

ENGINEERING LARGE DNA VIRUSES FOR ENHANCED ONCOLYTIC
VIROTHERAPY

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Abstract

Oncolytic viruses (OVs) offer a promising approach to cancer immunotherapy, but challenges in safety, efficacy, and engineering complex platforms remain. This thesis addresses these hurdles with novel tools and strategies. We designed an array of chemically inducible systems, controlled by FDA-approved small molecules, that grant unprecedented precision over both viral replication and transgene expression. We demonstrate the application of these chemically inducible systems for modulating viral replication dynamics, providing a potential strategy to mitigate cytokine release syndrome (CRS) associated with cytokine delivery, and regulating the activity of fusogenic proteins within the tumor microenvironment (TME). This allows for a more nuanced optimization of therapeutic outcomes. We illustrate the combined power of these systems by engineering a virus with multiple control levels for replication and expression of the potent immune modulator IL-12. Furthermore, we introduce an innovative antibiotic-based selection method that significantly improves the efficiency of OV engineering. This approach facilitates rapid platform discovery through random mutagenesis and demonstrates its utility in the parallelized incorporation of 20 different cytokines in two distinct viral backbones, enabling the assessment of their *in vivo* efficacy. This work advances the development of next-generation OVs, designed for precise control, potent immune activation, and enhanced therapeutic efficacy in cancer immunotherapy.

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List of Abbreviations

AAV	Adeno-associated Virus
AdV	Adenovirus
BAC	Bacterial Artificial Chromosome
BCA	Bicinchoninic Acid Assay
BFP	Blue Fluorescent Protein
CAR	Coxsackie-Adenovirus Receptor
CF33	a Chimeric Oncolytic Vaccinia Virus Platform
CMC	Carboxymethylcellulose
CMV	Cytomegalovirus
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats
CRVV	Conditionally Replicating Vaccinia Virus
CuO	Cumate operator
CymR	Cumate Repressor
DAMP	Damage-Associated Molecular Pattern
Dox	Doxycycline
dsDNA	Double-stranded DNA
eGFP	Enhanced Green Fluorescent Protein
FDA	Food and Drug Administration
FKBP	FK506 binding protein
FRB	FKBP-rapamycin binding domain
GFPLuc	Green Fluorescent Protein – firefly Luciferase fusion protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HDR	Homology Directed Repair
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
HSV-1	Herpes Simplex Virus Type 1
ICP	Infected Cell Protein
ICI	Immune Checkpoint Inhibitor
IFN	Interferon
IFN- γ	Interferon gamma
IL	Interleukin
IL-2	Interleukin-2
IL-12	Interleukin-12
IRES	Internal Ribosome Entry Site
IP	Intraperitoneal
IV	Intravenous
IVIS	In Vivo Imaging System
Lag3	Lymphocyte-activation gene 3
MDSC	Myeloid-Derived Suppressor Cell
MeV	Measles Virus
MOI	Multiplicity of Infection
MVA	Modified Vaccinia Ankara

Nab	Neutralizing Antibody
NHEJ	Non-Homologous End Joining
NK	Natural Killer (cell)
OV	Oncolytic Virus
P2A	"Thosea asigna virus 2A" self-cleaving peptide
PBS	Phosphate Buffered Saline
PD1	Programmed cell death protein 1
PEL	Vaccinia virus promoter (synthetic, early-late)
Pexa-Vec	previously known as JX-594, an oncolytic vaccinia virus platform
PFU	Plaque Forming Unit
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RBD	Receptor Binding Domain (SARS-CoV-2 spike protein region)
RNAP	RNA polymerase
RPMI	Roswell Park Memorial Institute (cell culture medium)
SARS-CoV2	Severe Acute Respiratory Syndrome Coronavirus 2
SB	Sleeping Beauty (transposon system)
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
ST7	Split T7 RNA polymerase system
ST7-TetR	Split T7/Tetracycline repressor system
T2A	"Ectocarpus siliculosus virus 2A" self-cleaving peptide
TAMV-GP	Tamiami Virus Glycoprotein
Temsi	Temsirolimus (mTOR inhibitor)
TetO	Tetracycline operator
TETR	Tetracycline repressor
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM3	T cell immunoglobulin and mucin domain-containing protein 3
TK	Thymidine Kinase
TME	Tumor Microenvironment
TNF- α	Tumor necrosis factor alpha
tSNE	t-Distributed Stochastic Neighbor Embedding
TT	Specific Vaccinia Virus Strain
T-VEC	Talimogene Laherparepvec (an oncolytic virus therapy)
UL	Unique long gene regions in HSV
US	Unique short gene regions in HSV
VR-1508	Designation of the strain of MVA
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
VV- Δ TK	Vaccinia Virus with a deletion in the Thymidine Kinase gene
WHO	World Health Organization
YFP	Yellow Fluorescent Protein

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Chapter 1 - Introduction

1.1. Oncolytic Virus Therapy: Past and Present

1.1.1. The origins of oncolytic virotherapy

The origins of oncolytic virus (OV) therapy date back to the mid-19th century, coinciding with the first documented cases of tumor regression following viral infections such as influenza, rabies, and West Nile virus^{1,2}. A notable example is the observation of brief clinical remissions in leukemia patients who contracted influenza². These early observations, though not leading to complete cures, suggested the therapeutic potential of infectious agents and laid the groundwork for the concept of using viruses against cancer. This occurred even before the formal identification of viruses. While preclinical research and clinical trials with natural viruses showed signs of tumor regression, particularly in blood malignancies, challenges such as a lack of long-term remission, virus-related toxicities, and the rise of chemotherapy treatments temporarily hampered progress in the field¹.

1.1.2. Direct cell lysis and immunogenicity

Advances in cell culture techniques and rodent cancer models revitalized research into oncolytic virotherapy. This progress allowed for rigorous testing and, crucially, the genetic engineering of natural viruses to enhance their safety and therapeutic potential. As these engineered viruses demonstrated tumor regression in models, their direct lytic effect on cancer cells was seen as the primary mechanism of action³. However, the

observation of antigen-specific anti-tumor responses following cell lysis suggested a more complex interaction between virotherapy and the immune system⁴.

Seminal studies, such as that by Andreansky et al., explored this interplay between oncolytic viral therapy and immune modulation. They engineered herpes simplex virus type 1 (HSV-1) recombinants expressing either the immunostimulatory cytokine IL-4 or the immunosuppressive cytokine IL-10. In preclinical glioma models, IL-4 expressing HSV-1 significantly prolonged survival compared to controls. Conversely, IL-10 expressing HSV-1 shortened survival, suggesting that therapeutic outcomes depend on the balance between viral oncolysis and the elicited immune response⁵. This demonstrated that the impact of OV's extends beyond direct cancer cell lysis; the cell death caused by the virus appears to initiate a broader immune response against the tumor. These findings highlight the dual nature of oncolytic virotherapy – the direct destruction of tumor cells and the modulation of the immune system – ultimately forging a link with immunotherapy strategies⁶.

1.1.3. Toward developing modern oncolytic viruses

The 20th century and early 2000s brought challenges for OV therapy, particularly those related to viral toxicity and limited tumor specificity. This spurred research in several directions: the search for less pathogenic natural viruses (e.g., vaccinia virus (VV)⁷ and adenovirus⁸), the discovery of functional mutants with reduced virulence⁹ (e.g., herpes simplex virus HF10), and the exploration of non-human viruses capable of replicating in human tumor cells (e.g., maraba virus¹⁰).

To improve safety and efficacy, genetic modifications were employed. These included the deletion of virulence genes (e.g., thymidine kinase (TK) in VV¹¹ and ICP34.5 in HSV-1¹²). By targeting pathways differentially active in cancer cells, these modifications allowed OV^s to selectively replicate within tumors while minimizing harm to healthy tissues. However, these safety improvements didn't always translate into significantly improved clinical outcomes.

Meanwhile, breakthroughs in immune checkpoint inhibitors (ICIs)¹³ and cytokine therapy¹⁴ transformed the field of immunotherapy, emphasizing the need to overcome tumor-induced immune suppression for successful cancer treatment¹⁵. Researchers began to see the potential of OV^s as powerful immune primers, capable of synergizing with ICI therapies¹⁶. Modern OV platforms are designed with safety, strong immunogenicity, and the ability to produce immune modulators within the tumor as primary goals, often achieved by incorporating these elements directly into the viral genome¹⁷.

1.1.4. From T-VEC to the current combinational therapy approaches

The early 2000s witnessed a surge of clinical trials, but most failed to demonstrate significant survival benefits for OV^s used as monotherapies¹⁸. One notable exception was Talimogene Laherparepvec (T-VEC), an engineered HSV-1 expressing the Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) transgene, which received FDA approval for the treatment of unresectable melanoma¹⁹. Despite limited success with OV^s alone, these trials confirmed the safety of modern OV platforms and hinted at the potential for synergy between OV^s and conventional therapies like chemotherapy²⁰. This

shift in focus toward combination therapies suggests that the true potential of OV_s may lie in their ability to complement and enhance existing cancer treatment strategies²¹.

1.2. Viruses used as oncolytic: Vaccinia Virus & HSV-1

1.2.1 Clinically relevant oncolytic virus platforms

A diverse range of viruses, each with unique properties, holds potential for oncolytic virotherapy. These platforms broadly fall into RNA and DNA virus categories. RNA viruses like coxsackievirus, reovirus, measles virus, Newcastle Disease virus, and vesicular stomatitis virus show promise as OV_s²². However, DNA viruses – particularly adenoviruses, poxviruses, and herpesviruses – have dominated clinical trials, offering extensive safety and efficacy data²³.

Adenoviruses, among the earliest explored for OV therapy, remain clinically relevant due to their favorable safety profile. However, limitations hinder broader use of the most common adenovirus vector, human adenovirus type 5 (HAdV5)²⁴. Pre-existing neutralizing antibodies against HAdV5 in a significant portion of the adult population can compromise efficacy, particularly with systemic administration²⁵. Additionally, intravenous HAdV5 can lead to liver toxicity due to sequestration²⁶. Furthermore, HAdV5 relies on the coxsackie-adenovirus receptor (CAR), often downregulated in cancer cells, limiting infection and replication within tumors²⁷.

Despite challenges in preclinical development due to the species-specific nature of adenoviruses, efforts to compare adenoviruses with other platforms are ongoing. While

one Syrian hamster study suggested AdV had superior therapeutic potency over VV and HSV-1²⁸, dosage differences and model-specific factors make definitive conclusions challenging. Additionally, adenoviruses typically have a smaller genome compared to poxviruses and herpesviruses. This smaller genome size simplifies engineering adenoviruses, but it also limits their capacity to carry multiple therapeutic genes – a key factor as OV therapy evolves toward strategies combining potent immune modulators, tumor-targeting molecules, and cytotoxic payloads for optimal anti-tumor efficacy.

1.2.2 Vaccinia Virus

Among the diverse poxviruses explored for their oncolytic potential, VV is the most extensively studied. VV has a remarkably well-characterized safety profile thanks to its extensive use in the smallpox eradication campaign²⁹. Additionally, the availability of FDA-approved antivirals (e.g., Tecovirimat³⁰) further enhances its safety profile. Beyond safety, its unique biology offers several advantages for therapeutic applications.

VV is a large, enveloped virus with a complex structure and a double-stranded DNA (dsDNA) genome approximately 190-200 kilobase pairs in size, encoding around 200 genes³¹. This substantial genome allows VV to carry a wide range of genetic payloads and effectively manipulate host cell machinery³². Its genome is distinctly organized into central and terminal regions. The central region houses essential genes for viral replication and transcription, while the terminal regions contain genes involved in host immune evasion and virulence. This organization contributes to the virus's ability to modulate host immune responses. Furthermore, VV employs a multi-stage transcriptional process involving early, intermediate, and late gene expression. Early genes initiate viral replication, followed by intermediate and late genes responsible for structural proteins

and virion assembly, ensuring efficient viral propagation³³. Finally, VV exhibits a remarkably rapid replication cycle compared to other DNA viruses, completing a full cycle within 6-8 hours³⁴. This allows for swift viral spread within tumor tissues, leading to potent tumor cell destruction.

A number of attenuation strategies, primarily through gene deletion, have been discovered to increase VV tumor selectivity and limit off-target replication³⁵. Deletion of the TK gene is one of the most common modifications in VV to ensure tumor-selective replication. The TK gene plays a crucial role in nucleotide salvage pathways, facilitating the incorporation of thymidine into viral DNA. In cancer cells, where nucleotide pools are elevated due to increased metabolic activity, the TK gene is dispensable for viral replication. This makes it attractive for selective attenuation in oncolytic VV development³⁶. This approach led to platforms like JX-594 (Pexa-Vec)³⁷, one of the most clinically tested VV-based OVVs. However, the field is evolving towards next-generation VV platforms like SKV³⁸ and CF33³⁹. SKV, derived from the Copenhagen strain, features major deletions that enhance its growth and immunogenicity by removing immune suppressor proteins⁴⁰. CF33, a chimeric virus generated through directed evolution, also exhibits potent oncolytic and immunostimulatory properties⁴¹. Despite these advancements, neither SKV nor CF33 has progressed to FDA approval. In case of SKV, its increased immunogenicity, caused by deletions that target immune suppressor genes, leads to faster clearance by the immune system. This can limit the virus's persistence within the tumor and hinder its ability to elicit a prolonged anti-tumor immune response.

Overall, VV's advantages include its large transgene capacity, allowing for the incorporation of multiple therapeutic payloads, and its rapid cell-to-cell spread, which facilitates tumor destruction. Additionally, its wide host range makes it suitable for various tumor types, and most individuals lack pre-existing immunity to it. However, VV's complex genome poses engineering challenges^{42–44}. Modifications often rely on specialized vaccinia-based promoters, a time-consuming selection process, and cannot directly utilize developments in mammalian expression systems^{45–47}.

1.2.3 HSV-1

The herpesvirus family encompasses a diverse range of viruses, with HSV-1 emerging as the most commonly used herpesvirus for OV development⁴⁸. This enveloped, double-stranded DNA virus is a widespread human pathogen. It's known for its neurotropism and complex life cycle that alternates between lytic and latent phases within the nervous system⁴⁹.

HSV-1's potential use as an oncolytic agent faced a significant hurdle: its inherent neurovirulence. Extensive genetic engineering has addressed this challenge, with a primary focus on the ICP34.5 gene⁵⁰. ICP34.5 is a key neurovirulence factor and plays a vital role in evading cellular antiviral defenses. It counteracts the PKR pathway, which normally inhibits viral protein synthesis, by dephosphorylating eIF2 α . Additionally, ICP34.5 disrupts interferon-beta production by interacting with TBK1 and inhibits the protective process of autophagy by binding to Beclin 1⁵¹.

Early HSV-1 based OV_s, such as HSV1716⁵², incorporated deletions of both copies of ICP34.5 to enhance safety. Subsequent platforms refined safety profiles by removing additional genes:

- G207: Deletion of both copies of ICP34.5 and insertion of LacZ in place of ICP6 (involved in viral DNA replication)⁵³.
- G47Δ: Deletion of both copies of ICP34.5, removal of ICP47 (which blocks antigen presentation), and insertion of LacZ in place of ICP6⁵⁴.

These early modifications led to the development of T-VEC, the first OV approved by the FDA for unresectable melanoma¹⁹. T-VEC replaces ICP34.5 copies with the GM-CSF transgene to enhance its immunostimulatory properties. However, researchers discovered that removing ICP34.5 can compromise viral replication and production yields, highlighting the delicate balance between safety and therapeutic efficacy⁵¹.

A more recent and promising approach involves using a tissue-specific promoter (pNestin) to control ICP34.5 expression, as seen in rQnestin34.5⁵⁵⁻⁵⁷. This strategy aims to restrict ICP34.5 activity within tumor cells, preserving neurovirulence attenuation while enhancing oncolytic potential. Interestingly, while a large percentage of the population is seropositive for HSV-1, recent clinical data from rQnestin34.5 trials suggest that pre-existing immunity may be beneficial in certain contexts, such as glioblastoma treatment. This benefit likely stems from enhanced immune infiltration and survival⁵⁵.

HSV-1's success is evident with FDA approval for T-VEC⁵⁸, clinical approval for G47Δ in Japan for glioblastoma⁵⁴, and the promising results from rQnestin34.5 trials⁵⁵.

However, challenges remain with HSV-1 engineering. Its large genome poses complexities. Traditional recombinant approaches are laborious, while techniques like

bacterial artificial chromosomes (BACs)⁵⁹ and CRISPR-Cas9⁶⁰ offer promise but come with their own limitations – the potential for mutations with BACs and lower efficiency of CRISPR-Cas9 triggered homology-directed repair for larger transgenes^{61,62}.

1.3. Oncolytic Virus Platform Development: Strategies and Techniques

1.3.1 The need for novel OV Platforms

The evolution of OV development reveals a shifting focus. Initially, the emphasis was on safety and tumor specificity, achieved by attenuating known viruses. However, researchers recognized that excessive attenuation could limit therapeutic efficacy. The goal evolved towards striking a balance between potency and tumor specificity⁶. A notable example of this shift is seen in a clinical trial with ACAM2000, the reserve smallpox vaccine. Patients exhibiting signs of pox lesions and associated viral toxicities demonstrated significantly better responses (longer survival). This suggests that a degree of viral virulence might lead to a more robust systemic immune response⁶³.

These objectives, while logical, pose inherent contradictions. Increased tumor specificity often leads to greater attenuation, reducing oncolytic potential. This highlights three primary approaches to platform development:

1. Rational Engineering for Attenuation: Modifying inherently less safe human pathogens (e.g., HSV-1) to reduce neurovirulence while maintaining tumor-killing potential⁵⁰.

2. Chimeric Viruses and Directed Evolution: Improving viruses with established safety profiles, like Vaccinia⁶⁴ and Adenovirus⁶⁵, through strain mixing or serial passaging in cancer cells to evolve enhanced platforms.
3. Novel Viral Species: Screening for OV's with unique properties, like the immune evasion capabilities of Orf^{66,67} or the potentially increased safety and potency of HSV-2 compared to HSV-1 in certain tumors^{68,69}.

The ultimate challenge lies in developing platforms that are potent, tumor-specific, persistent enough to facilitate lasting immune responses, and suitable for IV delivery⁷⁰.

1.3.2 Evolutionary approaches to virus engineering

While directed evolution strategies have been a major driver in developing novel serotypes of adenoviruses^{71,72} (Uil et al. 2011; Berman et al. 2018) and Adeno-associated viruses (AAVs)^{73,74}, applying similar approaches to larger DNA viruses within the poxvirus and herpesvirus families presents unique challenges. Primarily, the inherently higher safety concerns associated with poxviruses⁷⁵ and herpesviruses limit the scope of permissible mutations. Additionally, their large genome size makes it difficult to use common mutagenesis techniques (e.g., error-prone PCR) typically applied to adenoviruses and AAV.

Efforts to evolve VV have largely relied on leveraging natural genomic variation among existing strains⁷⁶ or, less safely, recombination of closely related poxviruses under selective pressure in cancer cell lines³⁹. Examples include deVV5, derived from recombination between four major VV strains (WR, Cop, Wyeth, and MVA) through serial passaging in resistant cancer cell lines⁷⁶. A more recent attempt, the CF33 platform,

involved serial passaging of Cowpox, Raccoonpox, Rabbitpox, and multiple VV strains (WR, IHD, and Lister) in CV-1 cells⁶⁴. Despite the intent to generate a mixed-species hybrid, CF33's genome arose primarily from recombination between the VV strains. This demonstrates the difficulty of inter-species poxvirus recombination⁷⁷.

Our group also explored serial passaging of the Copenhagen VV strain, with repeated sequencing of isolated viral plaques as a mutant discovery method. This led to the discovery of variants with significant deletions at their 5' and 3' ends. Intentional recombination of these variants with unique deletions subsequently gave rise to the SKV platform^{38,40}.

These results highlight the challenges of directed evolution for large DNA viruses. In vitro screening promotes the loss of viral DNA that, while crucial for in vivo function, is dispensable in cell culture. This can lead to promising in vitro results but less potent clinical outcomes, as seen with CF33⁷⁸ and SKV. Ideally, novel approaches would facilitate in vivo screening of diverse viral mutant libraries. However, a key bottleneck remains the lack of high-throughput methods for generating the recombinant libraries needed for screening.

1.3.3 Rational design of oncolytic viruses

Rational engineering plays a crucial role in OV development. Examples include modifications to VV (e.g., TK gene deletion for tumor selectivity³⁷), VSV (e.g., IFN beta insertion to modulate the type I interferon response⁷⁹, and the delta 51M mutation for attenuation^{80,81}), and HAdV5 (e.g., E1B and E3 deletions for attenuation⁸²). However,

HSV-1 stands out as one of the most extensively modified platforms through rational design. This emphasis on targeted modifications stems from safety concerns with non-specific approaches and the well-characterized nature of the HSV-1 genome^{50,51}.

HSV-1 modifications address several key areas:

- Preventing Neurovirulence: Primarily achieved through deletion of ICP34.5 (G207⁵³, G47 Δ ⁵⁴), modification of its expression (rQnestin34.5⁵⁷), or functionally modified mutants (HSV-68H-6⁵¹).
- General Attenuation: Reducing replication in normal cells (e.g., deletion of ICP6, involved in viral DNA replication⁸³).
- Improved IV Delivery & Tumor Targeting: Modifications to the gD protein (important for viral entry) with targeting moieties like scFvs against HER2⁸⁴ or EGFR⁸⁵.
- Enhanced Cell-to-Cell Spread: Mimicking the naturally fusogenic HF10 strain through mutations in the gB gene (involved in viral fusion)⁸⁶.
- Improved Immunogenicity: Deletion of ICP47 (which blocks antigen presentation) to increase visibility to the immune system⁵⁴.

These concerted efforts have significantly boosted the oncolytic potency of HSV-1 in clinical trials, leading to successful platforms like T-VEC, G47 Δ , and rQnestin34.5.

1.4. Armed oncolytic viruses: Transgenes

1.4.1 Different types of transgenes

While late 20th-century OV development prioritized safety and tumor-specificity, modest clinical outcomes prompted a strategic shift towards arming OVs with therapeutic

transgenes. These transgenes offer diverse functionalities: they can enhance viral replication within tumors⁸⁷, encode prodrug-activating enzymes⁸⁸, modulate the immune response⁸⁹, restore tumor suppressor genes⁹⁰, and disrupt the tumor microenvironment (TME)⁹¹.

A crucial consideration in transgene selection is the delicate balance between efficacy and safety. Ideally, transgenes should enhance viral replication within the tumor without compromising patient safety. Conversely, transgenes that don't enhance replication and solely target other pathways in infected cells may have minimal therapeutic effect due to the virus's inherent lytic nature. This highlights the importance of identifying transgenes that promote a potent "bystander effect," primarily affecting uninfected neighboring cancer cells or other cells within the TME. By amplifying the therapeutic impact without compromising safety or hindering viral propagation, these transgenes hold significant promise.

Transgenes that directly trigger apoptosis or those encoding cell-penetrating peptides could be potent options. However, their potential toxicity towards healthy cells and negative impact on manufacturing (due to harm to virus-producing cells) pose significant challenges. Consequently, the field has shifted its focus towards transgenes that modify the TME. While fusogenic proteins⁹² and extracellular matrix modifiers⁹¹ hold promise, immune modulators have emerged as a primary focus⁸⁹. This emphasis aligns with the successes seen in cytokine therapies¹⁴. Importantly, OV's offer localized delivery of these immune modulators, potentially reducing the systemic toxicity often associated with cytokine treatments.

Research is increasingly focused on OV_s encoding diverse immune modulators. These include immune checkpoint blockers, bispecific T-cell engagers, cytokines, and other agents that can enhance anti-tumor immunity within the TME⁸⁹. Platforms like T-VEC¹⁹ and JX-594³⁷ exemplify this trend. Both encode GM-CSF, highlighting the potential synergy between OV_s and immunostimulatory molecules for a more potent therapeutic effect.

1.4.2 Oncolytic Viruses encoding cytokines

OV_s disrupt the immunosuppressive TME, priming it for immune-mediated destruction. Direct tumor cell lysis by OV_s releases tumor antigens and damage-associated molecular patterns (DAMPs). This triggers an initial innate immune response involving natural killer (NK) cells, neutrophils, and macrophages, accompanied by a pro-inflammatory cytokine surge that further recruits immune cells. OV_s can also shape the adaptive immune response for sustained tumor eradication and potentially generate immunological memory against recurrence^{93–95}.

To amplify the immunomodulatory effects of OV_s and enhance therapeutic efficacy, researchers have armed them with genes encoding immunostimulatory cytokines. This approach allows precise manipulation of the TME, promoting T-cell activation, recruitment, and shifting the balance from immunosuppression to active tumor elimination. Importantly, the timing and context of cytokine release from the virus play a crucial role in effectiveness^{6,16,17}.

A wide range of cytokines have been explored, each with unique advantages. IL-2, IL-12, IL-15, and IL-18 are potent stimulators of T and NK cells. GM-CSF and type I/II interferons (IFN- α , IFN- β , IFN- γ) enhance antigen presentation and boost anti-tumor immunity. The success of T-VEC, which expresses GM-CSF, highlights the potential of this approach^{22,89}. Ongoing research investigates combinations of cytokines and pairings with ICIs to further maximize therapeutic benefits for patients^{96,97}.

1.4.3 IL-12 encoding OV

Among the various cytokines explored in OV platforms, IL-12 holds a prominent position. Its frequent use across diverse viral backbones, along with extensive preclinical and clinical studies, highlights its potential in improving OV therapy⁹⁸.

IL-12 is a potent immune regulator, bridging innate and adaptive anti-tumor responses. It promotes the growth and cytotoxicity of NK and T cells, drives CD4⁺ T cell differentiation towards a Th1 phenotype, enhances IFN- γ production, and inhibits angiogenesis⁹⁹.

While systemic delivery of IL-12 showed promise in preclinical models, severe toxicity limited its clinical translation^{100,101}. To mitigate these side effects, localized IL-12 expression within the TME emerged as an attractive strategy. OVs with their tumor tropism and inherent immune recruitment capabilities, offer an ideal vehicle for localized IL-12 delivery.

Numerous studies demonstrate the efficacy of IL-12-expressing OV. Common observations include increased IFN- γ levels, reduced tumor vasculature, enhanced Th1 differentiation, and decreased Treg populations in the TME. Despite these successes, IL-12-OV monotherapy has limitations. One potential issue is accelerated antiviral immunity, which can lead to premature clearance of the virus and limit the duration of IL-12 expression⁹⁸. Additionally, while IL-12 boosts immune cell cytotoxicity, tumor cells can evolve mechanisms to evade this effect, such as upregulating immune checkpoints¹⁰². The anti-angiogenic effects of IL-12 may also vary depending on the specific tumor model¹⁰³.

To fully realize the potential of IL-12-expressing OV, strategies to enhance viral persistence are needed. Additionally, exploring synergistic combinations with other cytokines or checkpoint inhibitors holds promise for overcoming tumor resistance and achieving more durable therapeutic responses.

1.5 Limitations and Frontiers in Oncolytic Virotherapy

1.5.1 The Path to the "Immunotherapeutic Battleship"

A fundamental challenge in oncolytic virus therapy is balancing viral clearance with tumor control. While the immune system's response to a viral infection can enhance anti-tumor activity, it can also prematurely eliminate the virus. This limits its spread and its ability to create a sustained shift in the TME⁶.

The concept of the OV as an "immunotherapeutic battleship"⁴³ envisions a therapy that goes beyond direct tumor cell killing. An ideal immunotherapeutic battleship would

persist within the TME long enough to reshape the TME and trigger a robust and coordinated anti-tumor immune response. This persistence is crucial for prolonged control of tumor growth and the sustained expression of any therapeutic payloads encoded within the virus. To achieve this, sophisticated control over the timing, intensity, and sequence of immune function is needed – primarily through carefully selected immune modulators. This fine-tuning would ensure a dominant anti-tumor response while minimizing premature antiviral activity.

Large DNA viruses like Vaccinia and HSV-1 have the capacity to encode multiple immune modulators. However, our limited understanding of their gene expression systems makes it difficult to precisely control both transgene expression and viral replication. Additionally, current engineering methods lack the high-throughput capacity needed to screen large libraries of transgenes or viral backbones. This hinders our ability to systematically optimize viral persistence and immunogenicity – two crucial factors for creating effective immunotherapeutic battleships.

1.5.2 The Need for Speed: Accelerating Viral Engineering

Engineering DNA viruses with large, complex genomes, such as VV and HSV-1, poses significant obstacles for developing oncolytic therapies. Their high GC content, repetitive sequences, and overlapping genes complicate genetic manipulation. Traditional methods, like plaque purification for virus rescue, are time-consuming and inefficient, particularly for larger modifications. Even with the advent of CRISPR-Cas9, which has revolutionized genome editing, limitations persist within viral systems^{60,104}. These

include the dominance of error-prone NHEJ repair over the more precise HDR, rapid viral replication, and complexities in sgRNA design¹⁰⁵.

While alternative approaches like genome segmentation¹⁰⁶ or BAC-based manipulation⁵⁹ offer potential improvements, they come with technical challenges. One concern is the increased mutational load the virus may acquire when grown in a non-native host¹⁰⁷. Additionally, none of these methods are currently optimized for high-throughput, automated mutagenesis, which would significantly accelerate the development process.

The use of transposons for viral engineering holds promise, particularly when employed within the virus's natural host. This approach could facilitate the generation of diverse recombinant libraries specifically tailored for cancer therapy. However, this area requires further exploration to unlock its full potential.

To accelerate the discovery and optimization of novel oncolytic therapies, it's crucial to develop innovative engineering systems that overcome existing limitations. These systems should enable rapid, high-throughput manipulation of complex viral genomes. This will facilitate the creation of diverse libraries, allowing for the screening and selection of the most potent immunotherapeutic candidates.

1.5.3 Spatiotemporal control over viral gene expression

The unique replication and transcription mechanisms of OV's necessitate different approaches to gene expression control. VV with its cytoplasmic replication, relies on its own specialized machinery. This feature allows scientists to leverage native viral

promoters for transgene expression in VV. In contrast, HSV-1, although it replicates within the nucleus, exhibits complex interactions with host factors during its lytic and latent phases. This complexity often necessitates the use of common mammalian engineering promoters, like CMV or EF1-alpha, to control transgene expression in HSV-1.

Previous efforts have explored chemically inducible systems (like Tet repressors)^{108,109}, hypoxia-specific promoters^{110,111}, and tissue-specific promoters⁵⁷ within both VV and HSV-1. Yet, limited data exists on optimizing these systems for controlled gene expression. Factors like efficiency, leakage, and functionality within complex, multimodal platforms that simultaneously modulate viral replication and multiple transgenes need further exploration.

1.6 Rationale and Objectives

Historically, virology has been a major driver of breakthroughs in cell and molecular biology¹¹². Now, as viruses themselves become powerful therapeutic tools, particularly in oncolytic virotherapy, there's a unique opportunity to reciprocate this knowledge exchange. By adapting and optimizing molecular biology tools developed for cellular studies, we can engineer sophisticated viral platforms for significantly enhanced therapeutic efficacy.

This thesis explores the characterization, optimization, and application of two fundamental yet powerful molecular biology tools – chemically inducible promoter elements¹¹³ and mammalian cell selectable antibiotics¹¹⁴ – within the context of OV

engineering. We hypothesize that these tools can significantly address limitations in current OV platforms. Specifically, they offer the potential for precise control of viral replication and transgene expression through inducible systems. Additionally, mammalian cell selection antibiotics will facilitate platform discovery and accelerate transgene insertion. This combined approach could unlock the development of complex, multi-component OVs with enhanced safety, optimized therapeutic payloads, and ultimately, a greater impact in cancer immunotherapy.

1.6.1 Precision Control of Transgene Expression

Chapter 2 focuses on developing and optimizing chemically inducible systems for precise control of transgene expression within OVs. We demonstrate the successful application of rapamycin, doxycycline, and cumate-inducible systems in VV, and provide examples of their adaptation to HSV-1. Importantly, we showcase the potential of combining these systems into multi-component platforms, allowing for unprecedented flexibility in controlling expression levels. This flexibility is demonstrated in the regulation of viral replication, cytokine expression, and even fusogenic proteins. These tools significantly expand the potential of oncolytic virotherapy by enabling fine-tuned and dynamic control over therapeutic payloads and viral functions.

1.6.2 A Multi-Pronged Therapeutic Platform

Chapter 3 builds upon the multi-component inducible systems developed in Chapter 2. Here, we demonstrate their power in creating conditionally replicating OVs with superior therapeutic potential. We present a novel system for tightly controlling essential VV gene

expression, enabling precise modulation of viral replication. This allows for a direct comparison of high-level wildtype-like replication to the more attenuated replication characteristic of many clinical trial viruses – a comparison conducted within the same platform. This analysis reveals the profound impact of viral replication on both treatment efficacy and immune response modulation.

Furthermore, we integrate our inducible system with the potent cytokine IL-12, creating a virus capable of simultaneous control over both replication and transgene expression. By fine-tuning IL-12 levels while also modulating replication, we gain valuable insights into the dose-dependent effects of IL-12 on the anti-tumor response and viral clearance dynamics. This underscores the importance of controlled cytokine expression for optimal therapeutic outcomes.

1.6.3 Accelerating Viral Engineering and Discovery

Chapter 4 reimagines the concept of antibiotic-based selection for viral engineering. By harnessing the antiviral properties of mammalian cell selection antibiotics, we developed a novel positive selection system to isolate recombinant viruses. This approach is applied in several key ways:

1. Creation of diverse mutant libraries for functional genomics and platform discovery.
2. Efficient engineering of viruses with large transgenes, outperforming the current CRISPR-Cas9 standard.
3. Rapid generation and in vivo screening of cytokine-expressing OVIs in both VV and HSV-1 backbones.

This work highlights the significant potential of established techniques when adapted for complex, modern virotherapy applications. It offers a promising way to accelerate the discovery and optimization of novel oncolytic therapies.

Chapter 2 - Synthetic virology approaches to improve the safety and efficacy of oncolytic virus therapies

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Contributions

J.C.B. proposed and supervised the project. T.A., R.R., R.S., S.B., K.A.O., N.T.M., V.H., M.G., M.M., M.J.F.C., H.D.H., A.N.A., M.A., N.K.Z. conducted in vitro experiments. T.A., R.R., J.P., R.M., X.H. performed mouse experiments. T.A., R.R., J.C.B. engineered the viruses. T.A., R.R., R.S., A.P. performed data analyses. T.A., R.R., R.S. wrote the manuscript. A.G., T.A., P.G., M.A., B.C.D., L.H.T., C.S.I., J.C.B. contributed to the design of studies.

Abstract

Vaccinia virus (VV) is known for its large coding potential, making it an attractive oncolytic or vaccine vector. However, controlling viral replication and transgene expression timing and dosing is crucial for safe and effective payload delivery. Limited VV-adapted chemogenetic switches exist for dose-dependent, temporal regulation of promoter activity. We present three drug-controlled gene switches to add regulatory layers to virally encoded transgene expression. Two safety-switches use FDA-approved small molecules: rapamycin and doxycycline. Using ribosome profiling, we design synthetic promoters by fusing the operator element of drug-inducible systems with VV promoters, resulting in robust inducible expression with undetectable baseline levels. Novel chimeric promoters are also generated for additional regulatory layers. These chemogenetic switches are applied in various applications, such as inducible-expression of fusogenic proteins, dose-controlled delivery of toxic cytokines, and chemical regulation of VV replication. This enables complex tunable transgene circuitry incorporation in VV-vectored oncolytic virus design.

Introduction

Tumors are heterogeneous cellular ecosystems that continuously evolve in the face of therapeutic intervention limiting the efficacy of an array of conventional treatments for metastatic disease. Replicating cancer therapeutics like engineered bacteria¹¹⁵, immune cells¹¹⁶ and viruses¹¹⁷ comprise an emerging class of modalities that, in principle, can work in harmony with a patient's immune system to initiate robust, systemic anti-tumor immune responses capable of keeping pace with and attacking evolving tumor ecosystems. These forms of therapies can be thought of as biological machines that to be optimally deployed, will require control strategies coordinating and regulating their activity within the patient^{118,119}. Oncolytic viruses (OVs) have the potential to be replicating cancer gene therapy vectors that reproduce in and destroy tumor cells (oncolysis) and in addition have the capacity to dispense therapeutic payloads to remodel the TME¹¹⁷ by impacting tumor vasculature¹²⁰ and reshaping extracellular matrix components¹²¹. However, this remodeling process, especially the generation of robust adaptive and innate immune responses, can sometimes be at odds with optimal virus spread within and between tumors. Incorporation of genetic circuits into oncolytic virus backbones that regulate the timing/magnitude of expression of virulence genes and therapeutic payloads could facilitate safer, more effective therapeutics. In this study, we used synthetic virology approaches to develop a new generation of OVs equipped with multiple inducible systems, which can be used as "safety switches" for providing user defined control of both virus replication and transgene expression. To facilitate clinical translation of our products we tested orally available FDA approved small molecule responsive transcription systems. We show through combinatorial application of discrete regulatory elements we can increase the safety and efficacy of an oncolytic vaccinia virus in xenograft and syngeneic mouse tumor models. These novel systems will pave the way for developing a new generation of

OVs in which the timing and level of both OV replication and their therapeutic payloads can be regulated.

Results

Generation of a rapamycin-inducible expression system in an oncolytic vaccinia virus vector.

We designed a series of chemogenetic switches to enable temporal control of the expression of multiple therapeutic payloads. Vaccinia virus (VV) is a cytoplasmic virus and while is able to infect and replicate in mammalian cells, its transcriptional machinery and genomic structure is not optimized for the use of mammalian promoters as reported in the previous publications. Vaccinia virus has its own set of regulatory sequences and transcription factors that are adapted to its specific needs, and the use of mammalian promoters is not compatible with these requirements (**Supplementary Fig. 2.1a**). Previous studies have demonstrated that the VV-encoded T7 polymerase can drive the expression of gene cassettes under the control of a T7 promoter element and so we incorporated a recently developed small-molecule activated, proximity dependent split T7 RNA polymerase (RNAP)¹²². This system consists of T7 RNA polymerase divided into two domains (N-1-29 and C-30-181 term T7 RNAP), that assemble into a functional polymerase in a proximity-dependent fashion. The T7 components were fused to a rapamycin-induced dimerization system, consisting of FKBP-rapamycin binding domain (FRB) and FK506 binding protein (FKBP). Upon introduction of rapamycin, FRB is recruited to FKBP, which brings the N-terminus in close proximity to C-terminal T7 RNAP. T7 RNAP is then assembled and able transcribe a gene regulated by a T7 promoter (**Fig. 2.1a**). We engineered VV to constitutively express mCherry and the rapamycin-controlled, split T7 RNA polymerase-based transcription system (ST7). In the same locus (Thymidine Kinase

coding region-TK), we also engineered a T7 promoter-driven firefly luciferase and GFP (GFPLuc) cassette (VV-ST7-iGFPLuc; **Fig. 2.1a**). In VV-ST7-iGFPLuc infected U2OS osteosarcoma cells, GFP and luciferase expression were induced dramatically in the presence of 10 nM rapamycin over a range of different virus concentrations (**Fig. 2.1b-c**). Infected U2OS cells showed a greater than ten-fold increase in luciferase activity 24 h post-infection relative to uninduced infected controls (**Fig. 2.1c**). Similar results were observed in HeLa cervical carcinoma cells, and A549 lung carcinoma cells (**Fig. 2.1d**). In order to confirm that rapamycin and the inducible split T7 polymerase system did not impact viral growth kinetics, we performed multi-step virus growth curves. Rapamycin and ST7 expression did not have a significant effect on VV-ST7-iGFPLuc growth in U2OS, HeLa, or A549 cells (**Fig. 1e; Supplementary Fig. 2.1b-c**).

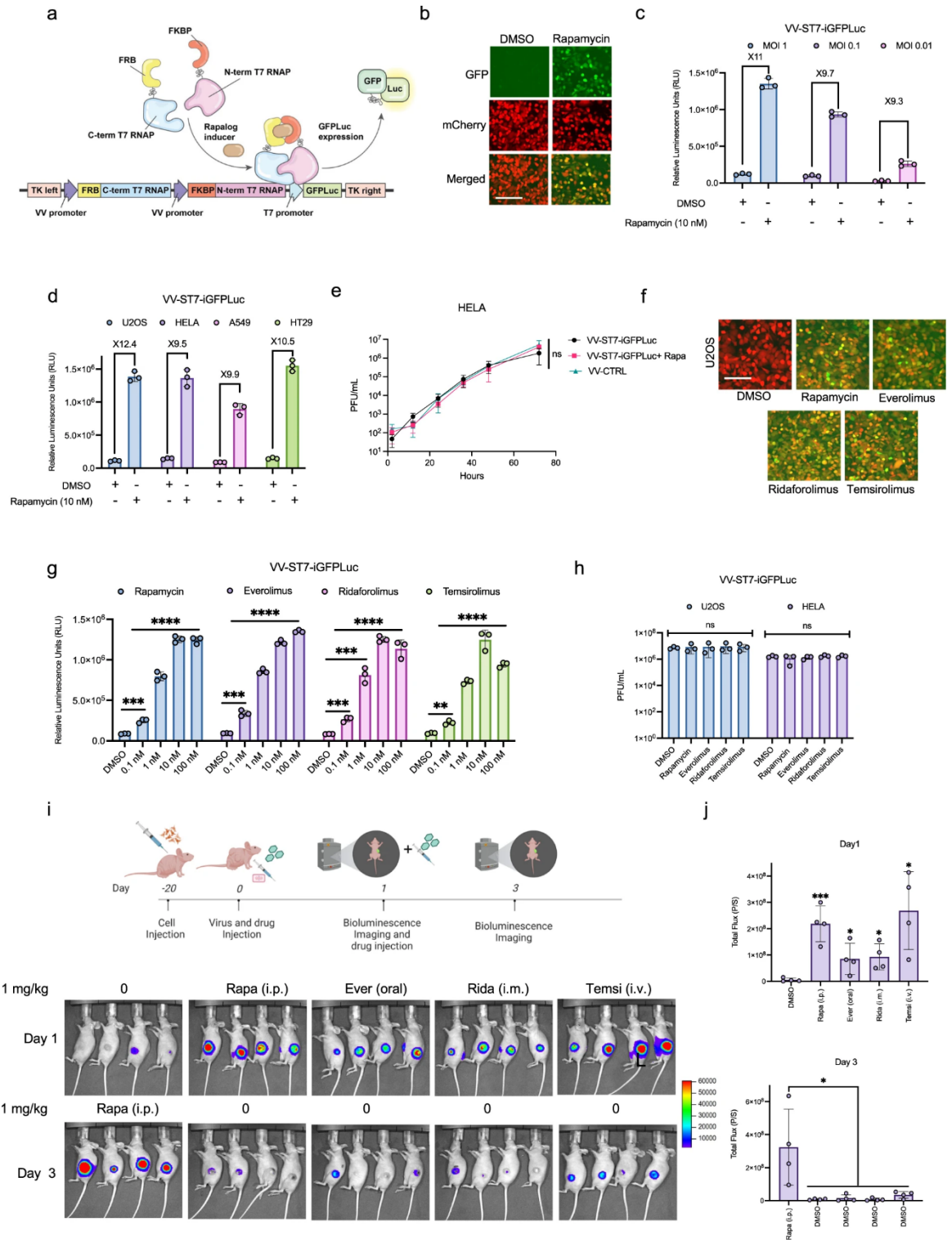


Figure 2.1. Integration, functional characterization, and optimization of the split T7 inducible system in vaccinia virus (A) Schematic illustration of the split T7 inducible expression cassette inserted into Thymidine Kinase (TK) open reading frame. The FRB linked N-terminal portion of the T7 RNAP and the FKBP linked C-terminal portion of the T7 RNAP were expressed under continuous vaccinia virus promoters. A Fusion protein consisting of GFP, and firefly luciferase (GFPLuc) was incorporated under T7 promoter and induced by the addition of Rapalogs. mCherry fluorescent protein is expressed from the virus to detect virus infection in cells. (B, C) Representative fluorescent images and quantitation of luciferase signal (RLU) of the T7 inducible system from U2OS cells 24 hours after infection with VV-ST7-iGFPLuc in the presence or absence of 10 nM rapamycin. mCherry indicates virus infection. (D) Comparison of the luciferase signal from various cell lines 24 hours after infection at MOI 0.1 with VV-ST7-iGFPLuc in the presence or absence of 10 nM rapamycin. (E) Multistep growth curve of VV-ST7-iGFPLuc compared to the control vaccinia virus at different time points from Hela cells infected at MOI 0.01 in the presence or absence of 10 nM rapamycin. (F) Representative fluorescent images of the T7 inducible GFP system from U2OS cells 24 hours after infection with VV-ST7-iGFPLuc at MOI 0.1 in the presence of different Rapalogs at 10 nM concentration. (G) Relative luminescence emitted after 24 hours from U2OS cells infected with VV-ST7-iGFPLuc at MOI 0.1 in the presence of different concentrations of Rapalogs. (H) Comparison of vaccinia virus growth in the presence of Rapalogs at 10 nM in U2OS and HELA cells 24 hours after infection at MOI 0.1. (I, J) IVIS imaging of HT-29 tumors in CD-1 nude mice. Tumors were injected with VV-ST7-iGFPLuc (1E7 PFU/tumor) when they reached ~150 mm³ in size. Different Rapalogs were administered at 1 mg/kg by their preferred route. 24 hours later, luciferase signal was measured, and the control group received rapamycin intraperitoneally. Luciferase signal was measured in all groups again at 72 hours. Scale bars = 40 μ m in B & F. Data indicate means $\pm$ SD of three (1c-e, g, and h) to five (1j) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

Rapamycin (sirolimus) is approved for use as an immunosuppressant and treatment of lymphangioleiomyomatosis, a rare lung disease¹²³. Its actions are mediated through inhibition of the mTOR protein kinase and is associated with anti-proliferative and anti-cancer activity. Rapamycin suffers from poor solubility, stability and pharmacokinetic properties. However, rapamycin analogs, or “rapalogs”, (e.g. everolimus, temsirolimus, and ridaforolimus) have been approved for the treatment of a wide spectrum of cancers^{124,125}. We examined if these approved rapalogs were compatible with the VACV-adapted ST7 expression system. In infected U2OS cells, we observed a robust increase in GFP and luciferase expression during treatment with rapalogs, in line with our observations with rapamycin (**Fig. 2.1f-g**). We also demonstrated a dose-dependent increase of luciferase activity in infected U2OS cells treated with rapalogs, suggesting the system is tunable (**Fig. 2.1g**). Similar to rapamycin, rapalogs did not significantly influence viral growth (**Fig. 2.1h**). To examine whether rapamycin and rapalogs can be used *in vivo* to control the virally encoded transgenic RNA polymerase system, we injected HT-29 xenograft bearing mice with VV-ST7-iGFPLuc intratumorally and treated mice with rapamycin or rapalogs via different routes of administration. Temsirolimus¹²⁶ is more water soluble and suited for intravenous injection, whereas everolimus¹²⁷ is available for oral administration. In this study, we infected tumours and applied a single dose of the rapalog at the time of infection using the clinically appropriate route for each rapalog. As shown in **Fig. 2.1i-j**, we observed robust stimulation of luciferase 24 hours later. The animals received no further treatments with the rapalogs and then at 72 hours post initial infection the tumours were once again imaged to determine the level of luciferase expression. The level of expression of luciferase driven by the initial induction with the rapalog has decreased significantly (**Fig. 2.1i-j**). This decrease in signal is not related to a clearance of virus as we determined the level of infectious particles at day 10 and it was comparable in

all of the tumours sampled (**Supplementary Fig. 2.1d**). Furthermore, in animals that were infected but not treated with rapalog there was minimal luciferase signal which was robustly induced upon rapalog induction at 48 hours. Among the conditions tested, rapamycin (i.p.) and temsirolimus (i.v.) produced the highest induction of luciferase signal in the tumors. These results demonstrate that the rapamycin inducible ST7 system can be used to temporally control viral transgene expression *in vivo*.

Control of vaccinia virus promoter elements with doxycycline.

We investigated the utility of repressor/operator systems to create virally-encoded chemogenetic switches. Doxycycline (Dox) is an approved orally delivered tetracycline-class antibiotic with well-established pharmacokinetic and pharmacodynamic properties. The Dox-inducible expression system has previously been applied in the context of a VV genome to enable conditional expression of viral factors¹²⁸. We carried out a more detailed analysis of the application of this technology to a variety of vaccinia promoter elements. Vaccinia genes have stage-specific transcription factors and corresponding promoter elements which mediate temporal regulation of their expression. We screened a diverse set of VV promoters encompassing the range of temporal expression patterns exhibited by VV proteins. Using ribosome profiling in VV infected HeLa cells, we identified viral promoters associated with genes encoding transcripts that are efficiently translated, and thus highly expressed, at different times during the virus replication cycle (**Fig. 2.2a**). We generated a series of recombinant VV strains encoding tet operator controlled VV candidate promoters to conditionally control the expression of the GFP_{Luc} cassette (**Fig. 2.2b**). In the same locus (TK), the strains encoded blue fluorescence protein (BFP) and the tetracycline repressor protein (TetR), which binds TetO in the absence of Dox to impair

expression. We performed an arrayed screen of these viruses to identify which TetO controlled VV promoter resulted in the highest level of induced GFP and luciferase expression (**Fig. 2.2c-d**). There was considerable variability in the ability to control vaccinia virus promoters using the Dox-inducible gene cassettes. We found that the p11 and PEL promoters were most amenable to this form of transcriptional control with robust induction in the presence of Dox and minimal background expression in its absence. A closer examination of the luciferase assay results (**Fig. 2.2d**) revealed that the use of p11 promoter resulted in maximal reporter gene expression in the presence of Dox; therefore, we selected the TetO-controlled P11 promoter for further characterization studies. We first tested the kinetics of inducible expression with the newly developed VV strain encoding TetR and p11-TetO driven GFPLuc gene cassettes (VV-TetR-iGFPLuc). We observed induction of a GFP signal in infected U2OS cells within 3 hours of Dox treatment (**Supplementary Fig. 2.2a**) and similar high inducibility (>30-fold) in a panel of other cancer cell lines (A549, HeLa, HT-29, and SKOV-3) (**Fig. 2.2e and Supplementary Fig. 2.2b**). We conducted a comparison of luciferase expression at various time points using the wild-type vaccinia virus promoter and the doxycycline-controlled promoter with and without induction and demonstrated that both promoter systems achieve a similar maximum level of reporter expression (**Supplementary Fig. 2.2c**). Neither expression of TetR, incorporation of the TetO controlled expression system, or Dox influenced viral growth – illustrating compatibility of the Dox-controlled chemogenetic switch in VV-based vectors (**Fig. 2.2f and Supplementary Fig. 2.2d-e**). Under *in vitro* conditions, 10 ng/mL of Dox was sufficient to induce detectable levels of luciferase activity in VV-TetR-iGFPLuc infected U2OS cells, while 25 ng/mL produced the maximal signal (**Supplementary Fig. 2.2f**).

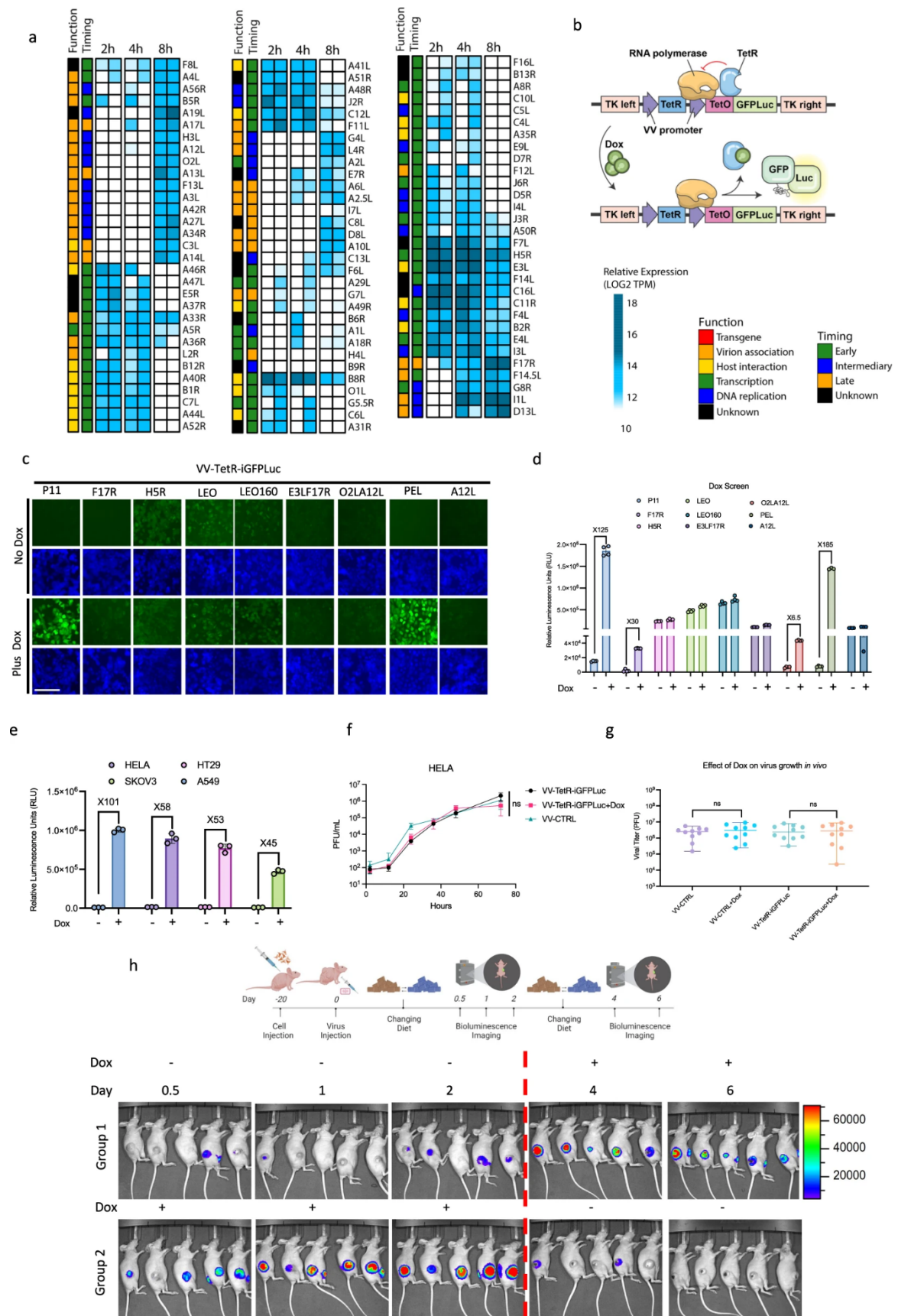


Figure 2.2. Optimization of the doxycycline-inducible system for controlling gene expression in vaccinia virus (A) Hela cells were infected with vaccinia virus at MOI 10 and RNA was collected at 2, 4, and 8 hours post infection. The heatmap, categorized by gene function and reported timing of gene expression, depicts results of the ribosome profiling after cDNA synthesis and sequencing. (B) Schematic illustration of the tetracycline inducible expression cassette inserted to TK locus. The TetR protein was expressed under a continuous vaccinia virus promoter and various vaccinia virus promoters preceding a TetO element, which binds TetR protein, were incorporated upstream of the GFPLuc fusion protein. The dissociation of the TetR from TetO upon doxycycline (Dox) administration leads to initiation of transcription and gene expression. Blue fluorescence protein (BFP) is continuously expressed from the virus to detect viral infection (C, D) Representative fluorescent images and quantitation of luciferase signal (RLU) of U2OS cells 24 hours after infection with viruses expressing the GFPLuc fusion protein (VV-TetR-iGFPLuc) at MOI 0.1 under the control of various native and synthetic vaccinia promoters. Expression of GFPLuc was induced with 100 ng/ml Dox. BFP images indicate virus infection (E) Comparison of luciferase signal (RLU) from different cell lines 24 hours after infection at MOI 0.1 with VV-TetR-iGFPLuc in the presence or absence of 100 ng/ml Dox. (F) Multistep growth curve of VV-TetR-iGFPLuc compared to the control vaccinia virus at different time points using Hela cells infected at MOI 0.01 in the presence or absence of 100 ng/ml Dox. (G) Quantitation of viral titers within HT-29 tumors from CD-1 nude mice five days after Dox (625 mg/kg) was introduced into the diet of mice. Tumors were infected with control vaccinia virus (VV) or VV-TetR-iGFPLuc at 1E7 PFU/tumor prior to the introduction of Dox. (H) IVIS imaging of HT-29 tumors following injection with VV-TetR-iGFPLuc at 1E7 PFU/tumor when tumors reached ~150 mm³ in size. Mice were given Dox (625 mg/kg) in their diet 2 days post viral infection (Group 1) or immediately following viral infection (Group 2) of tumors. For Group 2, Dox was removed from the diet after 2 days. Luciferase signal was monitored at 12 hours, 1, 2, 4 and 6 days post viral infection. Scale bars = 40 μ m in panel C. Data indicate means $\pm$ SD of three (2e-f), four (2d), or ten (2g) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

We then characterized VV-TetR-iGFPLuc *in vivo* using a HT-29 colon cancer xenograft model. We infected the mice intratumorally with VV-TetR-iGFPLuc and performed 1 mg/kg intraperitoneal treatments. *In vivo* imaging of bioluminescence, 24 hours post-infection, demonstrated robust luciferase activity in the tumors (**Supplementary Fig. 2.2g**). We repeated the experiment with one alteration – introduction of Dox via diet to simulate the potential use of oral Dox in combination with a VV vector bearing Dox-controlled transgene expression (**Fig. 2.2g-h, Supplementary Fig. 2.2h**). We were able to conditionally control transgene expression through feeding and withdrawal of a Dox-containing diet (**Fig. 2.2h; Supplementary Fig. 2.2g**). We noted that Dox had no influence on viral growth *in vivo*, in line with our *in vitro* results (**Fig. 2.2g**), and no negative effect on luciferase activity *in vivo* (**Supplemental Fig. 2.2i-j**). Collectively, these results establish the utility of a Dox-controlled gene cassette as a tool for temporal control of payload expression in VV vectors *in vivo*.

A cumate-controlled chemogenetic switch for vaccinia virus.

We sought to identify a novel repressor-based operator system to generate another VV-adapted inducible expression system to enable further independent temporal control of multiple payloads. The cumate-controlled operator system derived from the *Pseudomonas putida* p-cmt and p-cym operons^{113,129}, has been used in gene therapy vectors^{130,131} and is similar to the tetracycline-controlled operator system. It consists of the cumate operator (CuO) sequence which is bound by a repressor (CymR) in the absence of cumate. We engineered VV to constitutively express CymR and BFP. In the same locus (TK), we incorporated the coding sequence of GFPLuc under the control of the cumate operon fused to a VV promoter (**Fig. 2.3a**). Similar to the development of the Dox-based chemogenetic

switch, we screened VV promoters to identify which worked best in combination with the CuO to mediate cumate-inducible expression in infected U2OS cells (**Fig. 2.3b-c**). As with the Dox system, there was considerable variability in the control of vaccinia promoters by the cumate operon. While the early-late promoters H5R, LEO, and LEO160 resulted in maximal luciferase activity and GFP expression in the presence of cumate, these promoters also resulted in the highest basal reporter signal in the absence of cumate. The use of the synthetic early-late promoter PEL in tandem with CuO resulted in the highest inducibility (450X) while maintaining minimal basal expression (**Fig. 2.3c**); therefore, we selected the PEL-Cu promoter/operator combination for further evaluation.

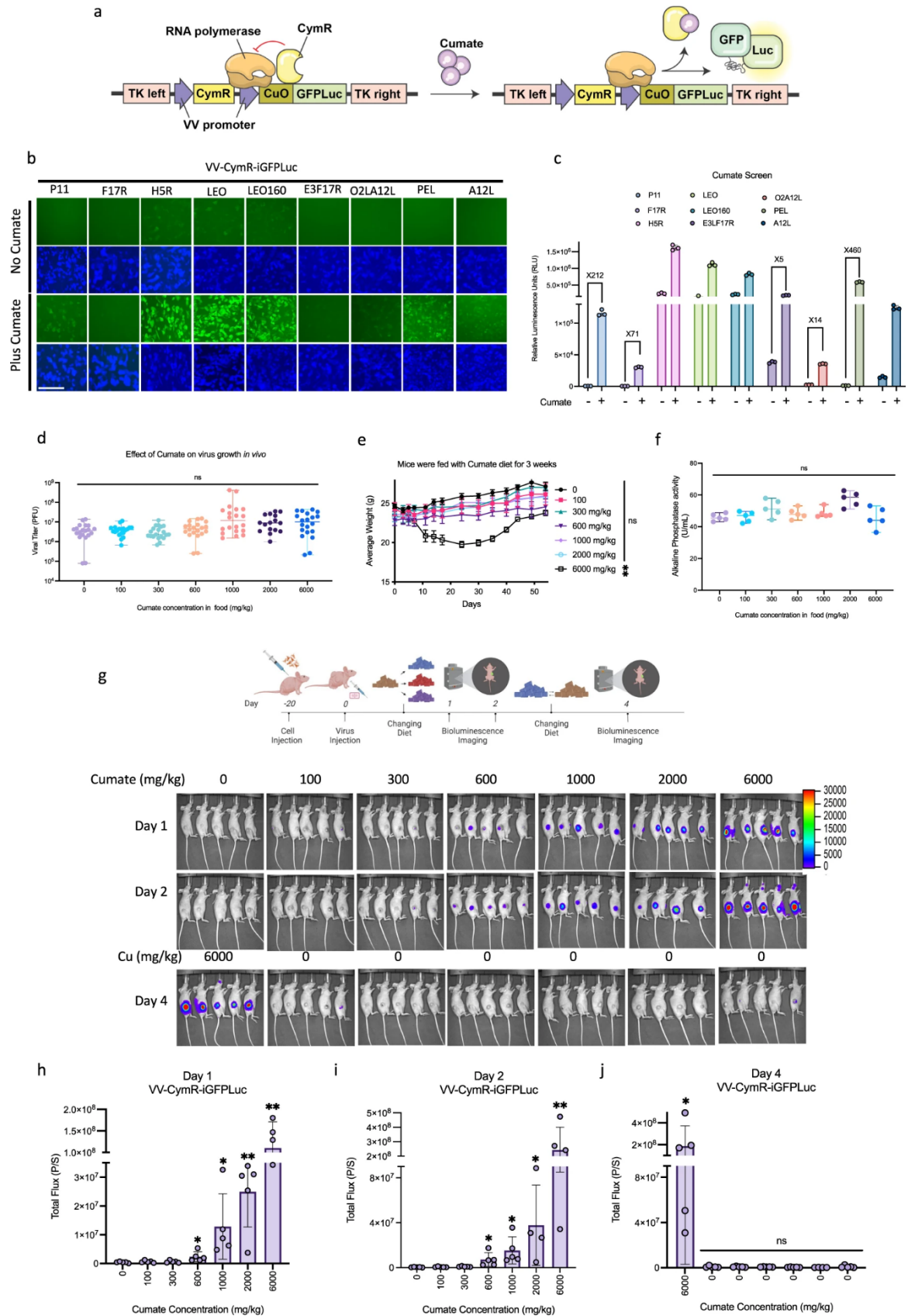


Figure 2.3. Cumate inducible system controls viral gene expression *in vitro* and *in vivo*

(A) Schematic illustration of the cumate inducible expression cassette inserted into TK locus. The CymR protein was expressed under a continuous vaccinia virus promoter and a GFPLuc fusion protein incorporated under various vaccinia virus promoters preceding a CuO element which binds CymR protein. The dissociation of the CymR from CuO upon cumate administration leads to initiation of transcription and gene expression. Blue fluorescence protein is expressed from the virus to monitor its presence in infected cells.

(B, C) Representative fluorescent images and quantitation of luciferase signal (RLU) from U2OS cells 24 hours after infection at MOI 0.1 with viruses expressing the GFPLuc fusion protein (VV-CymR-iGFPLuc) under the control of various native and synthetic vaccinia promoters. Expression of GFPLuc was induced with 100 ug/ml cumate.

(D) Quantitation of viral titers within HT-29 tumors from CD-1 nude mice five days following infection with VV-CymR-iGFPLuc (1E7 PFU/tumor) and treatment with varying amounts of cumate.

(E, F) C57BL/6 mice were fed with varying amounts of cumate in their diet for 25 days and weighed at regular intervals for up to 55 days to measure cumate toxicity. Serum was collected at days 25 and 55 to measure different toxicity indicators.

(G-J) HT-29 tumors from CD-1 nude mice were infected with VV-CymR-iGFPLuc (1E7 PFU/ml) and mice fed cumate diets according to the schedule and amounts shown. Bioluminescence images were taken, and luciferase activity quantified 1 and 2 days following initial virus treatment. After day 2, cumate diets were switched to a normal rodent diet and the control group received a diet containing 6000 mg/kg of cumate. Additional images were acquired following the diet switch, at day 4 post virus infection. Bar graphs show the total flux signal measured in the tumor area at the indicated days. Scale bars = 40 μ m in panel B. Data indicate means $\pm$ SD of three (3c), twenty (3d), and five (3e-j) five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

We examined the kinetics of inducible expression with our VV strain encoding CymR and PEL-CuO driven GFPLuc gene cassettes (VV-CymR-iGFPLuc). With cumate concentrations as low as 10 $\mu\text{g/mL}$, GFP signal is observed within 6 hours of cumate treatment of VV-CymR-iGFPLuc infected cells (**Supplemental Fig. 2.3a**). Consistent with this, significant luciferase activity is also observed in infected cells with 10 $\mu\text{g/mL}$ cumate (**Supplemental Fig. 2.3d**). We confirmed that cumate treatment does not influence viral growth in U2OS cells at concentrations up to 500 $\mu\text{g/mL}$ (**Supplemental Fig. 2.3b-c**). Similarly, multi-step viral growth studies confirmed that cumate or CymR expression does not impact VV replication in U2OS, HeLa, and A549 cells (**Supplemental Fig. 2.3e-g**). We next compared head-to-head the cumate-inducible expression system with the ST7 and Dox-inducible systems (**Supplemental Fig. 2.3h-k**). In infected U2OS cells, the Dox-inducible system produced the highest reporter signal in the presence of its inducer; however, the cumate-inducible system provided the greatest inducibility in the presence of its ligand with minimal basal expression in the absence of cumate (**Supplemental Fig. 2.3h**).

HT-29 xenografts were injected intratumorally with VV-CymR-iGFPLuc and cumate (1 mg/kg) was administered intraperitoneally. *In vivo* imaging of bioluminescence 24 hours post-infection revealed robust tumor-localized reporter signal in a cumate-dependent fashion (**Supplementary Fig. 2.3l**). In parallel, we demonstrated that cumate treatment *in vivo* did not influence VV replication (**Supplementary Fig. 2.3l – lower panels**). To examine the potential for more accessible administration of cumate, we repeated the experiment and delivered cumate (6000 mg/kg) to mice via their diet. We observed cumate-dependent activation of luciferase activity in the tumor, and, confirmed that cumate does not influence VV replication (**Supplementary Fig. 2.3m-n**). As this is the first report to our knowledge of feeding mice cumate, we examined the effect of feeding

cumate on mouse health and viral growth. At all concentrations tested 100-6000 mg/kg), cumate did not impact viral replication *in vivo* (**Fig. 2.3d**). Treatment for more than twenty-five days with a diet containing less than 2000 mg/kg cumate did not cause significant loss of weight in immunocompetent mice (**Fig. 2.3e**). For a more comprehensive toxicity analysis, we also examined the activity of the serum enzymes alkaline phosphatase, aspartate aminotransferase, and glutamate dehydrogenase. Twenty-five days of cumate diet, at all cumate concentrations tested (100-6000 mg/kg), did not impact on the levels of these enzymatic markers of liver toxicity was observed (**Fig. 2.3f and Supplemental Fig. 2.3o-r**). The data suggests that the cumate-inducible system has an adequate safety profile for use *in vivo*.

Lastly, we examined the sensitivity of the cumate-inducible system *in vivo* (**Fig. 2.3g-j**). We were able to rapidly induce luciferase expression in VV-CymR-iGFPLuc infected mice within 1 day of treatment. Upon removal of the cumate, the luciferase signals dissipated within 96 hours. Overall, the data establish VV-CymR-iGFPLuc as recombinant virus enabling tunable expression of a target transgene or viral factor.

Combinatorial application of chemogenetic switches.

We noted that relative to the TetO or CuO controlled VV promoters, the VV-adapted T7 system resulted in higher basal expression of transgenes. We reasoned that combining the ST7 system with either operator would result in a more selective safety switch controlled by the use of two small molecules. We generated a virus encoding a TetO immediately downstream of a T7 promoter driving GFPLuc expression (**Fig. 2.4a**). The virus also encoded the TetR and ST7 (VV-ST7-TetR-iGFPLuc) to mediate Dox- and rapamycin-inducible expression of the reporter genes, respectively. In VV-ST7-TetR-iGFPLuc

infected U2OS cells, we demonstrated that treatment with Dox alone increase luciferase activity 7-fold, whereas treatment with rapamycin alone induced luciferase activity by 19-fold (**Fig. 2.4b**). A combination of Dox and rapamycin treatment synergized to activate luciferase activity by 48-fold. Similar trends were observed using the GFP reporter system (**Fig. 2.4c**).

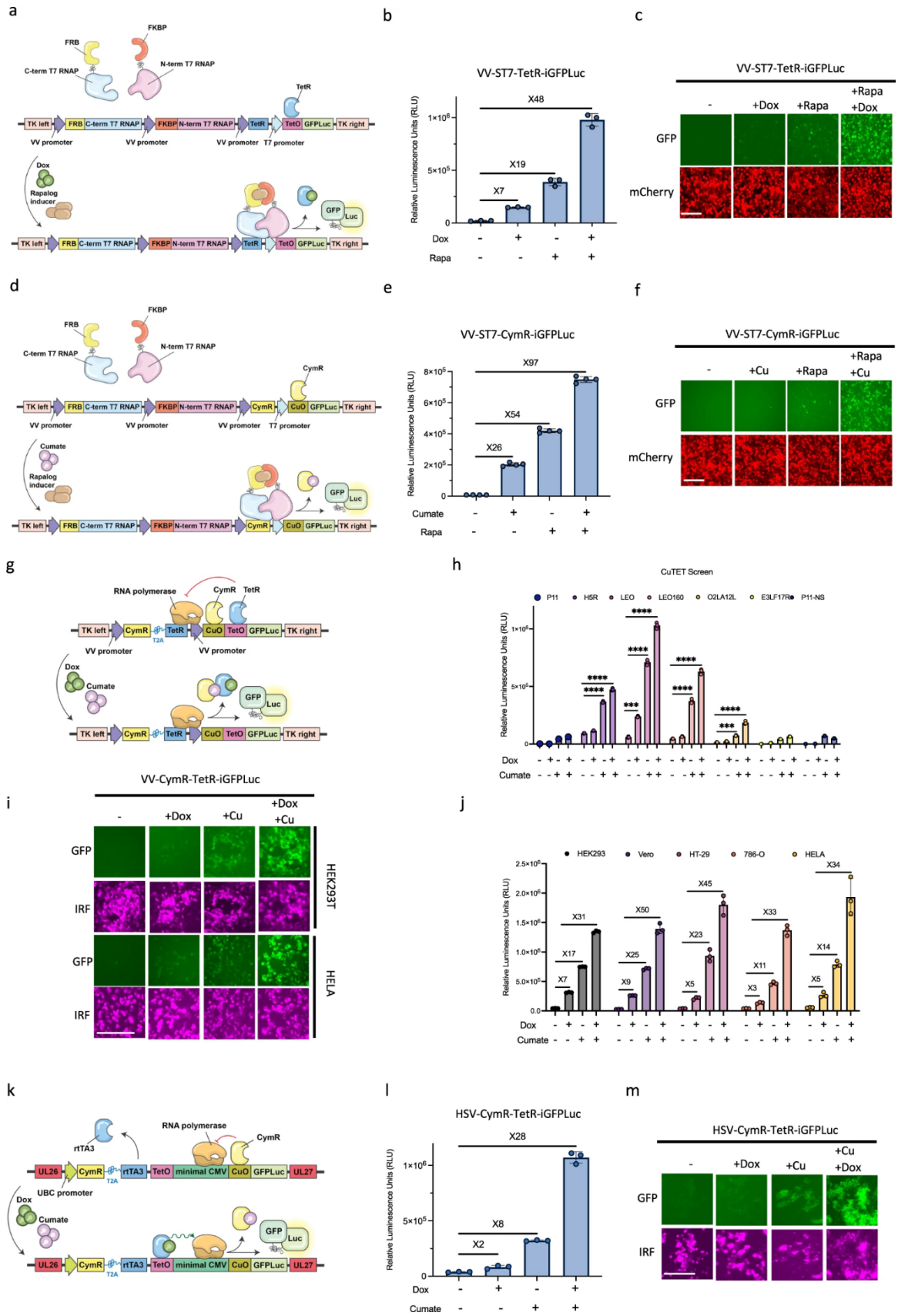


Figure 2.4. Double inducible chemogenetic switches overcome major challenges with single molecule systems. (A) Schematic illustration of the ST7-Tet double inducible expression cassette inserted into TK locus. The TetR and split T7 fragment proteins were expressed under continuous vaccinia virus promoters and a GFPLuc fusion protein was incorporated under T7 promoter preceding a TetO element which binds TetR protein. The binding of split T7 fragments in the presence of Rapalogs and dissociation of the TetR from TetO upon Dox administration leads to initiation of transcription and gene expression. (B, C) Representative fluorescent images and quantitation of luciferase activity from U2OS cells 24 hours following infection with VV-ST7-TetR-iGFPLuc in the presence or absence of Dox and rapamycin. mCherry signal indicates virally infected cells. (D) Schematic illustration of the ST7-Cumate double inducible expression cassette inserted into the same locus as described in A. The CymR and split T7 fragment proteins were expressed under continuous vaccinia virus promoters and GFPLuc was incorporated under T7 promoter preceding a CuO element which binds CymR protein. The binding of split T7 fragments in the presence of Rapalogs and dissociation of the CymR from CuO upon cumate administration leads to initiation of transcription and gene expression. (E, F) Representative fluorescent images and quantitation of luciferase activity from U2OS cells 24 hours following infection with VV-ST7-CymR-iGFPLuc in the presence or absence of cumate and rapamycin. (G) Schematic illustration of the Cu-Tet double inducible expression cassette inserted into the same locus as described in A. The CymR and TetR proteins were expressed under continuous vaccinia virus promoters and GFPLuc was incorporated under different vaccinia virus promoters preceding a CuO element which binds CymR protein, and a TetO element which binds TetR protein. Dissociation of the CymR from CuO and TetR from TetO upon cumate and Dox administration leads to initiation of transcription and gene expression. (H) Luciferase signal (in RLU) was quantified 24 hours after infection of U2OS cells with viruses expressing GFPLuc under the control of CuTet and various native and synthetic vaccinia promoters in the presence or absence of 100 ug/ml cumate and 100 ng/ml Dox. (I, J) Representative fluorescent images and quantitation of luciferase activity from HEK293T, Hela, Vero, HT-29, 786-O cells infected with VV-CymR-TetR-iGFPLuc in the presence or absence of cumate and Dox after 24 hours. IRF signal indicates virally infected cells. (K) Schematic illustration of the CuTet double inducible expression cassette inserted in the intergenic location between UL26 and UL27 open reading frames of the HSV-1 genome. The CymR-T2A-rtTA3 fusion protein was expressed under the UBC promoter. For expression of the GFPLuc fusion protein, TetO followed by a minimal CMV promoter and CuO was used. In the presence of Dox, rtTA3 binds to the TetO and enhances CMV promoter activity which will be at maximum activity if CymR is also dissociated from CuO upon cumate administration. IRF is continuously expressed from the virus, to monitor viral infection. (L, M) Representative fluorescent images and quantitation of luciferase activity from U2OS cells infected with VV-CymR-TetR-iGFPLuc in the presence or absence of cumate and Dox after 24 hours. IRF signal indicates virally infected cells. Scale bars = 40 μ m in panel C, F, I, M. Data indicate means $\pm$ SD of three (4b, 4h, 4j, 4l) or four (4e) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

Analogous to VV-ST7-TetR-iGFPLuc, we developed a recombinant VV encoding ST7, CymR, and a CuO immediately downstream of a T7 promoter driving GFPLuc expression (VV-ST7-CymR-iGFPLuc; **Fig. 2.4d**). In VV-ST7-CymR-iGFPLuc infected U2OS cells, treatment with cumate alone, rapamycin alone, or both in combination induced luciferase activity 26-fold, 54-fold, and 97-fold respectively (**Fig. 2.4e-f**). Importantly, incorporation of either the TetO or CuO operators into the ST7 system significantly reduced basal “leaky” expression associated with the ST7 system as illustrated by the complete loss of basal luciferase activity in the absence of small molecule inducers.

During our generation of a VV-adapted cumate-inducible expression system, we noted that while H5R, LEO, and LEO160 promoters produce the strongest luciferase activity in the presence of cumate (**Fig. 2.3c**), but also produced the highest basal expression in the absence of cumate. We hypothesized that combining CuO with TetO would produce a more tightly regulated expression system (Cu-Tet double inducible expression system). We engineered seven different recombinant VV expressing CymR, TetR, as well as a CuO and TetO placed immediately downstream of unique VV promoters (p11, H5R, LEO, LEO160, O2LA12L, E3LF17R and P11-NS) driving GFPLuc expression (**Fig. 2.4g**). Of the VV promoters screened, LEO in concert with a TetO and CuO produced the highest inducible luciferase activity in the presence of Dox and cumate, while maintaining low basal activity in the absence of small molecule inducers (**Fig. 2.4h**) in infected U2OS cells. Treatment with Dox alone or cumate alone induced intermediate levels of luciferase activity. Analyses of GFP fluorescence analysis revealed similar trends (**Fig. 2.4i**). We utilized VV expressing CymR, TetR, and CuO-Tet-O-LEO driven GFPLuc (VV-CymR-TetR-iGFPLuc) for additional in vitro characterization studies. To confirm that this level of tunability was not specific to U2OS cells, we examined the inducibility of luciferase activity in VV-CymR-TetR-iGFPLuc infected HEK293, Vero, HT-29, 786-0,

and HeLa cells (**Fig. 2.4j**). In all tested cell lines, Dox induced 3-7-fold increase in reporter signal, whereas cumate induced a 11- to 23-fold activation of luciferase activity. Combined treatment with Dox and cumate produced maximal increases in luciferase activity ranging from 23 to 45-fold across the five tested cell lines. As a proof-of-concept, we also demonstrated that a 3rd generation Dox-inducible expression system (Tet-ON using reverse tetracycline-controlled transactivator 3, rTA3) can be combined with the cumate-inducible expression system (CymR/CuO) to generate a highly tunable expression system in an oncolytic herpes virus platform (HSV: **Fig. 2.4k-m**) – illustrating that the novel chemogenetic switches developed for VV have the potential to be adapted for other viruses.

The development of the VV-CymR-TetR, VV-ST7-TetR and VV-ST7-CymR expression systems enable the introduction of additional tunable functionality into poxviruses. Further, these novel inducible expression systems enable multiplexed control of multiple virally-encoded transgenes through the addition of various combinations of rapamycin, cumate, and doxycycline.

Chemically regulated control of oncolytic virus replication and spread.

Oncolytic viruses with deletions in critical virulence genes have a very strong safety record but this often comes at the price of compromised therapeutic potency and challenges to manufacturing. An alternative strategy for selective replication of an OV is to control expression of key replication gene products. Recent studies have described that deletion of the D13L gene in VV produces a severely attenuated vector incapable of producing infectious virus in healthy volunteers^{132,133}. Thus, we replaced the endogenous vaccinia virus D13L promoter with a TetO-controlled p11 promoter. (VV-TetR-iD13; **Fig. 2.5a**). As expected, in VV-TetR-iD13 infected U2OS cells, the addition of Dox increased plaque

size, as observe by GFP fluorescence (**Fig. 2.5b**) and Dox dependent cell killing (**Supplementary Fig. 2.4a**). These results were observed in a panel of infected human cancer cell lines of a variety of histologies (A549, HeLa, HT29, SKOV3: **Fig. 2.5c-d** and **Supplementary Fig. 2.4b-c**). While vaccinia replication as measured by a multi-step growth curve analysis was severely compromised in the absence of Dox, the growth rate of VV-TetR-iD13L was indistinguishable from wildtype vaccinia virus in the presence of the inducer (**Fig. 2.5e-f**). Furthermore, our observations indicate that this virus replicates 100 to 1000 times less efficiently in human primary cells when compared to cancer cells (**Supplementary Fig. 2.4d**).

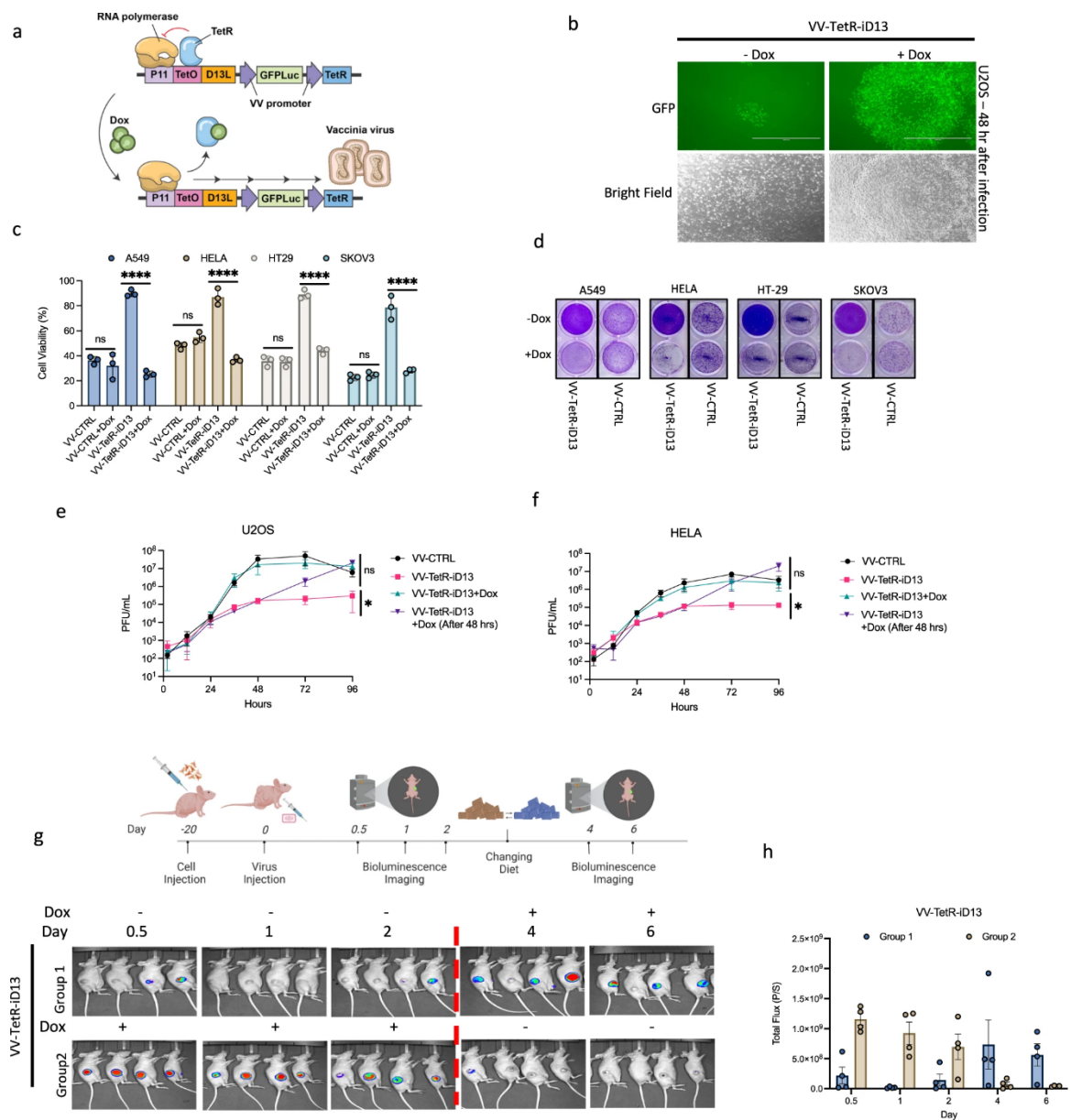


Figure 2.5. Application of the tetracycline inducible system as a safety switch to generate a conditionally replicating vaccinia virus. (A) Schematic illustration of the tetracycline inducible system controlling the expression of the D13L vaccinia virus gene which leads to the conditional growth of the vaccinia virus in the presence of Dox. (B) Representative images of U2OS cells 48 hours after infection with VV-TetR-iD13 (MOI 0.01) in the presence or absence of 100 ng/ml Dox. (C, D) A549, Hela, HT29, and SKOV3 cells were infected with control vaccinia virus or VV-TetR-iD13 at MOI 0.01 in the absence or presence of Dox at 100 ng/ml. After 48 hours, cells were stained with crystal violet, and cell viability assessed by measuring absorbance at 570 nm using resazurin. (E, F) Multistep growth curves of VV-TetR-iD13 compared to control vaccinia virus at different time points from U2OS and Hela cells when infected at MOI 0.01 in the presence or absence of 100 ng/ml Dox. (G, H) IVIS imaging of HT-29 tumors in CD-1 nude mice following injection with VV-TetR-iD13 (1E7 PFU/tumor) when tumors reached 150 mm³. Mice were given a Dox diet either 2 days post viral infection (Group 1, blue bars) or immediately following viral infection (Group 2, brown bars). For Group 2, Dox was removed from the diet after 2 days. Luciferase signal was measured at 12 hours, 1, 2, 6 and 6 Days after virus and drug administration. Bar graphs show the average total luciferase signal emitted in the tumor area. Scale bars = 200 μ m in panel B. Data indicate means $\pm$ SD of three (5c, 5e-f) or four (5h) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

We next examined the temporal control of VV-TetR-iD13 in HT-29 xenograft bearing mice *in vivo*. Bioluminescence imaging of HT-29 xenografts revealed that in the presence of Dox-containing diet, robust tumor selective viral replication was observed within 12h of viral infection (**Fig. 2.5g-h**). However, after changing back to normal diets viral replication was extinguished. Similarly, it was possible to delay the introduction of a Dox-containing diet to 2 days post-infection, and robust tumor-localized luciferase activity would be observed 4-6 days post-infection. Collectively, the data demonstrates that the chemogenetic safety switches (e.g., TetR/TetO) can be applied as a safety switch to generate VV vectors with chemically regulated replication kinetics *in vivo*.

While our primary interest is devising strategies to manipulate oncolytic virus replication and transgene expression, it seems reasonable to expect that conditional virus gene expression could be also used to create effective vaccine vectors for either infectious diseases or certain cancer indications. The use of replication competent VV-vectored vaccines in the clinic to-date has been limited due to potential adverse events, including progressive vaccinia and post-vaccination encephalitis. We hypothesized that the use of the conditionally replicating VV-TetR-iD13 as a backbone vector for immunogen delivery could help reduce safety concerns. To examine the safety of VV-TetR-iD13, we compared the effects of VV-TetR-iD13 and VV-CTRL infection in SCID mice after intravenous injection. VV-TetR-iD13 infection was followed by two days of Dox treatment to allow for initial replication to enhance any antigen expression in the context of a vaccine vector. Five days post-infection, we noted a significant number of pox lesions in VV-CTRL infected mice; however, VV-TetR-iD13 infected mice had drastically lower number of lesions (**Fig. 2.6a, d**). This result was consistent with bioluminescent imaging of virus localization (**Fig. 2.6b, e**). Whereas in the VV-TetR-iD13 infected mice, there was no detected signal, the VV-CTRL infected mice had bioluminescent signals localized to tails,

paws, and snouts. Furthermore, VV-CTRL infected mice demonstrated increasing weight loss from 4 to 8 days post-infection (**Fig. 2.6c**). Virus biodistribution in known mouse VV reservoirs were analyzed via plaque assay and there was more a than tenfold decrease in viral titers in lungs, livers, and spleen. Collectively, the data demonstrate that conditionally replicating VV-TetR-iD13 represents a safer vector for antigen delivery.

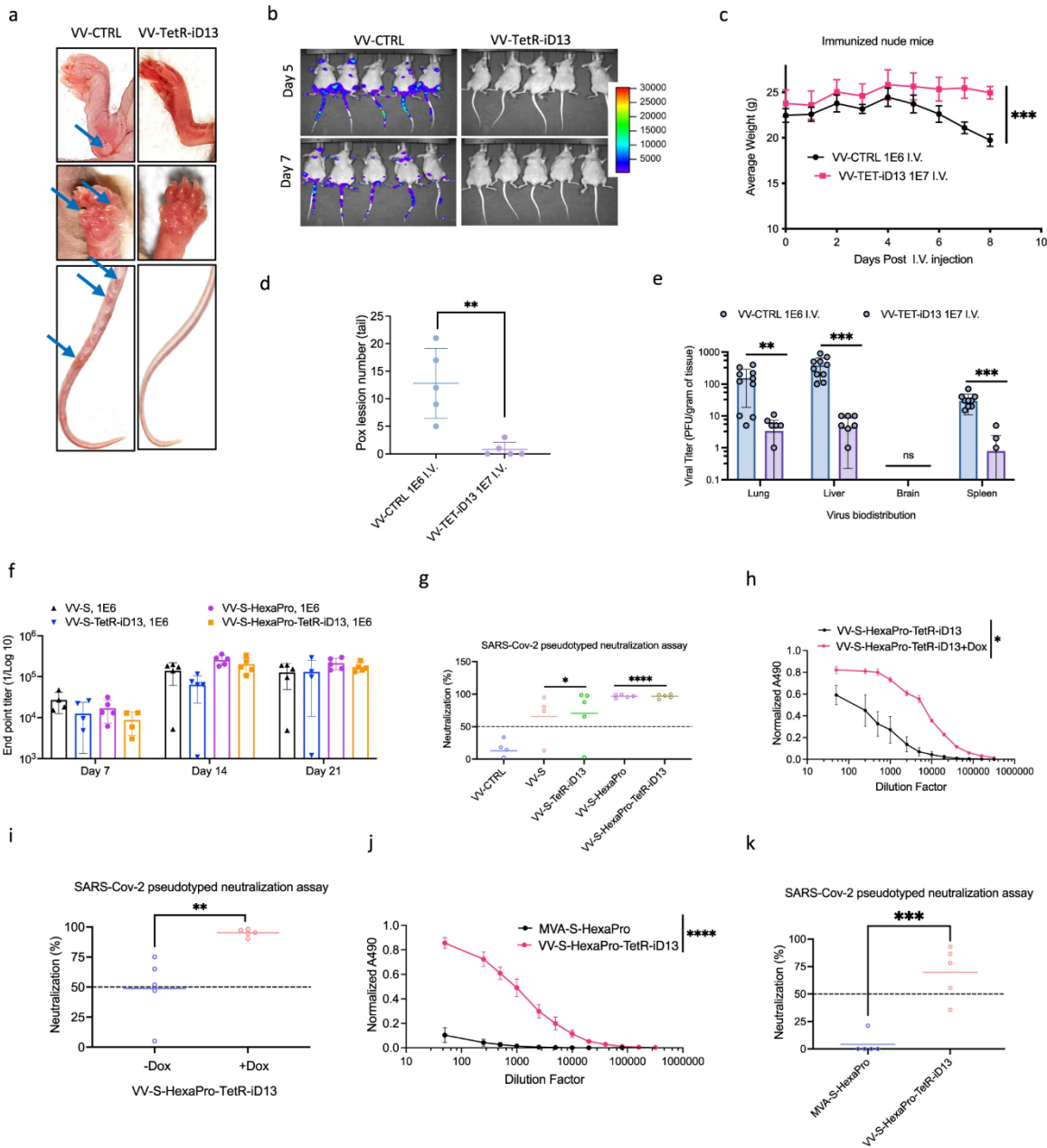


Figure 2.6. Conditionally replicating vaccinia virus as a safe alternative for vaccine development. (A-E) Evaluation of the safety of the conditionally replicating virus. Mice were injected intravenously with either control vaccinia virus at 1E6 pfu or VV-TetR-iD13 at 1E7 pfu. Formation of pox lesions on mice feet and tails was monitored (A) and quantified (D) after 5 days post i.v. injection. IVIS imaging was performed on Days 5 and 7 post virus injection (B) and body weight were measured at consistent intervals (C). Organs were harvested at Day 10 and tissues processed for quantifying viral titer (E). (F, G) Mice were intraperitoneally injected with 1E6 pfu of control and vaccine viruses with serum collected at Days 7, 14, and 21. Subsequently, a RBD ELISA assay was performed and endpoint titer for all the samples and days were quantified (F). A SARS-CoV-2 pseudotyped neutralization assay was performed on Day 14 samples (G). (H, I) Mice were intraperitoneally injected with 1E6 pfu of the VV-S-Hexapro-TetR-iD13 in the presence or absence of 625 mg/kg Dox in the diet for two days. Serum was collected at Day 14 and was used for a RBD ELISA to quantify the normalized ELISA absorbance versus dilution factor in different conditions (H). Serum was also used for a SARS-CoV-2 pseudotyped neutralization assay. (J, K) Mice were intraperitoneally injected with 1E6 pfu of the VV-S-HexaPro-TetR-iD13 or MVA-S-HexaPro. Serum was collected at Day 14 and was used for a RBD ELISA to quantify the normalized ELISA absorbance versus dilution factor in different conditions (J). Serum was also used for a SARS-CoV-2 pseudotyped neutralization assay (K). S and S-HexaPro represent SARS-CoV-2 wildtype spike protein and proline stabilized version of the SARS-CoV-2 spike respectively. Data indicate means $\pm$ SD of five (6c-d, 6f-j) or ten (6e) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

We also evaluated the immunogenicity of VV-TetR-iD13 delivering a clinically relevant immunogen. The SARS-CoV-2 spike glycoprotein (VV-S-TetR-iD13) or a prefusion-stabilized spike variant (VV-S-HexaPro-TetR-iD13) was encoded into VV-TetR-iD13 to generate novel SARS-CoV-2 vaccine candidates. We confirmed that these viruses maintained Dox-inducible replication (**Supplementary Fig. 2.5a-b**) and expressed their respective antigens (**Supplementary Fig. 2.5c**). Subsequently, we analyzed the humoral responses induced in mice infected with these vaccine candidates in the presence of 48 h Dox treatment. A single dose of VV-S-TetR-iD13 or VV-S-HexaPro-TetR-iD13 was sufficient to induce robust Spike-targeted IgG response (**Fig. 2.6f**). However, an examination of neutralizing antibody titer levels via SARS-CoV-2 S pseudovirus neutralization assay suggested that HexaPro more consistently elicited a stronger response (**Fig. 2.6g**). In the absence of Dox treatment, there was a detectable, but significantly impaired antibody response elicited by VV-S-HexaPro-TetR-D13 (**Fig. 2.6h-i**). Further, we confirmed that VV-S-HexaPro-TetR-D13 induced higher antibody responses relative to a non-replicating vaccinia virus vector (MVA) delivering the same antigen (**Fig. 2.6j-k**). Taken together, these results demonstrate that chemogenetic switches can be applied to increase the safety of replicating vaccine VV vectors while maintaining their ability to induce strong immunogen specific antibody responses.

Chemogenetic control of virus directed membrane fusion.

There is accumulating evidence that encoding membrane fusion proteins into an oncolytic virus backbone can improve therapeutic activity by enabling neutralizing antibody-resistant viral spread, immunogenic cell death, and minimizing the release of virus into healthy tissue or systemic circulation¹³⁴. However, expression of heterologous fusogenic

proteins can also be counter-productive to OV replication, impacting virus manufacturing and in some circumstances causing off-target toxicity^{92,135–137}. We hypothesized that integration of a chemogenetic switch to mediate temporal control of fusogenic protein expression would overcome some of these limitations. To identify an optimal fusion protein, we performed an overexpression screen in HEK293 cells of arenavirus and reovirus fusion proteins. To this end, HEK293 cells expressing GFP were transfected with a mammalian expression plasmid encoding a range of fusion proteins and then co-cultured with HEK293 cells stably expressing mCherry. Twenty-four hours post-transfection cells were analyzed for co-localization of mCherry and GFP (indicative of fusion between heterotypic cells). (**Fig. 2.7a; Supplementary Fig. 2.6a**). The screen revealed that two fusion proteins produced significant amounts of syncytia – the Tamiami virus glycoprotein (TAMV-GP) and reovirus p14 in a variety of cell lines (U2OS, Vero, A549 and HeLa, **Supplementary Fig. 2.6b**). We performed an Alamar Blue assay to analyze the effects of the GPs on cell viability (**Fig. 2.7b**). In a panel of cell lines (HEK293, U2OS, Vero, A549, and HeLa), overexpression of TAMV-GP or p14 resulted in a greater than 70% reduction of cell viability, whereas overexpression of p15, a reovirus fusion protein produced low levels of syncytia and resulted in minimal cell death (**Fig. 2.7b**). Previous work has demonstrated that expression of p14-induced cell fusion results in increase caspase 3 activation⁹². Consistent with this, we observed increased caspase activity in cells overexpressing p14 or TAMV-GP (**Supplementary Fig. 2.6c**). As both TAMV-GP and p14 showed robust induction of fusion and cell death, we generated recombinant VV strains encoding each fusion protein under the control of our Dox-inducible expression system (VV-TetR-iP14 and VV-TetR-iTAMV-GP; **Supplementary Fig. 2.6d**). Vaccinia virus has a complex replication cycle with multiple viral isoforms that contribute to its ability to disperse throughout an infected individual. To examine the contribution of membrane

fusion to local virus spread within the TME, we analyzed virus-induced syncytia formation in the presence of ST-246, an established inhibitor of extracellular isoforms of orthopoxvirus¹³⁸ (**Fig. 2.7c**). While both viruses induced robust cell fusion in all tested cell lines in the presence of Dox, the addition of ST-246 significantly impaired the ability of VV-TetR-iP14 to induce syncytium. On the other hand, VV-TetR-iTAMV-GP's ability to induce syncytia was not impacted by ST-246 treatment – suggesting VV-TetR-iTAMV-GP, in the presence of Dox treatment, has an enhanced ability to spread via cell fusion relative to VV-TetR-p14. We analyzed the impact of TAMV-GP expression on the growth of the recombinant virus. Multistep viral growth curve analysis in A549 and HeLa cells showed that VV-TetR-iTAMV-GP infection with simultaneous Dox treatment results in decreased viral growth (**Fig. 2.7d-e**); however, delaying expression of TAMV-GP by treating with dox 48 hours post-infection resulted in similar viral growth kinetics to untreated VV-TetR-iTAMV-GP infected cells. This suggests that while constitutive expression of the fusion protein impairs viral growth, the use of a chemogenetic switch will enable temporal control of TAMV-GP expression allowing productive virus infection of the tumor before initiating membrane fusion and its downstream therapeutic sequelae. To test this hypothesis, we evaluated the anti-tumor effects of VV-TetR-iTAMV-GP *in vivo* using the A549 xenograft model infected in the presence or absence of Dox (**Fig. 2.7f-i**). Without a Dox-containing diet, VV-TetR-iTAMV-GP had decreased anti-tumor activity which was significantly enhanced in mice fed a Dox-containing diet 48 hours-post infection. Tumor volumes and weight were significantly reduced in mice co-treated with VV-TetR-iTAMV-GP and Dox relative to the virus alone. Immunohistochemistry analysis of tumors revealed Dox treatment-specific induction of TAMV-GP and caspase activation in virus infected cells, consistent with our *in vitro* results (**Fig. 2.7i, Supplementary Fig. 2.6e**). We repeated the study utilizing intravenous delivery, and our results demonstrated

that systemic delivery of VV-TetR-iTAMV-GP in the presence of a Dox-containing diet was superior to virus alone (**Supplementary Fig. 2.6f-h**). Collectively, our results demonstrate that the application of chemogenetic switches in VV enables the chemically regulated delivery of virus-inhibitory payloads strategically delaying transgene expression while optimizing therapeutic effect.

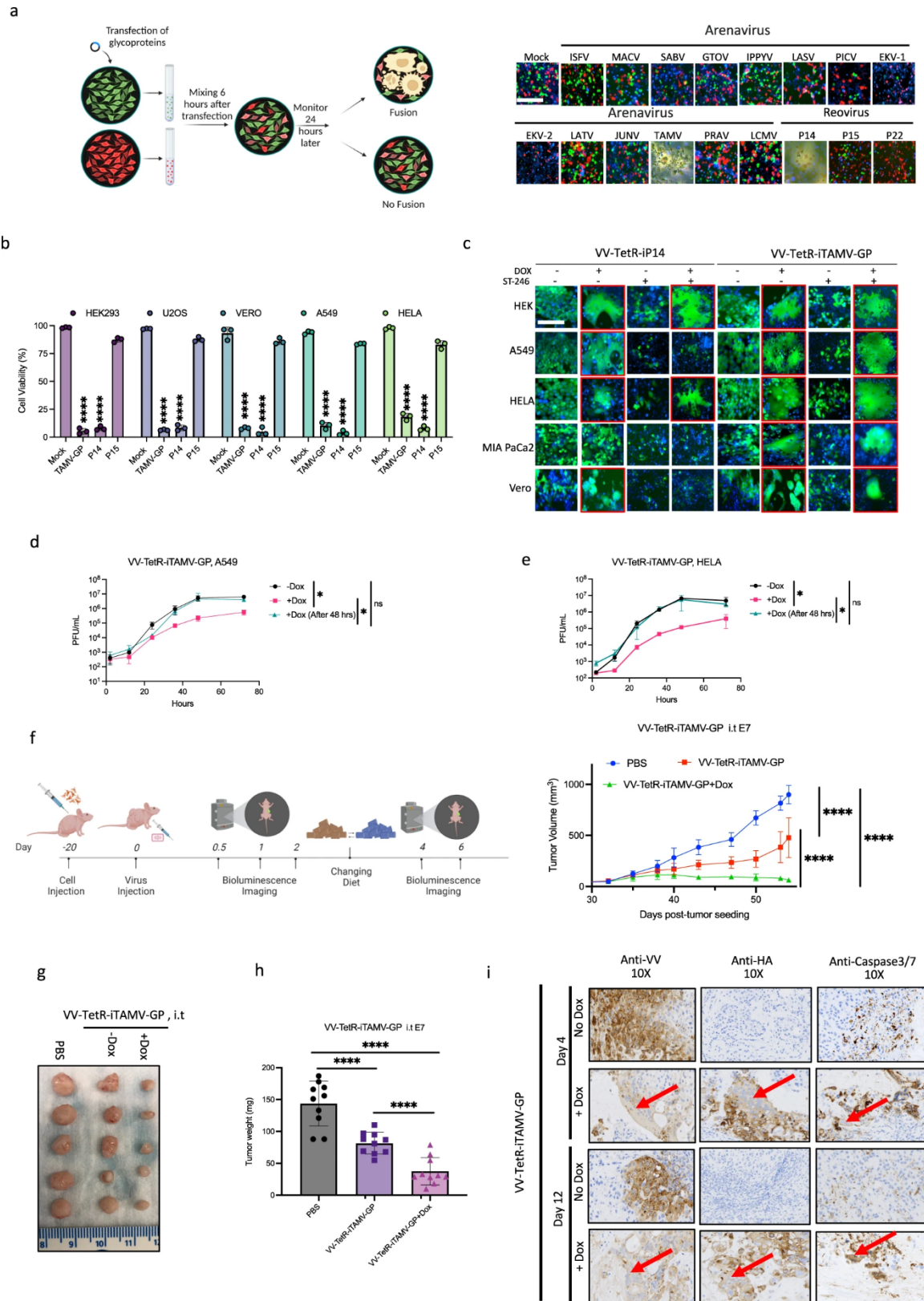


Figure 2.7. Doxycycline inducible system as an effective strategy to regulate expression of a toxic fusogenic proteins. (A) Schematic illustration and representative images of GFP expressing cells transfected with Arenavirus glycoproteins including Isfahan virus (ISFV), Machupo virus (MACV), Sabiá virus (SABV), Guanarito virus (GTOV), Ippy virus (IPPYV), Lassa virus (LASV), Pichinde virus (PICV), Ekpoma virus-1 (EKV-1), Ekpoma virus-2 (EKV-2), Latino virus (LATV), Junín virus (JUNV), Tamiami virus (TAMV), Paraná virus (PRAV), lymphocytic choriomeningitis virus (LCMV) and Reovirus P14, P15, P22 FAST proteins. 6 hours post transfection of GFP expressing cells, cells were mixed with a separate population of mCherry expressing cells. After 24 hours, cells were monitored for signs of cell-cell fusion. **(B)** HEK293, U2OS, Vero, A549, and Hela cells were transfected with TAMV-GP (Tamiami virus glycoprotein), P14, and P15 FAST proteins. 24 hours after transfection, an Alamar blue viability assay was performed. **(C)** HEK293, A549, Hela, MiaPaCa2, and Vero cells were infected with either VV-TetR-iP14 or VV-TetR-iTAMV-GP (MOI 0.1) in the presence or absence of 100 ng/ml Dox or ST-246, an anti-poxvirus drug. Representative images were taken 24 hours post infection. **(D, E)** Multistep growth curves of Hela and U2O2 cells infected with VV-TetR-iTAMV-GP (MOI 0.01), in the presence or absence of 100 ng/ml Dox added at the same time, or 48 hours after virus infection. **(F-H)** A549 tumors were injected with either PBS or VV-TetR-iTAMV-GP (1E7 PFU/tumor) at a size of ~150 mm³. Dox was given in the diet of mice (625 mg/kg) as indicated two days after virus injection. Tumors were measured at regular intervals **(F)**. The image shown compares tumor size across all treatment groups and mice in the study **(G)**. Tumor weight was also measured **(H)**. **(I)** A549 tumors were injected with VV-TetR-iTAMV-GP (1E7 PFU/tumor) at a size of ~150 mm³ in size and depending on the treatment group received 625 mg/kg of Dox in their diet two days after virus injection. Tumors were harvested at Day 4 and Day 12 post virus injection and processed for immunohistochemistry staining using vaccinia virus, HA and caspase 3/7 antibodies. Scale bars = 40 µm in panel A, C. Data indicate means ± SD of three (7b, 7d-e) or ten (7f, 7h) biological replicates. ns P > 0.05, *P < 0.05 **P < 0.003841, ***P < 0.000125, ****P < 0.001 in unpaired two-samples t-test. Source data are provided as a Source Data file.

Orchestrating transgene expression for safety and efficacy.

Several studies have attempted to deliver cytokines using viral vectors^{139,140}, however, for a subset of potent, pro-inflammatory cytokines, the safety and efficacy of cytokine therapy is heavily influenced by the timing and intensity of dosage. Our chemogenetic switches provide an opportunity to adjust both *timing* and *magnitude* of payload expression. For these experiments we used the cumate-based operator as we had established the dynamic range of the VV-adapted cumate-regulated expression system *in vivo* (**Fig. 2.3c-j; Supplementary Fig. 2.3h**). Numerous lines of evidence indicate that IL-12, IL-2 and IL-18 give complementary immunoregulatory signals^{141–144}. It has been demonstrated that these three cytokines exert synergistic anti-tumor action in preclinical tumor models by activating the innate immune response, increasing the cytotoxicity of natural killer cells, natural killer T cells and granulocytes, and potentially shutting down the tumor vasculature and reversing the immune suppression mediated by dysfunctional myeloid cells in the tumor^{141,145–147}. We generated several different PEL-CuO driven gene cassettes for combinatorial expression of the three cytokines, including a multi-cistronic construct with internal ribosome entry sites (VV-CymR-iIL2IRES-2-DIRES-18; **Supplementary Fig. 2.7a**), 2A self-cleaving peptide-based multi-gene expression system (VV-CymR-iIL12-P2A-2-T2A-18; **Supplementary Fig. 2.7b**), as well as independent PEL-CuO promoters driving each cytokine (**Fig. 2.8a**; VV-CymR-iIL-12-2-18). Of the three tested systems, ELISA analyses revealed that three separate promoter system resulted in the most robust, cumate-inducible expression of all three cytokines (**Fig. 2.8b, Supplementary Fig. 7c**). All three cytokines are known to activate IFN- γ and TNF- α expression in immune cells and so we cultured mouse splenocytes with the virus-filtered supernatants of VV-CymR-iIL2-2-18 infected U2OS cells and examined the levels of both cytokines in splenocyte supernatants. In the presence of cumate, VV-CymR-iIL12-2-18 infected cells' supernatant

produced robust increases in IFN- γ and TNF- α production (**Fig. 2.8c-d**). The functional role of each cytokine was examined through the use of neutralizing antibodies against IL-2, IL-12 or IL-18. Blocking of IL-2 or IL-12 in the supernatants impaired activation of IFN- γ and TNF- α production while blocking of IL-18 only impaired IFN- γ production from splenocytes. These *in vitro* results demonstrated that VV-CymR-iIL12-2-18 encodes a cumate-regulated multi-expression system for IL-2, IL-12, and IL-18.

We examined whether the chemical regulation of cytokine expression by VV-CymR-iIL12-2-18 circumvents the toxicity associated with systemic administration of these cytokines. For our toxicity studies, we utilized CD-1 nude mice to mimic an immunocompromised cancer patient, who may be more susceptible to cytokine-induced immunopathology due to persistence of the VV vector after administration. Whereas infection with VV-CTRL or VV-CymR-iIL12-2-18 in the absence of cumate resulted in no mouse mortality after 5 days, infection with VV constitutively expressing the three cytokines (VV-IL12-2-18) resulted 100% mortality (**Fig. 2.8e**). This is consistent with constitutive high levels of expression of all three cytokines being toxic. 80% mortality was observed in the mice infected with VV-CymR-iIL12-2-18 and fed a 6000 mg/kg cumate diet to induce comparable levels of cytokine production in mouse sera (**Fig. 2.8f**). Similar to VV-IL12-2-18, the 6000 mg/kg cumate combined with VV-CymR-iIL12-2-18 infection induced lung edema (**Fig. 2.8g**), and liver toxicity as evidenced by increases in serum alkaline phosphatase activity levels (**Fig. 2.8h**). In order to determine whether cumate dosage could be used to limit toxicity of VV-CymR-iIL12-2-18, we fed infected mice with a range of cumate doses (1000 mg/kg, 2000 mg/kg and 6000 mg/kg). At 1000 mg/kg cumate, we observed no mortality after 5 days in VV-CymR-iIL12-2-18 infected CD-1 nude mice (**Fig. 2.8i**). The 1000 mg/kg cumate diet induced a lower level of cytokine production in infected mice relative to the other tested doses and caused no significant

differences in lung edema (**Fig. 2.8k**), or elevated liver markers (serum alkaline phosphatase or aspartate aminotransferase activity (**Fig. 2.8l-m**). These results demonstrate that 1000 mg/kg is safe dosage of cumate in VV-CymR-iIL12-2-18 infected mice. Finally, we examined the anti-tumor effects of the combination of VV-CymR-iIL12-2-18 and 1000 mg/kg cumate in immunocompetent mice with peritoneal murine cancer (MC38) (**Fig. 2.8n**). As expected, mice treated with VV constitutively expressing the three cytokines while providing some limited benefit over VV lacking the transgenes, ultimately succumbed likely due to toxicity of the three cytokines. In contrast, with controlled cytokine expression using the VV-CymR-iIL12-2-18 virus and 1000 mg/kg cumate we observed increased mouse survival from the tumor challenge to 40%. These results demonstrate that the chemogenetic switch enables dosage control to mediate delivery of potentially toxic payloads at safe and efficacious levels.

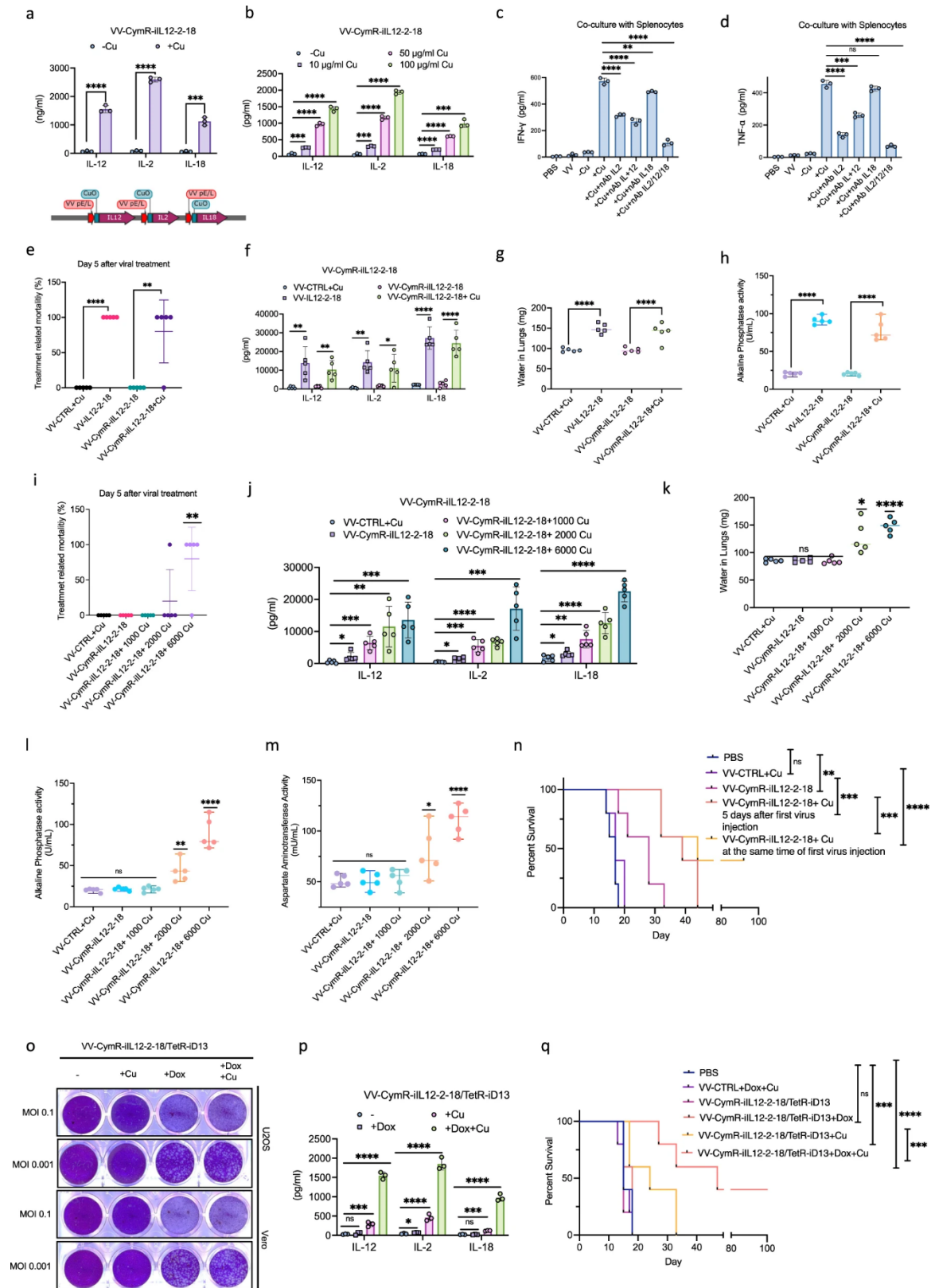


Figure 2.8. Applications of the cumate “safety switch” in regulating expression of potentially toxic cytokines (A) IL-2, IL-12 and IL-18 concentrations measured by ELISA from supernatants of U2OS cells 6 hours after infection with VV-CymR-iIL12-2-18 (MOI 1) and treated with 100 ug/ml cumate or PBS. (B) IL-2, IL-12 and IL-18 concentrations measured by ELISA from supernatants of U2OS cells 6 hours after infection with VV-CymR-iIL12-2-18 (MOI 1) and varying concentrations of cumate. (C, D) IFN-gamma and TNF-alpha levels measured by ELISA. Supernatants were taken from mouse splenocytes cultured ex vivo with conditioned media from U2OS cells treated with 100 ug/ml cumate or PBS following infection with VV-CymR-iIL12-2-18 (MOI 1). Neutralizing antibodies against IL-2, IL-12 and IL-18 were added to conditioned media for 1 hour prior to transferring them to splenocytes. (E-H) Treatment-related mortality and toxicity were assessed 5 days after intratumorally injection of HT-29 tumors with control vaccinia virus, VV-IL12-2-18 or VV-CymR-iIL12-2-18 (1E7 PFU/tumor). Tumors were ~150 mm³ in size at the time of injection. Mice treated with VV-CymR-iIL12-2-18 were given a cumate (6000 mg/kg) or regular diet. Water in the lungs (an indication of pulmonary edema) (G) and alkaline phosphatase activity (H) in serum were measured as indicators of toxicity. (I-M) Treatment-related mortality and toxicity were assessed 5 days after intratumoral injection of HT-29 tumors with control vaccinia virus, VV-IL12-2-18 or VV-CymR-iIL12-2-18 (1E7 PFU/tumor). Tumors were ~150 mm³ in size at the time of injection. Mice treated with VV-CymR-iIL12-2-18 were given a regular diet, or a diet with varying amounts of cumate (1000, 2000 or 6000mg/kg; panel J). Water in the lungs (K), and serum levels of alkaline phosphatase (L) and aspartate aminotransferase activity (M) were assessed. (N) Survival analysis of C57BL/6 mice injected intraperitoneally with 5E5 MC38 cells and treated with either PBS, control vaccinia virus, or VV-CymR-iIL12-2-18 at 3E7 pfu/mouse. Treatments were given at days 6, 8, and 10 post cell injection. A cumate diet (1000 mg/kg) was given to the VV-CymR-iIL12-2-18 treatment groups the same day, or 5 days after virus injection as indicated. (O) Crystal violet staining of U2OS and Vero cells 48 hours after infection with VV-CymR-iIL12-2-18/TetR-iD13 at different MOIs in the presence of 100 ug/ml cumate (+Cu), 100 ng/ml doxycycline (+Dox), or both (+Dox +Cu). (P) IL-12, IL-2, and IL18 concentrations measured by ELISA in supernatants of U2OS cells 6 hours following infection with VV-CymR-iIL12-2-18/TetR-iD13 (MOI 1) in the presence or absence of 100 ng/ml Dox, 100 ug/ml of cumate or both. (Q) Survival analysis of C57BL/6 mice injected intraperitoneally with 5E5 MC38-WT cells and treated with either PBS, control vaccinia virus, or VV-CymR-iIL12-2-18/TetR-iD13 at 3E7 pfu/mouse. viruses were injected at days 6, 8, and 10 post cell injection, with mice receiving either Dox (625 mg/kg), cumate (1000 mg/kg) diets, or both for 5 days. Data indicate means $\pm$ SD of three (8a-d, 8p) or five (8e-n, 8q) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test. Source data are provided as a Source Data file.

Finally, we demonstrated that both virus replication and payload delivery can be placed under separate controls to increase safety of the VV vector (VV-CymR-iL12-2-18/TetR-iD13). This vector replicates conditionally in the presence of Dox (**Fig. 2.8o**) and expresses the three cytokines in the presence of cumate (**Fig. 2.8p**). It should be noted that both cumate and dox treatment were required for VV-CymR-iL12-2-18/TetR-iD13 infected cells to produce the same levels of cytokine as VV-CymR-iL12-2-18 in the presence of cumate. This demonstrates that Dox-conditional replication adds another level control on payload delivery in addition to dox-inducible replication. Lastly, we examined the anti-tumor effects of VV-CymR-iL12-2-18/TetR-iD13 in the murine model with peritoneal MC38. In line with the results obtained with 1000 mg/kg cumate and VV-CymR-iL12-2-8 (**Fig. 2.8n**), VV-CymR-iL12-2-18/TetR-iD13 infection with induced replication (Dox treatment) and payload delivery (cumate treatment) resulted in 40% mouse survival (**Fig. 2.8q**). Taken together, our results demonstrate the application of two chemogenetic switches in VV enables the independent chemical regulation of both replication and payload expression.

Discussion

Challenges to the development of replication competent viral vectors as therapeutics include safety concerns related to uncontrolled viral dissemination in cancer patients and healthcare workers as well as off-target delivery of potentially toxic payloads. Here we develop three VV-adapted chemogenetic switches: the rapamycin-inducible ST7 RNA polymerase expression system (FRB/FKBP; **Fig. 2.1**), Dox-inducible expression (TetR/TetO; **Fig. 2.2**), and cumate-inducible expression (CymR/CuO; **Fig. 2.3**) that can be used alone or in combination to control VV replication and/or transgene expression. This

study describes, to the best of our knowledge, the first application of the cumate- or rapamycin inducible expression systems in a replicating viral vector. These systems will facilitate the development of a range of vectors that can potentially deliver potent transgenes in a safe and controlled fashion to enhance therapeutic outcomes. The use of combinatorial chemogenetic switches is particularly appealing as they provide the clinician with opportunities to tune both the level of virus replication and therapeutic transgene expression. One of the challenges in the development of oncolytic virus vectors is finding the balance between safe selective replication and potency through expression of viral virulence gene products. A strategy that has shown promise is the development of vectors that control certain viral virulence genes using tumor selective promoter elements. For instance oncolytic herpes viruses have been developed for selective replication in glioma cells using the nestin promoter to control the virulence gene ICP 34.5⁵⁷. Our system has the advantage of providing control over the virus replication and gene expression in any tumor type as well as being able to both turn on and off virus gene expression. The cumate controlled system demonstrated good dynamic range. However, it is not currently an FDA approved compound thus limiting its rapid application into the clinic. On the other hand, both doxycycline and the rapalogs used in this study are FDA approved drugs and thus their corresponding chemogenetic switches could have a more straight forward regulatory approval path. Ultimately, the expansion of the available toolkits to regulate payload delivery will enable the incorporation of more complex synthetic circuits within viral vectors. Towards this goal, we combined different conditional expression systems (ST7-CymR, ST7-TetR, and Cu-Tet) to create expression systems with two levels of control and decreased basal level of expression. These systems will prove critical to the delivery of potent toxic payloads that can be locally therapeutically effective but have significant toxicity when delivered systemically. Lastly, while our studies have been focused on

engineering tunable expression systems in VV, these conditional expression systems can be rapidly adapted to other viral vectors, as we demonstrate for HSV (**Fig. 2.4k-m**). We anticipate that each viral vector, with its own unique replication kinetics and replication cycle will have different selectivity and robustness of inducible transgene expression; therefore, expanding the available systems will enable the selection of an expression system tailored to the viral vector or application. Overall, the application of viral expression switches described herein should enable the development of conditionally replicating viral vectors or inducible transgene expression system. This technology will enable testing of highly potent transgenes in oncolytic virus vectors and expand their utility in the clinic.

Methods

The study discussed in this document adheres to all pertinent ethical guidelines at OHRI and the University of Ottawa (certificate for biohazardous material utilization GC317-125-12). All samples from patients were acquired via the Global Tissue Consenting committee at OHRI. The University of Ottawa's institutional animal care committee approved all animal experiments (Protocol ID: OHRI2870 and MEe-2258) which were conducted following the National Institutes of Health and the Canadian Council on Animal Care standards.

Viruses- MVA (VR-1508) and VV Copenhagen strain were purchased from the ATCC. The Vaccinia TT strain was a gift from Dr. David Evans. HSV strain KOS was used.

Cell culture- All cell lines were purchased from the American Type Culture Collections (Manassas, VA). The cells were grown in Dulbecco's Modified Eagle Medium/DMEM (GE Healthcare Life Sciences; ON, CAN) or Roswell Park Memorial Institute/RPMI 1640 Medium (Gibco; MA, USA), supplemented with 10% fetal bovine serum/FBS (Gibco). Cells were kept at 37°C in a humidified environment with 5% CO₂. Cells co-cultured with effector cells were cultivated in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin (volume/volume) (Gibco). Co-cultures were kept at 37°C in a humidified environment with 5% CO₂. Cells were regularly checked for mycoplasma contamination using PCR (e-Myo VALiD Detection Kit, 25239, LiliF Diagnostics; South Korea) and were shown to be mycoplasma-free.

Construct Design- The promoter constructs were designed using Snapgene software. codon-optimized inserts were ordered from GenScript (Piscataway, NJ, USA).

Bioluminescence Imaging-After lysis of virus infected cells with passive lysis buffer, the lysate was moved to 96 well plates and 1:1 ratio of the luciferase reagent (Promega) was added. A Synergy microplate reader (BioTek, Winooski, VT, USA) was used to measure luminescence. Results are presented as RLU normalized to control. The data presented are the mean of three independent experiments.

In vitro co-culture cell viability assays- The resazurin assay was used to determine cell cytotoxicity caused by viruses or transgenes. The metabolic activity of the cells was measured using the manufacturer's protocol and resazurin sodium salt (R12204;

ThermoFisher Scientific). In each well, treated and/or infected cells were given 10% (v/v, final) resazurin and incubated for 2-4 hours, depending on the cell line. Using a BioTek Microplate Reader, fluorescence was measured at 590 nm after excitation at 530 nm (BioTek, Winooski, VT, USA). In a nutshell, freshly isolated splenocytes were co-cultured with target viruses in a 24-well plate or a flat-bottom 96-well plate with just media. In short, 24 hours before, 200,000 (24-well plate) or 15,000 (96-well plate) target cells were seeded. Virus infections were performed on cells in serum-free media at the indicated MOIs. After 6 hours, the media was removed and splenocytes in RPMI supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin were added.

Reagents- Cumate solution (QM150A-1) was purchased from System Biosciences, doxycycline (D9891-25G) was purchased from Sigma-Aldrich, and Rapamycin analogs were purchased from Selleck Chemicals.

ELISA- Nunc Maxisorp 96-well flat-bottom plates were coated with 125 ng of RBD per well overnight at 4°C (prepared in-house [see supplemental materials and methods]). The RBD solution was removed the next day, and the plates were washed three times with PBS-Tween (0.1% Tween 20) before being blocked for one hour with a 3% skim milk solution. Vaccinated mouse sera were then serially diluted in 1% skim milk and added to the plates to incubate for 2 hours at room temperature. In addition, a positive and negative control consisting of a monoclonal RBD antibody (1 g/mL; Cat No: MBS434247, Anti-RBD Domain [SARS-CoV-2 spike], monoclonal antibody, MyBioSource, CA, USA) and a pool of sera taken from mice prior to vaccination were added to each plate. Following the 2-hour incubation, plates were washed with PBS-Tween and incubated for 1 hour at room temperature with anti-mouse immunoglobulin (Ig)G conjugated to horseradish peroxidase

(HRP) (1:3000; Cat No: 314930, Goat anti-Mouse IgG [H + L] Secondary Antibody, HRP, Invitrogen). After that, plates were developed with SigmaFast OPD solution and measured at 490 nm with a BioTek microplate reader.

All experimental absorbance measurements were normalised to the blank and the positive control (monoclonal RBD antibody at 1 g/mL) and fitted with a quadratic binding polynomial assuming 1:1 binding. A Monte Carlo simulation with the nonlinear curve-fitting tool in QtGrace was used to perform the fitting. The reciprocal antibody titer (LDF) was calculated by interpolating the dilution factor that intersected with a minimum detection threshold defined by 10x the standard deviation of responses from TT WT-vaccinated mice, or to a fixed value of 0.025 (whichever was larger).

Mouse experiments- Six- to 8-week-old female C57BL/6 or CD-1 nude mice (The Jackson Laboratory, Bar Harbor, ME) were obtained for studies. All experiments were approved by the University of Ottawa animal care and veterinary services (MEe-2258-R5, or OHRIe-3340-A), including periodic saphenous vein bleeds for serum collection.

Pseudovirus neutralization assay- At the time of infection, Vero E6 cells were seeded in 96-well plates with 40,000 cells per well. Serum was first diluted in serum-free DMEM at a 1:10 dilution in a separate 96-well plate, followed by a serial 1 in 2 dilution series. VSV pseudotyped with the SARS-CoV-2 spike glycoprotein and co-encoded with eGFP was then added to serum in an equal volume of serum-free DMEM for a final dilution of 2000 pfu per well and incubated at 37°C for 1 hour. After 1 hour, the media on the cell was replaced with 60 L of the virus/serum and incubated at 37°C for 1 hour. The wells were

then filled with carboxymethylcellulose (CMC) in DMEM (supplemented with 10% fetal bovine serum) to a final concentration of 3% CMC and incubated at 34°C for 24 hours. Using a Cellomics ArrayScan VTI HCS Reader, GFP foci were imaged and counted.

Western Blot- Whole cell lysates were prepared in Cell Lysis Buffer II (Invitrogen™) with protease/phosphatase inhibitors (NEB). The BCA assay was used to determine protein concentrations (Pierce). Samples were loaded into precast SDS-PAGE gels after being mixed with NuPage LDS sample buffer (Invitrogen) (Bio-Rad). Proteins were transferred onto nitrocellulose membrane after SDS-PAGE, blocked for 1 hour with 5% skim milk in TBS-Tween, and incubated with primary antibody. The following antibodies SARS-CoV-2 Spike (GeneTex, 1A9), HA (Thermo Fisher Scientific, 26183), VV (Abcam, ab117453), and GADPH (Cell Signaling, 2118L) antibodies, which were used at a dilution of 1:1000. Cell Signaling supplied HRP conjugated anti-mouse (7076S) and anti-rabbit (7074S) IgG secondary antibodies, which were used at a dilution of 1:2000. Westerns were created using Bio-Rad Clarity or Clarity Max ECL substrates and imaged with a Bio-Rad GelDoc imaging system. Uncropped western blots are shown in supplementary Fig. 9.

Virus Production and Titration- HeLa cells in 850 cm² roller bottles were infected with virus at a MOI of 0.03 without removing inoculation media to produce vaccinia virus. Cells were cultured at 37°C in 5% CO₂ for 72 hours, or until a sufficient cytopathic effect was observed. Cells were pelleted and resuspended in 1 mM Tris at pH 9.0. Cells were frozen/thawed three times before being centrifuged at 2000 rpm for 10 minutes at room temperature. Before centrifugation at 11500 rpm for 1 hour and 30 minutes at 4°C, supernatant was collected and overlaid onto 36% sucrose cushions. Reconstituted viral

pellets in 1 ml of 1 mM Tris and stored at -80°C. Titration was used to determine viral titers. Virus stocks were serially diluted tenfold before infecting U2OS cells in 12-well plates. After 2 hours at 37°C in 5% CO₂, the media was replaced with overlay medium (1:1 of 3% CMC and 2x DMEM + FBS) and incubated for 48 hours at 37°C in 5% CO₂. Plaques were stained with crystal violet after 48 hours and plaque forming units per ml were calculated. Vero cells were used for HSV-1 virus production and titration under the same conditions as described above. Schematic illustration of VV-CTRL, VV-GFP^{Luc} is shown in **supplementary Fig. 2.8**.

Statistical analysis- GraphPad Prism v9 was used to create all graphs and statistical analyses. When appropriate, unpaired two-samples t-test was used. In figure legends, the n value represents all biological replicates. As indicated in the figure legends, error bars represent standard deviation (SD) or standard error of the mean (SEM). For all statistical tests, ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$.

Chapter 3 - Tunable IL12 Enhances Safety and Efficacy of Conditionally Replicating Vaccinia Virus

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Abstract

Oncolytic viruses (OVs), such as Vaccinia Virus (VV), hold promise for cancer immunotherapy, but their clinical efficacy is hindered by safety concerns, limitations in replication, and overcoming antiviral immune responses. This study introduces a novel approach to address these challenges by developing a chemically-inducible platform for precise control of VV replication and cytokine expression. Unlike traditional gene deletion strategies, this system allows for switchable virulence, offering greater safety without compromising therapeutic efficacy. We demonstrate the importance of potent viral replication in driving superior antitumor immune responses in multiple syngeneic models. Furthermore, we integrate controlled expression of the potent immune modulator IL-12, linked to viral replication, and investigate its impact on therapeutic outcomes and the antiviral response. Our findings reveal that high IL-12 levels enhance viral clearance, particularly in immunocompromised models. Surprisingly, in the B16-F10 model, high IL-12 expression combined with viral replication leads to increased efficacy, despite decreased tumor immune infiltration, suggesting alternative IL-12-mediated antitumor mechanisms. Overall, this study introduces a versatile oncolytic platform that offers unprecedented control for personalized cancer therapy, enhancing safety while improving therapeutic efficacy.

Introduction

Oncolytic viruses (OVs) are a new frontier in cancer immunotherapy that exhibit a dual mechanism of action: direct oncolytic activity that kills cancer cells and the enhancement of immune responses, transforming 'cold' tumors into 'hot', more susceptible to immunotherapy^{22,148,149}. OVs have demonstrated excellent safety profiles in clinical trials. However, critical to this safety, is the attenuation of the virus, typically achieved through methods such as gene deletion—targeting genes detrimental to viral growth in healthy cells but not essential for propagation in cancer cells^{37,51}. Other strategies include using tissue or tumor-specific promoters to control these genes¹⁵⁰, employing tissue-specific microRNAs to inhibit off-target viral activity¹⁵¹, and various other methods to ensure specificity and minimize virus related toxicities⁸⁹.

The vaccinia virus (VV) stands out as a promising OV⁴². Its large genome offers ample room for adding therapeutic genes, and it replicates outside the cell nucleus, reducing the chance of affecting the host's DNA³¹. VV is also not linked to human diseases and has a long history of use in smallpox vaccination, highlighting its excellent safety profile³³. However, to further ensure safety even in patients with weakened immune systems, strategies like removing the Thymidine kinase gene often are used in VV based platforms in clinical development^{37,152}.

Observations of tumor eradication following natural viral infections have raised questions about the balance between a virus's oncolytic potency and immune activation. Clinical trials have shown that patients with a more pronounced immune response against the virus often exhibit better treatment outcomes⁶³, possibly due to the bystander effect of infecting healthy cells and thus activating a stronger immune response. This underscores the need for alternative safety measures that do not compromise the virus's oncolytic activity. Therefore, it's crucial to create technologies that allow for the controlled

weakening of the virus if needed due to toxicity. However, reducing the virus's strength prematurely, even in the absence of notable toxic effects, could lead to its rapid elimination from the body before it can effectively lyse cancer cells and activate the immune system¹⁴⁸.

Cytokines, particularly IL2 and IL12, have gained a lot of attention in cancer immunotherapy due to their potent preclinical data^{153–155}. However, their clinical applications have been limited by systemic toxicities and lack of optimal delivery methods¹⁰¹. The integration of these cytokines into OV's like vaccinia has shown promise, potentially enabling a single agent to effectively kill cancer cells and enhance immune activity^{98,156,157}. However, an often-overlooked aspect of this strategy is the enhanced immune response primarily directed against the virus as a foreign entity, rather than against the tumor^{158,159}. This phenomenon, rather than being a setback, could present an opportunity to create a safer viral platform. It could provide a natural safety mechanism, where the immune system's heightened response to the virus can act as a regulatory factor. This reduces the risk of off-target effects or systemic toxicity without diminishing the virus's ability to replicate and exert its oncolytic effects. Therefore, this approach could allow for a balance where the virus can remain potent against cancer cells while the immune system concurrently manages its activity, potentially leading to a more controlled and effective oncolytic virotherapy^{148,149}.

Our recent work has optimized the application of multiple chemically inducible promoters in VV gene expression, including a tetracycline-based repressor system and a rapamycin-controlled split T7 promoter, allowing for multi-level control over virus gene expression¹⁶⁰. In this study, we have developed a Conditionally Replicating Vaccinia Virus (CRVV) and demonstrated its controlled replication both *in vitro* and *in vivo* by FDA approved small molecule Doxycycline. We evaluated its efficacy across multiple syngeneic models, comparing the immune response elicited by CRVV to that of a common vaccinia

virus platform based on the deletion of the TK gene. Furthermore, we engineered the virus to encode IL-12 under our recently developed dual inducible promoter system - a split T7, rapamycin-controlled, coupled with a tetracycline, doxycycline-controlled operator promoter (ST7-TetR)¹⁶⁰. This design allowed us to show the multi-level control of IL-12 expression in vitro. Interestingly, we observed tumor-restricted growth of the novel virus in immunocompromised mice, contrasting with off-tumor growth of the CRVV. Finally, our results demonstrate the superior efficacy of IL12 expressing virus in B16-F10 model, underlining its potential as a more effective and safer option in oncolytic virotherapy.

Results

Developing a Novel Conditionally Replicating Vaccinia Virus (CRVV) Platform with Higher Safety

To engineer a chemically inducible, conditionally replicating Vaccinia Virus (CRVV) platform, we focused on the E1L gene, the catalytic subunit of the vaccinia virus poly(A) polymerase enzyme, as our gene of choice. This selection was driven by the dual essentiality of E1L^{161,162}: its critical role in the viral replication cycle and its incidental activity in polyadenylating and degrading host miRNAs, some of which are key to the antiviral response³². To achieve conditional replication, we redesigned the promoter region of the E1L gene to incorporate the optimized doxycycline (Dox)-inducible promoter, and co-encoded the tet repressor (TetR) adjacent to it¹⁶⁰. This design ensured that in the presence of Doxycycline, which inhibits TetR activity, the virus replicated almost at the level of the parental wildtype virus. Conversely, in the absence of Doxycycline, the

replication was markedly slower, thus successfully establishing a CRVV recombinant with replication contingent upon the presence of Doxycycline (**Fig 3.1A-B**).

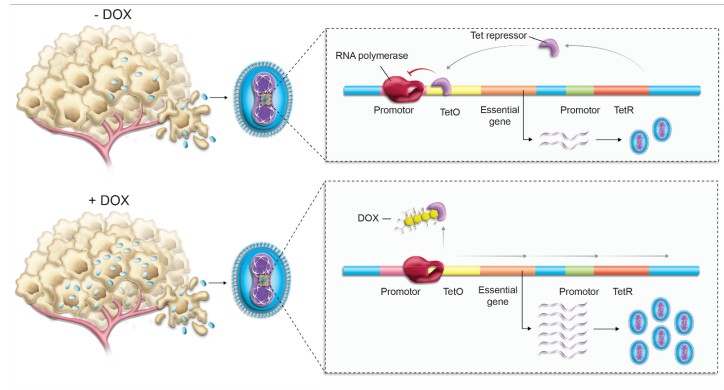
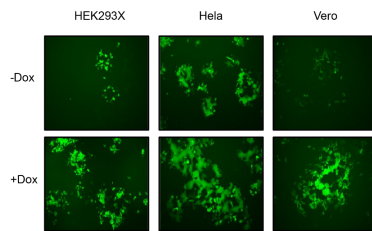
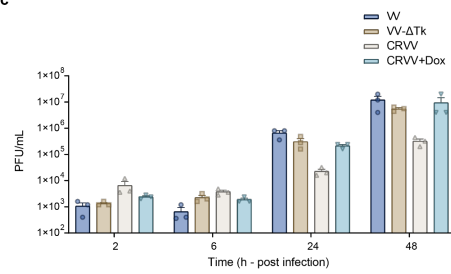
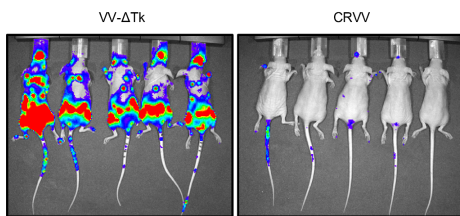
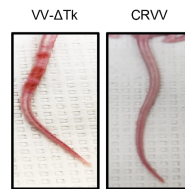
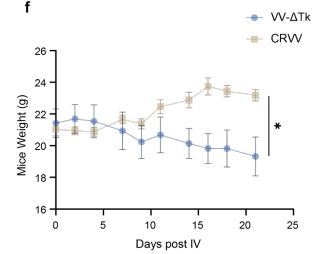
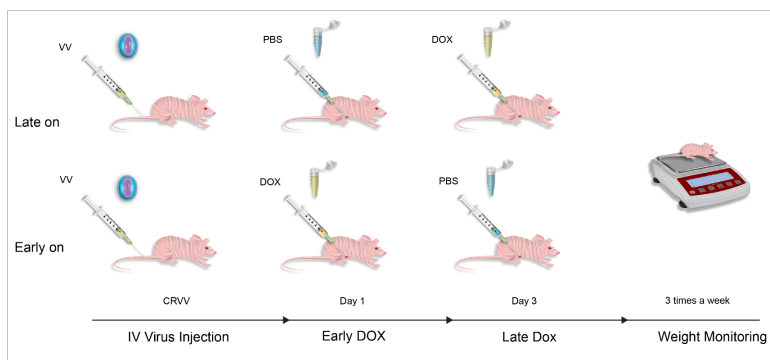
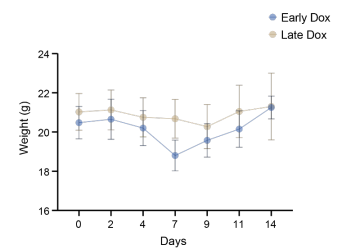
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Figure 3.1: Inducible Replication and Safety Profile of Conditionally Replicating Vaccinia Virus (CRVV)

a. Schematic representation of doxycycline-mediated control over viral replication, depicting an essential virus gene regulated by the TetO/TetR system. b. Fluorescent microscopy images of Hela, Vero, and HEK293 cells infected with CRVV at an MOI of 0.01, comparing conditions with and without doxycycline in the media, 48 hours post-infection. c. A bar graph depicting a multi-step growth curve in Hela cells infected with VV, VV- Δ TK, or CRVV (both in the presence and absence of Doxycycline in the media). Cells were collected at designated time points and viral titers were determined on U2OS cells. d. Bioluminescence imaging (IVIS) of CD1 nude mice at 48 hours post intravenous injection with 1E7 PFU of either VV- Δ TK or CRVV. e. Tail images of the CD1 nude mice taken at the study's endpoint on day 21 post-infection. f. A graph illustrating the weight monitoring of CD1 nude mice at regular intervals following intravenous injection with VV- Δ TK or CRVV. g-h. Graphic depiction and weight monitoring data for CD1 nude mice intravenously injected with 1E7 PFU of CRVV, with intraperitoneal doxycycline administration on either day 1 or day 3 post-virus injection, and weight tracked at regular time points.

First, using common human cancer cell lines, we demonstrated a significant decrease in viral spread and plaque formation in the absence of Doxycycline, highlighting the CRVV's conditional replication efficacy (**Fig 3.1B**). Next, we compared the growth kinetics of CRVV with both parental VV and the commonly used oncolytic vaccinia virus with thymidine kinase deletion (VV- Δ TK), currently the standard VV based OV in clinical trials, in Hela cells. Here, CRVV showed a growth rate very similar to VV and VV- Δ TK when Doxycycline was present. But the growth rate of CRVV was dramatically reduced, by nearly 100-fold, in absence of Doxycycline (**Fig 3.1C**).

To assess the safety of CRVV platform in comparison to the VV- Δ TK, we conducted experiments using immunocompromised CD1 nude mice. These mice were injected intravenously with either VV- Δ TK or CRVV. Bioluminescent imaging conducted 48 hours post-injection revealed a notable contrast in virus spread: VV- Δ TK had disseminated throughout the body of the mice, whereas CRVV infection, indicated by luciferase expression, was primarily localized to the injection site with significantly lower nonspecific infection (**Fig 3.1D**). Over an extended observation period, pox lesions began to manifest in mice injected with VV- Δ TK, but no such lesions were observed in the CRVV-injected mice (**Fig 3.1E**). Additionally, we noted a significant weight loss in the mice injected with VV- Δ TK, in contrast to those injected with CRVV, which exhibited no weight loss (**Fig 3.1F**). These observations indicate that CRVV is a safer platform compared to VV-TK.

To demonstrate the control over viral replication and virulence of CRVV in vivo, we conducted an experiment with immunocompromised mice injected intravenously (IV) with CRVV. The mice were then given intraperitoneal (IP) injections of Doxycycline either early (1 day post-virus injection) or late (3 days post-virus injection) (**Fig 3.1G**). A few days following the Doxycycline administration, we observed significant weight loss

in the early Dox injection group, indicative of viral activation, increased replication, and enhanced virulence. However, approximately 7 days after the initial virus injection, the mice's weight began to increase and stabilize. The group injected late with Dox did not show such a dramatic decrease in weight indicating the importance of timing of viral activation in controlling its virulence (**Fig 3.1H**). These experiments effectively highlighted the ability to modulate the replication and virulence of CRVV in vitro and in vivo, showcasing its potential as a versatile and controllable therapeutic agent. Moreover, the data indicates that viral attenuation by means of controlling its gene expression is significantly safer than removing nonessential genes in immunocompromised settings.

Differential Treatment Outcomes as a Function of Viral Replication

Our conditionally replicating vaccinia virus (CRVV) platform offers a unique tool to directly investigate how viral replication dynamics influence oncolytic therapy outcomes. Unlike VV- Δ TK, with its gene deletion, CRVV allows us to modulate replication without affecting overall viral structure^{37,152}. This enables a more precise assessment of how controlling viral replication and the associated oncolytic activity impacts treatment efficacy and potential off-target effects.

We first examined viral persistence and localization in a subcutaneous B16-F10 model using IVIS imaging. Mice received intratumoral injections of CRVV either with or without Dox, or VV- Δ TK. As expected, CRVV without Dox demonstrated lower luciferase signal, indicating reduced persistence. Interestingly, active CRVV did not exhibit significantly higher bioluminescence in the tumor or off-target sites compared to VV- Δ TK

(Fig 3.2A). This suggests that in immunocompetent settings, viral persistence and off-target activity may be influenced by factors beyond replication rate alone.

To assess therapeutic efficacy, we monitored tumor growth in the subcutaneous B16-F10 model with intratumoral injections. Both active CRVV and VV- Δ TK significantly delayed tumor growth compared to CRVV without Dox, highlighting the importance of robust viral replication for optimal treatment outcomes **(Fig 3.2B).**

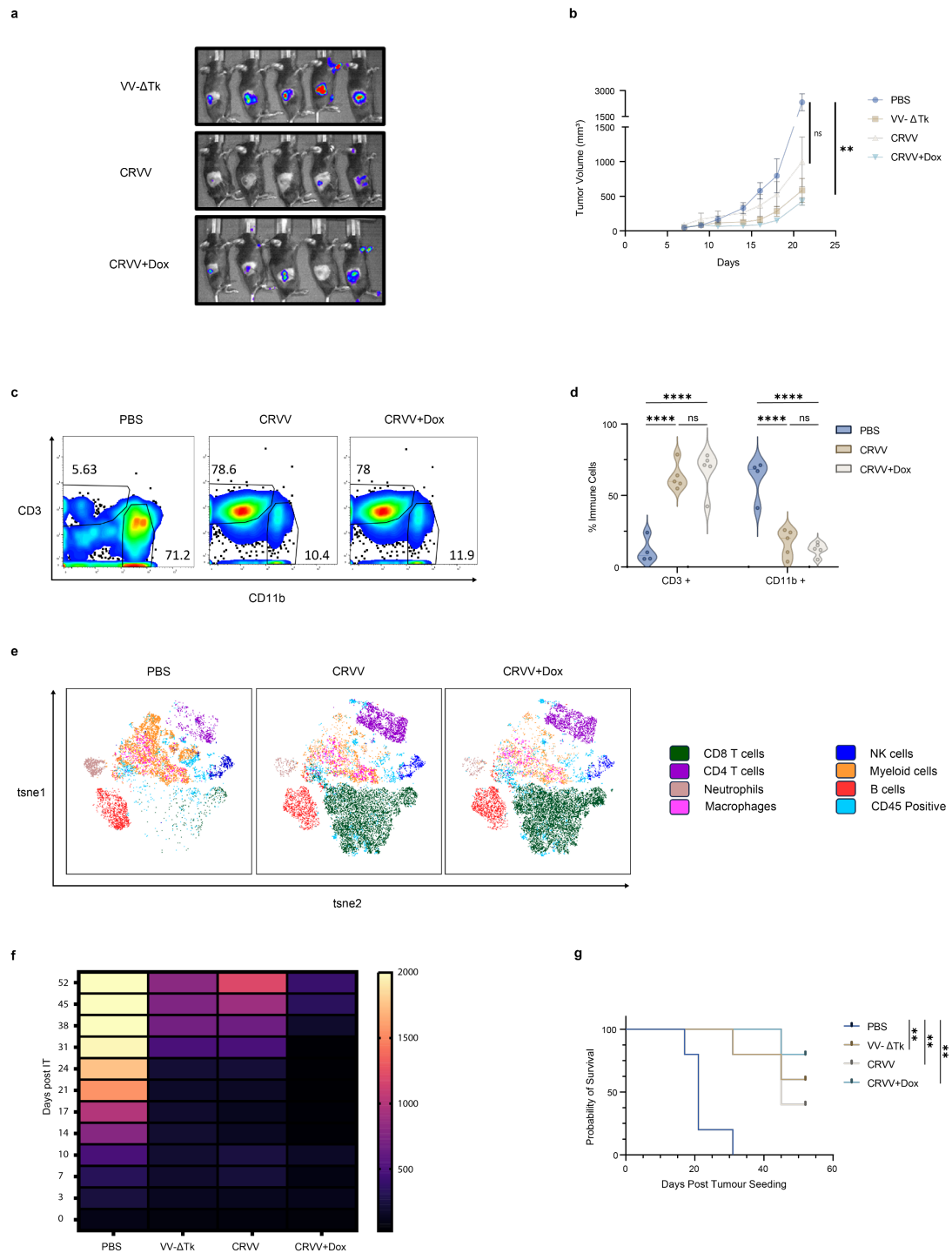


Figure 3.2: Oncolytic Efficacy of Dox-Inducible CRVV Enhances Treatment Outcomes in B16-F10 Melanoma and CT26-LacZ Carcinoma Models

a-b. B16-F10 melanoma tumors were established subcutaneously in C57/BL6 mice and subsequently received intratumoral injections of 1×10^7 PFU of CRVV or VV- Δ TK on specified days post-tumor seeding, with one CRVV group receiving a doxycycline-supplemented diet. a. Bioluminescence imaging was performed 5 days after the initial virus injection to assess viral activity. b. Tumor volumes were measured at regular intervals to monitor progression. c-e. A similar setup was used for another group of B16-F10 model mice, which were intratumorally injected with CRVV, with or without a doxycycline-containing diet, on day 9 post-tumor seeding. Tumors were collected for analysis 7 days after virus injection. c-d. The graphs present the ratio of CD3 to CD11b positive immune cells, indicating the immune response to each treatment condition. e. A t-SNE plot illustrates the overall immune cell population distribution resulting from each treatment. f-g. For the CT26-LacZ carcinoma model in Balb/c mice, intratumoral injections of 1×10^7 PFU of CRVV or VV- Δ TK were administered on subsequent days post-tumor seeding, with a subset of the CRVV group receiving a doxycycline-containing diet. f. A heatmap displays the average tumor volume for each group, with a maximum threshold of 2000 mm³. g. A survival plot tracks the lifespan of mice in each group for up to 60 days post-tumor seeding, demonstrating the impact of treatment on survival rates.

Next, we examined how viral replication influences tumor immune response. Subcutaneous B16-F10 tumors injected with CRVV (with or without Dox) were compared to PBS controls 7 days post-injection. Analysis of tumor-infiltrating immune cells revealed a dramatic shift from a myeloid-dominant environment to a lymphoid-rich infiltrate following CRVV injection, regardless of replication rate. CD3⁺ lymphoid cells comprised over 70% of the tumor immune population in both the Dox-activated and slower-replicating CRVV groups (**Fig 3.2C-D**). tSNE analysis of major immune populations within the tumor confirmed these observations, indicating a similar immune landscape despite the differences in viral replication kinetics (**Fig 3.2E**).

Finally, employed a CT26-LacZ murine colorectal cancer model with intratumoral viral injections. A significant survival benefit was observed with doxycycline-activated CRVV, increasing cure rates from 40% (CRVV without Dox) to 80%. This therapeutic advantage was even greater than that of VV-ΔTK, which achieved a 60% cure rate. This finding underscores the critical role of rapid viral replication and robust oncolytic activity in maximizing treatment outcomes (**Fig 3.2F-G**).

Differential Therapeutic Outcomes and Immune Responses of CRVV and VV-ΔTK in MC38 Model

To assess treatment outcomes in a different setting without direct injection of virus into the tumor, we utilized an intraperitoneal MC38 colorectal cancer model with IP viral injections. While neither CRVV without Dox nor VV-ΔTK demonstrated a survival

advantage compared to the PBS control, CRVV with Dox significantly extended survival (**Figure 3.3A**).

Given the comparable in vitro replication of active CRVV and VV- Δ TK (**Supplementary Figure 1**), we hypothesized that CRVV's superior therapeutic efficacy may result from a more robust immune response. To test this, we employed a subcutaneous MC38 model to compare systemic and tumor-specific immune profiles following CRVV and VV- Δ TK treatment.

Initial analysis of splenocytes six days post-treatment revealed significantly higher immune cell counts in both virus-treated groups compared to PBS, with CRVV trending towards a higher count than VV- Δ TK (**Figure 3.3B**). Interestingly, Ly6G⁺ cell proportions (associated with MDSCs) were lower in both CRVV-treated spleen and tumor samples compared to VV- Δ TK and PBS controls (**Figure 3.3C**). Additionally, a significant increase in total splenic T cells was observed only in the CRVV group. While both virus treatments induced a significant increase in tumor T cell count compared to PBS, no substantial difference was seen between the CRVV and VV- Δ TK groups (**Figure 3.3D**).

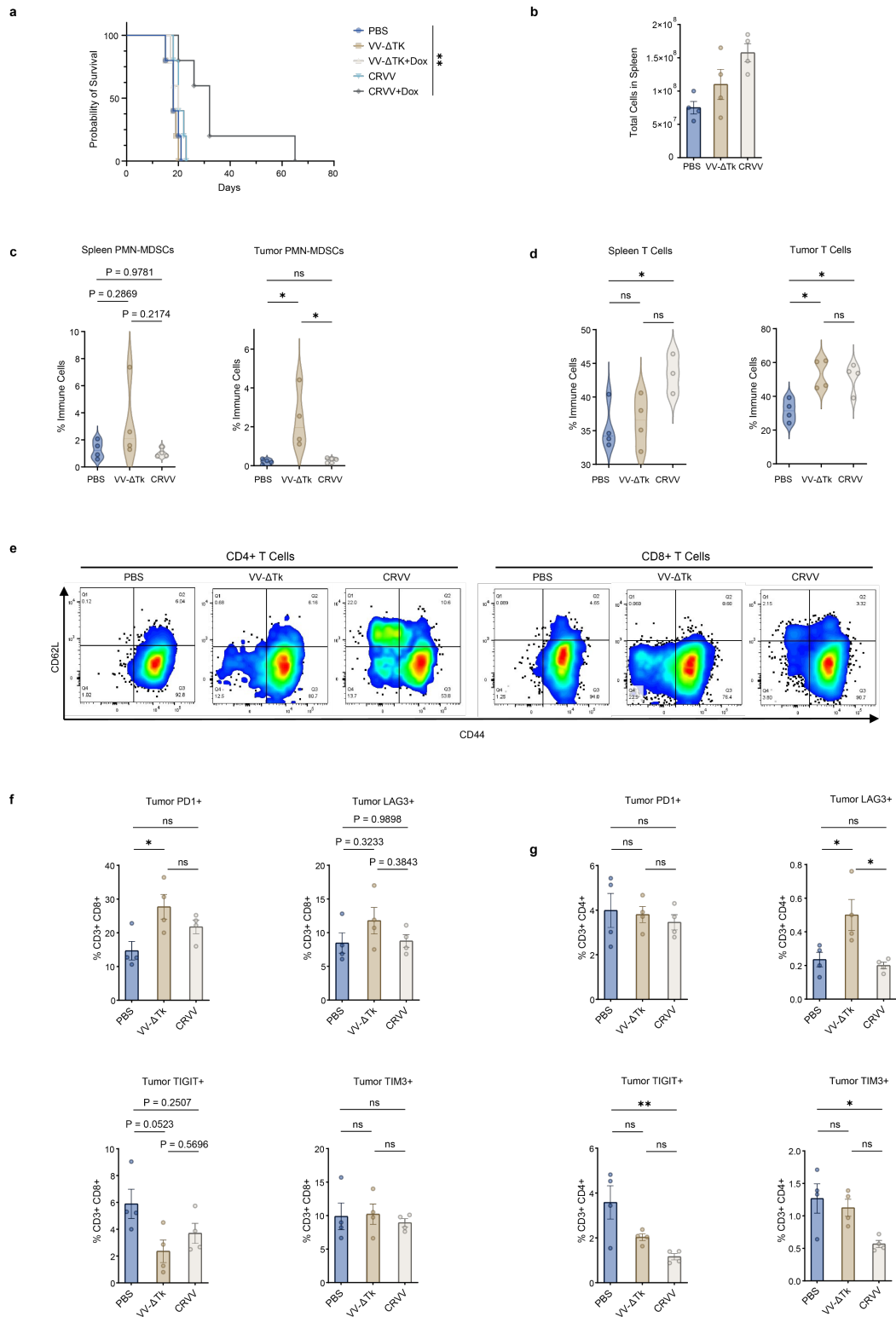


Figure 3.3: Survival Enhancement and Immune Landscape Modulation by Dox-Diet Augmented CRVV in MC38 Tumor Model

a. Survival curve of C57/BL6 mice with intraperitoneal MC38 tumors, followed by IP treatments of PBS, VV-ΔTK, or CRVV with or without a dox-containing diet, administered on days 7, 9, and 11 post-tumor injection. b-g. The following panels represent the immune profiling data from C57/BL6 mice with subcutaneously injected MC38 tumors, followed by intratumoral (IT) injections of PBS, VV-ΔTK, or CRVV 7 days post tumor seeding. Tumor and spleen were collected 6 days post virus injection, and all mice were maintained on a dox-supplemented diet: b. Bar plot indicating the total cell count in the spleen from each treatment group. c. Violin plots showing the percentage of PMN-MDSCs in the spleen and tumor for each group. d. Violin plots presenting the percentage of T cells within the spleen and tumor tissues across different treatment groups. e. Flow cytometry plots illustrating the expression patterns of CD62L and CD44 on CD4⁺ and CD8⁺ T cells in tumors from each treatment group. f. Bar graphs quantifying the percentage of CD3⁺ CD8⁺ T cells expressing PD1, LAG3, TIGIT, and TIM3 in tumors from each group. g. Bar graphs detailing the percentage of CD3⁺ CD4⁺ T cells expressing PD1, LAG3, TIGIT, and TIM3 in tumors for each treatment condition.

However, a key distinction emerged in the phenotype of tumor-infiltrating T cells. CRVV treatment led to a marked increase in CD4⁺ and CD8⁺ T cells expressing both CD62L⁺ and CD44⁻ markers (characteristic of naive T cells). This phenotype was virtually absent in the PBS and VV-ΔTK groups (**Figure 3.3E**). Furthermore, CRVV treatment significantly downregulated the exhaustion markers PD1 and Lag3 in CD8⁺ T cells (**Figure 3.3F**) and reduced Lag3, TIGIT, and TIM3 in CD4⁺ T cells (**Figure 3.3G**).

CRVV-R12, A Novel Oncolytic Virus with a Dual Regulatory System for tunable IL-12 Expression and Viral Replication.

Interleukin-12 (IL-12) is a potent cytokine with promising preclinical efficacy in cancer treatment^{153,154}. However, it is associated with systemic toxicity and cytokine release syndrome in clinical settings. To mitigate these risks, incorporating IL-12 into oncolytic viruses for localized tumor expression, coupled with viral oncolysis, has been explored in various viral platforms⁹⁸. However, to fully address concerns regarding cytokine release syndrome, a controlled modulation of IL-12 expression, tailored to individual patient responses, is essential. Our previously developed Vaccinia Virus (VV) promoter system, combining a split T7 promoter and tetracycline repressor systems (ST7-TetR), has demonstrated the capacity for varying transgene expression levels influenced by the presence or absence of a FDA approved rapalog, doxycycline, or both¹⁶⁰. In the context of the CRVV, controlled by a TetR/TetO Dox-based system, we explored if it could be a synergy between virus replication and IL-12 expression. This synergy offers a method to finely tune IL-12 expression at multiple levels. The new virus, referred to as conditionally replicating vaccinia virus with regulated IL-12 expression (CRVV-R12), will

give us the possibility of simultaneously modifying IL-12 expression and antitumoral response, alongside virus replication and antiviral response (**Figure 3.4A**).

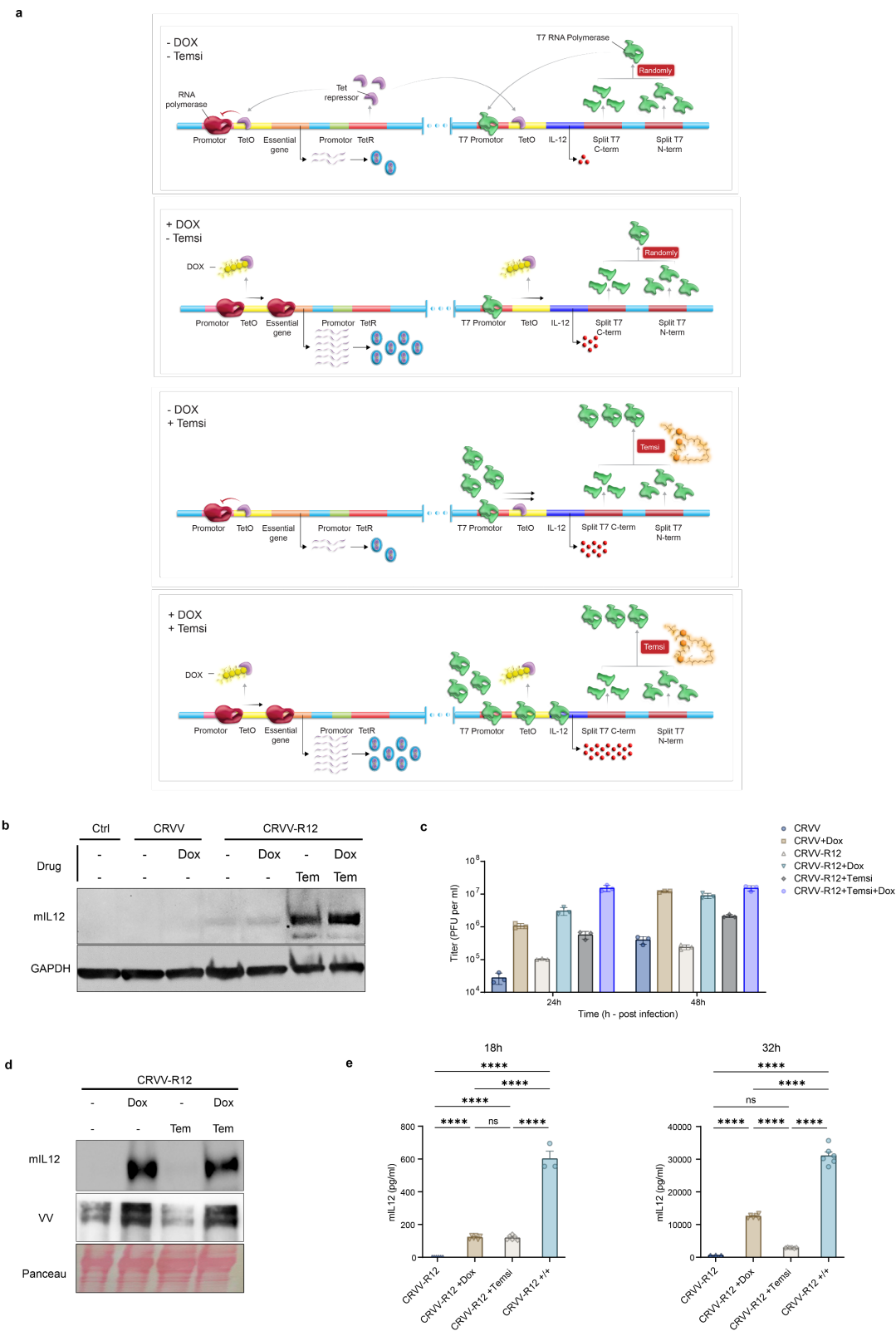


Figure 3.4: Regulation and Kinetics of IL12 Expression in Conditionally Replicating Vaccinia Virus (CRVV-R12)

a. Schematic diagram illustrating the control of IL12 expression in a conditionally replicating vaccinia virus (CRVV-R12), where IL12 is regulated by a rapalog-responsive split T7 system and a doxycycline-responsive TetO system. The schematic shows the system's response to different drug treatments, with or without the presence of doxycycline and temsirolimus. b. Western blot analysis displaying IL12 protein levels at 18 hours post-infection in cells infected with either CRVV or CRVV-R12 at an MOI of 0.5, under various conditions of doxycycline and temsirolimus administration. c. Growth kinetics of CRVV-R12 in Hela cells infected at an MOI of 0.01 with media containing different drug treatments. Cells were collected at 24- and 48-hours post-infection and viral titers were quantified through titration on U2OS cells. d. Western blot showing IL12 protein levels in U2OS cells infected with CRVV-R12 at an MOI of 0.01 under different drug treatment conditions, with cell lysates collected 48 hours post-infection. e. In a similar setup to (d), supernatants from the infected U2OS cells were collected at 18- and 32-hours post-infection, and the levels of murine IL12 were measured using an ELISA assay.

To assess IL-12 expression independently of viral replication, we infected U2OS cells with CRVV-R12 at an MOI of 1. IL-12 expression increased incrementally across treatment conditions, ranging from no drug to the combined presence of Dox and Temsi (**Figure 3.4B**). Notably, Temsi alone induced a significant increase in IL-12, with combined Dox and Temsi yielding the highest levels.

We evaluated viral titers 24 and 48 hours post-infection to assess the impact of the TetR system on CRVV-R12 replication. As expected, Dox significantly enhanced viral replication. Interestingly, Temsi alone also increased viral titers compared to the no-drug condition, though not to the same extent as Dox treatment (**Figure 3.4C**).

To explore the effect of viral replication on IL-12 expression, we infected cells at a lower MOI (0.1). Western blot analysis of cell lysates demonstrated a marked increase in IL-12 protein in the presence of Dox (**Figure 3.4D**). ELISA analysis of the supernatant confirmed these findings. While IL-12 levels were similar across single-drug conditions at 18 hours, the cumulative effect of viral replication over time led to significantly higher IL-12 levels at 32 hours in conditions with active viral replication (**Figure 3.4E**).

IL-12 Expression Enhances Safety of CRVV-R12 Compared to CRVV

Previous studies have shown the efficacy of IL-12-expressing OV_s^{156,157}. However, the impact of varying IL-12 levels and potential effects on viral replication in vivo remain unexplored. To address these questions, we used a subcutaneous HT-29 human colorectal adenocarcinoma model in CD1 nude mice, with intratumoral injections of either CRVV or CRVV-R12 under different treatment conditions.

Bioluminescent imaging revealed a significant difference in viral spread. While CRVV exhibited nonspecific spread beyond the tumor in 3 out of 4 mice, CRVV-R12 demonstrated reduced spread and greater tumor specificity, even with Dox-induced replication (**Figure 3.5A**).

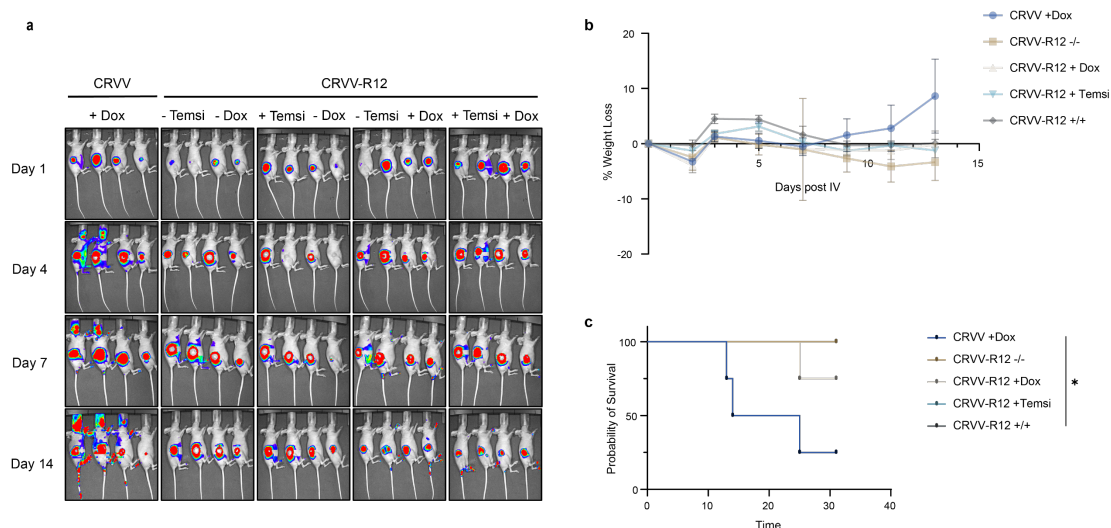


Figure 3.5: Safety and tropism of CRVV-R12 in an immunocompromised model

Mice bearing HT-29 human colorectal tumors received intratumoral injections of either CRVV or CRVV-R12 (n=4/group). Treatments were provided with or without doxycycline (Dox) and temsirolimus (Tmsi) to evaluate the effects on viral spread and safety. a. Bioluminescent imaging was performed on mice at designated days following treatment. b. Body weight of all mice was monitored regularly throughout the experiment. c. The survival of mice was tracked throughout the experiment.

This enhanced safety profile was further supported by health monitoring. CRVV-R12 treated mice experienced no significant weight loss (**Figure 3.5B**). Strikingly, the highest survival rate (100%) was observed even in mice treated with CRVV-R12 with both Dox and Tmsi, the condition with the highest IL-12 expression and active viral replication (**Figure 3.5C**), indicating that higher IL-12 expression may act as a safety mechanism, even in conditions of fast viral growth.

Therapeutic Efficacy and Immune Response of CRVV-R12 in B16-F10 model

To evaluate the therapeutic efficacy of CRVV-R12, we employed a subcutaneous B16-F10 melanoma model with intratumoral viral injections. CRVV-R12 in its replicating state (Dox + Temsi), with the highest IL-12 expression, demonstrated superior tumor control compared to all other conditions, including CRVV with active replication (**Figure 3.6A-B**). Conditions with lower IL-12 expression exhibited significantly reduced efficacy compared to actively replicating CRVV.

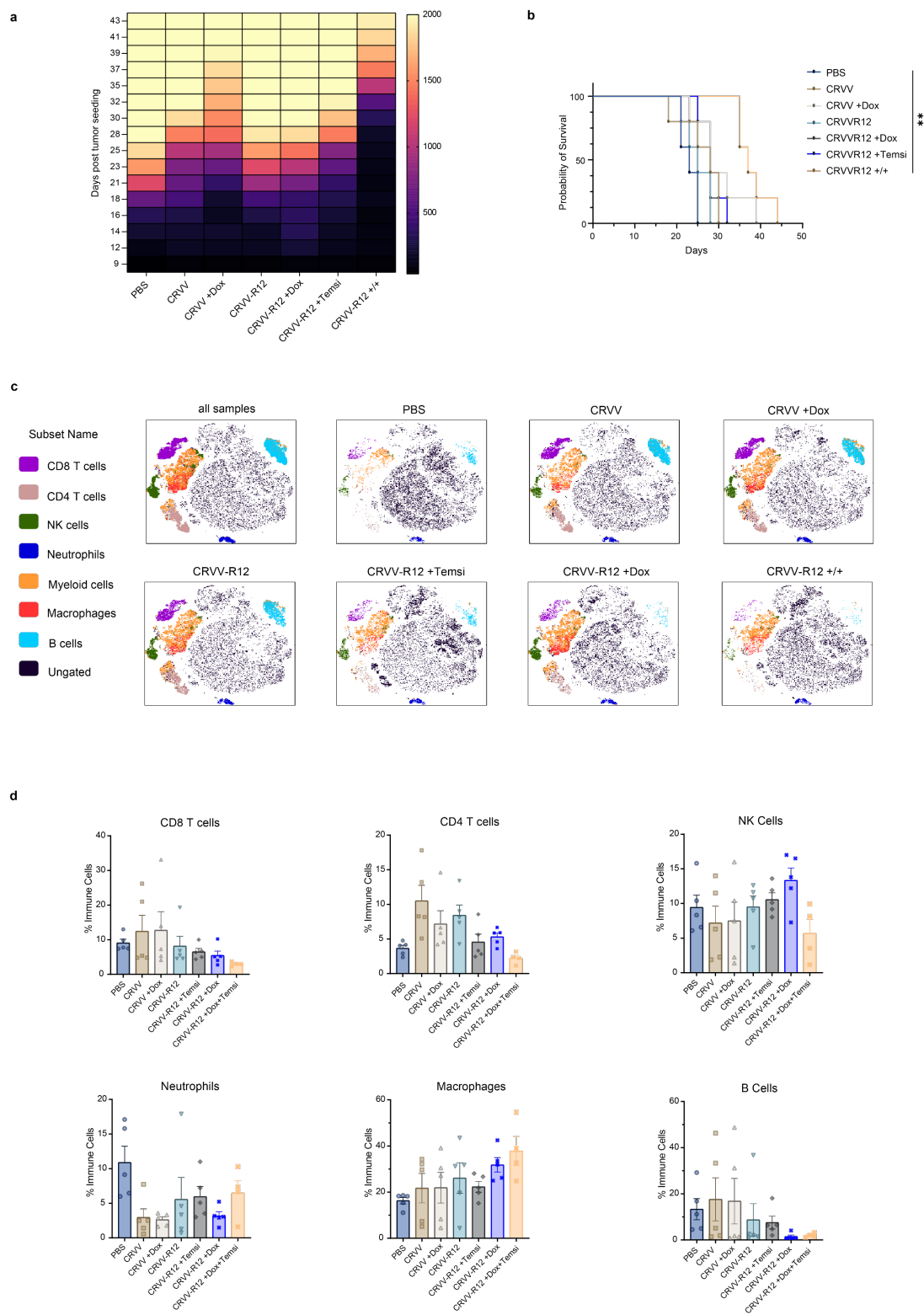


Figure 3.6: Efficacy and immune profiling of CRVV-R12 in B16-F10 model

Mice bearing subcutaneous B16-F10 melanoma tumors received intratumoral injections of CRVV or CRVV-R12 variants with or without doxycycline (Dox) and temsirolimus (Tems) treatment. a. Heatmap showing average tumor volume for each treatment group over time (n=5/group). b. Survival graph of mice following treatment (n=5/group). c. t-SNE plot illustrating the overall immune cell population distribution within tumors 4 days post-injection (n=5/group). d. Bar plots quantifying the percentage of various immune cell populations within tumors: CD8⁺ T cells, CD4⁺ T cells, NK cells, neutrophils, macrophages, and B cells (n=5/group).

To investigate how different IL-12 levels and viral replication rates affect immune infiltration, we repeated the experiment and analyzed tumors 4 days post-injection. With no drug treatment, both CRVV and CRVV-R12 triggered a substantial increase in total tumor immune cells, confirming low basal leakage within the system. Surprisingly, IL-12 expression reversed this effect, with maximum IL-12 levels leading to a significant decrease in immune cells, particularly lymphoid cells. Interestingly, CRVV-R12 with Dox (moderate IL-12 expression and active replication) showed an intermediate phenotype, with higher immune cell counts than drug-induced IL-12 conditions, but still lower than the no-drug groups. These findings suggest that in the B16-F10 model, IL-12 may exert its primary therapeutic effect through mechanisms other than increasing immune cell infiltration (**Figure 3.6C-D**).

Discussion

The clinical efficacy of oncolytic viruses, including VV as a major oncolytic platform, is influenced by multiple factors. Key among these are challenges like intravenous delivery, the virus's ability to replicate and spread effectively in condense tumor microenvironments, and most crucially, overcoming OV resistance due to antiviral response^{22,42,43,149}. This response encompasses both cellular and extracellular immune reactions to virally infected cells^{148,149}. VV is sophisticated in evading these responses, countering cellular antiviral defenses while not being a human pathogen³². However, in immunocompromised patients there is a small chance of off-target viral replication and toxicity. To address safety concerns in clinical settings, researchers have been focusing on deleting non-essential genes for growth in cancer cells from the Vaccinia Virus (VV)^{37,42}. This strategy aims to weaken the virus, thereby enhancing its safety for therapeutic use. Studies from clinical trials have suggested that such deletions reduce the treatment efficacy in patients significantly^{63,163}. Furthermore, the initial antiviral immune response following viral oncolysis and tumor antigen release which draws the immune system to the tumor underscores the importance of viral replication in treatment outcomes.

In this study, we leveraged our knowledge of VV promoters and previously developed chemically inducible promoter cassettes to control VV replication using doxycycline¹⁶⁰. By blocking the expression of an essential gene with the tetracycline repressor protein, we created a switchable virulence system with two states: replication akin to wildtype virus and attenuation significantly greater than currently active clinical trial stage oncolytic VV. Our findings indicate that external chemical control over viral replication is significantly more effective, in ensuring safety, than the typical gene deletion approach (such as Thymidine kinase³⁷) (**Figure 3.1**).

We then explored the importance of viral replication in treatment efficacy across multiple syngeneic mouse models, revealing the substantial impact of high-level virus replication on treatment outcomes (**Figure 3.2**). Investigation into the immune response to virulent viruses versus the Tk-deleted clinical virus version showed that the switchable virulent virus significantly alters the immune cell profile within tumors, favoring less protumoral cells and more naïve T cells, while enhancing systemic immune activation (**Figure 3.3**).

Building on this, we utilized our recently developed promoter system to control cytokine IL-12 expression, linking its expression to viral replication. This approach not only modulates viral replication, using TetR/TetO Dox controlled system, but also tunes IL-12 expression via a novel dual inducible promoter (ST7/TetR) (**Figure 3.4**). IL-12, known for its potent immune-activating properties, has been used with oncolytic viruses, including VV, but the effects of varying IL-12 levels on the antiviral immune response have not been previously addressed. Using CD1 nude mice model with human HT-29 xenografts, intratumorally injected with our novel virus with tunable IL-12 expression (CRVV-R12), we observed significantly improved survival and restriction of viral replication to the tumor area, especially at high IL-12 expression levels (**Figure 3.5**).

Our results with the CRVV-R12 platform in the B16-F10 model further highlight the complex interplay between viral replication, IL-12 expression, and the tumor immune response. CRVV-R12's superior efficacy at high IL-12 levels, coupled with the observation that higher IL-12 appears to decrease tumor immune cell infiltration, suggests an alternative IL-12-driven antitumor mechanism. This could involve direct cytotoxicity,

modulation of tumor vasculature, or the recruitment of other immune cell subsets not analyzed in this initial study^{98,103} (**Figure 3.6**). This finding warrants further investigation into the specific mechanisms through which IL-12 enhances therapeutic efficacy in this model.

In summary, this study presents a novel approach to enhance the safety and therapeutic potential of oncolytic vaccinia viruses. Our conditionally replicating platform with its chemically-inducible regulation of both viral replication and IL-12 cytokine expression provides unparalleled control and adaptability for personalized cancer therapy. Our results demonstrate that this system affords greater safety than traditional gene deletion approaches while retaining superior therapeutic efficacy, even in immunocompromised settings. Moreover, the ability to modulate IL-12 expression offers both enhanced safety and reveals a potential new avenue of IL-12-mediated antitumor activity. Overall, these findings highlight the versatility of our platform and its potential to significantly advance oncolytic virotherapy through fine-tuned control of viral replication and cytokine delivery, paving the way for safer, more targeted, and effective cancer treatments.

Methods

Ethics

This study strictly adheres to the ethical principles established by the Ottawa Hospital Research Institute and the University of Ottawa (certificate for biohazardous material utilization GC317-125-12). All animal procedures were conducted under the approval of the University of Ottawa's institutional animal care committee (Protocol IDs: OHRI2870 and MEe-2258) and followed the strict guidelines provided by the National Institutes of Health and the Canadian Council on Animal Care.

Viruses

Our work primarily utilized the Vaccinia Tian Tan strain as our viral backbone. For propagation purposes, the virus was maintained in HeLa cells.

Cell Culture

All cell lines were acquired from the American Type Culture Collections (Manassas, VA). Cell growth and maintenance occurred in either Dulbecco's Modified Eagle Medium (DMEM) from GE Healthcare Life Sciences (ON, CAN) or Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco; MA, USA), with both types of media supplemented with 10% fetal bovine serum (FBS) (Gibco). Cells were grown at 37°C in a humidified environment containing 5% CO₂. Regular PCR tests (e-Myo VALiD Detection Kit, 25239, LiliF Diagnostics; South Korea) ensured cultures remained free from mycoplasma contamination.

Construct Design

The IL-12 encoding construct was designed using Snapgene software. Codon-optimized inserts were ordered from GenScript (Piscataway, NJ, USA).

Reagents

Doxycycline (D9891-25G, Sigma-Aldrich) and Temsirolimus (Selleck Chemicals) were used in our experiments.

Mouse Experiments

This study involved three tumor models: CT26LacZ (Balb/c), B16-F10 (C57BL/6), and MC38 (C57BL/6). To establish tumors, six- to eight-week-old female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and received subcutaneous injections on the right flank. All animal protocols were approved by and conducted in accordance with the guidelines set by the University of Ottawa's animal care and veterinary services (MEe-2258-R5, or OHRle-3340-A).

Virus Production and Titration

Vaccinia virus production was performed using HeLa cells in 850 cm² roller bottles. Cells were infected at an MOI of 0.03, without removing the inoculation media. Incubation proceeded for 72 hours at 37°C with 5% CO₂, or until sufficient cytopathic effects were

observed. Afterward, cells were harvested and resuspended in TNE buffer (pH 8.0). Following three freeze-thaw cycles, the mixture underwent centrifugation at $700 \times g$ for 10 minutes at room temperature. Supernatant collection and overlay onto 36% sucrose cushions was followed by centrifugation $20,666 \times g$ for 1.5 hours at 4°C . Viral pellets were resuspended in 1 ml of TNE buffer and stored at -80°C . Titration was performed on U2OS cells, using the established plaque assay method.

Flow Cytometry

Splenocytes were isolated, or tumors were isolated using the Miltenyi Biotech mouse tumor dissociation kit (Cat# 130-096-730). Following isolation, cells were prepared for flow cytometric analysis. The following fluorophore-conjugated antibodies were used: CD45 (BUV395, Cat# 564279, BD), CD3 (BV786, Cat# 740854, BD), CD8 (APC-R700, Cat# 564983, BD), CD4 (BV510, Cat# 563106, BD), CD11b (FITC, Cat# 101206, BL), CD11c (BUV737, Cat# 612797, BD), MHCII (PerCPCy5.5, Cat# 107626, BL), CD86 (BV650, Cat# 740501, BD), NK1.1/CD49b (APC, Cat# 1.21E+05, BL), Ly6C (PECy7, Cat# 128018, BL), Ly6G (BV711, Cat# 563979, BD), F4/80 (PE, Cat# 123110, BL), CD206 (BV421, Cat# 141717, BL), CD19 (PE-TR/PECF594, Cat# 562291, BD), Live/Dead (APCCy7, Cat# 423106, BL), PD1 (BV650, Cat# 748266, BD), TIM3 (PE, Cat# 119704, BL), CTLA4 (FITC, Cat# HMCD15201, TF), TIGIT (BV421, Cat# 142111, BL), LAG3 (APC, Cat# 125210, BL), CD44 (PECy7, Cat# 103030, BL), and CD62L (BUV737, Cat# 367-0621-82, TF). Samples were stained as per standard protocols and analyzed on a BD LSRFortessa flow cytometer with four lasers and 18 color detection capabilities.

ELISA

Standard ELISA protocol was followed to measure IL-12 levels. Briefly, Nunc Maxisorp 96-well flat-bottom plates were coated with capture antibodies overnight at 4°C. Samples and standards were then added and incubated. After washing, detection antibodies conjugated to HRP were added. Following further incubation and washes, a TMB substrate solution was used for colorimetric development. The reaction was stopped, and absorbance was measured at 450 nm. IL-12 concentrations were determined by interpolation from a standard curve.

Western Blot

Cell lysates were prepared using a suitable lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using the BCA assay. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, membranes were incubated with primary antibodies against IL-12 and a loading control protein. HRP-conjugated secondary antibodies were then applied. Chemiluminescent substrates were used for detection, and images were captured using an appropriate imaging system.

Statistical Analyses

All graphs and statistical analyses were generated using GraphPad Prism v9. When appropriate, unpaired two-samples t-test or two-way ANOVA were used. The number of biological replicates (n value) is noted in the figure legends. Error bars in the figures represent either the standard deviation (SD) or the standard error of the mean (SEM), as

specified in the legends. Statistical significance was determined with the following thresholds: ns ($P > 0.05$), * $P < 0.05$, ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$).

Chapter 4 - Harnessing Antibiotics as Tools for High-throughput Insertional Mutagenesis and Generating Cytokine-Expressing Oncolytic Viral Libraries

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Abstract

Oncolytic or cancer lysing viruses (OVs) are biological agents selected or engineered to replicate selectively in tumour cells. The optimization of OV platforms is a blend of strategic removal/mutation of virulence genes coupled with insertion of transgenes that enhance replication, spread and immunogenicity of the therapeutic. For Herpes Simplex Virus-1 (HSV-1) and Vaccinia virus, the complexity of their genomes coupled with challenging and time-consuming recombinant systems has slowed the advancement of their development as therapeutic agents. We optimized a selection strategy using antibiotics to accelerate virus genome engineering, identification of viral genes dispensable for virus replication in tumour cells and isolation of novel stable intergenic insertion sites. The toolbox we developed facilitates the rapid creation of transgenic virus libraries that can be screened in in vitro systems or animal models to identify more potent therapeutics. Finally, we demonstrate the potential for sequential application of immunologically distinct transgenic OVs to enhance therapeutic outcomes.

Introduction

Oncolytic Viruses (OVs) were originally conceived/designed to selectively grow in and lyse cancer cells, acting as self-replicating cytolytic agents¹. Over the last 20 years our understanding of these agents has completely changed and while “oncolysis” is an important, and essential component of these agents, it is clear that an OV’s ability to stimulate anti-tumour immune responses creates a unique biological tool for remodeling the tumour microenvironment (TME)^{42,148}. Designing a replicating cytolytic agent that also expresses multiple payloads could create a “Mobile Pharmacy” that can selectively dispense therapeutic proteins when and where they are needed^{43,164}. While this is an attractive idea, it has become clear that a simple “cut and paste” of immune stimulating genes into a virus backbone is complicated by the observation that most viruses are inherently immune suppressive^{4,22,148}. Large DNA viruses like Herpes and Pox Viruses have complex genomes (200+ genes) and determining which viral genes are redundant or even counterproductive for generating therapeutic immune responses is challenging and has slowed the development of more effective OV therapeutics^{31,50,165}. Indeed, the only FDA approved oncolytic virus is a herpes virus with very limited deletions of viral virulence genes and encoding a single therapeutic transgene, human GM-CSF^{19,58}. The combinatorial impact of multiple transgenes in the context of an immune suppressive TME needs to be done empirically through testing of libraries of mutagenized and modified viruses^{43,148,164}. The potential to explore multiple unique combinations of therapeutic payloads and viral gene deletions has been hampered by complex and time-consuming virus engineering strategies^{165,166}. We and others have shown that application of CRISPR-Cas9 technology while improving the efficiency of recombinant virus generation^{60,104,167} is still limited by challenges in simultaneously targeting diverse genomic regions to create virus libraries¹⁶⁸, labor-intensive process of recombinant

selection¹⁶⁶ and the diminishing efficiency of recombination and selection with increased insert length¹⁶⁹. Sleeping Beauty transposon mediated insertional mutagenesis has been used extensively as a functional genomics tool in mammalian cells^{170,171}. We adapted this system to randomly insert transposons throughout the genomes of both vaccinia virus and herpesvirus with the goal of identifying which viral gene products are essential or dispensable for virus replication and spread in tumour cells. To enhance the speed with which we can isolate recombinant viruses and to provide the opportunity to sequentially mutagenize viral backbones we engineered Sleeping Beauty transposons to encode a variety of drug resistance genes focused primarily on protein translation inhibitors^{114,172}. This choice was motivated by the observation that viruses, as obligate parasites, are strictly dependent upon the cellular protein synthesis machinery¹⁷³. Indeed, viruses have evolved multiple strategies to rapidly usurp cellular protein synthesis providing them with the ability to evade cellular innate anti-virus responses, enhance the production of viral proteins, skew cellular resource allocation towards virus replication and in some cases delay cellular apoptosis programs^{32,174}. Given the critical importance of protein translation to the virus life cycle, we reasoned that drugs which target protein synthesis could be particularly effective at blocking virus replication at the earliest stages perhaps at concentrations that only minimally impact mammalian cell viability. This approach allowed us to generate large complex libraries of mutagenized Vaccinia Virus and Herpesvirus vectors that can then be used in selection strategies to identify phenotypes of interest. We further exploited our drug selection system to enhance virus recombinant rescue efficiency even with large transgenes and short homology arms. Finally, we simplified the protocol to create viruses expressing a range of cytokines, compared their efficacy in animal models, and explored the potential of adaptive sequential therapy with these oncolytic agents.

Results

Antibiotics inhibit the growth of oncolytic virus platforms

We sought to identify compounds that would facilitate the rapid isolation of virus recombinants and could be potentially used alone or in combinations on a number of different virus families. We initially focused on the application of the aminonucleoside antibiotics puromycin¹⁷⁵, hygromycin¹⁷⁶, neomycin¹⁷² as well as blasticidin¹⁷⁷, an inhibitor of peptidyl transferase, as these drugs are known to impair protein synthesis¹¹⁴. As discussed, a key feature of the life cycle of many viruses is to usurp host cell protein synthesis and thus we hypothesized that a temporary inhibition of cellular protein synthesis would be more detrimental to an invading virus than to the host cell. To test this idea, we infected U2OS cells (human osteosarcoma cell line) with a spectrum of oncolytic virus backbones in the presence and absence of puromycin, an antibiotic that induces premature chain termination during mRNA translation. At concentrations that did not impact cell viability we observed that a variety of clinically relevant OV platforms are significantly impaired by puromycin with decreases in virus infectivity revealed by either virus encoded green fluorescent protein (GFP) or virus titre at 24 hours post infection (**Fig. 4.1a-b**). We then tested individual antibiotics for their impact on four different OV platforms (**Fig. 4.1c, Supplementary Fig. 4.1**). In these experiments we included the glycopeptide antibiotic zeocin that induces double strand DNA breaks. All of the protein synthesis inhibitors reduced virus titres from 10 to 1000-fold while zeocin as expected, did not impact the RNA based viruses (vesicular stomatitis virus or VSV and measles virus or MeV) but significantly impaired vaccinia virus (VV), a DNA virus. At the concentrations tested, zeocin did not impact HSV replication. In animal models, blasticidin was the most effective at attenuating virus infections and impacted the growth

of the pathogenic viruses SARS-CoV2, Zikavirus and Mayaro virus (**Supplementary Fig. 4.2**).

Sleeping Beauty Mediated Mutagenesis of Oncolytic Vaccinia and Herpes Viruses

Large DNA viruses like vaccinia and herpes viruses have highly evolved genomes that allow them to be successful pathogens in a variety of species and cell types. However, cancer cells represent a unique niche for virus growth¹⁷⁸ where many viral virulence genes may be redundant¹⁷⁹. To probe the genetic landscape that is sufficient for oncolytic virus growth we employed the Sleeping Beauty (SB) transposon system¹⁷¹ engineered to express an antibiotic resistance gene, initially under the control of a vaccinia virus promoter element¹⁶⁰ (**Fig. 4.2a**). The SB transposon and transposase were co-transfected into vaccinia virus infected cells and then virus progeny were rescued that: (1) were resistant to puromycin and (2) were able to form plaques on tumour cells. The subsequent library of viable mutagenized vaccinia viruses was subjected to long-read nanopore sequencing to identify individual insertion sites¹⁸⁰. Using this approach, we mapped the frequency of transposon insertions within the vaccinia virus genome, as illustrated in the circos plot demonstrating the location of more than 1300 retrieved insertion sites (**Fig. 4.2b**). Of particular interest to us, were intergenic insertion sites since we predicted placement of transgenes into some of these regions may not compromise virus growth whereas intragenic insertions may ultimately come at cost of viral fitness¹⁸¹. Indeed, we discovered six such loci (A25L-A24R, A43R-A42R, F1L-K7R, G1L-I8R, G6R-G5R, J5L-J4R), notably in the central region of the genome which is highly conserved between poxvirus family members and in general has much fewer intragenic insertions (**Fig. 4.2b**). To assess the suitability of the identified intergenic sites in the VV for stable transgene expression, we targeted each identified locus independently with a YFP/puromycin fusion

gene. Fluorescent microscopy of recombinant viral plaques consistently revealed unstable YFP expression at the J5L-J4R site leading to its exclusion from further study. However the other five other loci—A25L-A24R, A43R-A42R, F1L-K7R, G1L-I8R, G6R-G5R, demonstrated strong and consistent transgene expression (**Fig. 4.2c**). To assess the influence of each genomic alteration on VV replication, the plaque size among recombinant VV viruses, the wild-type parental strain and a variant in which we selectively deleted the viral thymidine kinase gene (Δ TK) were compared. As shown previously, the Δ TK variant consistently produced smaller plaques and lower virus output at 24 hours than the wild-type virus¹⁸² (**Fig. 4.2d-e**). In contrast, with the exception of the G6R-G5R recombinant, intergenic insertions displayed plaque sizes and virus titers, 24 hours post infection, that are comparable to the wild-type strain, suggesting minimal adverse effects on viral replication (**Fig. 4.2d-e**). Finally, we determined if particular intergenic insertions in the genome impacted the expression of neighboring genes using a qRT-PCR strategy and found minimal to no impact on adjacent gene transcription (**Fig. 4.2f**) indicating that at least four of these intergenic loci represent insertion targets for stable transgene expression without compromising virus fitness in tumour cells. We explored the potential of creating VV populations with multiple transposon insertions by introducing a second transposon harbouring the neomycin resistance gene and mCherry markers into our existing YFP/Puromycin-resistant transposon library. The vast majority of second insertion sites mirrored the initial YFP/puromycin selected sites (**Fig. 4.2g**) and interestingly new plaque phenotypes featuring fused cells began to appear following dual antibiotic selection (**Fig. 4.2h**). These observations suggest that our compound insertion library provides an opportunity to search for phenotypes dependent upon disruption of at least two different viral genes. In the case of an oncolytic virus a cell fusion phenotype

may enhance virus spread within the tumour and create a more immunogenic form of cell death^{137,183}.

We applied our antibiotic-coupled SB approach to wild type HSV-1 and mapped transposon insertion sites again using nanopore sequencing. Since HSV uses cellular transcription machinery, we used the CMV promoter to drive the expression of the antibiotic resistance gene. Our data revealed two regions with a high density of insertions: one encompassing the US2, US3, and US4 genes, and another encompassing the UL39, UL40, and UL41 genes (**Fig. 4.3a**). Each of these genes have individually been reported as nonessential for in vitro virus replication in permissive cells¹⁷⁹. We tested whether compound deletions of these regions impacted growth on tumor cells to determine if these regions could potentially be replaced by therapeutic transgenes. To this end we used the antibiotic selection methodology described below to create two novel HSV1 recombinants: HSV-ΔUS24, featuring deletions spanning the US2 to US4 region, and HSV-Δ3941, with deletions across the UL39 to UL41 region. We compared plaque morphology and size between the parental HSV-KOS strain, the clinical candidate HSV-G47 delta (G47Δ)⁵⁴, HSV-ΔUS24, and HSV-Δ3941, in Vero cells. Notably, HSV-ΔUS24 exhibits both plaque morphology and size comparable to the parental HSV-KOS strain, suggesting viral spread akin to the wild-type virus (**Fig. 4.3b-c**). This data indicates that the deletion of multiple genes in HSV-US24 does not adversely affect viral spread in Vero cells. In contrast, the ICP34.5 deleted vector G47Δ and HSV-Δ3941 displayed a trend towards a smaller plaque phenotype suggesting a diminished ability to spread in vitro. A multi-step growth curve revealed that HSV-Δ3941 and HSV-ΔUS24 produced significantly higher numbers of infectious virions than G47Δ over a 72-hour period (**Fig. 4.3d**) although both consistently produced lower titers than the parental HSV-KOS strain. The G47Δ virus like many of the oncolytic HSV strains being tested in the clinic, has a

deletion of the neurovirulence gene ICP34.5 which although increases the safety of the virus does compromise its ability to replicate in tumour cells^{12,50,51,184} compared to wild type HSV strains that have a functional ICP34.5 gene. We tested whether our novel deleted HSV strains, which are ICP34.5 replete, would be safe and have improved oncolytic activity over the G47 Δ backbone. We conducted an in vivo study to assess the safety profiles of HSV- Δ 3941, HSV- Δ US24, HSV-KOS and G47 Δ using intra-cerebroventricular injection of each virus into BALB/c mice. Animals injected with HSV-KOS at 1E5 PFU experienced significant weight loss within 3 days and consistent with its established neurovirulence (**Fig. 4.3e**) whereas even at ten times higher dose mice injected with HSV- Δ 3941, HSV- Δ US24 and G47 Δ experienced little to no weight loss and were alive and well 100 days post-injection (**Fig. 4.3e**). Brain isolation for immunohistochemistry was performed on a cohort of animals on day 3 (**Fig. 4.3f**) and while HSV-KOS was found to be widely spread throughout the brain, there was only minimal evidence of virus infection with HSV- Δ 3941, HSV- Δ US24 and G47 Δ . To evaluate the oncolytic potency of HSV- Δ 3941 and HSV- Δ US24 compared to parental KOS and G47 Δ , we used a subcutaneous CT26 (murine colorectal cancer) model with intratumoral injection of each virus. HSV- Δ US24 exhibited the most significant reduction in tumor volume (**Fig 4.3h**). The combined safety and potency of HSV- Δ US24 in this mouse model suggests that this multigene deletion could be an alternative to ICP34.5 deletion for generation of novel HSV-1 based oncolytic viral platforms.

Strategic use of antibiotics facilitates rapid engineering of VV and HSV

Common approaches for the engineering of recombinant HSV and VV strains are time-consuming and labor-intensive. Recently, we and others have attempted to increase the efficiency of the traditional methods by developing diverse CRISPR-Cas9

systems^{60,104,167}. Although CRISPR-Cas9-associated systems can accelerate the isolation of recombinant viruses, they show very limited efficiency as the complexity and size of transgene insertions increase¹⁸⁵. We explored whether an optimized antibiotic schedule can be used along with conventional homologous recombination strategies, to overcome this challenge (**Fig. 4.4a**). To this end we incorporated an antibiotic resistance marker into a homology directed repair (HDR) vector to facilitate the rapid outgrowth of transgene-accepting viral particles under our optimal antibiotic concentrations. After virus infection, coupled with plasmid transfection, we used an optimized strategy of sequentially harvesting and passaging viruses on fresh cells in the presence of antibiotics for 5 days. At this time pure clones of recombinant viruses could be isolated. This contrasts with the longer duration, often weeks, required for plaque-picking enriched recombinant viruses after selection by the CRISPR-Cas9 method (**Fig. 4.4a**). We evaluated the efficacy of both CRISPR/Cas9 and antibiotic selection methods in engineering VV and HSV-1 with increasing transgene sizes. The VV and HSV-1 vectors contained fluorescent reporters and antibiotic resistance genes (Zeocin for VV, Puromycin for HSV-1). We measured recombination success by the presence of fluorescent positive plaques at 5 days post-rescue. Our findings indicated that while CRISPR/Cas9 efficiency rapidly decreased with larger transgenes, the antibiotic selection method performed at a high level, exhibiting 50-80 times greater efficiency for 5 kb transgenes (**Fig. 4.4b-c**). We examined the influence of homology arm length in the HDR vector on the efficiency of recombinant virus isolation. Linear DNA constructs with a range of homology arm lengths and inserts carrying fluorescent marker and antibiotics resistant genes were designed. Transgene integration efficiency was quantified as the percentage of fluorescent positive plaques over 5-days post-targeted rescue. The efficiency was more than 80 percent for homology arms as small as 100 base pairs.

However, a significant reduction in recombination efficiency occurred when homology arm lengths were 50 bp or smaller (**Fig. 4.4d**). To determine if antibiotic selection efficacy is dependent on the genomic insertion site, we examined four common insertion loci in the VV genome (B8R, B19R, J2R, A46R) and three in HSV-1 (US9, ICP34.5, UL55), each using an antibiotic and fluorescent protein selection marker. The results demonstrated a consistent rate of 80% or higher purity in VV and HSV-1 across all tested sites (**Fig. 4.4e**). We applied our optimized HDR design and antibiotic selection strategy to rapidly create a library of VV and HSV recombinants expressing immune modulating cytokines. The cytokines were inserted into the thymidine kinase gene in vaccinia virus, an often used site for inclusion of therapeutic genes¹⁸², and in the intergenic region between UL26 and UL27 in HSV which has been shown not to impair HSV growth¹⁸⁶. Our initial library included interleukins known for immune stimulation (IL2, IL7, IL12, IL15, IL18) and those associated with immune suppression (IL33, IL1B, IL6, IL10, IL17A), alongside chemokines linked to recruiting anti-tumour immune cells (CXCL9, CXCL10, CXCL11, CXCL14, CCL20) and those associated with promoting tumor suppressive immune cells (CCL2, CCL3, CCL5, CXCL12, CCL22)¹⁸⁷. Our strategy significantly shortened the engineering time for making transgenic virus libraries to less than a week from an estimated several months (**Fig. 4.5a**). Quantitative reverse transcription PCR (qRT-PCR) from infected cells confirmed both the presence of each cytokine gene within the viral genome and their active expression (**Supplementary Fig. 4.3**). We used two different mouse tumour models to carry out an initial test of the efficacy of our individual engineered viruses. In Figure 5, we present the survival data for groups of mice (n=5) harbouring either B16 melanoma or CT26 colon tumours following direct intratumoural injection with either VV or HSV recombinants. By comparing and contrasting these two models we hoped to identify immune modulators

that enhance the efficacy of these two OV platforms. For VV clear trends were evident in swimmer plots of tumour bearing animals (n=5) treated with recombinant viruses.

Unmodified VV had minimal therapeutic activity compared to vehicle (PBS) in both the CT26 and B16 tumour bearing animals. In contrast VVs encoding a number of different cytokines impacted tumour growth in both models (**Supplementary Fig. 4.4**). A cadre of cytokines appeared to enhance the therapeutic benefit of vaccinia virus in the CT26 model notably the IL2, IL12, IL18 and CCL5-expressing groups which achieved complete tumour response in multiple animals. In the B16-F10 melanoma model, known for its rapid growth and lower immunogenicity, VV on its own has robust growth compared to the CT26 model. However, the addition of immune-stimulating transgenes is overall less effective in the B16-F10 model. For instance, IL2, IL18 and CCL5 enhanced VV therapy in the CT26 model but had little to no effect in the B16 model. In contrast, IL12 and IL15 showed some therapeutic benefit in both models (**Fig. 4.5b,d**).

HSV lacking any therapeutic transgene was effective in the CT26 model (**Fig. 4.5c**) curing 2 of 5 mice and making it challenging, in this initial study, to see the benefit of incorporation of interleukin genes into the virus backbone. However, IL12 expressed from HSV did cure 4 of 5 mice in the CT26 model and 3 of 5 mice in the B16 model (where control virus was ineffective (**Fig. 4.5e**) suggesting that this cytokine can enhance the therapeutic activity of both VV and HSV in two different animal models. The clear differences between the two virus platforms in different animal models tested, argue that more detailed studies will be required to decipher the complex interactions between virus backbones, therapeutic transgenes and the tumour context while highlighting the value of our virus library approach for selecting optimal candidates.

One of the challenges in oncolytic virus therapy is the generation of anti-viral antibodies that tends to preclude repeat dosing after tumour recurrence. The sequential

administration of two immunologically distinct virus products could be a strategy to allow repeat dosing to address tumour relapse. Since we consistently found that IL12 expressing viruses provided the most therapeutic benefit compared to other cytokines, we used VV and HSV encoding IL12 in an adaptive therapeutic strategy. We reasoned that administration of a second immunologically distinct virus at the point that tumour recurrence was clearly evident may be a more productive approach than using virus therapy on a fixed schedule (**Fig. 4.6a-b**). To test this hypothesis, we conducted a proof-of-concept experiment using HSV-IL12 followed by VV-IL12 in an adaptive sequential injection strategy (n=5), tailored based upon tumor size (**Fig. 4.6c-d**). This approach was compared to reinjection of HSV-IL12 and a PBS control in the B16-F10 tumor model. The adaptive sequential injection of HSV-IL12 and VV-IL12 achieved a 100% complete response rate, a first in our study with the B16-F10 model (**Fig. 4.6e-f**). This outcome underscores the potential of strategically timed sequential, cytokine armed oncolytic virotherapy guided by tumor response dynamics, in achieving enhanced therapeutic efficacy.

Discussion

Random mutagenesis serves as a powerful tool for exploring functional genomics and advancing directed evolution studies. Nonetheless, applying this approach to poxviruses and herpesviruses is hindered by the complex and time-consuming nature of generation and enrichment of recombinant viruses. We combined the antiviral properties of anti-eukaryotic antibiotics with the Sleeping Beauty (SB) transposon, a widely used tool for creating genome-wide mutations in mammalian systems, to develop and implement a simplified, efficient insertional mutagenesis technique for large DNA viruses such as

poxviruses and herpesviruses. Rapid analysis of the libraries was made possible through the application of long-read nanopore sequencing, producing a comprehensive mutagenesis map in both Vaccinia Virus (VV) and Herpes Simplex Virus-1 (HSV-1). Mining this data allowed us to identify promising intergenic insertion sites in the Vaccinia virus genome that we showed facilitated stable introduction of transgenes without compromising virus growth. This may be particularly important for the design and construction of multiplex virus therapeutics^{42,43,164}. The creation of a second SB transposon with a different antibiotic resistance gene allowed us to generate compound libraries of mutagenized viruses. This dual antibiotic selection process resulted in an increased total number of insertions, particularly near the two ends of the VV genome. These 5' and 3' regions are known to encode host range genes and are quite diverse between members of the pox virus family^{31,188} whereas the central region of the genome encodes essential genes (e.g. polymerases, structural proteins) and is less tolerant of gene insertions¹⁸⁸.

Deletion or mutation of the ICP34.5 gene in oncolytic HSV-1 platforms is thought to be crucial for mitigating neurovirulence^{12,184}, however it significantly compromises virus fitness in both normal and tumour cells^{51,150}. Through our SB approach we observed particular regions of the HSV-1 genome that were very tolerant of transposon insertion suggesting that these could be targets for deletion that may not compromise virus growth. Indeed, two novel viruses: HSV-3941, with deletions from UL39 to UL41, and HSV-US24, with deletions from US2 to US4 demonstrated superior growth compared to viruses harbouring ICP34.5 deletions but remained safe following direct intracranial installation.

To fully exploit the potential of oncolytic viruses we argue that multiple, co-operative transgenes will be necessary to achieve therapeutic success in a spectrum of tumour

types. This may include the incorporation of cytokines^{93,187}, immune checkpoint blockers^{13,189}, T cell engagers^{190,191}, extracellular matrix degrading enzymes^{102,192} and/or and fusogenic proteins^{160,193}. However simple plug and play strategies may not identify optimum transgene combinations. This notion is supported by our observation that different cytokines can enhance therapeutic efficacy in two separate mouse models and argues for a library scanning approach to identify co-operative, broadly active transgene combinations. We and others have investigated the application of CRISPR-Cas9 to engineer recombinant viruses and while this is effective for small gene insertions, its efficacy drastically decreases for fragments larger than 3 kb^{104,167,185}. However, using conventional HDR vectors and our optimized antibiotic selection strategy we observed consistently, high efficiency insertion rates of large transgenes (5 kb or more) 70-80 times greater than using CRISPR-Cas9 technology. We also pushed the boundaries in homology arm length for virus engineering, reducing each arm to 100 bp without significant loss of efficacy.

To our knowledge, this is the first study that explored an array of VV and HSV-1 cytokine-expressing viruses. A number of different cytokines enhanced therapeutic efficacy of the two platforms with IL12 emerging as a top candidate in both viruses and two different tumour models. In previous studies using two different viruses expressing specific tumour antigens we and others have demonstrated that heterologous prime:boost strategies provided potent anti-tumour T cell responses and improved outcomes in mouse tumour models^{194–197}. We reasoned that using two immunologically distinct oncolytic viruses expressing IL12 could help to focus T cell responses on tumour antigens liberated during viral oncolysis. This concept of an in situ heterologous prime:boost strategy was tested using sequential HSV-IL12/VV-IL12 therapy. Remarkably we found this strategy led to complete responses in all treated animals.

Methods

Ethics

This study was conducted in accordance with the ethical guidelines set by the Ottawa Hospital Research Institute and the University of Ottawa, with due approval for the use of biohazardous materials (certificate GC317-125-12). All animal experiments were approved by the University of Ottawa's institutional animal care committee (Protocol IDs: OHRI2870 and MEe-2258) and were carried out in strict adherence to the standards of the National Institutes of Health and the Canadian Council on Animal Care.

Viruses

For our experiments, we utilized the Vaccinia TT strain, kindly provided by Dr. David Evans, and the Herpes Simplex Virus strain KOS.

Cell Culture

The cell lines employed in our study were sourced from the American Type Culture Collections (Manassas, VA). We cultivated these cells in either Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare Life Sciences; ON, CAN) or Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco; MA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco). The cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For co-cultures with effector cells, we used RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin (volume/volume) (Gibco), maintained under the same conditions. Regular PCR tests (e-Myo VALiD Detection Kit, 25239, LiliF Diagnostics; South Korea) ensured the cell lines were free from mycoplasma contamination.

Construct Design

Constructs were designed using Snapgene software, following protocols similar to our previous studies. Codon-optimized inserts for this research were synthesized by GenScript (Piscataway, NJ, USA).

Reagents

The following reagents were utilized in our experiments: Doxycycline (D9891-25G, Sigma-Aldrich), Puromycin (Thermo Scientific A1113803), Neomycin-Geneticin (Fisher Scientific 10-131-035), Zeocin (Invivogen ANT-ZN-05), Hygromycin B (Thermo Scientific 10687010), and Blasticidin (Thermo Scientific A1113903).

Immunohistochemistry

Immunohistochemistry (IHC) staining was conducted on formalin-fixed paraffin-embedded brain sections using the Leica Bond™ system. For rabbit antibodies, we used a modified protocol F, which omits the post-primary step when using rabbit antibodies on mouse tissue. Sections underwent heat-mediated antigen retrieval with citrate buffer (pH 6.0, epitope retrieval solution 1) for 20 minutes. The sections were then incubated at a 1:625 dilution for Rabbit HSV-1 (Abcam ab9533) and detected using an HRP-conjugated compact polymer system. Staining was done using DAB as the chromogen, followed by counterstaining with Hematoxylin, mounting, and cover slipping. The control for this experiment was cells +/- infected with HSV-1.

Mouse experiments

For animal studies we utilized Balb/c mice for the CT26WT model and C57BL/6 mice for the B16-F10 model. Both models, involving six- to eight-week-old female mice sourced from The Jackson Laboratory, Bar Harbor, ME, underwent subcutaneous injections on the right flank for tumor establishment. All procedures were conducted under the ethical guidelines and approval of the University of Ottawa animal care and veterinary services (MEe-2258-R5, or OHRIe-3340-A).

Virus production and titration

For Vaccinia virus production, HeLa cells in 850 cm² roller bottles were infected at an MOI of 0.03, maintaining the inoculation media. These cells were incubated at 37°C with 5% CO₂ for 72 hours, or until sufficient cytopathic effect was observed. Post incubation, cells were collected and resuspended in TNE buffer (pH 8.0), followed by three freeze-thaw cycles and centrifugation at $700 \times g$ for 10 minutes at room temperature. The supernatant was overlaid onto 36% sucrose cushions and centrifuged at $20,666 \times g$ for 1.5 hours at 4°C. The resulting viral pellets were resuspended in 1 ml of TNE buffer and stored at -80°C. For HSV-1 production, similar methods were used with Vero cells, but cell suspensions were resuspended in Phosphate Buffered Saline (PBS) post-harvest. Viral titers for both VV and HSV-1 were determined using U2OS cells. After tenfold serial dilutions of viral stocks and infection of U2OS cells in 12-well plates for 2 hours at 37°C and 5% CO₂, the medium was replaced with a 1:1 mix of 3% CMC and 2x DMEM supplemented with FBS. Following a 48-hour incubation, plaques were stained with crystal violet, and plaque-forming units per milliliter were calculated based on these observations.

Sequencing Library Data Analysis

The viral genome was sequenced using the Nanopore technology on the MinION sequencer, employing an R9.4.1 Flow Cell. The sequencing process generated raw .fast5 files of the viral genomes, which were then subjected to basecalling using Guppy v6.0.1. This step produced sequencing files complete with corresponding quality scores. The Sleeping Beauty transposon plasmid sequence was aligned with the viral sample sequence reads using BLAST command lines. We then pinpointed the insertion sites by analyzing the overlapping regions between the transposon and the viral genomes, employing the R programming language v4.3.2 for this purpose. The alignment and overlap data were visualized on the viral genome map using a custom script developed in the circlize package v0.4.15, facilitating a comprehensive view of the insertion landscapes within the viral genomes.

Statistical Analyses

All statistical analyses in this study were performed using GraphPad Prism version 9. Statistical tests applied included unpaired two-sample t-tests and two-way ANOVA, as appropriate to the data. The number of biological replicates (n value) for each analysis is indicated in the figure legends. Error bars in the figures represent either the standard deviation (SD) or the standard error of the mean (SEM), as specified in the legends. The significance levels for statistical tests were set as follows: not significant (ns) for $P > 0.05$, $*P < 0.05$, $**P < 0.003841$, $***P < 0.000125$, and $****P < 0.001$. These thresholds were consistently applied to assess the statistical relevance of the findings.

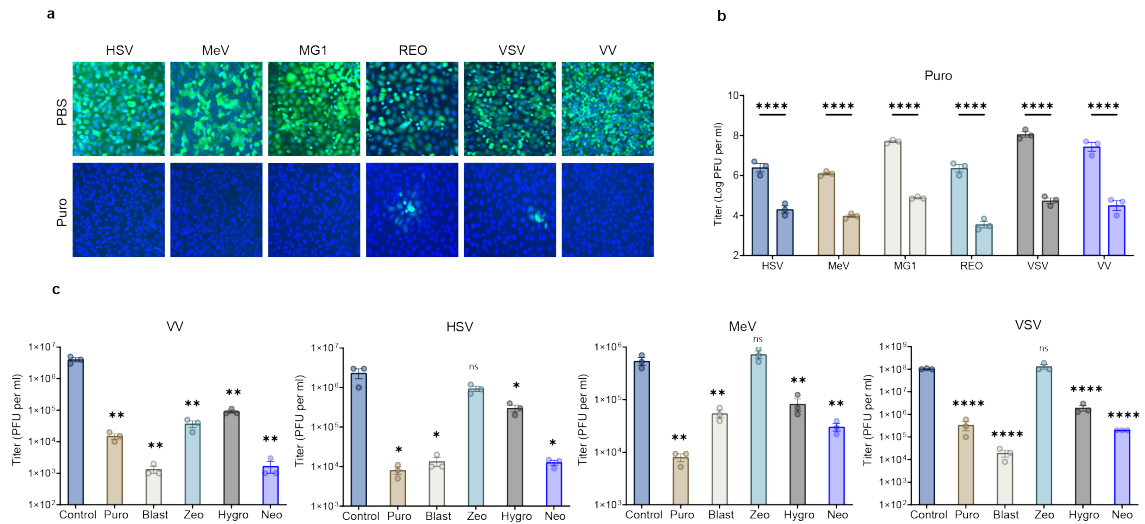


Figure 4.1: Using antibiotics to inhibit viral gene expression and replication.

(A) Fluorescence microscopy images captured at 10x objective 24 hours post treatment of U2OS cells (human osteosarcoma; blue) with 1 μ g/ml of puromycin (puro) or PBS (vehicle), either alone or in conjunction with GFP-expressing herpes simplex virus-1 (HSV), vesicular stomatitis virus (VSV), maraba virus (MG1), measles virus (MeV) or vaccinia virus (VV) at 0.1 MOI. (B,C) Viral titer (plaque forming unit per millilitre) in culture supernatants from U2OS cells 24 hours post antibiotics treatment with Puromycin (Puro; 1 μ g/ml), Blastcidin (Blast; 20 μ g/ml), Zeocin (Zeo; 1600 μ g/ml), Hygromycin (Hygro; 400 μ g/ml), Neomycin (Neo; 4000 μ g/ml), or PBS and virus, as described in (A), conducted on a monolayer of the respective producer cell line. Scale bars = 100 μ m in (a). Data indicate means $\pm$ SEM of three (b, c), four (h), or five (f) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

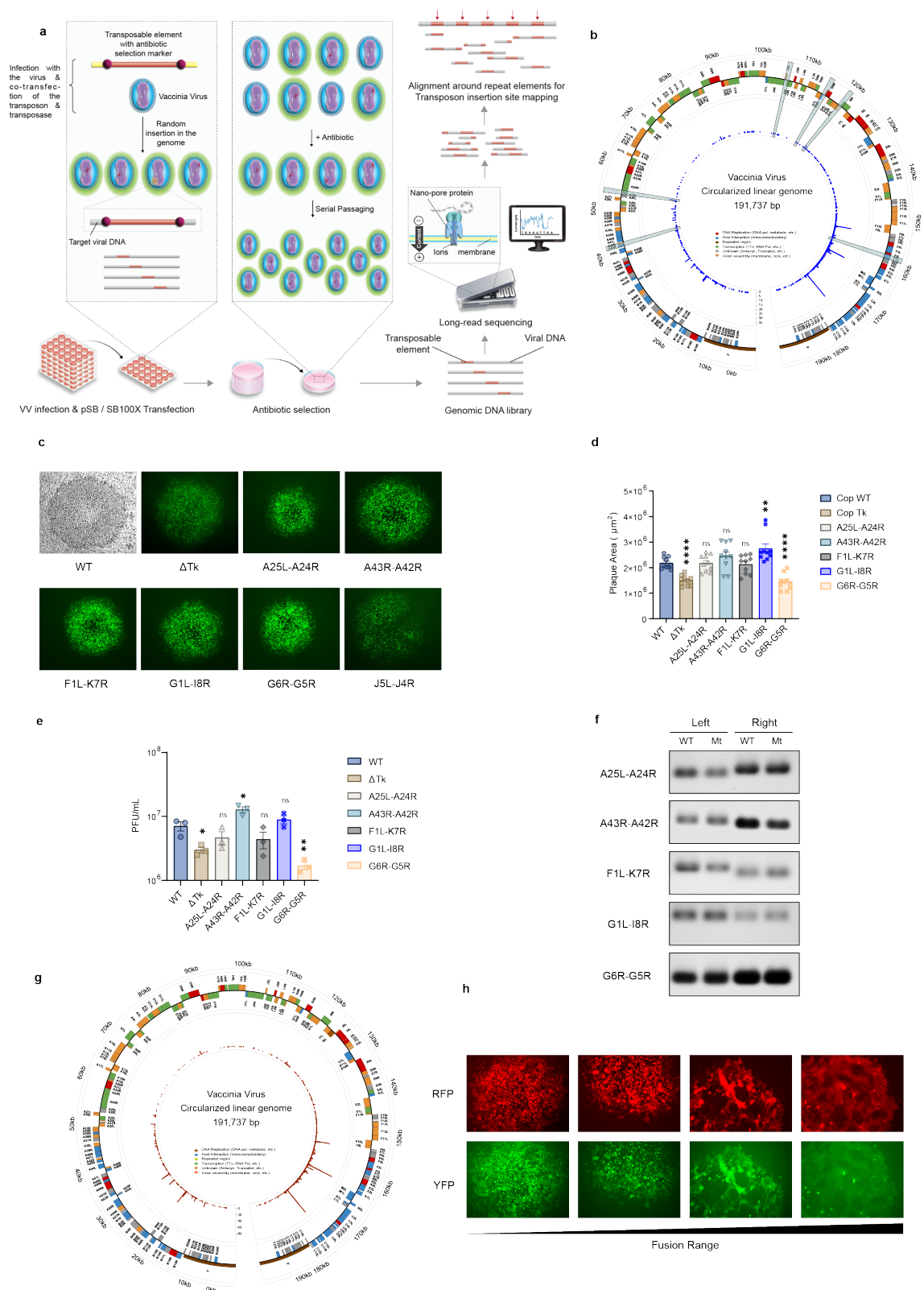


Figure 4.2: Exploring Novel Intergenic Insertion Sites and Multi-Transposon Integration in Vaccinia Virus Using a Sleeping Beauty Transposon and Antibiotic Selection Method.

(A) Schematic workflow of random mutagenesis in the VV using the Sleeping Beauty transposon system, followed by antibiotic selection and long-read nanopore sequencing to map the transposon insertion sites. (B) Circos plot mapping the distribution of Sleeping Beauty transposon insertions across the VV genome, with a focus on intergenic insertion sites. The blue-highlighted areas denote six regions with multiple insertions between adjacent genes or near gene stop codons. (C) Fluorescence microscopy images captured at 4x objective 48 hours post treatment of U2OS cells displaying yellow fluorescent protein (YFP) expression in Vaccinia virus plaques, following targeted insertion at different intergenic loci, compared to wildtype and Δ TK vaccinia virus variants. (D) Histogram comparing the viral plaque area of wild-type Vaccinia, Δ TK variant, and viruses with intergenic insertions. (E) Viral titer (PFU/mL) in culture supernatants from U2OS cells 24 hours post infection at MOI 0.01 with Vaccinia virus with transgene insertions at various intergenic loci, alongside wild-type and Δ TK variants. (F) Electrophoresis image displaying qRT-PCR products of genes adjacent to intergenic transgene insertion sites in wild-type and recombinant Vaccinia viruses. (G) Circos plot depicting the distribution of transposon insertions in the Vaccinia virus genome after two sequential insertion events. (H) Fluorescence microscopy images captured at 4x objective 48 hours post treatment of U2OS cells. Scale bars = 100 μ m in (a). Data indicate means $\pm$ SEM of three (e) to ten (d) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

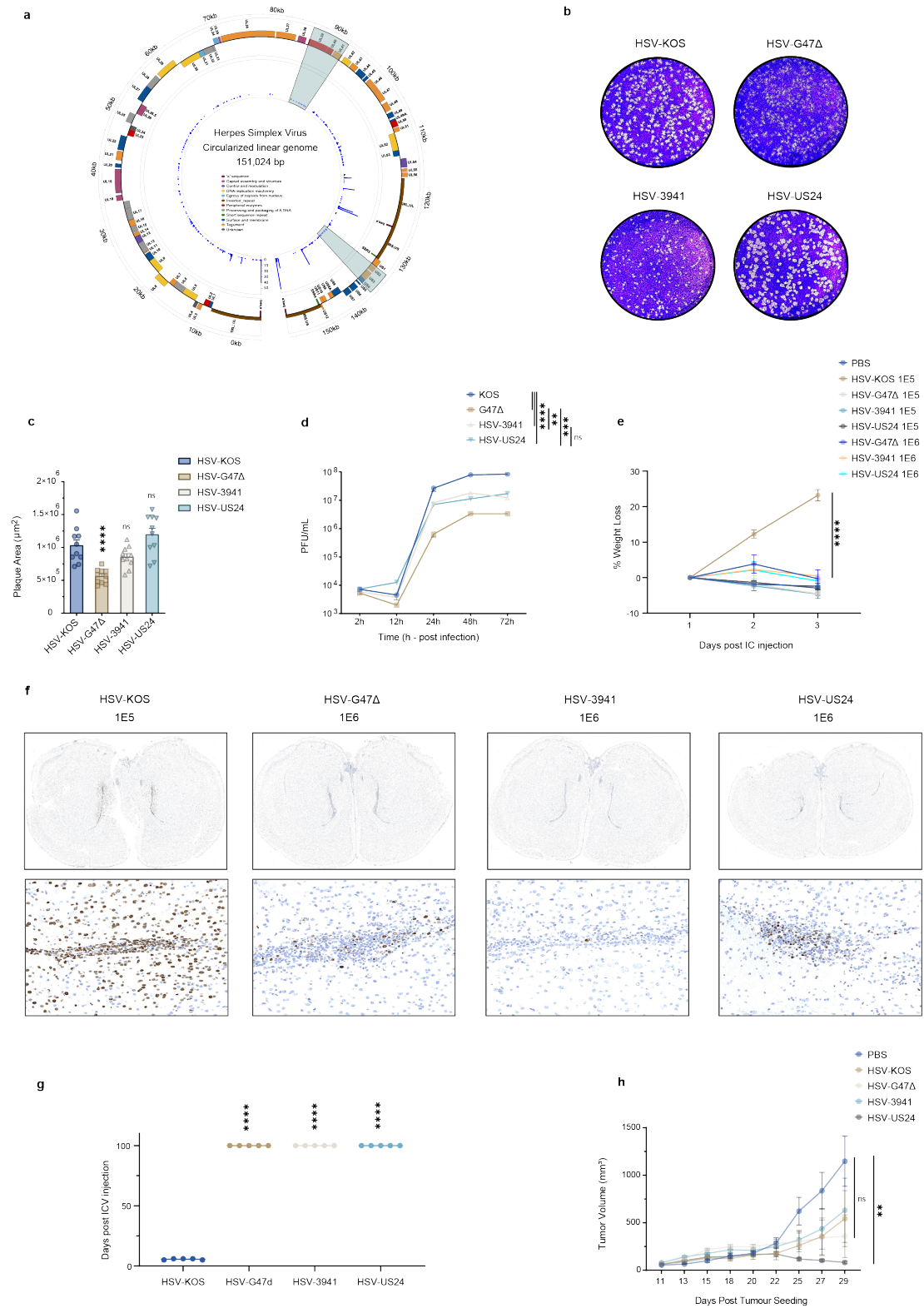


Figure 4.3: Identification and Evaluation of Novel HSV-1 Variants with Multiple Gene Deletions Using Antibiotic-Based Transposon Mutagenesis

(A) Circos plot illustrating the distribution and frequency of Sleeping Beauty transposon insertions in the HSV-KOS strain genome. Two dense insertion regions (blue), namely US2 to US4 and UL39 to UL41, were strategically utilized to generate novel oncolytic HSV1 variants, HSV-US24 and HSV-3941, through targeted genomic deletions. (B) Crystal violet-staining reveals variations in plaque size and morphology among HSV-KOS, HSV-G47 Delta (G47d), HSV-3941, and HSV-US24 cultured on a VERO monolayer. (C) The area of GFP positive HSV-KOS, G47d, HSV-3941, and HSV-US24 plaques on a VERO monolayer. (D) Multi-step growth curve measuring the number of HSV-KOS, G47d, HSV-3941, and HSV-US24 infectious virion (PFU/mL) produced by VERO infected at an initial MOI 0.01, before titering on a VERO monolayer. (E, F) The % of weight loss in BALB/c mice following intracranial injection of HSV-KOS, G47d, HSV-3941, or HSV-US24 at various MOIs, or PBS control (n= 4 animals). Animal weights were monitored over a three-day period, starting the day after the injections, providing an indication of the safety and neurovirulence of each strain. On day 3 brains were isolated and processed for immunohistochemistry staining using HSV-1 antibody. (G) Survival study of BALB/c mice following intracranial injection of G47d, HSV-3941, or HSV-US24 at 1E6, or KOS 1E5 control (n= 5 animals). (H) The volume of CT26 (murine colorectal cancer) tumors in BALB/c mice following intratumoral injection of HSV-KOS, G47d, HSV-3941, or HSV-US24 at 1E7 PFU, or PBS control. Data indicate means $\pm$ SEM of three (d), five (e-h) to ten (c) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

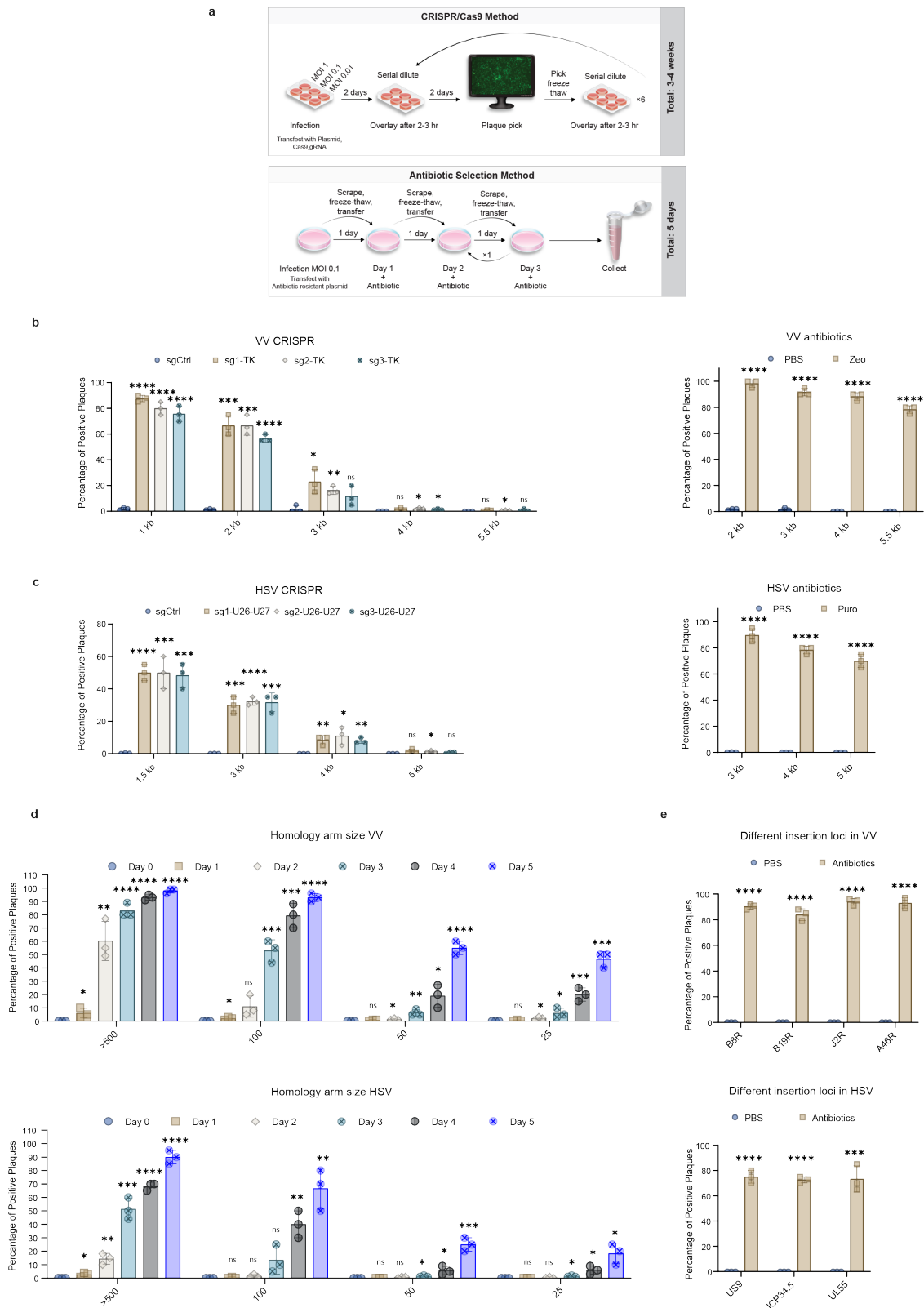
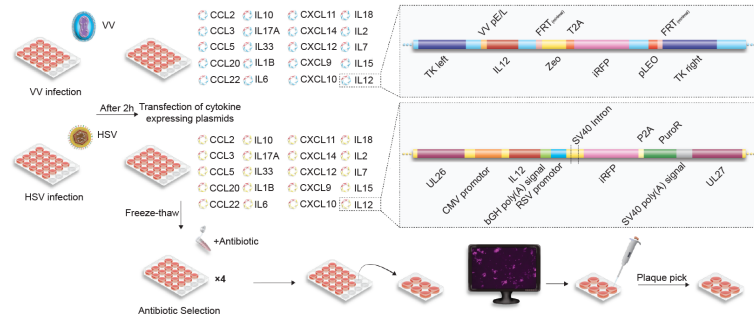


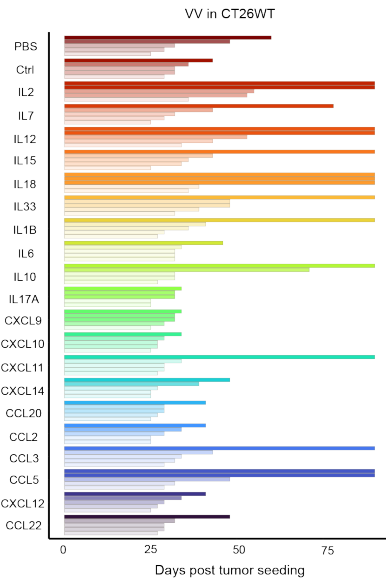
Figure 4.4: Rapid engineering of recombinant DNA viruses using antibiotic selection.

(A) Schematic workflow overview of CRISPR-Cas-9 mediated engineering of large DNA viruses and targeted rescue of viral constructs using an antibiotic selection method. (B,C) Percentage (%) of VV or HSV1 plaques positive for transgene expression after CRISPR/Cas-9 mediated gene insertion using distinct gRNAs alone, compared to the % of positive plaques achieved through targeted vector rescue via antibiotic selection over three consecutive days. Notably, the HSV1 vector employed a puromycin resistance marker, while the VV vector featured a Zeocin resistance marker. (D) The % of transgene positive plaques over a 5-days period post targeted rescue of recombinant VV or HSV1 engineered by the antibiotic-mediated selection method using linear DNA with varying homology arm lengths. Puromycin-resistant recombinant HSV1 expressed a transgene for yellow fluorescent protein (YFP), and Zeocin-resistant recombinant VV featured the expression of near-infrared fluorescent protein (iRFP) marker. (E) The % of recombinant virus plaques positive for yellow fluorescent protein (YFP) expression following 5 days of puromycin selection for transgene integration into various genomic locations of VV (B8R, B19R, J2R, A46R) and HSV-1 (US9, ICP34.5, UL55). Data indicate means $\pm$ SEM of three (b-e) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

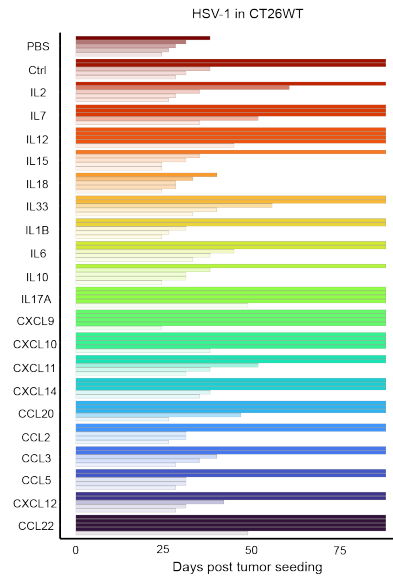
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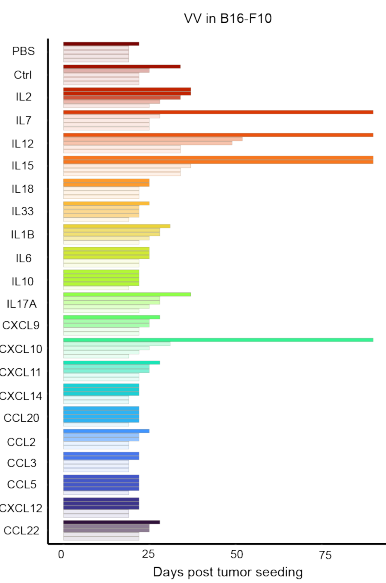
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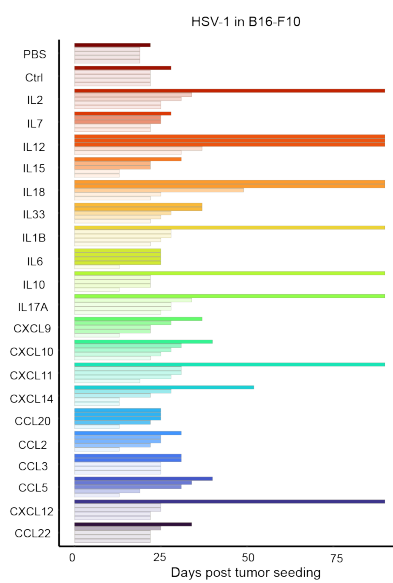


Figure 4.5: Targeted generation and In Vivo screening of a diverse library of HSV-1 and vaccinia viruses expressing cytokines

(A) A schematic detailing the process for parallel rescue of a cytokine library in Vaccinia and HSV-1 viruses. The diagram outlines the sequential steps of virus infection, plasmid transfection for individual cytokines, and iterative antibiotic selection leading to the isolation of pure viral populations expressing distinct cytokines. (B, C) Swimmer plots of the change in CT26 (murine colorectal cancer) tumor volume and survival in BALB/c mice. The animals received intratumoral injection (i.t.) of different cytokine-expressing VV or HSV-1 variants, control viral backbones, or PBS on days 11, 13, and 15 (n = 5 animals). (D, E) Swimmer plots of the change in the tumor volume and survival of C57BL/6J mice bearing subcutaneous B16-F10 (murine melanoma) tumors following i.t. Injection of different cytokine-expressing VV or HSV-1 variants, control viral backbones, or PBS on days 7, 9, and 11 post-tumor implantation.

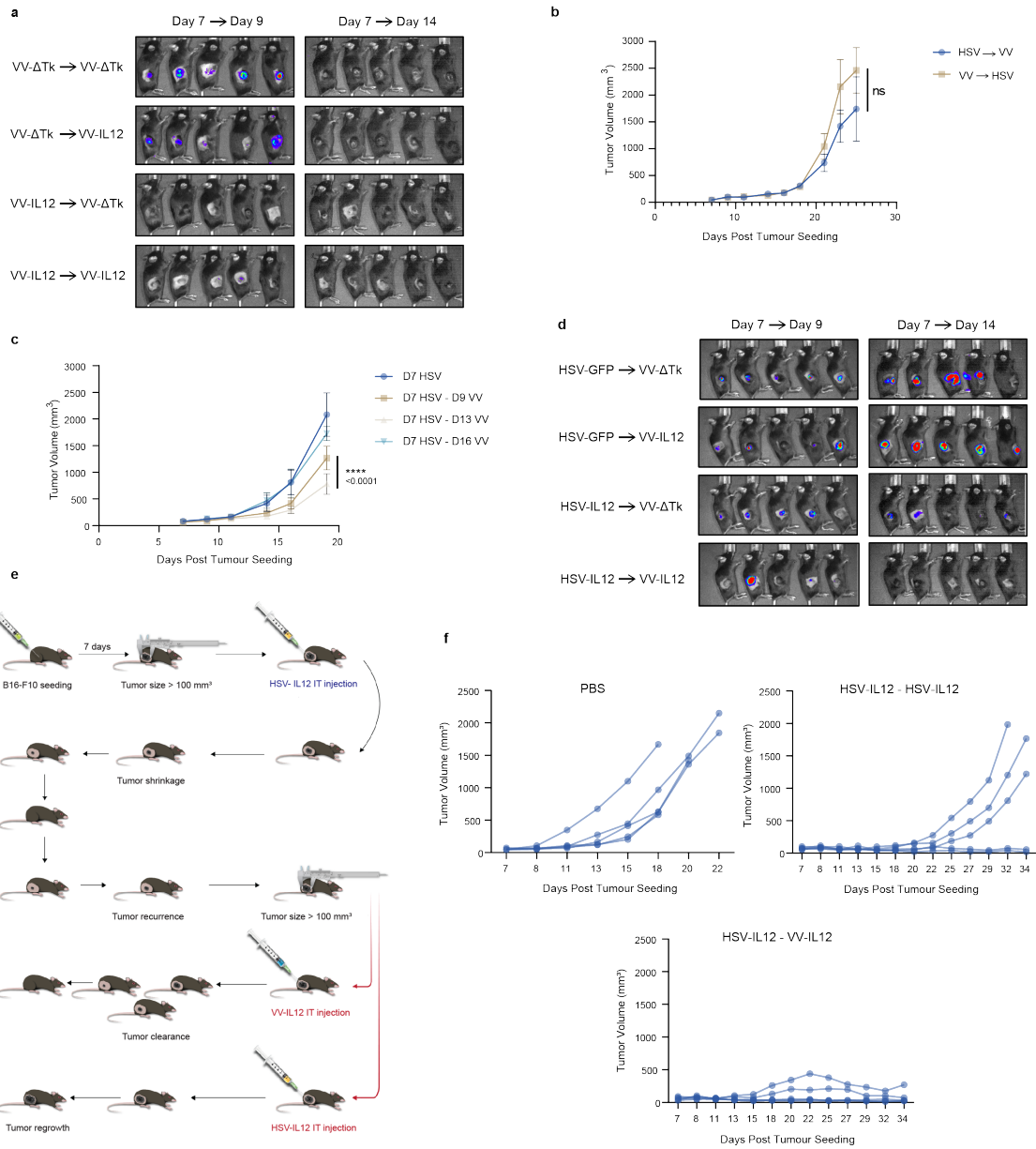


Figure 4.6: Sequential therapy of IL12 expressing viruses

(A) IVIS bioluminescent imaging showing C57BL/6J mice with subcutaneous B16-F10 tumors. Mice were initially injected with either VV-ΔTk or VV-IL12, both expressing luciferase, 7 days post-tumor seeding. A second injection was administered either on Day 9 or Day 14, with imaging conducted 24 hours after the second injection to assess the viral activity. (B) Graph depicting the volume of B16-F10 subcutaneous tumors in C57BL/6J mice post-intratumoral injection of control HSV and VV viruses. The injections were administered in alternating sequences of these viruses on days 7 and 9 post tumor seeding. (C) Tumor volume tracking in C57BL/6J mice with B16-F10 subcutaneous tumors, following a sequential intratumoral injection of HSV control virus on Day 7 and VV injections on day 9, 13, or 16 post-tumor seeding. (D) IVIS bioluminescent imaging illustrating the response in C57BL/6J mice with subcutaneous B16-F10 tumors. The initial injection was either HSV control or HSV-IL12, followed by a second injection of VV-ΔTk or VV-IL12, both expressing luciferase, at the same injection and imaging time points as in panel (A). (E) A schematic illustration depicting the tumor size adaptive sequential therapy approach using HSV-IL12 followed by VV-IL12. The diagram outlines the methodology and timing of injections, based on the adaptive response to tumor size changes, in the treatment of B16-F10 subcutaneous tumors in C57BL/6J mice. (F) Tumor volume measurements of B16-F10 subcutaneous tumors in C57BL/6J mice. The first injection, administered on Day 7 post-tumor seeding, consisted of either HSV-IL12 alone or a combination of HSV-IL12 and VV-IL12. The second injection, timed upon each tumor's recurrence and surpassing 100 mm³ in size, involved HSV-IL12, VV-IL12, or a combination of both. For comparison, a PBS control group received a single PBS injection on Day 7 post-tumor seeding. Data indicate means ± SEM of three (d, e) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

Chapter 5 – General Discussion

Challenges in Oncolytic Virus Development

The development of oncolytic viruses as cancer therapeutics has faced multifaceted challenges, including limitations in safety, efficacy, and the ability to engineer complex, multi-component platforms¹⁹⁸. This thesis advances the field by introducing novel tools and strategies to address these hurdles. We demonstrate the power of chemically inducible systems for precise control over viral replication and transgene expression, highlighting the importance of viral potency for optimal immune activation. Furthermore, we present a breakthrough methodology for rapid viral engineering and platform discovery, based on antibiotic selection for recombinant virus isolation. This versatile approach streamlines both random mutagenesis for functional genomics and targeted transgene insertion with increased efficiency. These advancements pave the way for the next generation of oncolytic viruses, poised to overcome limitations and unleash greater therapeutic potential in cancer immunotherapy.

Chemically Inducible Systems for Precise Control

As the field of oncolytic virotherapy progresses towards armed oncolytic viruses incorporating multiple transgenes^{50,199}, a critical challenge lies in the lack of well-characterized regulatory elements for precise control and tuning of transgene expression and viral replication. This control is essential for designing safe and effective treatment strategies. To address this, we developed three chemogenetic switches adapted for Vaccinia Virus (VV): the rapamycin-inducible ST7 RNA polymerase system, doxycycline-inducible expression, and cumate-inducible expression. These systems, individually or in combination, enable unprecedented control over both VV replication

and transgene expression. To the best of our knowledge, this is the first reported application of cumate- and rapamycin-inducible systems in a replicating viral vector, offering new avenues for tunable and precise application of therapeutic payloads in oncolytic viruses (OVs).

The versatility of these chemogenetic switches is evident in their diverse applications. For instance, they allow for the controlled expression of fusogenic proteins, which are beneficial for viral spread within the TME but could hinder virus growth if continuously expressed during production. Furthermore, these switches offer the ability to regulate viral replication itself by controlling essential viral genes, enabling applications such as generating safer vaccines (attenuated viruses that can replicate and induce an immune response but are rendered replication-incompetent if they escape the vaccination site) and creating conditionally replicating wild-type-like viruses (which can be turned off in case of safety concerns). Finally, these switches present a powerful tool for regulating the expression of cytokines, a crucial consideration to avoid the potentially life-threatening cytokine release syndrome (CRS) that can arise from continuous, uncontrolled expression of potent cytokines²⁰⁰. By combining tunable cytokine expression with localized delivery to the TME, we can harness the therapeutic potential of cytokines while minimizing the risk of systemic side effects. Our data demonstrates the feasibility of this approach, offering a promising strategy for incorporating cytokines into OV platforms.

While the cumate-controlled system demonstrated a favorable dynamic range, its lack of current FDA approval could hinder rapid clinical translation. Conversely, since both doxycycline and rapalogs are FDA-approved, their corresponding chemogenetic switches present a smoother regulatory path. Expanding the toolkit of regulatory elements will

empower the design of increasingly sophisticated synthetic circuits within viral vectors. To that end, we successfully engineered dual-control expression systems (ST7-CymR, ST7-TetR, and Cu-Tet), offering improved regulation and reduced basal expression. This has significant implications for the expression of highly potent payloads at effective but less toxic levels that could be toxic if expressed at high concentrations.

Though our focus was on VV, these conditional expression systems are readily adaptable to other viral vectors. We successfully demonstrated this in HSV-1. As each viral vector exhibits unique replication kinetics and cycles, a broader range of expression systems will allow researchers to tailor their choice to specific viral platforms and applications.

In conclusion, these viral expression switches represent a pivotal step towards the development of both conditionally replicating viral vectors and inducible transgene expression systems. This approach has the potential to significantly enhance clinical safety and efficacy by enabling the precise control of viral activity and transgene expression. As we continue to refine and expand this toolbox of regulatory elements, it will open up new ways of coordinating transgene expression and viral replication. This precise control could be one of the missing links to unlocking the full potential of currently used transgenes, allowing us to optimize their delivery and function within the TME. Imagine a scenario where a therapeutic cytokine is expressed only after successful viral infection and replication within the tumor, maximizing its efficacy while minimizing systemic side effects. Or, consider the possibility of tightly coordinating the expression of multiple transgenes in a defined sequence, creating a cascade of events that synergistically promote tumor cell killing and immune activation. These are just a few

examples of the exciting possibilities that lie ahead as we continue to refine this technology.

A Multi-pronged Platform: Controlled Virulence and IL-12 Expression

Chapter 2 introduced novel chemogenetic systems for dual regulation of transgene expression and viral replication. In Chapter 3, we capitalize on this flexibility by applying the TetR/TetO system to generate a conditionally replicating Vaccinia Virus (CRVV) platform. This approach allows for switchable virulence, demonstrating superiority over traditional gene-deletion attenuation strategies. Importantly, we provide compelling evidence that high-level viral replication, often downplayed in safety optimization, is crucial for optimal therapeutic outcomes. Our switchable CRVV alters the tumor immune landscape, favoring anti-tumor responses and increasing systemic immune activation.

Building upon the switchable virulence established with the TetR/TetO system, we further refined the platform by incorporating controlled expression of the potent cytokine IL-12, now under the regulation of the novel dual-control system (T7/TetR) developed in Chapter 2. This sophisticated design allowed us to investigate the interplay between viral replication dynamics and varying IL-12 levels in both immunocompetent and immunocompromised mouse models. We observe enhanced safety through increased immune clearance, particularly at high IL-12 expression levels. In multiple syngeneic models, this novel CRVV-R12 virus surpasses expectations, demonstrating both improved therapeutic efficacy and a remarkable safety profile. These studies underscore the potential of complex, chemically controlled oncolytic virus platforms for achieving potent immune activation alongside stringent safety considerations. Crucially, they emphasize the need to re-evaluate gene deletion as the means for improving viral safety.

Our findings suggest that controlled viral replication, achieved through inducible systems, offers a more nuanced and potentially safer approach compared to permanent attenuation via gene deletion.

Accelerating Viral Engineering and Platform Discovery

The development of effective oncolytic virus (OV) platforms has been hindered by the slow, laborious process of engineering complex viruses like HSV-1 and VV^{106,165}.

Chapter 4 addresses this bottleneck by introducing a breakthrough methodology that harnesses the antiviral properties of common mammalian cell selection antibiotics. This approach offers a two-pronged advantage. Firstly, by coupling antibiotics with the Sleeping Beauty transposon, we developed a rapid, high-throughput insertional mutagenesis method for functional viral genomics studies. This allows for the generation of large libraries of recombinant viruses, enabling platform discovery and the identification of novel phenotypes and optimal transgene insertion sites – a process previously unfeasible for these complex viruses.

Secondly, this antibiotic-based selection significantly accelerates targeted OV engineering. We demonstrate its efficiency by inserting diverse homology-directed repair (HDR) constructs into viral genomes, outperforming CRISPR-Cas9^{60,104} by a significant margin, especially for larger inserts. This technique empowers researchers to parallelize the engineering process. As proof of concept, we created a library of 40 cytokine-expressing OVs across both VV and HSV-1 backbones¹⁸⁷. Interestingly, our results showcase the context-dependent nature of cytokine activity within the tumor²⁰¹; even traditionally immunosuppressive cytokines²⁰² displayed unexpected efficacy in specific combinations and viral backbones. This work underscores the need for comprehensive

screens and highlights the value of our streamlined engineering method in facilitating such studies.

Focusing on IL-12, a highly potent cytokine with demonstrated efficacy in various OV settings, we explored combination therapies using our library of OVs. While mixed viral delivery produced no added benefit, a novel adaptive sequential therapy approach, employing both VV-IL12 and HSV-IL12 at optimized intervals, yielded remarkable results. This finding underscores the need for nuanced temporal control strategies in OV combination therapies to overcome limitations arising from antiviral responses and optimize synergy between different platforms and transgene activities.

Future Directions and Outlook

Addressing Leakage in Inducible Systems for Potentially Toxic Payloads

While the developed inducible systems offer significant control, addressing residual leakage is crucial for regulating potentially toxic payloads like apoptotic factors or antiviral genes. These factors are designed to kill cells, including the virus itself, and their unintended expression during production can significantly decrease viral load^{174,203}. Therefore, minimizing leakage is essential to ensure high viral titers during production, while still allowing for their activation within the TME where their cytotoxic effects can not only eliminate infected tumor cells but also induce bystander killing and enhance immunogenicity.

Immune Profiling of Multi-Component Platforms: Deep Dive Beyond IL-12

Deeper studies on complex, multi-component platforms are essential. IL-12 served as a powerful example in this thesis, but the potential extends far beyond this single cytokine¹⁸⁷. A vast array of immunomodulatory molecules hold immense promise for oncolytic virotherapy, including immune checkpoint blockers¹³, bispecific T cell engagers^{190,204}, and TME modifiers^{192,205}. These molecules can exert distinct effects on the tumor at different levels of expression, and their impact can be even more nuanced than that observed with IL-12. To fully understand their effects, we need to leverage the inducible control systems to study them across a range of expression and concentration levels. This will allow us to dissect their individual contributions, identify potential synergies, and optimize their therapeutic effects within the context of a multi-component platform.

Unveiling Mechanisms Through Deep Immune Profiling

Comprehensive immune profiling is crucial to address the intricate questions surrounding these multi-component platforms. One often-overlooked aspect is immune kinetic studies^{206,207}. Traditionally, researchers collect immune profile data at a single time point, often between 3-12 days post-virus injection^{157,208}. However, a more comprehensive understanding can be gained by examining the trajectory of the immune response over time. By tracking the dynamic changes in the TME and the systemic immune response, we can glean deeper insights into the effects of these transgenes and their combinations with the virus⁶. Furthermore, single-cell RNA sequencing (scRNA-seq) of the TME offers unparalleled resolution, delving beyond basic immune markers to reveal the heterogeneity of cellular populations^{209,210}. If possible, incorporating spatial scRNA-seq

would provide an even more detailed picture, allowing us to visualize the spatial distribution of these diverse cell types within the tumor²¹¹.

Balancing Immunogenicity and Viral Persistence

The short-lived transient nature of current OV_s in the TME presents a challenge when using inducible systems with transgenes that enhance immunogenicity. While these transgenes can potentially activate the immune response, they can also lead to faster clearance of the virus itself, creating a negative feedback loop that limits their therapeutic effect^{6,149}. To overcome this hurdle, strategies to extend viral persistence within the TME are crucial⁸⁹. A longer viral half-life would not only allow for sustained expression of immunomodulatory transgenes but also provide a window of opportunity for multi-day treatment regimens. These regimens could involve strategically turning transgenes on and off over time to optimize the immune response and maximize therapeutic efficacy.

Expanding and Optimizing Antibiotic-Based Engineering

The new antibiotic-based selection methodology developed in Chapter 4 offers a significant leap forward in viral engineering, ushering in an era of faster, more streamlined construction of complex OV_s. While this system represents a major breakthrough, there's still room for improvement. The ultimate goal remains the ability to synthesize entire viral genomes *de novo*^{212,213}. However, until we have gene synthesis machines capable of generating these large genomes in a single shot with an affordable cost²¹⁴, we must continue to refine current methods. One key area for advancement lies in expanding the selection of antibiotics employed. Utilizing antibiotics with smaller resistance genes will minimize the burden placed on the viral genome, ensuring the stability and safety of the engineered virus. Furthermore, streamlining antibiotic and marker removal with site-specific recombinases, potentially engineered into the

production cell line, could create a hands-free process, maximizing the efficiency and fidelity of OV production^{215,216}.

Harnessing Viral Libraries for Platform Discovery

Previous approaches to directed evolution of large DNA viruses often relied on mixing multiple naturally occurring strains and serial passaging in cancer cells, hoping for spontaneous emergence of cancer-specific variants^{38,39,72}. Apart from safety concerns, this method is inefficient for generating features relevant to in vivo performance⁷⁸. The antibiotic-based engineering tools introduced in this thesis provide a new way to overcome the bottleneck of generating complex viral libraries. Coupling these libraries with sophisticated artificial evolutionary environments opens up exciting possibilities for selecting mutants optimized for traits like IV delivery, viral persistence, enhanced immunogenicity, and potent tumor killing. This approach paves the way for the development of novel OV platforms with features specifically tailored for optimal therapeutic efficacy.

Adaptive Sequential Therapy and the Need for Translational In Vitro Models

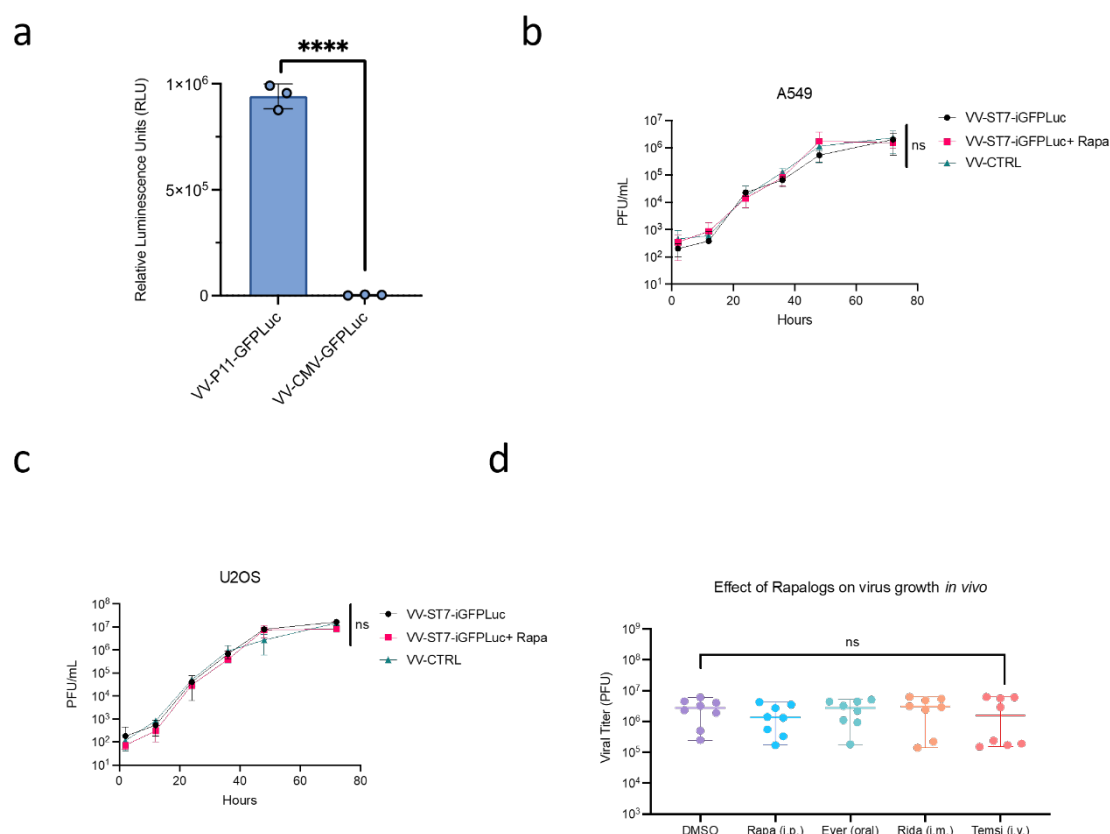
The adaptive sequential therapy approach, demonstrated with IL-12, holds immense potential for unlocking the power of combinational OV therapy, where multiple viruses with distinct functionalities are strategically combined to achieve synergistic anti-tumor effects^{156,217}. Future studies must fully leverage viral libraries to explore potent combinations of both viral backbones and transgenes, tailoring regimens to specific malignancies. However, conducting large-scale sequential therapy experiments remains challenging due to their time-consuming, labor-intensive, and expensive nature. This underscores the urgent need for better predictive in vitro models²¹⁸. Tumoroids, recapitulating tumor architecture and immune infiltrate, offer a promising solution.

Integrating tumoroid-based screening will accelerate the discovery process, making it faster, more cost-effective, and highly translational^{219–221}.

Conclusion

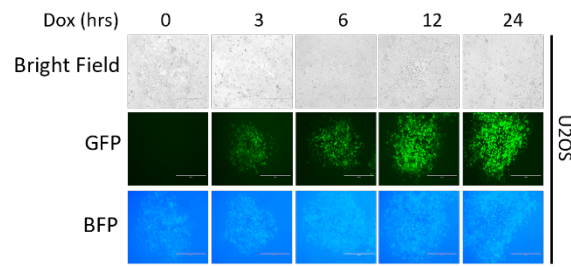
This thesis introduces a suite of novel tools and strategies in an effort to improve and accelerate the engineering of larger DNA viruses as OV platforms. By developing chemically inducible systems for precise control of viral replication and transgene expression, we establish a foundation for safer, more potent OV platforms. The ability to modulate viral activity and therapeutic payloads within the tumor creates opportunities for complex and nuanced treatment regimens. Furthermore, our antibiotic-based viral engineering methodology revolutionizes both platform discovery and the creation of sophisticated multi-component OVs. Taken together, the work presented in this thesis empowers researchers to build the next generation of oncolytic viruses. The future holds promise for precisely targeted therapies that expertly balance safety with potent immune activation, achieving transformative results in cancer immunotherapy.

Appendices I – Chapter 2 Supplemental information

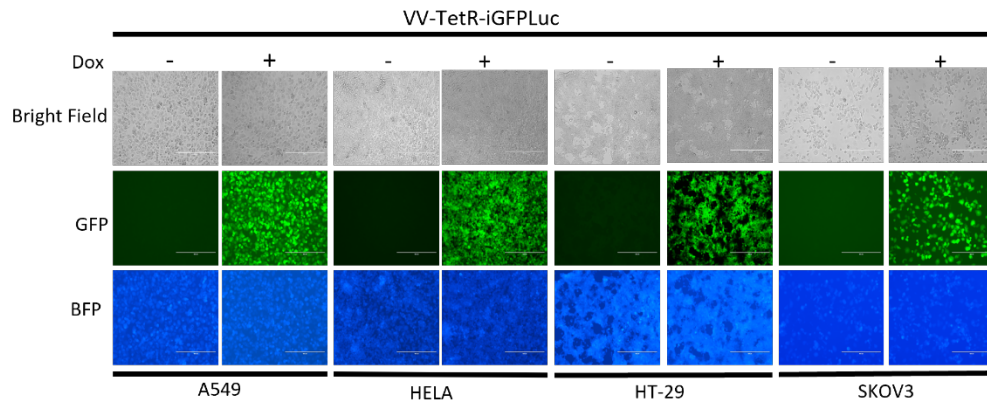


Supplementary Figure 1. (a) Compared to the P11 vaccinia virus promoter, the CMV promoter in vaccinia virus exhibited significantly lower activity. (b-c) The ST7 switch, and rapamycin have no significant effects on virus growth kinetics Multistep growth curves of VV-ST7-iGFP-Luc compared to control vaccinia virus at different time points from (b) A549 or (c) U2OS cells infected at MOI 0.01 in the presence or absence of 10 nM rapamycin. (d) Quantitation of viral titers within HT-29 tumors from CD-1 nude mice 10 days after Rapalogs were introduced into the mice. Tumors were infected with VV-ST7-iGFP-Luc at 1E7 PFU/tumor prior to the introduction of Rapalogs. Data indicate means $\pm$ SD of three biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

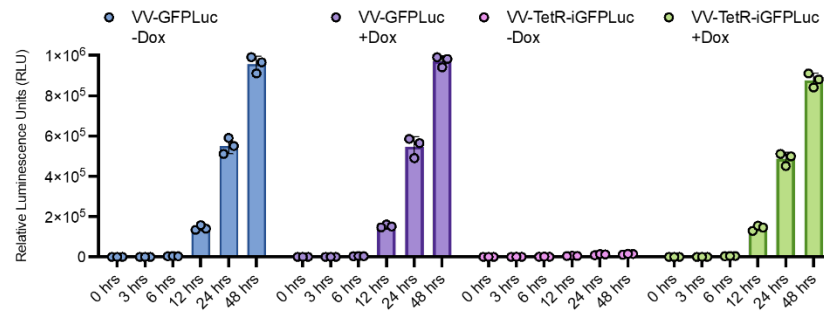
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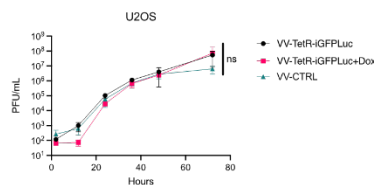
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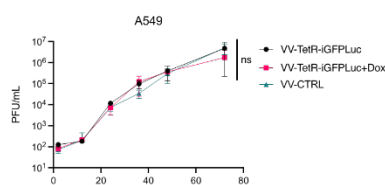
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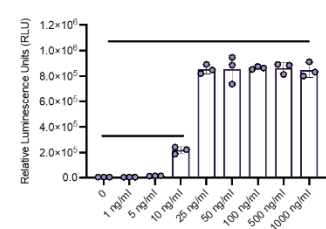
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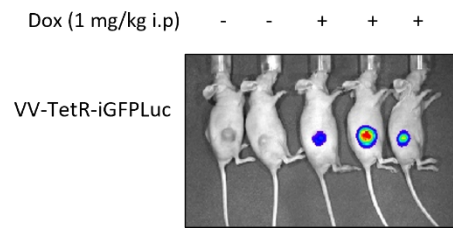
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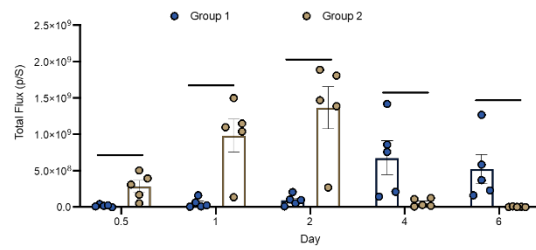
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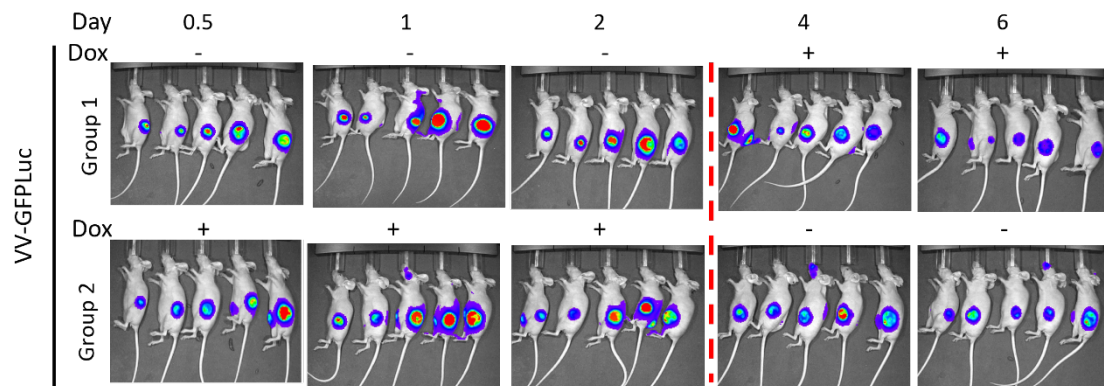
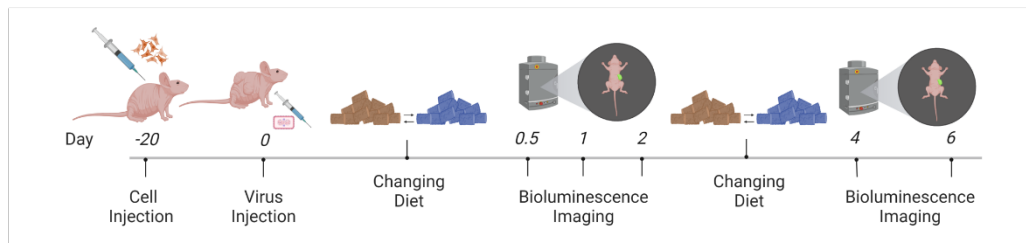
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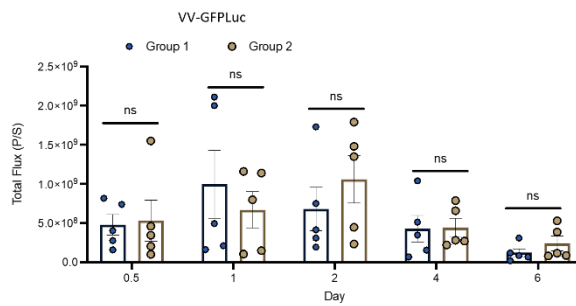
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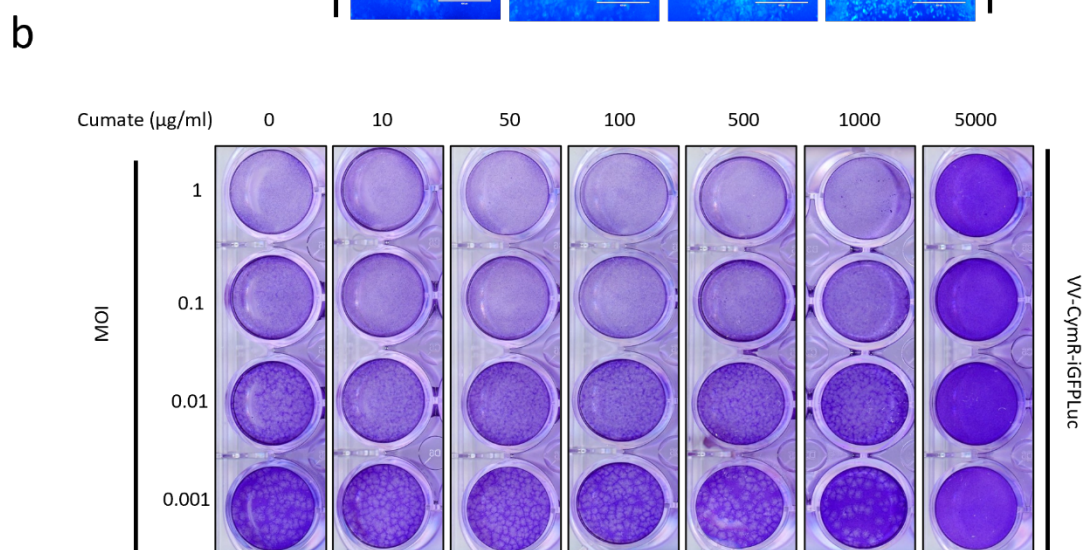
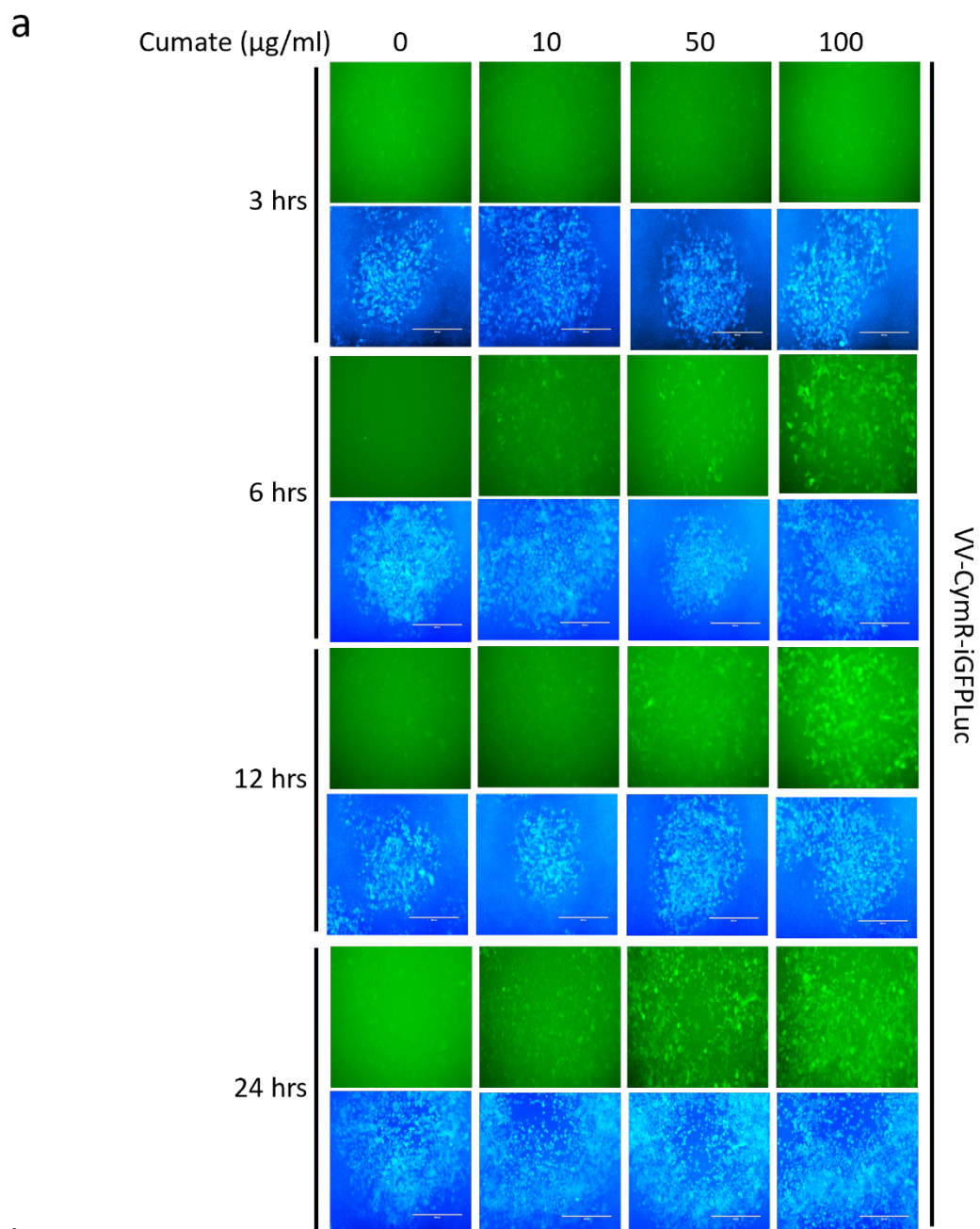
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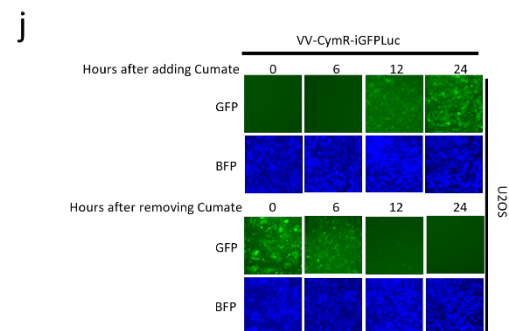
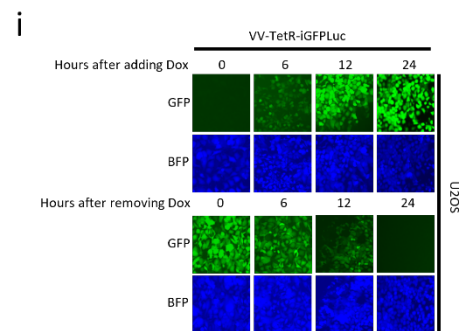
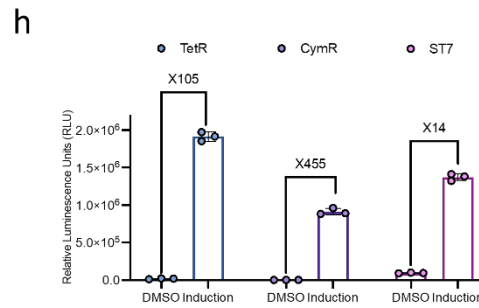
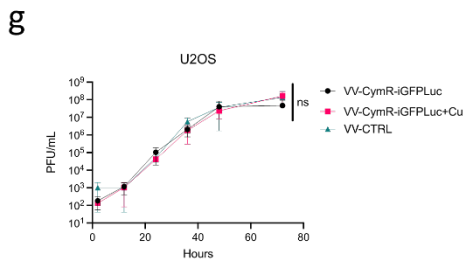
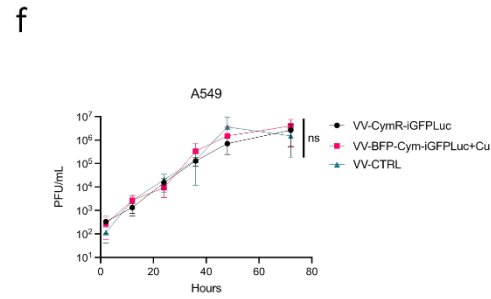
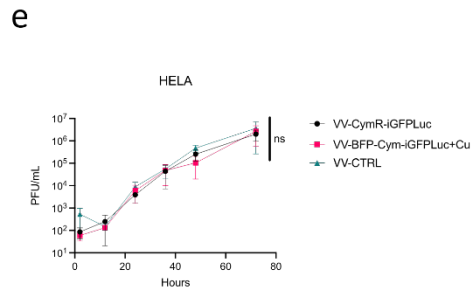
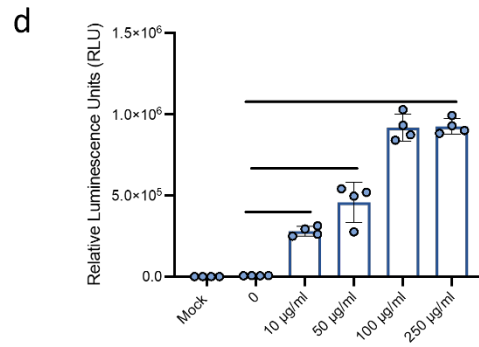
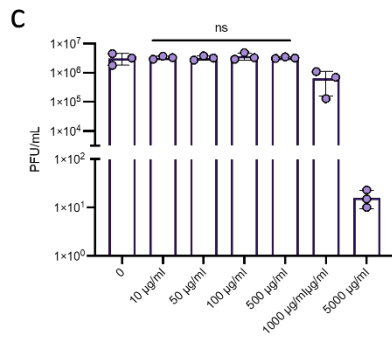


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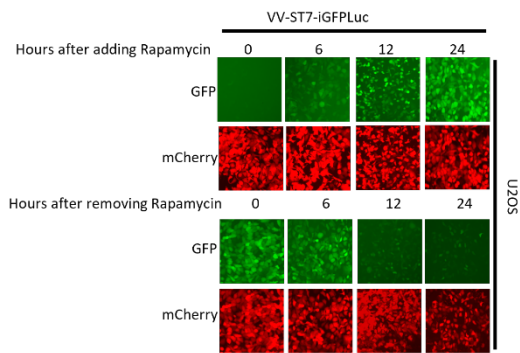


Supplementary Fig 2. *In vitro* and *in vivo* validation of the doxycycline chemogenetic switch (a) Representative images of U2OS cells at different time points following infection at MOI 0.01 with VV-TetR-iGFPLuc in the presence of 100 nM Dox. BFP signal, under continuous expression from vaccinia, indicates the presence of virus. (b) Representative brightfield and fluorescent images of different cell lines after infection at MOI 0.1 with VV-TetR-iGFPLuc in the absence or presence of 100 ng/ml Dox. (c) When comparing the expression pattern of GFPLuc directly transcribed by the wild-type vaccinia virus promoter with the doxycycline-controlled promoter, comparable levels of expression were observed between the two promoters in the presence of Dox. In some samples, Dox was added at the beginning of the experiment. (d, e) Multistep growth curves of VV-TetR-iGFPLuc compared to the control vaccinia virus at different time points from U2OS and A549 cells infected at MOI 0.01 in the presence or absence of 100 ng/ml Dox. (f) Quantitation of luciferase signal (RLU) from U2OS cells after 24 hours of infection with VV-TetR-iGFPLuc in the presence of different concentrations of Dox. (g) IVIS imaging 24 hours post viral infection and drug administration. HT-29 tumors were infected with VV-TetR-iGFPLuc at 1E7 PFU/tumor and were treated with 1 mg/kg of Dox. (h) Luciferase signal was quantified at various timepoints from HT-29 tumors infected with VV-TetR-iGFPLuc (1E7 PFU/tumor) following introduction of Dox into mice diets. Mice were given the Dox diet either 2 days post viral infection (Group 1, blue bars) or immediately following viral infection (Group 2, brown bars). For Group 2, Dox was removed from the diet after 2 days. Data shows the average total luciferase signal emitted from the tumor area. (i) IVIS imaging of HT-29 tumors following infection with VV-GFPLuc (1E7 PFU/tumor). Dox was introduced into the diet according to the schedule shown and groups assigned as described in panel G. (j) Quantitation of luciferase signal (measured as RLU) from HT-29 tumors following VV-GFPLuc infection and administration of Dox. Data shows the average total luciferase signal emitted from the tumor area. Scale bars = 40 μ m in A & B. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

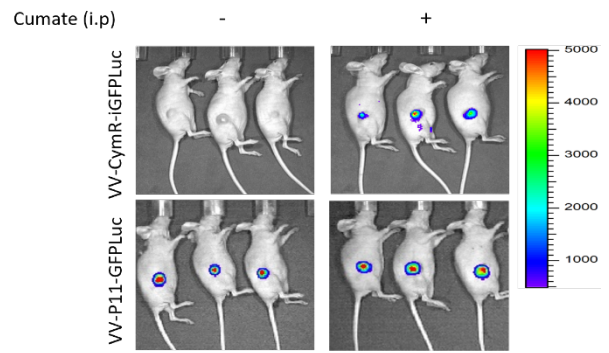




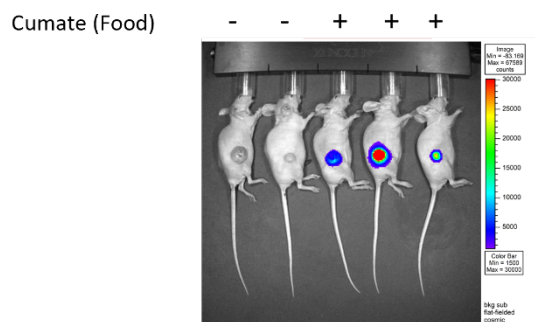
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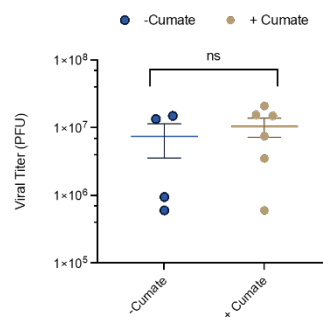
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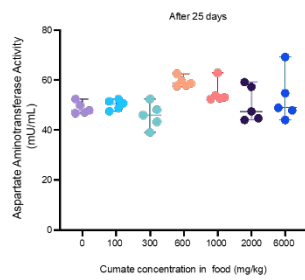
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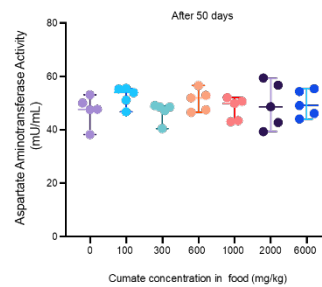
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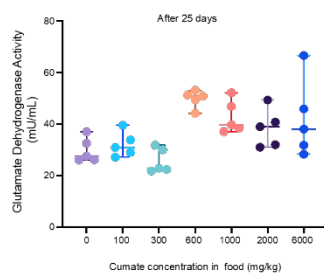
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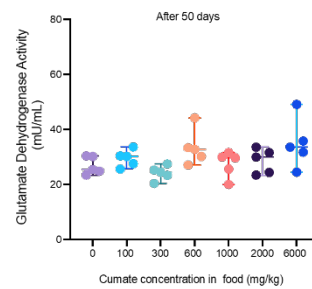
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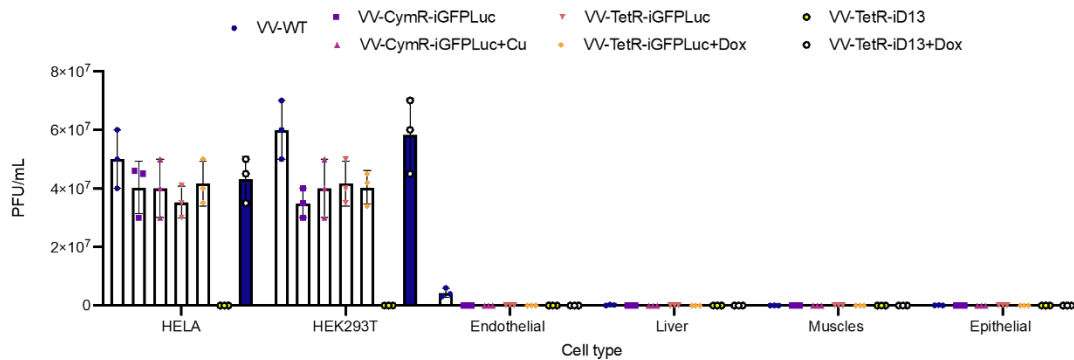


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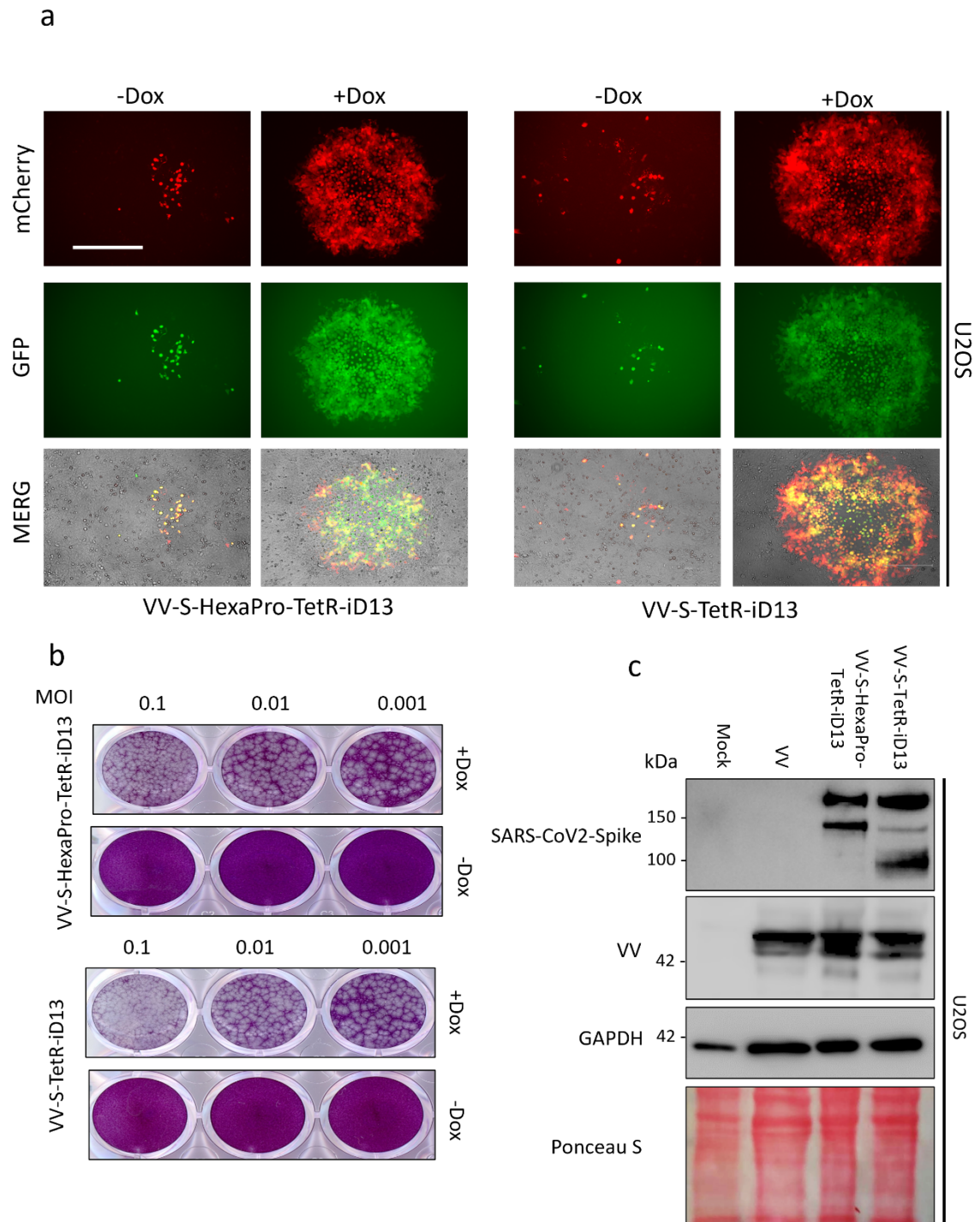


Supplementary Fig 3. Optimization of the CymR switch and comparison to ST7 and TetR systems. (a) Representative fluorescent images of U2OS cells at different time points after infection with VV-CymR-iGFPLuc (MOI 0.1) in the presence of different cumate concentration. BFP signal indicates viral infection. (b, c) U2OS cells were infected with VV-CymR-iGFPLuc at different MOIs in the presence of various concentrations of cumate for 24 hours. Cells were stained with crystal violet to detect viral plaques and viral load was determined for each group. (d) Luciferase activity was quantified in U2OS cells 24 hours following infection with VV-CymR-iGFPLuc in the presence of different concentrations of cumate. (e-g) Multistep growth curves of VV-CymR-iGFPLuc compared to the control vaccinia virus at different time points using Hela, A549, and U2OS cells infected at MOI 0.01 in the presence or absence of 100 ug/ml cumate. (h) Luciferase signal was quantified in U2OS cells 24 hours after infection with either VV-TetR-iGFPLuc, VV-CymR-iGFPLuc, or VV-ST7-iGFPLuc at an MOI 0.1 with or without induction. (i-k) Representative fluorescence images of U2OS cells infected with either VV-TetR-iGFPLuc, VV-CymR-iGFPLuc, or VV-ST7-iGFPLuc (MOI 0.1) and induced with Dox (100 ng/ml), cumate (100 ug/ml) or rapamycin (10 nM), respectively. Images were acquired at multiple time points and depict the presence of the inducible GFP fusion protein and viral infection (BFP or mCherry). (l-n) IVIS imaging and quantitation of viral load in HT-29 tumors injected with VV-GFPLuc or VV-CymR-iGFPLuc (1E7 PFU/tumor). When tumors reached ~150 mm³ in size, cumate was injected intraperitoneally (1 mg/kg) or administered via diet (6000 mg/kg). (o-r) C57BL/6 mice were fed a cumate diet with varying amounts of cumate. Serum was collected after 25 days, and 55 days post treatment and quantitation of Aspartate Aminotransferase and Glutamate Dehydrogenase activity by ELISA was performed. Scale bars = 40 μ m in panel B. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

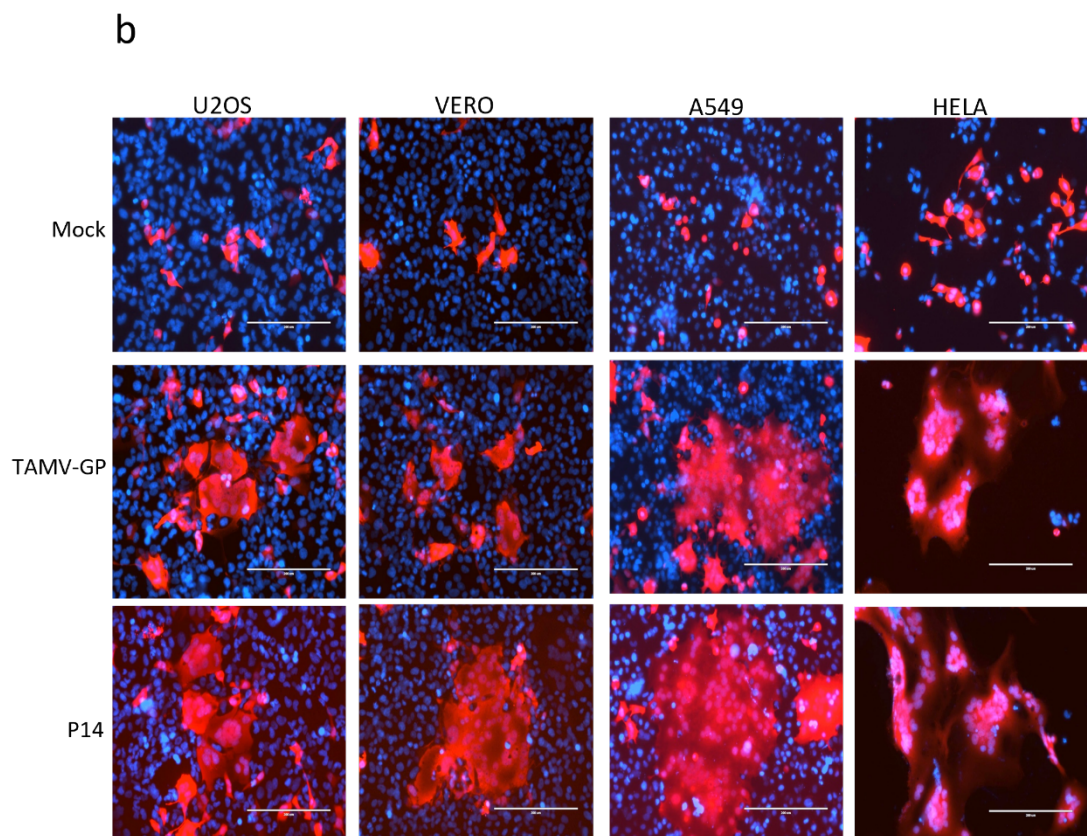
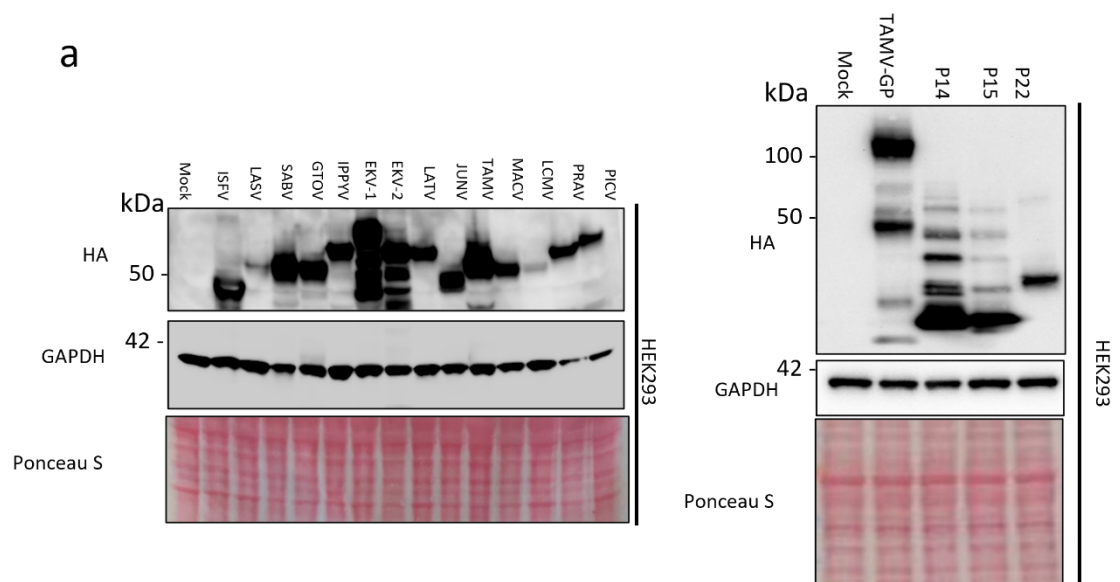
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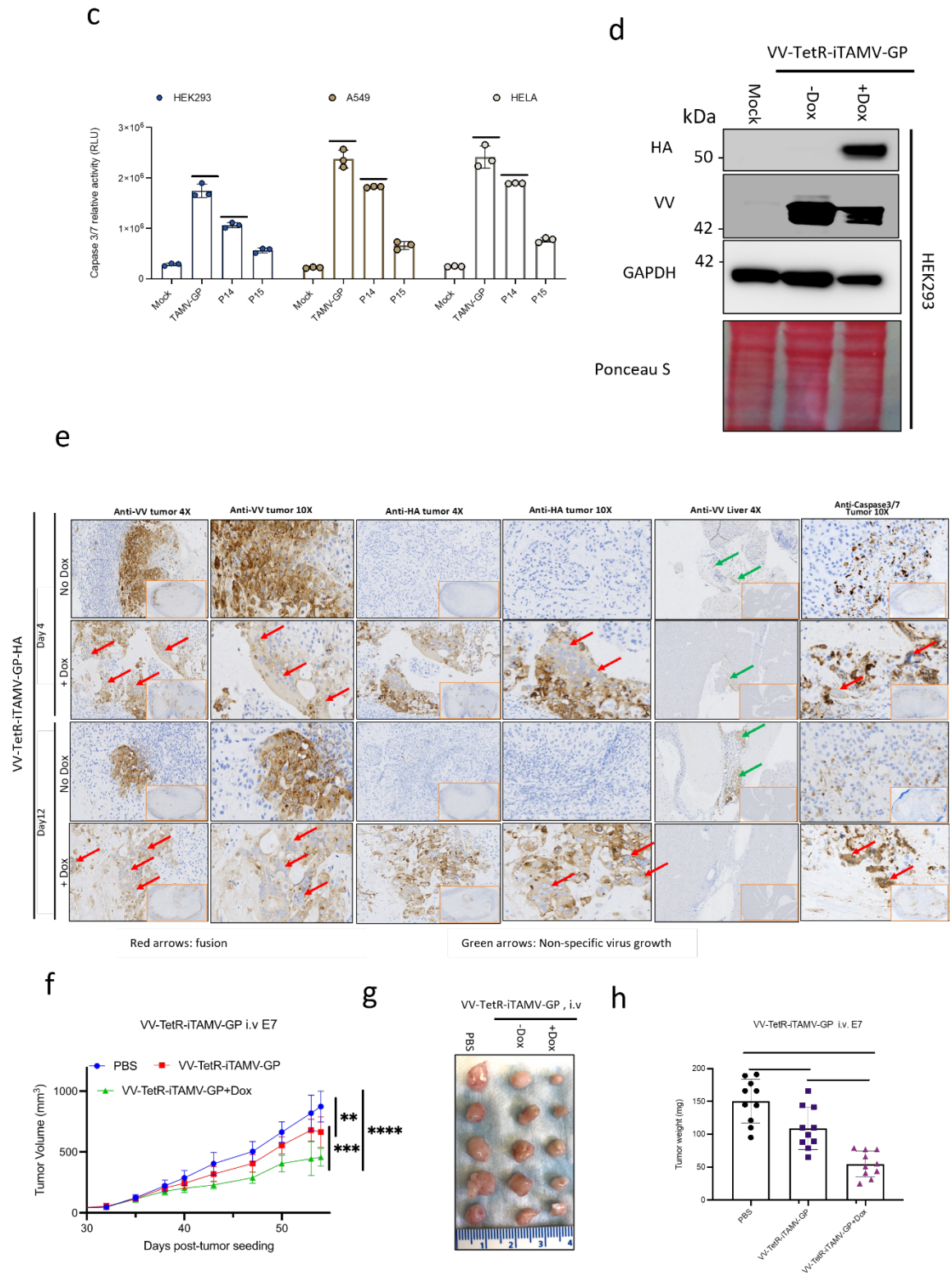


Supplementary Figure 4. In vitro validation of the conditional growth of the doxycycline inducible vaccinia virus (a) U2OS cells were infected at different MOIs with VV-TetR-iD13 or control vaccinia virus in the presence or absence of 100 ng/ml Dox. After 48 hours, plates were stained with crystal violet and representative pictures were taken. (b, c) A549, Hela, HT29, and SKOV3 cells were infected with control vaccinia virus or VV-TetR-iD13 at MOI 0.01 in the absence or presence of Dox at 100 ng/ml. After 48 hours, representative brightfield and fluorescent images were taken. Quantification of luciferase signal (RLU) is shown. (d) the viruses with chemogenic switch replicate 100 to 1000 times less efficiently in human primary cells when compared to cancer cells. Scale bars = 100 μ m in panel B. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

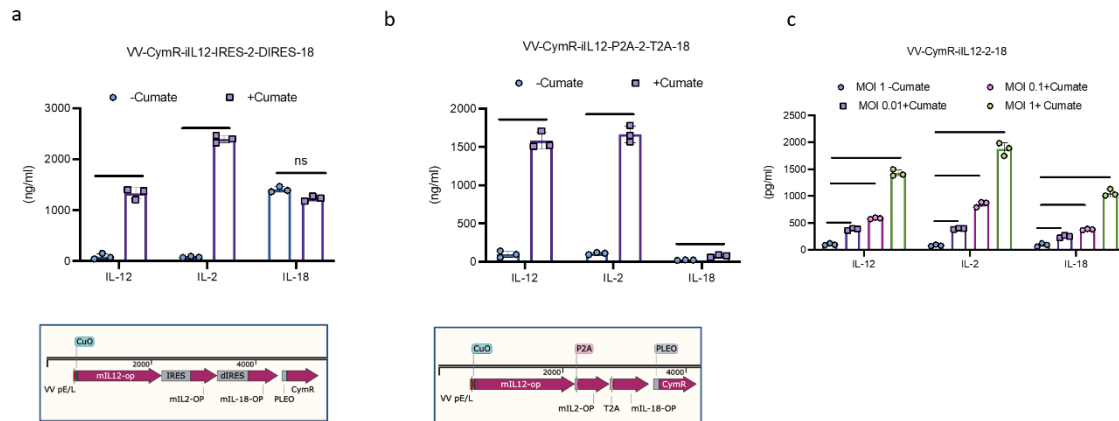


Supplementary Figure 5. In vitro validation of the growth and antigen expression of the vaccine candidates. (a) Images of U2OS cells infected with either VV-S-TetR-iD13 or VV-S-HexaPro-TetR-iD13 (MOI 0.01), in the presence or absence of 100 ng/ml Dox after 48 hours. The bottom row represents overlays of the red, green and brightfield channels. (b) U2OS cells were infected at different MOIs with either VV-S-TetR-iD13 or VV-S-HexaPro-TetR-iD13 in the presence or absence of 100 ng/ml Dox. After 48 hours, plates were stained with crystal violet and representative pictures were taken. (c) U2OS cells were infected with control or vaccine viruses at MOI 1 and samples were lysed and collected after 24 hours. Western blotting was performed to detect SARS-CoV2 Spike and vaccinia virus. Scale bars = 200 μ m in panel A. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.





Supplementary Figure 6. In vitro validation and in vivo application of conditionally expressed TAMV-GP (a) Western blotting analysis of HEK293 cells transfected with different arenavirus and reovirus glycoproteins. All glycoproteins expressed a HA tag for detection. (b) Representative fluorescent images of U2OS, Vero, A549, and Hela cells 24 hours following infection with TAMV-GP and P14 to show syncytia. (c) Quantitation of caspase 3/7 activity in HEK293, A549, and Hela cells 24 hours following transfection with TAMV-GP, P14, and P15. (d) Western blotting analysis of HEK293 cells infected with VV-TetR-iTAMV-GP (MOI 0.1) tagged with HA in the presence or absence of Dox at 100 ng/ml. (e) Full immunohistochemistry staining shown in Figure 7I. (f-h) Mice harboring A549 tumors were intravenously injected with VV-TetR-iTAMV-GP (1E7 PFU/mice) when tumors reached ~150 mm³ in size and depending on the treatment group received 625 mg/kg of Dox in their diet two days after virus injection. Tumor volume was measured at regular intervals (f). (g-h) Tumors were harvested after 60 days and weighed. Scale bars = 200 μ m in panel B. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.



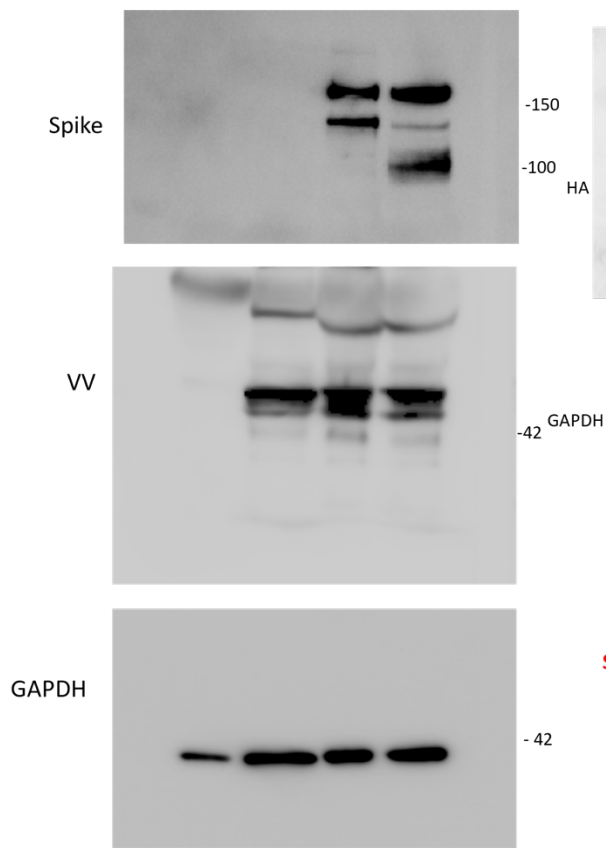
Supplementary Figure 7. Evaluating the optimal design for tandem expression of three interleukins controlled by a cumate switch. (a-b) Schematic representation of the constructs and ELISA analysis of IL-2, IL-12, IL-18 concentrations from supernatants of U2OS cells 6 hours after infection with VV-CymR-iIL12-IRES-2-IRES-18 (a) or VV-CymR-iIL12-2-18 (b). Cells were infected at an MOI of 1 and treated with 100 ug/ml cumate or PBS. (C) ELISA analysis of IL-2, IL-12, IL-18 from supernatants of U2OS cells 6 hours after infection with VV-CymR-iIL12-2-18 at different MOIs and treatment with 100 ug/ml cumate or PBS. Scale bars = 40 μ m in panel B. Data indicate means $\pm$ SD of three to five biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.



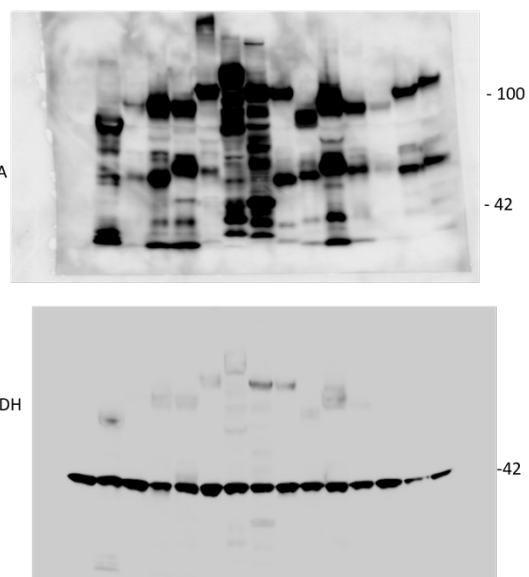
Supplementary Figure 8. Schematic illustration of VV-CTRL (left), VV-GFP-Luc (right).

Supplementary Figure 9. Uncropped western blots are shown thereafter.

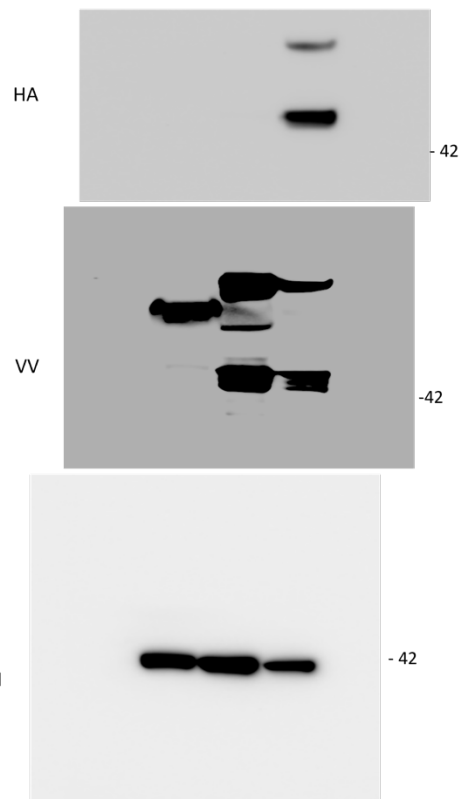
Supplementary Figure 5C



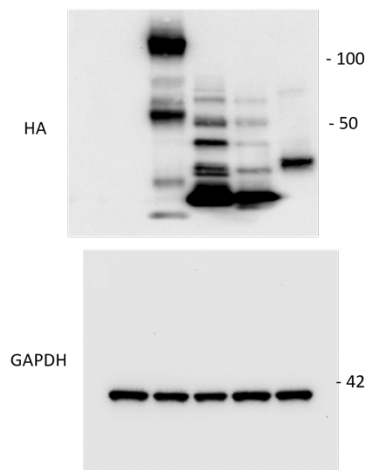
Supplementary Figure 6A



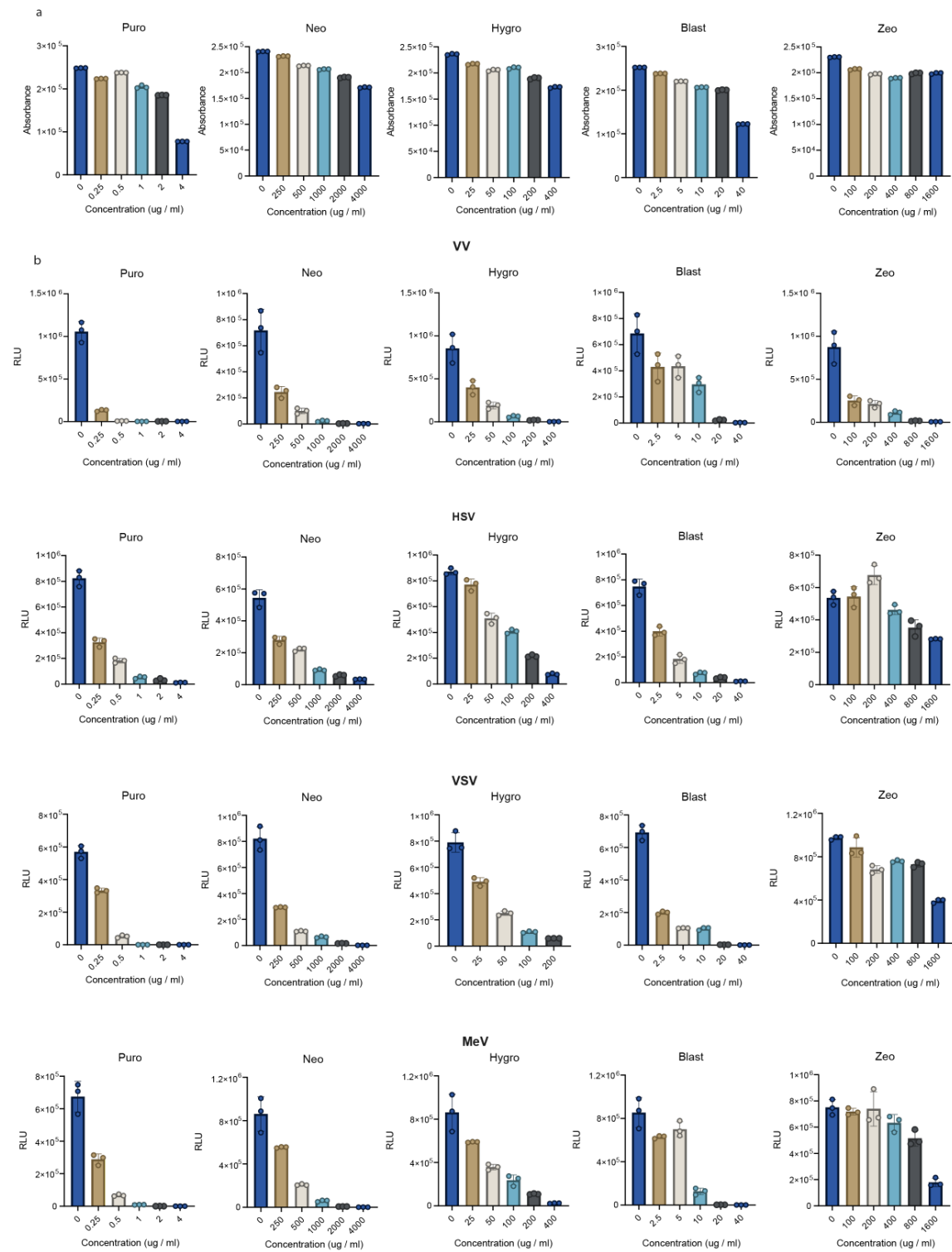
Supplementary Figure 6D



Supplementary Figure 6A

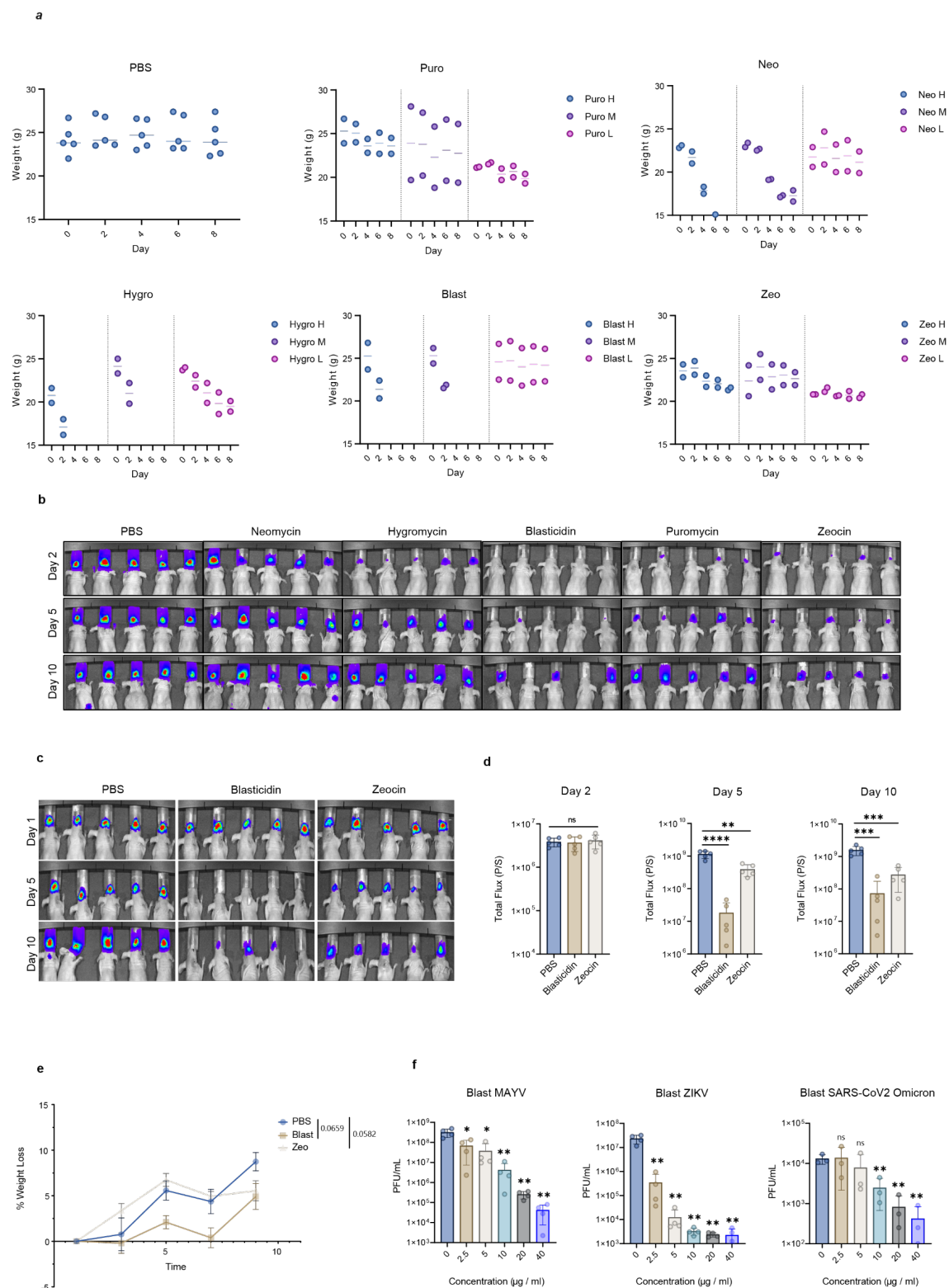


Appendices II – Chapter 4 Supplemental information



Supplementary Figure 1.

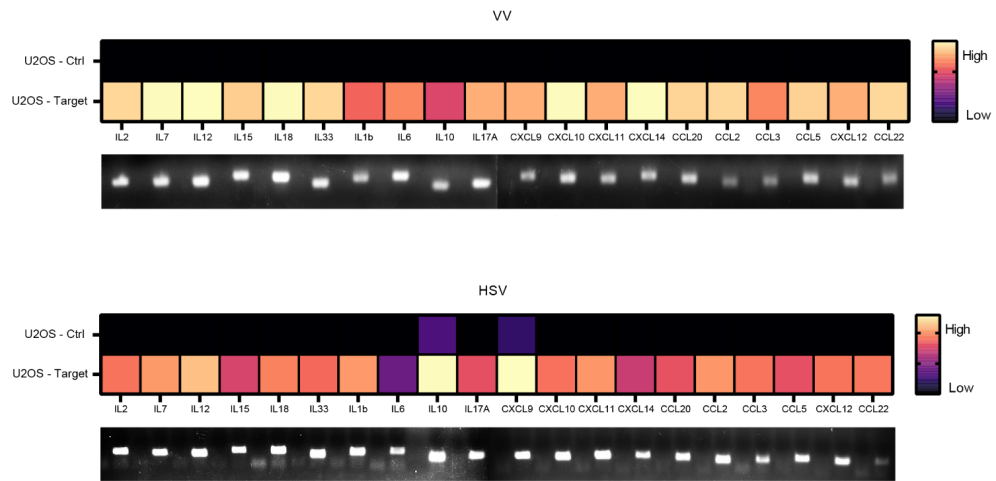
A. The viability of U2OS cells treated with each antibiotic concentration was measured 24h post infection. B. The luciferase expression from each virus treated with different antibiotic concentration are measured 24h post virus infection.



Supplementary Figure 2.

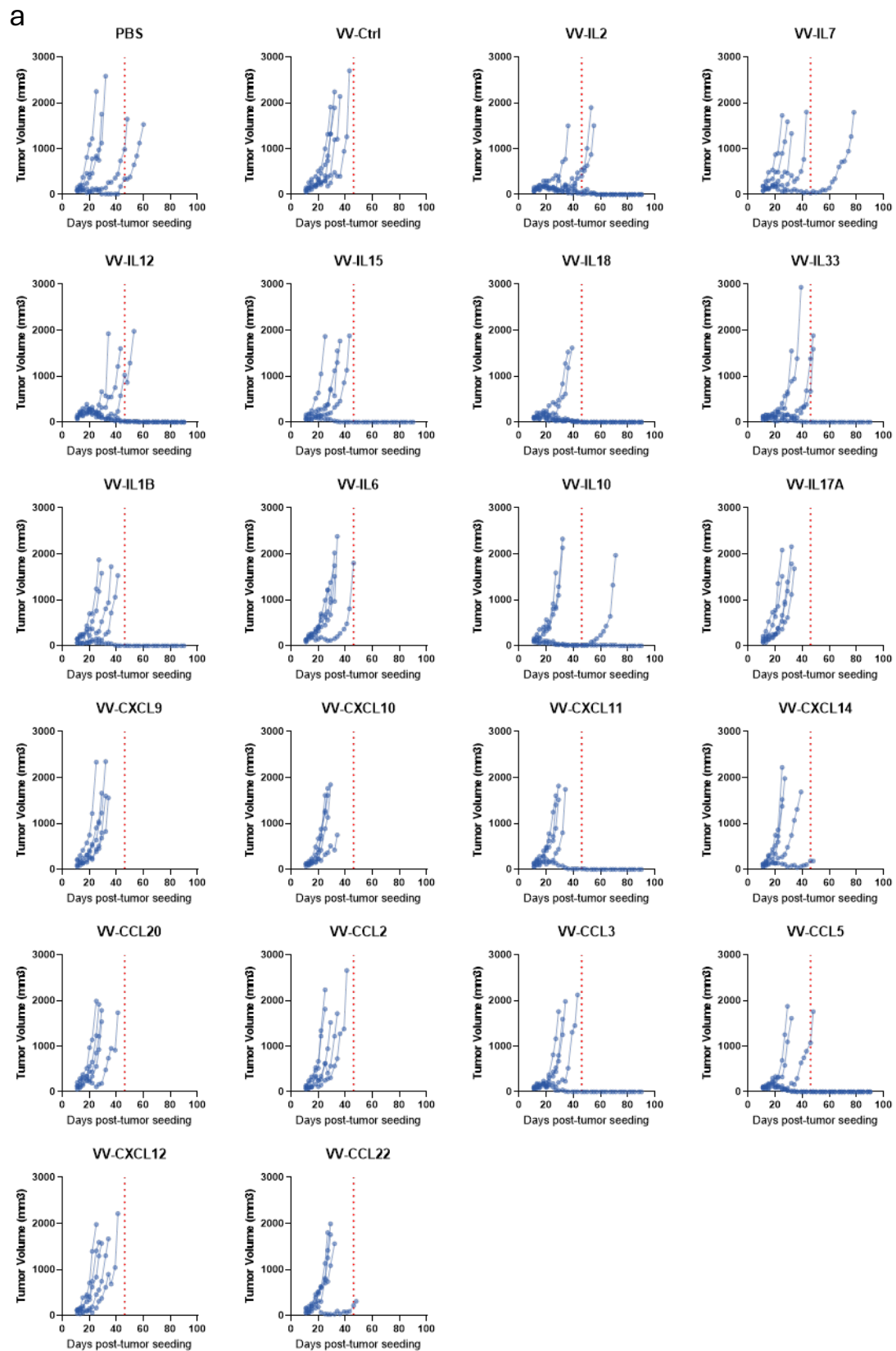
A. Mice weights were measured at regular intervals after 3 daily consecutive intranasal administration of each antibiotic at 10-fold dilutions (2 CD1 Nude mice per group). (B) CD1 nude mice received intranasal administration (i.n.) of luciferase-expressing VV-luc (1E6 PFU), followed by i.n. delivery of antibiotics. Antibiotics were administered for three consecutive days, and luciferase expression assessed by IVIS at 2-, 5-, and 10-days post-infection. (C,D) CD1 nude mice received i.n. administration of VV-luc (1E6 PFU) 24 hours before i.n. delivery of blasticidin or zeocin. Luciferase expression was captured 1-, 5-, and 10-days post infection. (E) Weight tracking of CD1 nude mice, treated as described in (E,F) over 10 days following initial viral challenge. (F) Viral titer (PFU/mL) in culture supernatants from U2OS cells 24 hours post treatment at increasing concentration of Blast with infection of Mayaro virus (MAYV), Zika and, and SARS-CoV-2 Omicron variant, conducted on a monolayer of the respective producer cell line. Scale bars = 100 μ m in (a). Data indicate means $\pm$ SEM of three (b, c), four (h), or five (f) biological replicates. ns $P > 0.05$, * $P < 0.05$ ** $P < 0.003841$, *** $P < 0.000125$, **** $P < 0.001$ in unpaired two-samples t-test.

a

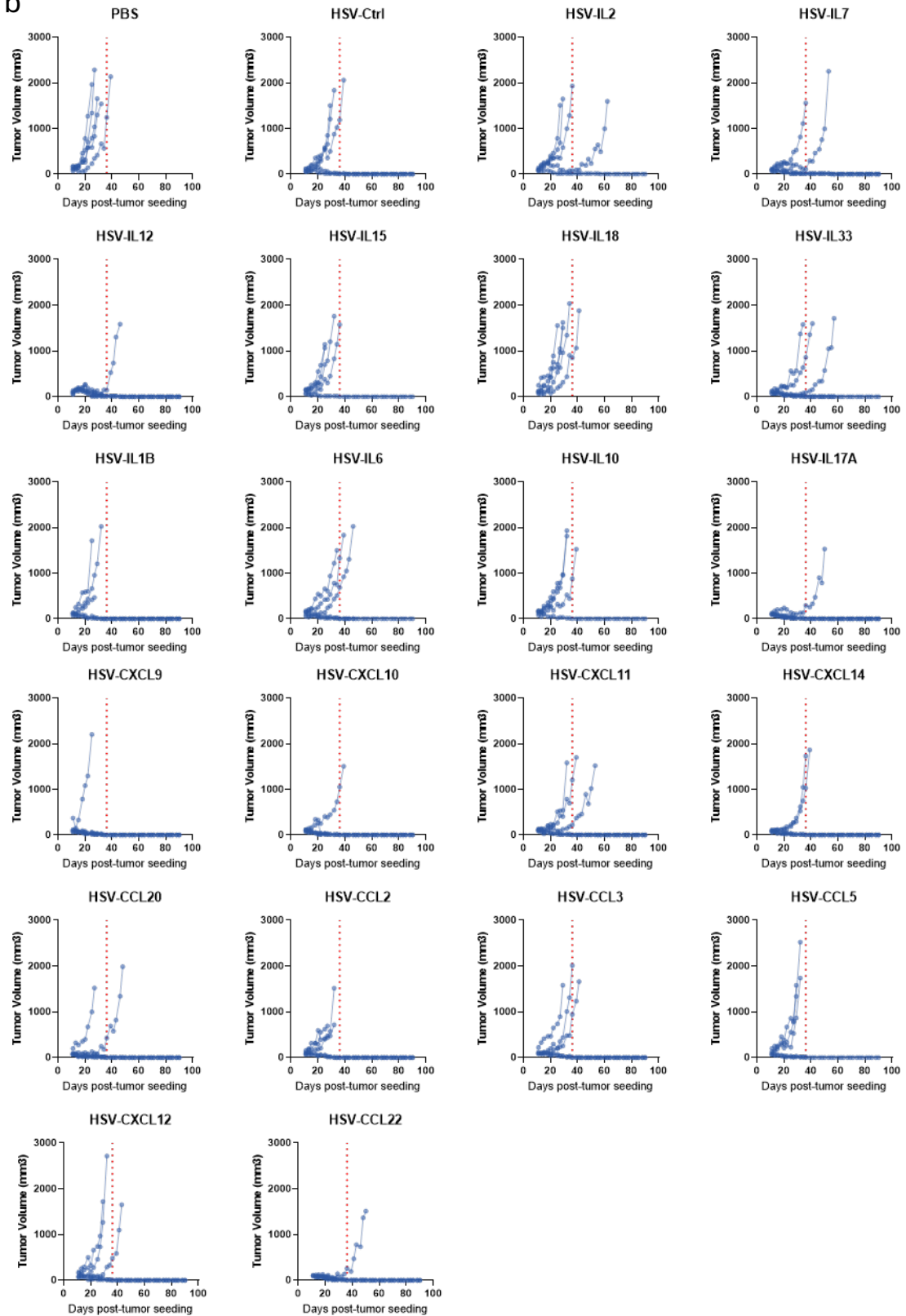


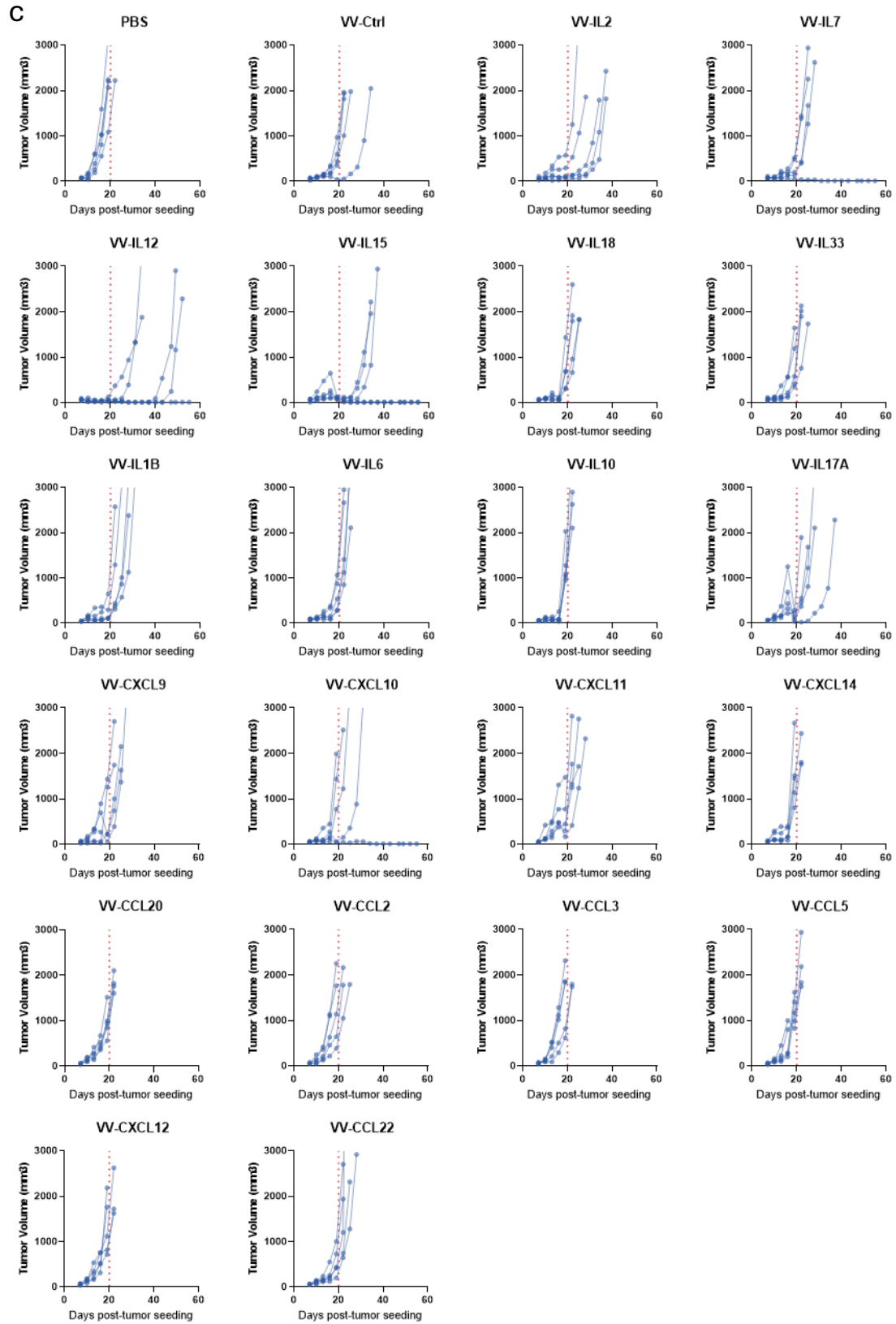
Supplementary Figure 3.

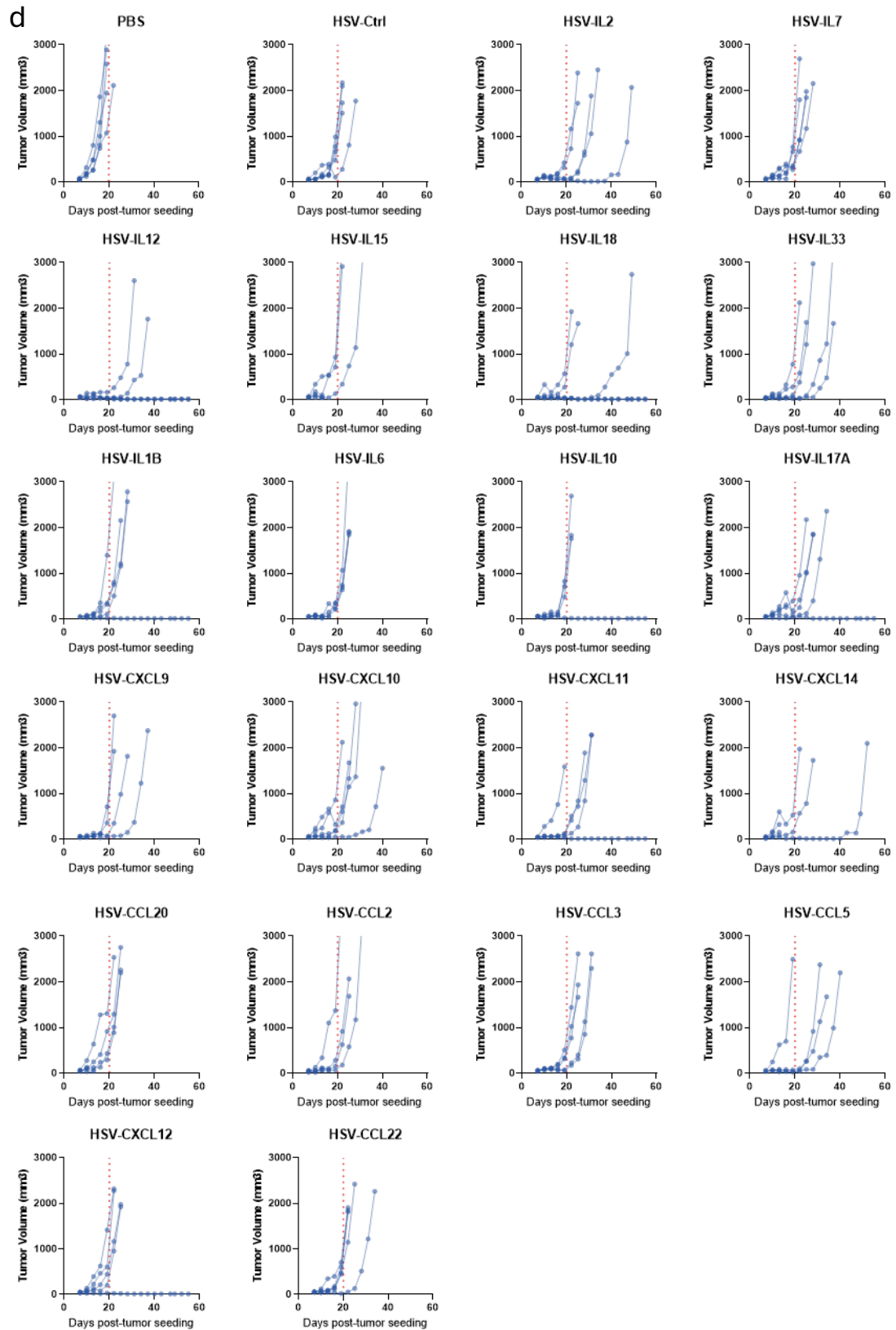
A. 24 well plates of U2OS cells were infected with MOI 0.1 of each cytokine expressing HSV and VV. Samples were prepared for qPCR 24h post infection. Heatmaps and gel electrophoresis pictures show the qualitative difference between Ct values of control and target samples.



b







Supplementary Figure 4.

A, B. Tumor volume graphs of subcutaneous CT26 (murine colorectal cancer) model in BALB/c mice. The animals received intratumoral injection (i.t.) of different cytokine-expressing VV or HSV-1 variants, control viral backbones, or PBS on days 11, 13, and 15 (n = 5 animals). (D, E) C, D. Tumor volume graphs of C57BL/6J mice bearing subcutaneous B16-F10 (murine melanoma) tumors following i.t. Injection of different cytokine-expressing VV or HSV-1 variants, control viral backbones, or PBS on days 7, 9, and 11 post-tumor implantation (n = 5 animals).

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