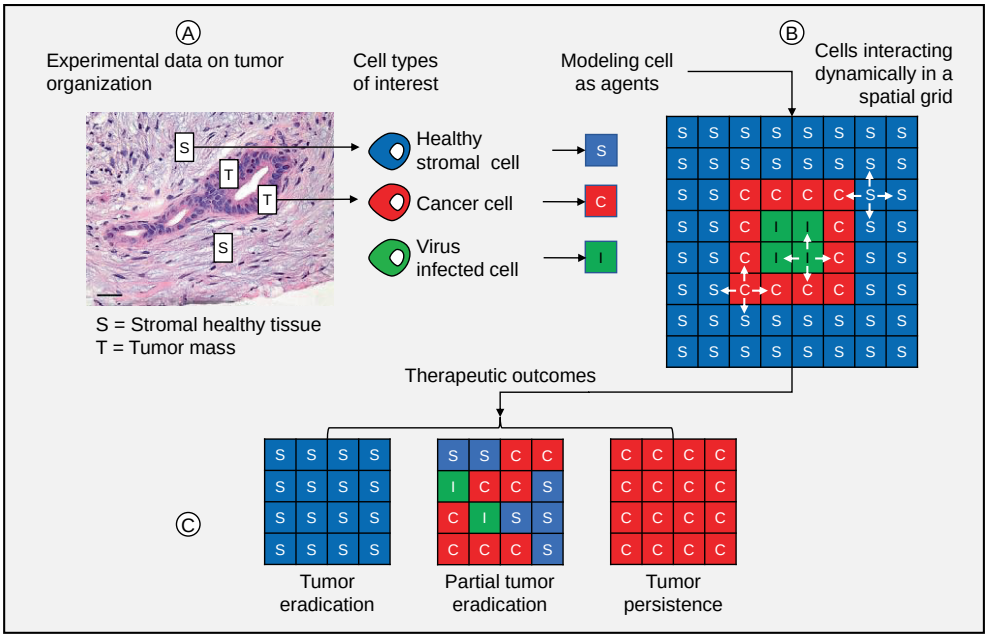

Figure 10: Computational modeling of the spatial dynamics of oncolytic virotherapy. (A) Modeling spatial organization of cancer and stromal cells as interacting entities in an agent-based model. (B) Infected cells, tumor cells, and healthy stromal cells interact dynamically in the model space which can result in (C) different therapeutic outcomes such as complete tumor eradication, partial tumor eradication, or persistence of tumor and therapy failure.

Through this approach one can study for example, under which conditions cancer resistance can undermine such therapy, and which factors are most relevant for this. A computational model can also be designed to model and analyze if and how the T cell-mediated immune responses play a role in the effectiveness of oncolytic virotherapy. Hence, computational modeling (a) will test whether, and to what extent, intuition and verbal reasoning are sound and consistent; it (b) will generate ideas on the conditions under which oncolytic virotherapy may fail (interestingly perhaps under qualitatively different conditions, for very different reasons) and the relative importance of the factors involved, and (c) it may point out possible therapeutic interventions that otherwise might have been overlooked. In this regard, the model can function as an explanatory model aimed at a better understanding of how the interaction between virus, tumor cells, and immune responses affects the therapeutic outcome.

Outline of the thesis

The scope of this thesis is to analyze, model, and develop strategies for the improvement of the safety and therapeutic efficacy of oncolytic virotherapy. The focus is on designing an oncolytic virotherapy based on genetically engineered Semliki Forest virus.

Chapter 1, the introduction starts with a summary of key observations supporting the use of viruses for treating cancer. Next, it describes the design of a safe and effective oncolytic virus based on Semliki Forest virus (SFV). Additionally, the chapter discusses the significance of studying tumor extracellular vesicle-mediated communication in therapeutic outcomes and introduces the advantages of a computational modeling approach in understanding the spatiotemporal dynamics of virotherapy and discovering strategies to develop effective oncolytic viruses.

Chapter 2 analyzes clinical efforts in evaluating the safety and efficacy of oncolytic virotherapy for cancer treatment. Through a systematic review, it explores the impact of oncolytic virotherapy compared to other cancer therapies, considering trial design, patient background, therapy design, delivery strategies, and study outcomes. The chapter highlights how genetically engineered viruses

enhance safety with tumor tropism, cancer-specific replication, and optimized delivery. The analysis discusses challenges in translating oncolytic viruses to clinical practice, which are further addressed in the thesis.

Chapter 3 reports on enhancing Semliki Forest virus-induced anti-tumor immunity by encoding cytokines in the viral genome. We engineered recombinant Semliki Forest virus (rSFV) replicon particles, encoding cytokines to promote immune cell recruitment and activation. Using real-time imaging and flow cytometry, we evaluated their immunogenic potential in human cancer cell-based monolayer and spheroid models. This study illustrates how oncolytic viruses can be rationally engineered to boost anti-tumor immunity.

Chapter 4 is a review of how tumor extracellular vesicles (EVs) regulate tumor progression by influencing cell communication in the tumor microenvironment. This review introduces the physical and biochemical features of EVs, explains how tumor EVs modulate tumor growth, angiogenesis, and metastasis, and discusses their role in creating an immunosuppressive environment.

Chapter 5 is a study of the role of extracellular vesicles (EVs) released by cancer cells upon oncolytic virotherapy. Through an interdisciplinary experimental approach, we characterized the physical and biochemical features of EVs released from cancer cells treated with oncolytic rSFV-based replicon particles. Here, we assessed their role in modulating the phenotype of tumor and immune cells receiving EVs. This study shows a novel mechanism through which oncolytic virotherapy can enhance anti-tumor immunity.

Chapter 6 is an analysis of resistance mechanisms influencing the efficacy of oncolytic virotherapy. It presents the challenges faced by oncolytic viruses, including humoral and cellular antiviral responses, tumor-associated interferon-mediated resistance, and other mechanisms that contribute to therapeutic failure. Overall, we emphasize the exploration of novel resistance mechanisms to enhance the effectiveness of oncolytic virotherapy.

Chapter 7 is a report on employing computational modeling to study the effect of infection-resistant cancer cells on oncolytic virotherapy. We designed a cell-based model to study virus-cell dynamics in tumor tissue, comparing monolayer and three-dimensional tumor variants to assess the effect of tissue spatial

configuration on virotherapy. We systematically investigated factors affecting therapeutic outcomes, including virus properties, tumor characteristics, healthy stromal cell resistance, and timing of treatment. This study aims to improve therapeutic strategies by understanding the mechanisms underlying failure.

Chapter 8 describes our work on computational modeling to find strategies to improve the immune responses to oncolytic virotherapy. Here, we build upon our model described in Chapter 7 by introducing immune responses to the model, focusing on T cell-mediated anticancer cytotoxicity. This study explores how the properties of inflammatory molecules released by infected cancer cells are pivotal for therapeutic success.

Chapter 9 showcases the rapidly growing synthetic biology community in the Netherlands. We show that Dutch academia and industry are actively involved in various domains of synthetic biology, with a focus on genetically engineering various organisms for various goals including improving health, nutrition, and economy.

Chapter 10, the general discussion provides a summarizing overview of the key findings from the research presented throughout the various chapters in this thesis. A major focus is on discussing if and how Semliki Forest virus-based replicons can fulfill the criteria of ideal oncolytic virotherapy. Finally, I provide a perspective on programming the next-generation Semliki Forest virus replicons for improved controllability and efficacy.

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