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Oncolytic virotherapy - analysis, design, models

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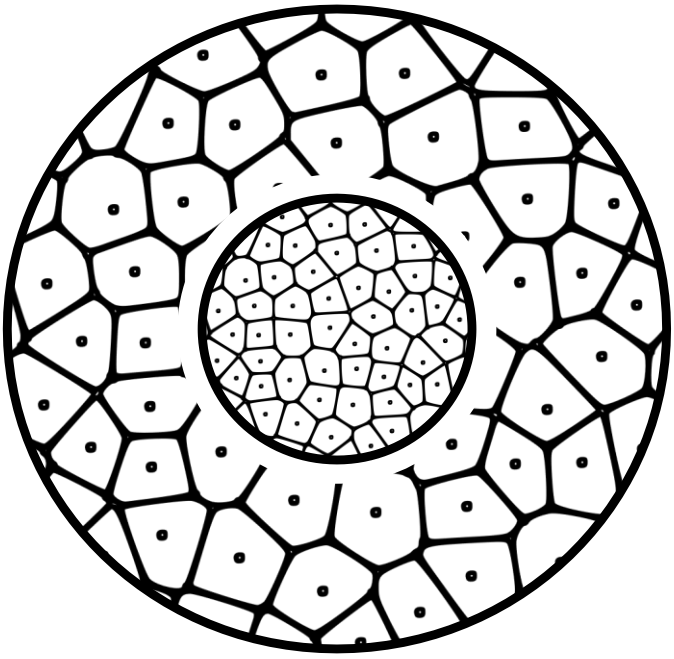
CHAPTER 10

Discussion and future perspectives



Summarizing discussion

Oncolytic virotherapy is a promising approach for cancer treatment where genetically modified or native viruses are employed to kill cancer cells and induce potent antitumor immune responses. Several oncolytic virotherapy candidates have been developed and clinically evaluated, demonstrating potential for treating advanced and metastatic tumors.[1–3] This thesis explored the use of oncolytic virotherapy and strategies to enhance clinical safety and efficacy. We employed different scientific methods such as systematic analysis of existing literature, the use of in vitro and in vivo experimental models, and a computational modeling approach to evaluate strategies aimed at developing safe and effective oncolytic viruses. As discussed further below in detail, we draw the following five key insights from our studies: (i) highlight the need for better controlled clinical studies and assessing immune responses, (ii) the possibility of using safe oncolytic Semliki Forest virus replicons to induce strong immune responses in tumors, (iii) the role of extracellular vesicles from virus-infected cells in promoting proinflammatory immune responses, (iv) draw attention to the importance of studying therapeutic resistance, and (v) to consider spatial mechanisms of resistance and immune regulation post virotherapy for optimal immune responses and complete tumor eradication.

We evaluated the clinical impact of oncolytic virotherapy by comparing cancer patients who received virotherapy with those who underwent other treatments (*Chapter 2*). First and foremost, our analysis underscored the safety of oncolytic virotherapy. This safety was a result of meticulous work in designing effective viral vectors, selecting appropriate therapeutic dosages, and developing efficient delivery strategies. Our analysis uncovered a diverse array of viral vectors used in treating a wide range of cancer types. Adenoviruses and herpesviruses were particularly prominent, with genetically modified adenoviruses, and oncolytic herpes virus (e.g., T-VEC) providing significant knowledge of oncolytic virotherapy use in clinical settings. Their versatility, spanning gene therapy, vaccine platforms, and synthetic biology, may have positioned them as frontrunners in cancer virotherapy. Interestingly, while genetic modifications have gained attention in recent years, some viruses like reovirus, were used in their native forms,

leveraging unique properties for tumor cell targeting. We found that skin cutaneous melanoma was the most studied tumor type, likely due to accessibility for intratumoral injections and high mutational burden. The studies were primarily dominated by patients with advanced cancer, potentially influencing the assessment results. To gain a more comprehensive understanding, it would be beneficial to include early-stage, high-risk, and refractory patients, which could lead to improved insights into the efficacy of oncolytic virotherapy. Our results showed that clinical trials have primarily focused on safety profiles, with manageable side effects reported such as fever, fatigue, and localized discomfort. Although few in number, controlled trials have generally shown positive or stable outcomes, underlining the potential of oncolytic virotherapy. It is intriguing, though, that immune responses have not been extensively measured, despite their significance in this context. The combination of oncolytic virotherapy with checkpoint immunotherapy, in particular, has shown great potential, again emphasizing the importance of understanding immune responses in this field. Moreover, we observed that comparative trials with conventional treatments have been limited. As we progress, it's important to consider a broader range of patients, assess immune responses, and evaluate the efficacy in different contexts.

The generation of antitumor immune responses is a critical requirement for the success of any oncolytic virotherapy. Therefore, we focused on enhancing the immunogenicity of the tumor microenvironment through engineered Semliki Forest virus (SFV)-based oncolytic virotherapy (*Chapter 3*). We employed human tumor cell-based monolayer and spheroid cancer models to demonstrate the ability of recombinant SFV (rSFV)-based replicon particles to express cytokines in cancer cells efficiently and to induce robust immune cell recruitment and activation. Surprisingly, even without encoding immunogenic cytokines, rSFV-infected cancer cells induced strong immune responses in terms of T cell activation and migration. This suggests that rSFV-infection itself can trigger immune responses by causing cancer cell death and various innate mechanisms to activate immune pathways. However, encoding specific cytokines, particularly IFN-γ, significantly amplified these responses. Notably, we observed differences in immune responses generated between different tumor cell lines which may be attributed to variations in their innate immune signaling pathways, highlighting

the intricate nature of immune interactions. Furthermore, our study revealed that the choice of cytokines encoded in rSFV-replicon particles can have distinct effects on immune modulation. While CXCL10 and Flt3L primarily enhanced immune cell recruitment, IFN-γ directly activated immune responses, making it a more potent immune activator. Our findings align with previous research indicating that rSFV-particles carrying immunogenic molecules can effectively induce antitumor immunity in animal models. In the future, we consider exploring additional factors (dendritic cells, NK cells) or alternative models, such as patient-derived organoids, that allow a comprehensive assessment of immune responses to oncolytic viruses.

We delved into the context of complex dynamics between tumor and stromal cells in the tumor microenvironment and how it influences disease progression and treatment outcomes. Specifically, we explored instances where extracellular vesicles (EVs) orchestrate cellular communication within the tumor microenvironment, promoting tumor progression (*Chapter 4*). Our review highlights the role of tumor-derived extracellular vesicles (TEVs) in malignant transformation and tumor progression. In this chapter, we recapitulate how TEVs influence the phenotype of stromal cells, promote tumor development, suppress anti-tumor immune responses, and prepare distant sites for pre-metastatic niches. Mechanistically, TEVs transfer microRNA, metabolites, and signaling proteins, to enhance recipient tumor cells' proliferative and migratory potential, heightening tumor aggressiveness. Similarly, TEVs impact early oncogene activation and carry pro-angiogenic factors to stimulate blood vessel formation and maintain nutrient supply to the tumor. Such a pro-tumoral role of TEVs has shown to be a relevant mechanism in hypoxic tumor microenvironments, where TEVs amplify angiogenesis, drug resistance, stemness, and invasiveness of tumor cells, thereby promoting disease progression. In the context of metastasis, TEVs influence distant stromal cells, induce metastatic behavior in recipient tumor cells, and foster an immunosuppressive TME by down-regulating cellular immune responses to support tumor progression. In the context of treatment, TEVs secreted post-therapy promote tumor regrowth and resistance by influencing cell survival and autophagy. The characteristic cargo carried by TEVs may allow their use as biomarkers, aiding diagnosis, prognosis, and treatment response monitoring. Alternatively, TEVs also hold the potential to be engineered as or for the delivery of therapeutic agents. Yet, gaps remain in understanding the nature

of TEVs, from their packaging mechanisms to standardized isolation methods and engineering platforms.

Considering that TEVs play an important role in tumor development, progression, and related therapeutic responses, we assessed their role in response to oncolytic virotherapy (*Chapter 5*). Specifically, we explored how rSFV infection influenced EVs released by melanoma cells, revealing their altered characteristics and functions. We found that rSFV infection resulted in changes in EVs' physicochemical properties, including increased concentration and size, and shifts in microRNA content, highlighting oncolytic virotherapy's impact on specific microRNA packaging within EVs. Furthermore, EVs from rSFV-infected melanoma cells had distinct features compared to those from uninfected cells. While EVs from infected cells enhanced the clonogenic potential of recipient melanoma cells, the cell sensitivity to rSFV infection remained unchanged. Intriguingly, rSFV-induced EVs displayed an immunogenic profile, suppressing a regulatory phenotype in macrophages and promoting lymphocyte proliferation, in contrast with the typical immunosuppressive role of melanoma EVs. These differences may stem from altered biogenesis or EV cargo, including virus-related components capable of activating immune cells.

Therapeutic resistance has been investigated as a mechanism through which tumor cells can evade and adapt to various forms of treatment and undermine efficacy. We performed a systematic analysis to explore resistance mechanisms influencing oncolytic virotherapy (*Chapter 6*). Our analysis showed that there was a predominant focus on studying mechanisms involving cellular signaling post-virus infection, with a focus on interferon responses mediated by tumor and stromal cells. Among various studies, tumor cell-mediated resistance was extensively assessed, particularly in pancreatic cancer, melanoma, prostate cancer, breast cancer, and glioblastoma. Our results allowed us to categorize resistance mechanisms as tumor-cell mediated, stromal cell-mediated, immune responses, and systemic responses. Mechanisms employed by tumor cells could be further classified in four stages (i) virus binding and entry, (ii) viral transcription–translation, (iii) cell survival, and (iv) cell signaling. We observed that stromal cell responses, while critical, were not the focus of a majority of studies. Antiviral immune responses, either humoral or cellular, affected

therapeutic efficacy and often protected tumor cells from viral infection. Systemic responses involved physical and soluble barriers; with factors such as vascular leakiness, extracellular matrix density, hypoxia, soluble factors, and unexpected players like erythrocytes affecting viral spread.

We employed a modeling approach to delve into the complexities of therapeutic resistance to oncolytic viruses within a spatial framework (*Chapter 7*). We aimed to gain insights into the dynamics of oncolytic virotherapy and resistance through our model. The findings of our model emphasized that therapeutic outcomes depend on factors such as virus replication parameters, tumor spatial architecture, and the presence of resistant cancer and stromal cells, which impede virus spread. Importantly, our study reveals strategies to enhance therapeutic outcomes. Improving virus dispersal within the tumor and sensitizing stromal cells to viral infection are potential avenues. Our model offers a systematic understanding of how various parameters influence oncolytic virotherapy's efficacy. Notably, our model acknowledges the stochastic nature of therapeutic outcomes, mirroring clinical variations. Our study highlights the significance of stromal cells in oncolytic virotherapy. Even if unaffected by the virus, stromal cells can hinder viral spread, impacting therapy. Sensitizing stromal cells enhances virus transmission to adjacent cancer cells, improving tumor eradication efficacy. However, this comes at the cost of stromal cell death and potential harm to healthy tissue. Additionally, we explore the influence of viral diffusion and grid size on therapeutic outcomes. Extending the infection distance, akin to improved viral diffusion, increases the likelihood of tumor eradication. While our model excludes certain complexities, such as intra-tumoral heterogeneity and immune responses, we believe it contributes to understanding the mechanisms underlying virotherapy resistance.

Next, we leveraged our computational modeling approach to investigate the intricate dynamics of T cell-based immune responses during oncolytic virotherapy (*Chapter 8*). Our focus encompassed tumor density-mediated regulation, cell-specific cytotoxicity, and the dynamics of spreading inflammatory molecules in the tumor. The outcomes of our research shed light on the interplay between these factors and their impact on treatment efficacy. Our findings emphasize that T cell responses can synergize with oncolytic viruses under specific conditions. Firstly, T cell cytotoxicity must be specific against cancer cells. Secondly, the inflammatory

molecules responsible for T cell recruitment need to be effective and spread rapidly. Conversely, strong antiviral T cell responses coupled with weak anticancer cytotoxicity due to tumor density effects can limit the effectiveness of oncolytic viruses. Overall, our study provides crucial insights into the role of T cell-mediated immune responses in oncolytic virotherapy. These observations are in line with previous experimental and computational studies, which have highlighted the disadvantages of triggering antiviral immune responses during virotherapy. Our model demonstrates that even a weak activation of antiviral T cells can lead to the clearance of infected cells, limiting virus spread. Interestingly, T cell cytotoxicity against cancer cells, while designed to eliminate infected cells, can unintentionally hinder virus spread within the tumor by targeting surrounding cancer cells, thus creating spatial barriers. Similarly, low diffusion rates of inflammatory molecules can hamper viral spread by confining T cell responses to localized areas surrounding infected tumor cells. Overall, our model introduces spatiality into the existing research on immune responses to oncolytic viruses, allowing us to capture the intricate dynamics within the tumor microenvironment.

The current state of synthetic biology (SynBio) and genetic engineering in the Netherlands reveals a growing interest and potential (*Chapter 9*). Dutch academia and industry have started recognizing the value of SynBio, as demonstrated by the accomplishments of Dutch teams participating in the International Genetically Engineered Machine (iGEM) competition and consortia-based project initiatives like Building A Synthetic Cell (BaSyC). However, there is an unmet need to connect the Dutch SynBio community. Funding mechanisms like research consortia, though beneficial, have limited lifetimes, prompting calls for more long-term, multi-stakeholder collaborations. Addressing these challenges, we propose the establishment of SynBioNL, the Dutch Association of Synthetic Biology aimed to foster connections and to advance SynBio in the Netherlands. Given the national nature of SynBio frameworks, having a Dutch association is essential. SynBioNL aims to bridge the gap, facilitating dialogue among local labs, industries, and policymakers while collaborating with EU and international SynBio communities. We envision that SynBioNL, created by PhDs and Postdocs, will play a pivotal role in addressing Dutch-specific SynBio issues, drawing from the experiences of other associations, and promoting collaboration, innovation, and responsible engagement.

Overall, our observations contribute new knowledge and perspectives in improving the effectiveness of oncolytic virotherapy. While not explicitly addressed in this thesis, the research findings and insights gained from preclinical studies contribute to the groundwork necessary for advancing oncolytic virotherapy into clinical practice. We hope that as more knowledge is gained and technical challenges are addressed, the path towards regulatory approval and clinical implementation becomes clearer. In this regard, we envision that a collaborative ecosystem like SynBioNL has the potential to drive scientific progress within society and could facilitate oncolytic viruses to become effective and viable cancer therapies.

Semliki Forest virus-based replicon particles - an ideal oncolytic virotherapy?

The quest for an ideal oncolytic virus has been a focus of recent research in cancer therapy, including this thesis. Our research indicates that oncolytic virotherapy based on rSFV replicon particles shows promise, but qualification as an "ideal" oncolytic virotherapy (see Box 1) would depend on its (i) safety, (ii) efficacy, and (iii) controlled management as discussed in detail below and illustrated in Figure 1.

Box 1: Characterizing an Ideal Oncolytic Virus: Drawing from Asimov's Laws

Isaac Asimov's three laws of robotics serve as governing principles guiding the development and ethical considerations of autonomous machines.[4–6] These laws prioritize the safety, efficiency, and controlled operation of robots, ensuring that their actions align with human values and well-being. Asimov notes, "The Three Laws are obvious from the start, and everyone is aware of them subliminally. The Laws apply, as a matter of course, to every tool that human beings use." and "analogues of the Laws are implicit in the design of almost all tools, robotic or not".[7] In the context of autonomous yet programmable biotherapies, capable of complex function and self-propagation,[8–12] these laws provide an interesting perspective to an optimal therapeutic design. Building on this notion, the concept of an ideal oncolytic virus harmonizes with Asimov's three laws of robotics, emphasizing non-harm (safety), efficient function (efficacy), and controlled function (controllability and feasibility), as discussed below:

Law 1, Safety: A tool must not be unsafe to use.

Echoing Asimov's first law, an ideal oncolytic virus should be safe for patient use and exhibit minimal or no pathogenicity in healthy cells. This principle aligns with the imperative of sparing healthy tissues while targeting cancer cells.

Efforts to engineer viruses for low virulence and enhanced tumor targeting demonstrate the application of this principle, guaranteeing that the therapy safeguards the patient's well-being.

Law 2, Efficacy: A tool must perform its function efficiently unless this would harm the user.

Asimov's second law, focusing on the efficient operation of robots, parallels the oncolytic virus's mission to efficiently eliminate cancer cells and cause tumor destruction. This requires the virus to target and replicate in cancer cells. Moreover, it emphasizes the significance of optimizing factors influencing the virus's potency, such as inducing potent antitumor immune responses and overcoming tumor resistance mechanisms. This emphasis on efficacy is by no means disregarding safe use or toxicity to patients.

Law 3, Controlled management: A tool must remain intact during its use unless its destruction is required for its use or for safety.

The third law of robotics emphasizes maintaining a robot's function while ensuring its actions remain controllable. Analogously, within oncolytic virotherapy, this criterion underscores the imperative of engineering viruses to possess controllable replication and gene expression. Moreover, it requires the possibility of meticulous fine-tuning or engineering of viruses to achieve the desired therapeutic outcomes without unforeseen repercussions.

(i) Safety

Nonpathogenic nature

The virus should have minimal or no pathogenicity in normal cells, ensuring safety for patients. Chapter 2 illustrates the efforts made in the rational use of native or attenuated viruses that have demonstrated a safe profile in clinics. Furthermore, next-generation oncolytic viruses have been genetically engineered for low virulence and better tumor targeting.[13,14] In this context, our design (Chapter 3) of a safe rSFV-based therapy is exemplary. Our approach offers several

advantages, including being an RNA-based non-integrative virus, which means it does not integrate its genetic material into the host cell's genome. Whereas, the single-round of infection feature reduces the risk of uncontrolled viral replication and enhances safety.[15,16] Furthermore, through a phase 1 clinical trial, our group has demonstrated the safety of rSFV-based replicon particles as a vaccine in cervical cancer patients.[17]

Selective Tumor Targeting

Ideally one would assume that the virus should only infect and replicate within cancer cells, sparing healthy tissues. Chapter 2 describes how through genetic engineering, researchers have modified wild-type viruses to control tumor tropism (e.g. modification of structural proteins) and tumor-selective replication (e.g. deletion of virulent genes). Interestingly, our observations of the spatiotemporal dynamics of virus and tumor cells modeled in Chapter 7 suggest that complete tumor tropism also hampers total cancer eradication due to resistance mediated by healthy stromal cells. This can be circumvented by sensitizing healthy cells for infection, but needs to be balanced against the risk of inducing toxicity to healthy tissue. An alternative could be to induce temporary or spatially limited sensitization of resistant stromal and cancer cells to improve viral spread in the tumor while maintaining safety. In this regard, rSFV-based therapy is promising as it is non-specific in infecting cancer cells and stromal cells, and it is not likely to cause virulence in patients due to its non-replicative nature. Moreover, through intra-tumoral injections, we aim to limit SFV infection in the tumor mass and prevent off-target infection in healthy tissues.

Genetic Stability

The virus should maintain its oncolytic properties without acquiring mutations that could reduce efficacy or safety. A major advantage of using rSFV-based replicon particles is its non-replicative nature. This property ensures that the patient will receive a new dose of genetically identical viruses that upon infection will not lead to the production of a mutated viral progeny.[16] An important consideration with the use of rSFV therapy is that it does not lead to recombination in patients infected with wild-type viruses. Research so far has shown that although a

possibility, it is rather unlikely to occur in clinical scenarios due to very few patients being infected with wild-type SFV and also due to molecular mechanisms preventing superinfection (infection by different viruses in an individual cell) of infected cells.[18]

Controllability

The virus should be engineered to have controllable replication and gene expression, allowing fine-tuning of its therapeutic effects. In Chapter 3, we employ genetic engineering to limit SFV infection to a single round. Additionally, we modify the rSFV-genome to introduce an enhancer sequence before the transgene to boost its expression and related therapeutic efficacy. Please read the next section for future perspectives on how to rationally engineer rSFV-based replicons for enhanced safety and efficacy.

(ii) Efficacy

Oncolytic Potency

The virus should efficiently kill cancer all cells, leading to tumor destruction. In general, our results show that the killing potential does not only depend on the properties of the virus, but also the characteristics of tumor cells such as the resistance mechanisms influencing virus infectivity and spread (Chapter 6,7), tumor extracellular vesicle-mediated cell communication (Chapter 4,5), and the spatiotemporal dynamics of virotherapy (Chapter 7). In line with previous research, our observations indicate that rSFV-based replicon particles show a high oncolytic potential in various cancer types.[19–23] However, the oncolytic potential of rSFV-therapy also depends on the dose and frequency of viruses injected due to their non-replicative nature. Furthermore, patient-specific factors such as tumor heterogeneity, innate or adaptive resistance to infection, limited viral spread, immune status, and genetic makeup, may also influence the oncolytic potential of viruses. As explained next, current research has shown that tumor destruction does not solely result from virus-mediated killing, but also involves antitumor immunity.

Immunogenicity

The virus should stimulate the immune system to recognize and attack cancer cells, enhancing the overall antitumor immune response. Various studies have now established that in addition to virus-mediated oncolysis, the activation of antitumor immunity plays an important role in systemic and long-term tumor clearance.[1,3] We have shown in Chapter 3 that it is possible to engineer rSFV-based replicon particles to encode cytokines to further enhance the immunogenicity of infected tumor cells. This strategy promotes the recruitment and activation of immune cells, potentially leading to a stronger and more effective antitumor immune response. Furthermore, by computationally modeling T cell activity in response to oncolytic virotherapy (Chapter 8), we find that a synergy between T cell responses and oncolytic viruses requires that inflammatory molecules produced by infected cells are effective at low concentrations, spread fast, and have a long half-life. These observations can help in engineering immunogenic proteins that can be encoded in rSFV-based replicons to improve their immunogenicity.

Replication and Spread

The virus should replicate within the tumor and efficiently spread to adjacent cancer cells. Modeling the spatiotemporal dynamics of oncolytic viruses with cancer and stromal cells (Chapter 7) shows that the rate of viral spread via contact coupled with the rate of infected cell death is a strong predictor of therapeutic efficacy. Moreover, therapeutic efficacy is significantly enhanced when viruses spread through diffusion to second-degree neighbors or further. As rSFV-based replicon particles are non-replicating in nature, it is not relevant to consider the spread of virus particles released by infected cells.

Tumor Penetration

The virus should be able to penetrate deep into solid tumors to target all cancer cells. This requirement is rather impossible to achieve as tumor mass is composed of spatially diverse microenvironments with a dynamic and wide range of cell populations including heterogeneous cancer cell clones. Successful tumor

penetration may not correlate with targeting all cancer cells, as virus infectivity and oncolysis also depend on features of tumor microenvironment such as metabolic activity (senescence),[24,25] necrotic areas, density of extracellular matrix,[26] and presence of stromal and antiviral immune cells.[27] In Chapter 7, our in-silico results also indicate that successful tumor eradication depends more on the spread of the virus rather than the site of infection. Therefore, a more realistic aim would be to improve the spread of rSFV-based replicon particles during the intra-tumoral injections in order to target as many cancer cells as possible. This for example can be done by either combining virotherapy with interventions that degrade the tumor extracellular matrix, or by encoding matrix-proteases as transgenes in the virus which can aid better virus delivery in subsequent injections.[26,28,29]

Low Pre-existing Immunity

The virus should not be commonly found in the population, reducing the chance of pre-existing immunity that could limit efficacy. Chapter 6 highlights the importance of considering antiviral immune responses as resistance mechanisms that impair the therapeutic efficacy of oncolytic viruses. Similarly, the in silico results from Chapter 8 demonstrate that even low-level activation of antiviral T cell responses has the potential to hamper chances of successful tumor eradication. Fortunately, wild-type SFV is not known to cause pathogenic infections in humans which might explain why there are relatively few records of pre-existing immunity in the population.[30,31] Upon therapeutic use, however, it is possible that patients develop neutralizing antibodies against SFV which may limit repeated use in an individual. On a positive note, our lab has shown that despite the development of neutralizing antibodies, repeated dosage of rSFV-therapy can successfully induce antitumoral specific T cell responses and contribute to tumor eradication as observed in animal models.[32,33]

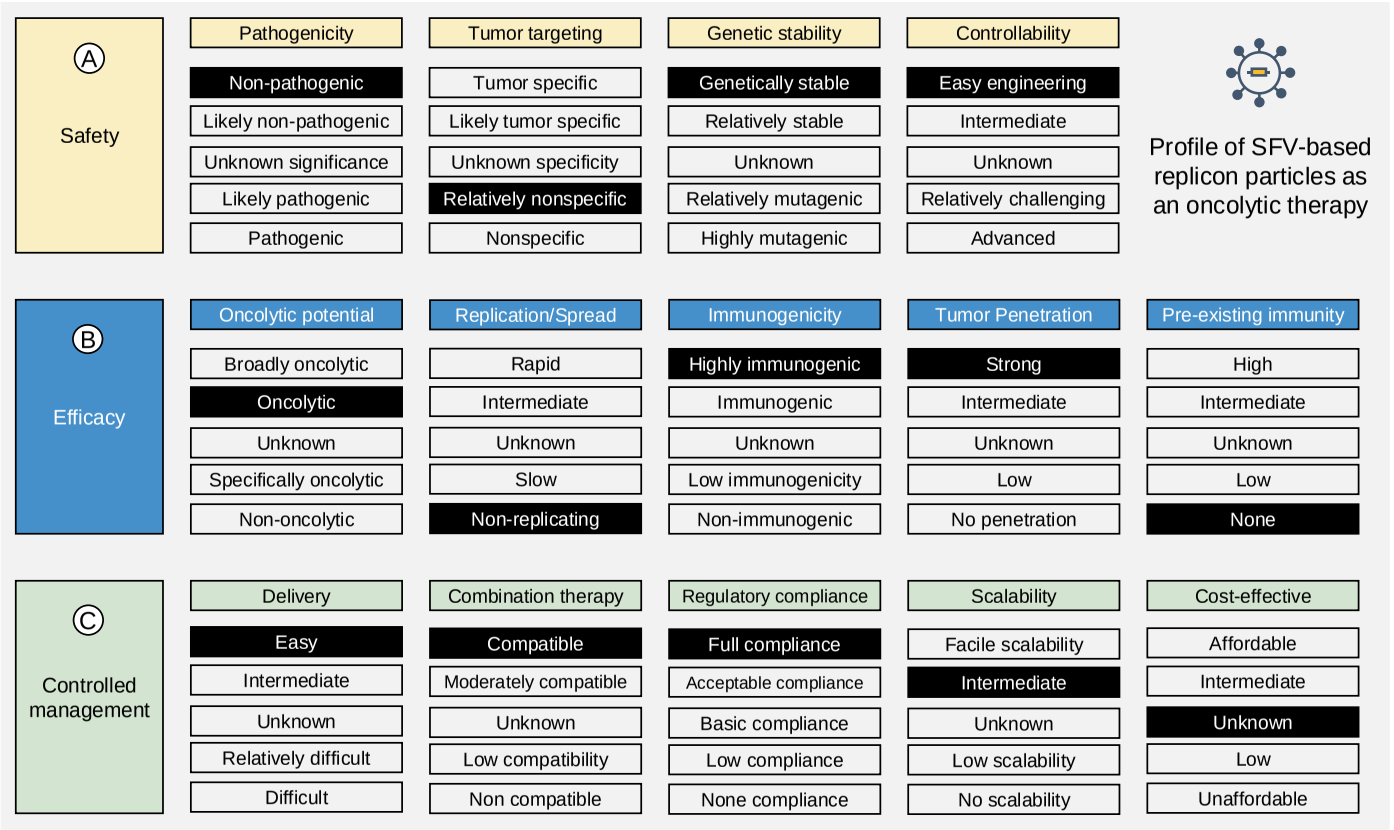


Figure 1: Framework of safety, efficacy, and controlled management to characterize the profile of SFV-based replicon particles as ideal for oncolytic virotherapy. The observed characteristic of SFV-based replicon particles is highlighted in black.

(iii) Controlled management

Easy Delivery

The virus should be easy to administer through various routes, such as intravenous, intratumoral, or systemic injection. Our lab and others have previously demonstrated that rSFV-based replicon particles can be delivered successfully through intramuscular, intravenous, and even tattoo-based intradermal routes in animal models.[15,20,34,35] A phase 1 clinical trial by our group also demonstrated the safe and easy delivery of rSFV-based replicon particles intramuscularly in cervical cancer patients.[17] The use of rSFV therapy as oncolytic virotherapy is also made easy for solid tumors through direct intratumoral injections with the aim of infecting part of the tumor mass and inducing immune responses.

Combination Therapy Compatibility

The virus should be compatible with other cancer treatments, such as chemotherapy, radiation therapy, or immunotherapy, to enhance overall therapeutic outcomes. Previous preclinical studies from our lab have demonstrated that rSFV-based replicon particles exhibit improved therapeutic efficacy in combination with chemotherapy and radiotherapy.[36,37] Moreover, we (Chapter 3) and others have shown that introducing immunogenic transgenes in the genome of rSFV boosts the antitumoral immune responses.[19–21]

Regulatory Approval

The virus should meet all regulatory requirements for clinical use and have a clear path to approval for patient treatment. Previously, our lab developed rSFV-based replicon particles as a vaccine platform and conducted a phase 1 clinical trial at the University Medical Center of Groningen.[17] This was possible through a Good Manufacturing Practice (GMP)-compliant manufacturing process to produce the clinical material.[38] The release testing verified viral titer and virus identity, biological activity, sterility, absence of bacterial endotoxins, absence of adventitious viruses, and absence of replication-competent virus. In conclusion,

the product complied with all regulatory requirements and was released for use as an investigational therapy. Thus, this makes us confident in envisaging the use of rSFV as an oncolytic virus in clinical settings.

Scalability

The virus production process should be scalable for large-scale manufacturing to meet clinical demand. As described above, our experience with the GMP-compliant manufacturing process is a promising step toward improving the production scale and distribution for a larger number of cancer patients. Further research could contribute to improving the yield, thus allowing for optimal scalability.

Cost-effectiveness

The therapy should be affordable and economically viable for widespread use. Current state-of-the-art technology allows the production of rSFV-based therapies at a relatively expensive rate when compared to conventional chemotherapeutic drugs. Interdisciplinary research in bio-manufacturing focused on improving the scalability and yield of rSFV-based therapies is likely to reduce the cost and contribute to better accessibility.[39] An alternative to SFV particle-based therapy could be the use of SFV RNA or DNA-based oncolytic replicons.[40,41] If RNA or DNA-based oncolytic replicons are equally effective as their virus-particle counterparts, this would prove to be a cheap alternative as it requires relatively simpler infrastructure and commonly used bio-processing techniques for production. DNA-based therapeutics also provide an advantage in the context of being independent of cold-chain storage.[42]

Overall, it is essential to validate safety, efficacy, and feasibility-associated factors when evaluating whether rSFV-based replicon particles are ideal for oncolytic virotherapy. Some critical considerations include their safety profile, immunogenic efficacy in different types of cancer, and the potential for resistance. Comparing rSFV-based replicon particles to other oncolytic viruses and therapeutic approaches may provide additional insights into assessing their overall effectiveness and suitability as ideal virotherapy. Considering patient-specific

factors would also prove to be crucial to improve efficacy. Finally, the promising potential of rSFV-based replicon particles as an ideal oncolytic virotherapy can be realized through further research in clinical trials, and by improving practical aspects related to production and distribution.

Programming SFV replicons for enhanced controllability and efficacy

The RNA genome of SFV is a replicon, meaning self-replicating, as the viral RNA encodes so-called non-structural proteins responsible for RNA replication and translation. Development in RNA technology[43],[44] makes it applicable to engineer synthetic or new-to-nature SFV-replicons for applications in vaccination and cancer treatment. So far, we have discussed how SFV-based replicon particles are safe for therapeutic use. Here we delve into how SFV-replicons can be programmed with the goal of enhancing controllability and efficacy as a cancer therapy.

Programming RNA: RNA is involved in various molecular processes in the cell including regulatory, enzymatic, and structural functions. Recent advances in synthetic biology have facilitated the development of RNA 'parts' or functional sequences as tools for reprogramming cellular machinery for therapeutic applications among various other purposes.[45] A repertoire of RNA parts is now available including ribosome binding sites, riboswitches, aptamers, translation ribo-regulators, transcriptional regulators. and CRISPR/Cas-based regulators. Taken together, these RNA parts can be used to regulate a wide range of genetic control functions and allow the design of advanced RNA programming. This holds true for the programming of replicon RNA as well. In the past few years, several research groups have demonstrated that replicon RNA can be programmed to improve controllability (Figure 2) as well as efficacy (Figure 3). Below are some examples.

Programming replicon RNA for improved controllability

Post-transcriptional circuits using RNA-binding proteins (RBPs)

Wroblewska et al. designed post-transcriptional circuits using RNA-binding proteins (Figure 2A).[46] These circuits could be encoded in a 'plug-and-play' fashion in modified mRNA or replicon RNA to build signaling networks of higher complexity. The study featured two RBPs that cross-repress the expression of each other. The 'switch' components include RBPs with specific binding sites that can be turned to an "on" or "off" state by introducing specific small-interfering-RNAs or endogenously expressed micro-RNAs (Figure 2C). These circuits help in tightly regulated transgene expression in specific scenarios, for example in the presence of certain target-cell specific micro-RNAs.

Small-molecule-based regulation

Wagner et al. developed a platform for regulating the expression of proteins from modified RNA and replicon RNA using small-molecule-responsive RBPs (Figure 2B).[47] This enabled external control over the timing and level of protein expression, facilitating the construction of switches with therapeutic applications. In 2021, this technology was further evaluated in vivo using trimethoprim (TMP), a US Food and Drug Administration-approved small-molecule drug.[48] The engineered replicon RNA in the study was demonstrated to show dose-dependent and reversible expression of encoded proteins upon oral administration of TMP in animal models.

MicroRNA-regulated circuits

In the study by Wroblewska et al., a multi-input microRNA sensing circuit was engineered as a simplified version of a HeLa cell classifier.[46] This circuit was shown to recognize specific microRNA expression profiles indicative of HeLa cells, resulting in cell-specific gene expression (Figure 2C). In an independent study in 2021, Martikainen et al. showed that replicon RNA can be engineered for expression in specific cells or tissues.[49] For example, a miR-124 de-targeted version of SFV-replicon was shown to selectively replicate in glioma cells, making it a promising approach for glioma treatment.

Riboswitch-based control

Bell et al. showed that replicon RNA can be engineered with riboswitches, which are RNA elements that modulate gene expression in response to ligand binding (Figure 2D).[50] Riboswitches integrated into replicon RNA were validated to regulate expression and viral replication, offering further control over vaccination strategies.

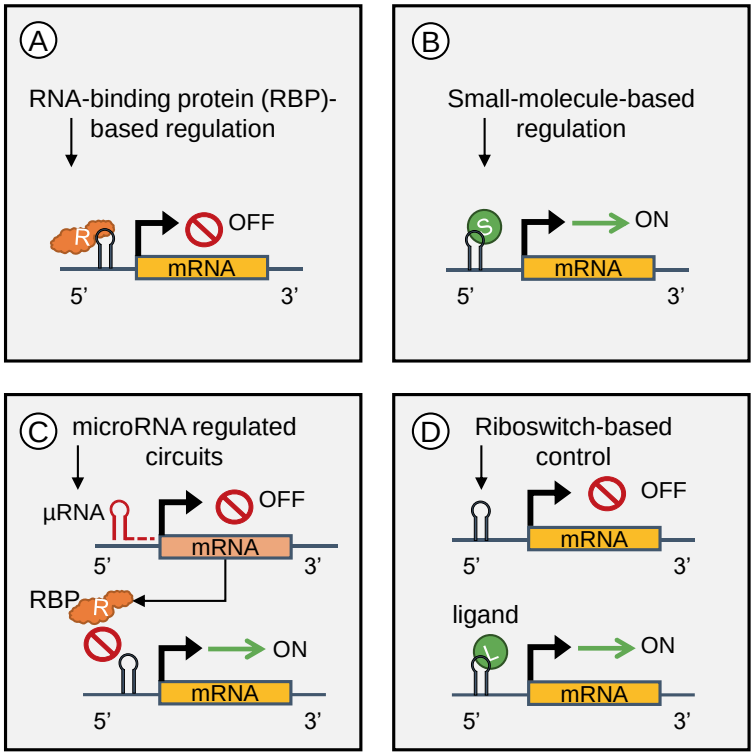


Figure 2: Genetic engineering of replicon RNA to improve controllability by regulating RNA expression using RNA-binding proteins (A), small molecule-based regulation (B), microRNA-based regulation (C), and riboswitch-based regulation (D).

Programming replicon RNA to improve efficacy

Engineering enhancer regions

Naturally derived or synthetically designed translation-enhancer regions can be incorporated into the replicon RNA to enhance its expression (Figure 3A). As early as 1994, Sjöberg et al. demonstrated that by utilizing a translational enhancer from the viral capsid gene, it was possible to achieve a ten-fold increase in the expression of encoded foreign proteins.[51] This feature can be applied to enhance the expression of transgenic cytokines or antigens encoded by the replicons to improve the immunogenicity of transfected tumor cells (Chapter 3).

Regulating replicon-induced cytotoxicity

Replicon RNA derived from viruses can induce cytotoxic effects in cells. This cytotoxicity can limit the efficacy of the long-term replicon RNA expression. To mitigate this limitation, Perri et al. demonstrated that persistent replication and expression of replicon RNA can be achieved by optimization of the replicon's non-structural proteins or by introducing specific mutations that attenuate its toxicity (Figure 3B).[52] This strategy has proven to be successful in enhancing transgene expression for replicons derived from other viruses as well.[53]

Blocking innate immune responses

Replicon RNA can trigger innate immune responses in cells, leading to the activation of antiviral defense mechanisms. These immune responses can interfere with the expression and function of the replicon. One strategy is to optimize the replicon’s non-structural proteins to escape interferon-mediated resistance, as demonstrated earlier by different groups.[49,53] Another successful strategy is to encode innate inhibiting proteins by the replicon RNA to enhance expression (Figure 3C).[54]

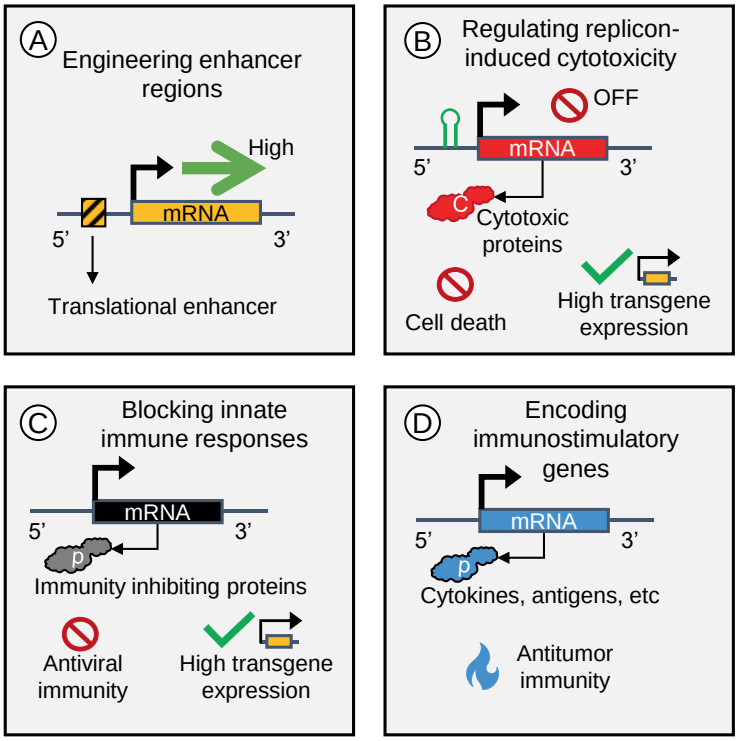


Figure 3: Genetic engineering of replicon RNA to improve efficacy. By (A) enhancing RNA expression under the regulation of a translational enhancer, (B) reducing replicon-induced cytotoxicity to prolong transgene expression, (C) blocking innate antiviral responses to prolong high transgene expression, and (D) encoding immunostimulatory transgenes to activate antitumor immune responses.

Encoding immunostimulatory genes

Replicon RNA can also be engineered to encode immunostimulatory genes. By incorporating genes that promote immune activation, such as cytokines or co-stimulatory molecules, the replicon can function as an in situ or personalized cancer vaccine (Figure 3D). The immunostimulatory genes encoded in the replicon can enhance the immune response against tumor cells, leading to improved efficacy in cancer treatment (Chapter 3).[19,20] Alternatively, tumor antigen(s) can be encoded by the replicons which upon presentation by infected cells or antigen-presenting cells can induce tumor-specific immune responses.[17,35]

In conclusion, there is a promising scope for programming SFV-replicon RNA to further enhance its efficacy and safety as an oncolytic therapy. RNA programming

can be done using various synthetic biology tools and parts that have been developed so far. We believe that these approaches offer promising strategies for enhancing the performance of replicon 'RNA machines' as a versatile platform for safe cancer therapy.

General conclusion

Oncolytic virotherapy stands as a promising avenue for cancer treatment, with significant potential to revolutionize current therapeutic paradigms. The studies presented in this thesis aimed to contribute to providing valuable insights and strategies to enhance the success of oncolytic virotherapy as a cancer immunotherapy, with a particular focus on the utilization of safe oncolytic Semliki Forest virus-based replicon particles. By addressing clinical gaps, understanding resistance mechanisms, optimizing immunological responses, and exploring innovative virus programming, this research hopefully sets the stage for more effective and safe cancer treatments. As the field of oncolytic viruses navigates through clinical translation and regulatory approval, the collaborative efforts of interdisciplinary research will continue to drive the development of oncolytic virotherapies that hold promise in transforming the landscape of cancer treatment.

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