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# EVALUATION OF HERPES SIMPLEX VIRUS TYPE 1-BASED ONCOLYTIC VIRUSES IN ADVANCED 3D MODELS

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# ABSTRACT

Glioblastoma, also known as glioblastoma multiforme (GBM), is the most aggressive and common type of primary malignant brain tumor in adults. [1] Despite some progress with chimeric antigen receptor (CAR)-T cell therapies, immunotherapy remains largely ineffective against GBM, due to the immunosuppressive tumor microenvironment, the target antigen heterogeneity, and the limited trafficking across the Blood-Brain Barrier (BBB), which often requires intraventricular administration or intracranial administration, relatively invasive methods of administration of therapeutics and not easily repeatable. [1] Furthermore, the infiltrative nature of the disease and its tendency to recur in distant brain areas make localized therapy insufficient. [1] In these experiments we focus on the production of cerebral organoids which represent an advanced 3D model composed of various types of cells including neurons, astrocytes, microglia-like cells, and oligodendrocytes precursor cells (OPCs) which can mature into myelin-producing oligodendrocytes, and others, to create an *in-vitro* environment that mimics the brain structures heterogeneity for testing oncolytic virotherapy. [3] Oncolytic viruses (OVs) are naturally or genetically engineered viruses which aim to boost the anti-tumoral response and exhibit cell bursting via oncolysis – they are designed to specifically target tumor cells and spare healthy cells. [5] The latter is in the core of the scope of the experiments, as we have tested one type of oHSV-1 oncolytic viruses (called oHSV1-mCherry), and compared it to the wild type virus (called HSV1-V41) in its ability to infect healthy organoids. To prove that, via confocal/fluorescence microscopy we evaluated the infectivity of the viruses regarding the healthy cells, exploiting their reporter genes production. Furthermore, we confirmed the previous results through a viral titration experiment, that permitted the quantification of viral load in each infected organoid. Interestingly, the neuroattenuation of oHSV1-mCherry, obtained through γ34.5 and Us12 deletions and miR-124 target sequence recognition, significantly reduced HSV-1 ability to replicate inside healthy neurons, confirming its neuroattenuation.

# INTRODUCTION

## 1. Herpes Simplex Virus type 1 replicative cycle

Herpes Simplex Virus type 1 (HSV-1) is a double-stranded DNA (dsDNA) virus belonging to class I of the Baltimore's classification system. This member of Alphaherpesvirinae subfamily is known for causing oral herpes (cold sores) and occasionally genital infections, with lifelong latency in sensory neurons. [7], [9] It features an icosahedral capsid (about 100-110 nm in size) enclosing a linear 152-kb genome, surrounded by tegument proteins and a lipid envelope in which are embedded

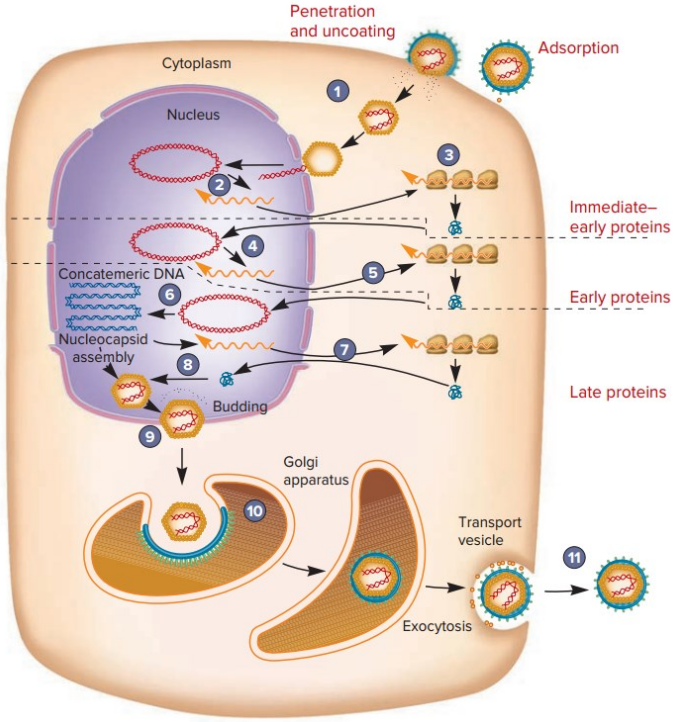



Figure 1: Replicative life cycle of HSV-1. Adopted from 9.

glycoproteins (gD, gB, and others), important for host cell attachment and entry. [9] During the first two phases of attachment and entry (Fig.1, step 1), HSV-1 targets cells of vertebrate hosts by binding to specific cell types through the binding of envelope spikes to specific host cell surface proteins (receptors), including nectin-1 or heparan sulfate. The glycoprotein-receptor recognition triggers HSV-1 through pH-dependent fusion (in the case of neurons) or endocytosis (in case of epithelial cells), causing the release of the capsid-tegument complex into the cytoplasm, from where they are transported by the host cell's microtubules to the nucleus, where genome replication and transcription occur. When the capsid docks to one of the nuclear pores of the nuclear membrane (Fig.1, step 2), the virus injects its dsDNA into the nucleus which follows a circularization process; after that – viral DNA is transcribed by host DNA-dependent RNA polymerase to form mRNAs, following a specific

temporal order. [9] The α genes, the first class of transcribed genes are translated to yield several early proteins (Fig.1, step 3 and 4). Among them, there is Infected Cell Protein 0 (ICP0) that synergizes with ICP4 to boost immediate early, early and late genes expression as well as blocking interferon activation pathways by inhibiting IRF3/IRF7 activity, preventing interferon production, crucial for the defense against the host's antiviral response. [8] The next step is followed upon 4 hours after infection and represents the expression of early beta genes (Fig.1, steps 5 and 6), like *UL30* encoding for a virus-specific DNA-dependent DNA polymerase responsible for viral replication process and other genes involved in early proteins expression, for example *UL29* that encodes for ICP8 (Infected Cell Protein 8), which is the major single-stranded DNA(ssDNA)-binding protein (SSB) essential for viral DNA replication – it protects the ssDNA from nucleases and helps in removing secondary structures to allow for the proper assembly of the replisome. [8], [9] Later on the DNA is forming a concatemer (Fig.1, step 7) from which late gamma genes are expressed, that are involved in the virion composition (Fig.1, step 8), like *GB* encoding for a glycoprotein (gB) for the surface receptor recognition, as described above, and many other structural proteins. [2], [9] Once the nucleocapsid is assembled, it buds into the space between the two nuclear membranes (Fig.1, step 9); this generates the primary viral envelope which is lost when it fuses with the outer nuclear membrane, releasing the herpesvirus nucleocapsid into the cytoplasm. Tegument proteins are then added in the cytoplasm, and the developing virion is enveloped by the Golgi apparatus (Fig.1, step 10), yielding a mature enveloped virion that is transported to the plasma membrane in a membrane vesicle. The membrane vesicle fuses with the plasma membrane and leads to the release of mature virions from the host cell into the surrounding environment, ready to infect other cells (Fig.1, step 11). [9]

## 2. HSV-1 neurotropism and establishment of latency

Herpes Simplex Virus type 1 causes both productive infections and latent infections. During productive infection, that usually occurs in epithelial cells, the virus follows the lytic life cycle, described above, and multiplies explosively (between 50,000 and 200,000 virions are produced from one infected cell), causing DNA degradation, cell's metabolism disruption and cell lysis. [7], [9] However, HSV-1 is able to infect also neurons, making it a neurotropic virus; indeed its neurotropism is explained by the capability of the virus in hitting the labile mucosa and subsequently moving in a retrograde manner via the axons of the neurons – reaching the nuclei where latency is established. During the latent infection the virions cannot be detected because the virus replication is deactivated

due to the restriction of lytic cycle viral gene expression. The only abundantly expressed viral RNA in latently infected neurons is the Latency Associated Transcript (LAT). Multiple regulatory processes govern the production of latency within the neuronal cells; both viral and host proteins play a role in making a latent human alphaherpesvirus 1 infection. [9] Regarding the lytic process, in presence of reactivation stimuli (like stress, UV or fever), HCF-1, a host protein called Host Cell Factor 1 (HCF-1) that is implicated in remodeling of HSV-1 chromatin structure, translocates from the cytoplasm to the neuronal nucleus, [9] where together with VP16 (virion tegument protein 16), and Oct-1 (POU homeodomain) forms the so called active VIC (VP16-induced complex), that stimulates expression of alpha, beta, and gamma genes for new viral production. [10]

## 3. Organization of the HSV-1 genome

The virus genomes are crucial for the design of innovative oncolytic viruses. In our case, we used oHSV-1-based oncolytic viruses that are engineered in a specific way to target tumoral tissues and microenvironments, as well as in spearing healthy cells and tissues. The HSV-1 genome features a distinctive organization comprising two unique regions – the Unique Long Region (ULR) and the Unique Short Region (USR) – each bounded by inverted repeat sequences. This configuration enables isomeric genome forms through recombination within the repeats. The ULR, the larger unique segment, encodes numerous essential genes responsible for viral replication, DNA synthesis, and virion assembly. [8] In contrast, the USR encodes genes associated with viral pathogenesis, immune evasion, and neurovirulence. [8] The inverted repeats flanking the UL and US regions permit recombination, yielding four equimolar isomeric genome arrangements across a virion population. [8] In general, in the case of HSV-1 there exists a 30-kb region from the genome of the virus that represents a total amount of intergenic regions dispersed across the genome, not involved in the replication process, so it can be used for recombination with other genes. [7]

## 4. Oncolytic viruses and mechanisms of action

Oncolytic viruses (OVs), viruses that selectively replicate within and induce cancer cells death, represent an innovative method for tumor treatments. As until now the term was known as oncolytic virotherapy, nowadays the ideas are shifting towards oncolytic viral immunotherapy, since the induction of the immune system, promoted by the OVs, plays a crucial role in eliminating the tumor. Indeed, OVs exploit two distinct antitumoral mechanisms: firstly, their replication in cancer cells induce tumor lysis. Secondly, the immunogenic cancer cells' death promote the activation of the host

immune system. Overall, these agents offer a multifaceted therapeutic approach that includes direct tumor lysis, immune activation, and gene delivery. Their selectivity towards malignant cells is the results of different factors. Firstly, tumor cells present defective antiviral responses (for example the tumors lack the expression of IFN alpha, beta and gamma genes), making cancer cells ideal for viral replication. Secondly, since the focus of cancer cells on their metabolism is to grow and proliferate via aerobic glycolysis (Warburg effect), the OVs use the cancer energy for viral proteins synthesis. Finally, the tumors express receptors that are recognized preferentially by OVs and this is useful for tumor delivery of the viruses and specificity. However, there is an insufficiency of the OVs alone to kill the tumor completely, so combination therapies – with for example CAR-T therapies, chemotherapy, Immune Checkpoint Inhibitors (ICI) therapy or radiotherapy, gained attention, showing significant improvement in cancer patients. Several viruses are employed as oncolytic viruses, including herpes simplex virus (HSV), adenovirus, reovirus, and vesicular stomatitis virus (VSV). For HSV-1-based oncolytics, the mechanisms of action involve direct oncolysis through selective viral replication in cancer cells (e.g., via ICP34.5 deletion), followed by tumor cell lysis releasing progeny virions, tumor-associated antigens, and danger signals (HMGB1, ATP, calreticulin, and others) that trigger immunogenic cell death and adaptive antitumor immunity (the involvement of T cells especially). What is more, HSV-1 exhibits tumor tropism via glycoprotein D binding nectin-1 receptors often upregulated on cancer cells (as it is in the case of GBM), enabling efficient entry and spread while sparing normal tissues. In the case of glioblastoma oHSV-1 is/are able to cross the BBB, but because of the diverse composition of brain structures this effect is limited and further analysis should be performed to understand and overcome the limitation. What happens corresponding the immune system activation is that basically oHSV-1 induces immunogenic cell death in GBM cells, where released tumor-associated antigens (TAA), danger-associated molecular patterns (DAMPs) like ATP, and viral pathogen-associated molecular patterns (PAMPs) like viral antigens activate dendritic cells (DC), natural killer (NK) cells, and CD8+ T-cells. This shifts immunosuppressive "cold" tumors to inflamed "hot" ones through the damaged integrity of the tumor, the increased expression of immunostimulatory receptors, and as the usage of immune checkpoints inhibitors antibodies that are added to oHSV-1 like anti-PD-L1, anti-PD1, and anti-CTLA4 to block the silencing of the immune response, also together with ICIs as a whole, enhancing T-cell infiltration. [5]

# MATERIALS AND METHODS

## Materials

### 1) Types of oncolytic viruses used during the experiments

In our case, we used oHSV-1-based oncolytic viruses that are engineered in a specific way to target tumoral tissues and microenvironments, as well as in spearing healthy cells and tissues. For reference, we used a wild-type HSV-1 virus called HSV1-V41, that is genetically modified to express the GFP (Green Fluorescent Protein) reporter gene for analyzing and following infection in the organoids. The gene is inserted in the UL55-UL56 Cytomegalovirus promoter region, just after the promoter, in order to ubiquitously allow for observation of the viral expression, independently of type of tissue or cell types. [8]

Then, we used the oHSV-1 oncolytic virus called oHSV1-mCherry that contains several modifications. Particularly, it presents:

- Two deletions of *γ34.5* genes: this gene encodes for the Infected Cell Protein 34.5 (ICP34.5), and it exists in a double copy (due to the inverted repeats, flanking the ULR) – so must be deleted from both strands of the DNA. ICP34.5 protein stimulates viral replication, through its ability to induce the dephosphorylation of eIF2α (through PP1 recruitment), an essential protein for eukaryotic translation, required for viral proteins production. Particularly, ICP34.5 induces the activation of PP1 phosphatase, that dephosphorylates the eIF2 α, normally inactivated by phosphorylation promoted by IFN/PKR, determining the overcoming of translational shutoff activated by the antiviral cellular response. [8] The deletion of gamma34.5, so, reduces oHSV1 neurotropism, by reducing its ability in replicating in normal neurons.
- The deletion of *US12*: this gene encodes for a viral inhibitor protein, called Infected Cell Protein 47 (ICP47). This protein typically limits the insertion of peptides into the context of Major Histocompatibility Complex class I (MHC-I) molecules on the surface of infected cells, limiting the presentation of viral antigens to T cells, in particular CD8+ T-cells. [8] The deletion of *US12* gene increases antigen presentation by infected tumor cells and makes them more visible and susceptible to the host's immune system thereby inducing immune-mediated

destruction of cancer cells, so infected cells can be recognized by the CD8+ T-cells and be destroyed.

- *MIR-124* target sites insertion below the essential gene *UL29*: the *UL29* gene encodes the multifunctional ICP8 protein, one of the most abundant early viral proteins with ssDNA binding activity, essential for viral genome replication. [8] The presence of miR-124 target sequences in this gene promoter, leads to a reduction in *UL29* gene expression, causing the partial inhibition of viral replication in healthy neurons, where miR-124 is abundantly expressed. Through this genetic modification, we aim to obtain a selective oncolytic HSV-1, able to replicate preferentially in tumor. The insertion of a gene that encodes for the red fluorescent protein mCherry: the intergenic UL55-UL56 region is used for the insertion of a single reporter gene, employed for viral infection visualization. [8]

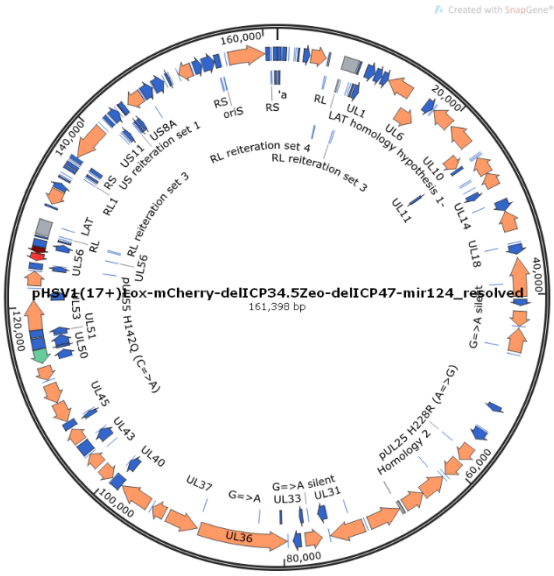



Figure 2. Map of the mCherry oncolytic virus with all the genetic information regarding it. Adopted with Snapgene viewer software.

## 2) Cell lines and culture medium

In this experiment were used the following cell lines:

- VERO CCL81 (ATCC ®): this cell line is obtained from a kidney tissue derived from an adult African green monkey. These cells were maintained at 37°C, in an atmosphere of 5% CO2 and 98% humidity. The cell culture medium used for their maintenance was DMEM, (Dulbecco's Modified Eagle Medium; Gibco), added with 10% v/v FBS (Fetal Bovine Serum). It provides nutrients, growth factors, glucose, amino acids, vitamins, salts, and pyruvate, to sustain cell growth. These cells, kept at 80-90% of confluency, were passed twice a week. [3]

For the cell passaging process, we considered the following steps:

- ✓ First, we removed the old exhausted medium;
- ✓ Second, we used PBS to wash the cells;
- ✓ Third, we added trypsin to allow the cells to detach and then we store them in the incubator for around 10 minutes;
- ✓ Fourth, we neutralized trypsin and diluted cells in fresh medium.

- H9 (WA09): it is a human embryonic stem cell line, derived from a blastocyst. It is a pluripotent cell line that can be differentiated into various cell types, including neural stem cells and hematopoietic progenitor cells, making it a valuable tool for studying development and disease modeling. These cells were maintained at 37°C, in an atmosphere of 5% CO2 and 98% humidity, in Multiwell-6 wells. [3]

Materials used for their maintenance include:

1. Geltrex: it is a reduced growth factor basement membrane extract used for attachment and maintenance of human embryonic stem cells, like H9. It is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma, and it contains laminin, collagen, and heparan sulfate proteoglycans. Its purpose is to minimize Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), and other mitogens to allow for a proper control of differentiation while supporting attachment and pluripotency; [3]
2. mTeSR-1 Basal Medium (STEMCELL Technologies): it is a serum-free maintenance medium, optimized for high-efficiency culture of human embryonic stem cells. It contains DMEM/F-12 base, L-ascorbic acid-2-phosphate magnesium, sodium bicarbonate, glucose, and other compounds, and needs to be supplemented with mTeSR™1 5X Supplement (provides various types of human recombinant growth factors and stabilizers); [3]
3. Rock Inhibitor (Aurogene): it is composed of cell-permeable small molecule inhibitor of Rho-associated coiled-coil containing protein kinases (ROCK1 and ROCK2). It acts as selectively binding ROCK kinases over PKA/PKC, blocking RhoA/ROCK in order to inhibit dissociation-induced apoptosis (anoikis). In cell passaging it further enhances

survival of human embryoid stem cells, like H9, and it improves embryoid body formation; [3]

4. Accutase (Thermofisher Technologies): it is an enzyme-based cell dissociation reagent, containing several proteolytic and collagenolytic enzymes. It is used to allow stem cells passage as a main function to minimize damage or stress to the cells. [3]

- For cell passaging process, we considered the following steps:
    1. First, aspirated and removed old medium;
    2. Second, washed with PBS;
    3. Third, detached cells, using Accutase that promoted its action at 37°C, for 10 min, pipetting to break aggregates;
    4. Then, neutralized Accutase with PBS;
    5. Pelleted cells with centrifugation at 1100 rpm for 5 min;
    6. Resuspension of cells in mTeSR-1 Basal, added with 10 µM ROCK Inhibitor;
    7. Added gently drops, containing cells to Geltrex-coated wells (pre-warmed 1h at Room Temperature);
    8. Changing medium daily with fresh mTeSR-1 + Supplement, to maintain pluripotency before organoid induction.

# Methods

## Production of cerebral organoids derived from H9 cells

Cerebral organoids are three-dimensional, self-organizing structures derived from pluripotent stem cells (PSCs) that reproduce key aspects of human brain structure and function *in vitro*. Neural tissue originates *in vivo* from the ectoderm germ layer, and PSCs can similarly be directed to form ectoderm within embryoid bodies (EBs) – multilineage aggregates – in defined media. Initial EBs formation occurs in ESC medium with reduced basic fibroblast growth factor (bFGF) and high-dose rho-associated protein kinase (ROCK) inhibitor to minimize apoptosis. Neural induction follows in minimal medium akin to neural rosette protocols, promoting uniform neuroectoderm on EBs exteriors while suppressing inner mesendodermal fates. After their growth, EBs are embedded in Matrigel droplets for neuroepithelial bud outgrowth, then transfered to spinning bioreactors or orbital shakers for enhanced nutrient/oxygen diffusion, fostering defined brain regions akin to fetal neocortex. Particularly, their subsequent maturation in Cerebral Organoid differentiation medium, composed of Neurobasal medium with B-27 supplement, insulin, and 2-mercaptoethanol supports radial neuroepithelia expansion into neural progenitor cells (NPCs) – multipotent precursors with limited

proliferation that give arise to diverse neural lineages (neurons and glia), yielding ventricular zones, cortical layers, and region-specific identities without early exogenous caudalizing agents like retinoic acid (it is added later on in the protocol). [3]

Materials used for the cerebral organoid generation include:

- Accutase;
- PBS;
- mTeSR™1 Basal Medium containing 50 μM of Rock inhibitor;
- ULA plates: these represent Ultra-low attachment MW 96 Round Bottom, U-like shaped plates for the proper generation of the cerebral organoids. These are specialized laboratory vessels featuring a hydrophilic, non-cytotoxic, or hydrogel-coated surface that prevents cell adhesion, fostering scaffold-free 3D spheroid and organoid formation; [3]
- Neural Induction Medium: this medium blocks the differentiation towards endoderm and mesoderm, promoting the ectoderm development from pluripotent stem cells (H9). A correctly developed organoid in neural induction medium should show clear radially organized optically translucent neuroectoderm. Its composition consists of: DMEM/F12 (Gibco), Supplement N2 (Thermofisher Scientific), GlutamaX (Thermofisher Scientific), Non-essential Amino Acids (Thermofisher Scientific), Heparin (Sigma Aldrich), and Antibiotic Antimycotic (Thermofisher Scientific); [3]
- Matrigel – (Corning): it acts as a 3D extracellular matrix scaffold that supports cerebral organoid development. Particularly, cerebral organoids are embedded into this membrane in a droplet to allow their proper differentiation and development; [3]
- Cerebral Organoid Differentiation Medium: it is formulated to promote the maturation of neural progenitors into specific neuronal subtypes or glial cells. It is composed of several compounds including: DMEM/F12, Neurobasal medium (Gibco), Supplement N2, GlutamaX, Insulin (Sigma Aldrich), Non-essential Amino Acids, 2-mercaptoethanol (Thermofisher Scientific), and B-27 supplement (Thermofisher Scientific) that is supplemented or not with vitamin A. Particularly, because of its caudalization activity, vitamin A is not included at early stages, but it is only added to the differentiation medium in later stages. [3]

The complete generation of mature cerebral organoids, follows the reported unguided protocol, that takes around 40 days. [3]

**Day 0 (Seeding H9 cells)**

1. Detach H9 cells from 12-MultiWell plate: Wash with 1 mL PBS, add 500 µL Accutase, incubate 10 min at 37°C, pipette to dissociate aggregates;
2. Neutralize Accutase with PBS, pellet cells through centrifugation 1100 rpm/5 min, and resuspend them in mTeSR-1 Basal Medium + 50 µM ROCK Inhibitor;
3. Count cells (in duplicate): trypan blue automated counter;
4. Seed 9,000 cells/well in 150 µL mTeSR-1 Basal + 50 µM ROCK in Thermofisher Sphera 96-well U-bottom ULA plates;
5. Incubate the plate at 37°C, in an atmosphere of 5% CO2 and 98% humidity.

**Days 3-9 (Media Changes, Every 2 Days)**

- **Day 3**: Aspirate old medium, and add fresh mTeSR-1 Basal Medium without the addition of ROCK Inhibitor;
- **Days 5,7,9**: Change cells medium with fresh Neural Induction Medium to promote ectoderm/neuroectoderm development (it suppresses endo/mesoderm).

**Day 11 (Matrigel Embedding)**

1. Prepare parafilm (ethanol-UV sterilized) on Petri dish;
2. Transfer organoids to parafilm, and dry excess medium;
3. Add 20 µL ice-cold Corning Matrigel per organoid, center it, and solidify for 20 min at 37°C;
4. Transfer embedded organoids to 6-Well plate, and add Cerebral Organoid Differentiation Medium without Retinoic Acid;
5. Incubate the plate in static condition at 37°C, in an atmosphere of 5% CO2 and 98% humidity.

**Days 13+ (Maturation)**

- **Day 13**: Change to Differentiation Medium (no retinoic acid).
- **Day 15+**: Switch to Differentiation Medium added with Retinoic Acid (promotes neuronal differentiation) and place organoids on orbital shaker; change twice a week.

This yields radially organized neuroectoderm progressing to NPCs, neurons, and glia, making it suitable for GBM modeling and HSV studies. The protocol followed in this experiment was unguided, meaning that there is no dedicated neural induction (SMADi/WNTi) in comparison to the guided protocol. As consequence, organoid cells recapitulate the retina, cerebrum, or medulla oblungata, so many different cells. [3]

## Protocol of infection of the cerebral organoids

Materials needed:

- Sterile 2 ml Eppendorf;
- Virus;
- 1000 tips;
- Sterile scissors;

The protocol focuses on the steps followed to allow for the viruses to infect the cerebral organoids, and it is as follows:

1. Transfer the organoid from the MW6 to a 2 ml Eppendorf: each Eppendorf must contain one single organoid. The transport is made through cut 1000 tips.
2. Remove the excess medium using a P200.
3. Add the virus: the virus is diluted in medium containing Neurobasal and DMEM F12 (50% and 50%; there are no growth factors). The virus must cover the organoid (300 µl are enough).
4. Perform centrifugation at 1100 rpm for 30 minutes.
5. Put the Eppendorf in the incubator for 1.30 hour at 37°C.
6. Put the organoid back inside a new MW6 plate: fill the new well of the MW with 3 ml of Cerebral organoid differentiation medium. For the transfer of the organoids, use 1000 tips with the tip cut off. Liquid should be taken from the well and placed into the Eppendorf; by pipetting gently up and down, the organoid rises.
7. Put on shaking in the incubator.

## Confocal/fluorescence microscopy

Confocal fluorescence microscopy relies on a laser that excites fluorophores (in our case mCherry/GFP dyes) in thin optical slices (~0.5-1 µm) of thick 3D samples like organoids, scanning point-by-point with a pinhole to reject out-of-focus light in order to establish sharp, z-stack images without blur, performed via the usage of Leica software with channels for multi-color to observe the difference in fluorescence. In our experiment we first took images with the confocal microscope to check the spreading of the infection of the viruses, then we analysed the morphology, focusing on how much area of these organoids is infected by the different viruses.

## Viral titration

Viral titration is a technique used to determine the concentration of infectious virus particles within a sample, typically expressed as plaque-forming units per milliliter (PFU/ml). In our case, the method plaque assay was employed to define the amount active virus present in cerebral infected organoids.

Particularly, it consists in infecting a confluent monolayer of host cells with serial dilutions of a virus, then covering them with a semisolid overlay (agarose/methylcellulose) to restrict viral spread to neighboring cells and analyzing the lysis of cells by the virus which creates visible clear zones (plaques). The supernatant of the organoids was used to infect VERO CCL81 cells. Particularly, we have prepared the dilutions of each virus of factor 10 in the media DMEM without serum (FBS 0%), ranging from $10^{-2}$ to $10^{-13}$ dilutions. Firslty, we aspirated and discarded the old medium from the VERO CCL81 cells and we washed cells with PBS to remove FBS residuals. Subsequently, we added the diluted virus in each of the VERO CCL81 cells wells, and we incubated for 1 hour at 37°C, 5% $CO_2$. The same procedure was followed for mock organoids. Then, after the incubation period has finished, we removed the medium and we washed cells with PBS. Finally, we added DMEM 2% FBS (it is diluted from 10% to 2% in order to allow for viral replication, but also sustain the cell line alive) added with carboxymethylcellulose, a substance required for the formation of plaques. When plaques were formed we dyed them with crystal violet. Particularly, we removed the medium, we washed with PBS, and then we added formaldehyde (PFA) at 4%, for cells fixation. After an incubation of 10 min at room temperature, we removed formaldehyde, and we added crystal violet, and we incubated for 5 min at room temperature. After incubation, we removed the crystal violet dye and washed with miliQ water, and finally we left the plates to dry. Viral plaques were then counted with fluorescent microscopy.

# Results

## 1. Cerebral organoids development

Cerebral organoids were followed with confocal microscopy for 40 days, to assess their structure and their development. Representative images are shown in Figure 3 and Figure 4.

Particularly, Figure 3 represents cerebral organoids at Day 25 post-production, highlighting the presence of several protrusions representing the rosettes formations. At this stage, moreover, the Matrigel constituted the majority of organoids surface, indicating the on-going development of these models. Figure 4 shows organoids at Day 38; at this time-point, organoids presented almost complete differentiation, in terms of structure and dimensions. Moreover, it can be seen that the Matrigel is covering less surface in comparison to the organoid, which is a sign of differentiation and proliferation of different cell types typical for this type of structures.

These organoids have been employed as models for the subsequent processes of infection and evaluation of the oncolytic viruses capacity to spare the healthy cells.

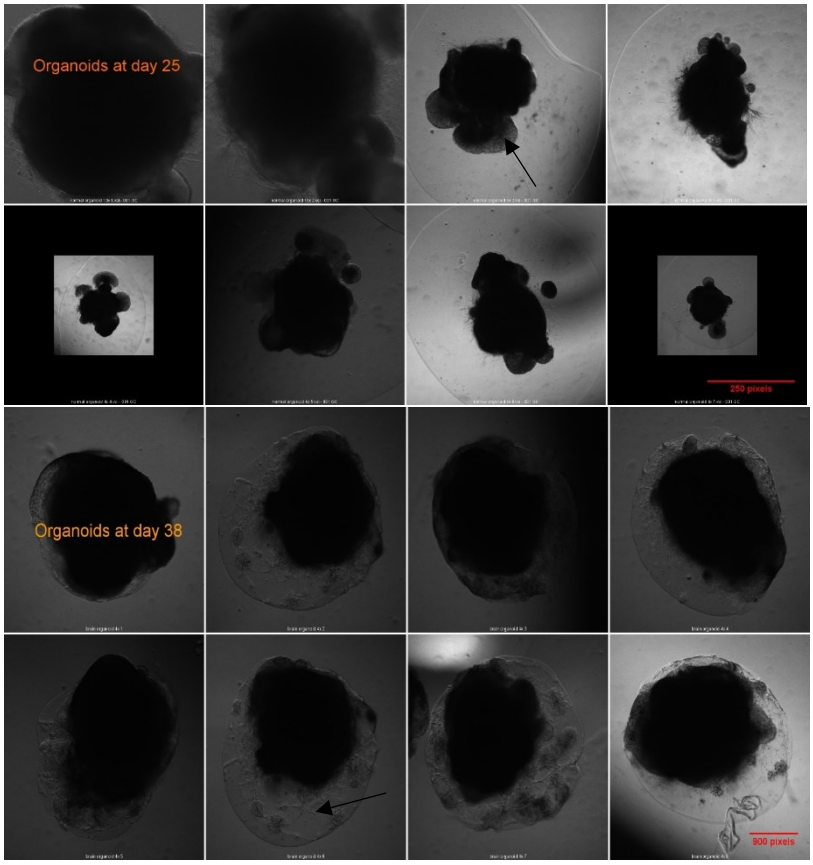



Figure 3. Confocal microscope image representing the formation of the cerebral organoids at day 25. Arrow is pointing at the rosettes protuberances. Adopted with Imagej software.

Figure 4. Confocal microscope image representing the formation of the cerebral organoids at day 38. Arrow is pointing at the Matrigel. Adopted with Imagej software.

## 2. oHSV-1-mCherry neuroattenuation in cerebral organoids

The HSV1-V41 and oHSV1-mCherry viral replication in H9 derived cerebral organoids was evaluated through confocal microscopy, exploiting the reporter proteins, EGFP and mCherry respectively, produced by these viruses. Particularly, three time-points were selected: Day 0, Day 1, and Day 3 post-infection, and Z-stacking were performed, to evaluate the estimated 3-D structure of the organoids by superimposing different images with different focusing. The idea is to get a better overall idea of the distribution of the viruses inside the organoids, and to capture in more detail the structural organization of the organoids. At Day 0 post-infection, as expected, no viral replication was observed, while organoids integrity was assessed. (Figure 5) At Day 1 post-infection, there was a significant difference in viral replication between organoids treated with wild-type virus compared to oncolytic virus infected organoids, as shown in Figure 6, panel A. Particularly, a higher level of replication of HSV1-V41 was observed, as demonstrated by the number of EGFP-positive cells, located especially in the periphery of the organoid, presented in Figure 6, panel B. Meanwhile, at the same timepoint, no viral particles were detected in oHSV1-mCherry treated organoids. Their difference was further confirmed at Day 3 post-infection, when HSV1-V41-treated organoids

appeared completely infected (the organoid resulted formed completely by green fluorescent cells), while oHSV1-mCherry replication resulted weaker and less homogeneous, part of Figure 7. Overall, these results confirmed the neuroattenuation of our oncolytic virus in healthy cerebral organoids compared to the wild-type isoform, opening the possibility to use this virus for glioblastoma therapy.

Figure 5. Images representing the three different conditions (presented in four images per condition) of cerebral organoids post infection at day 0: first, the mock, which is a control, without the viral infection, second is V-41, which is the wild-type virus, expressing the GFP reporter gene, to estimate fluorescence, and third is mCherry, which has further modifications aiming at diminishing the viral replication in neurons, and at boosting the oncolytic activity – it uses the mCherry reporter gene, in red fluorescence. Adopted with Imagej software.

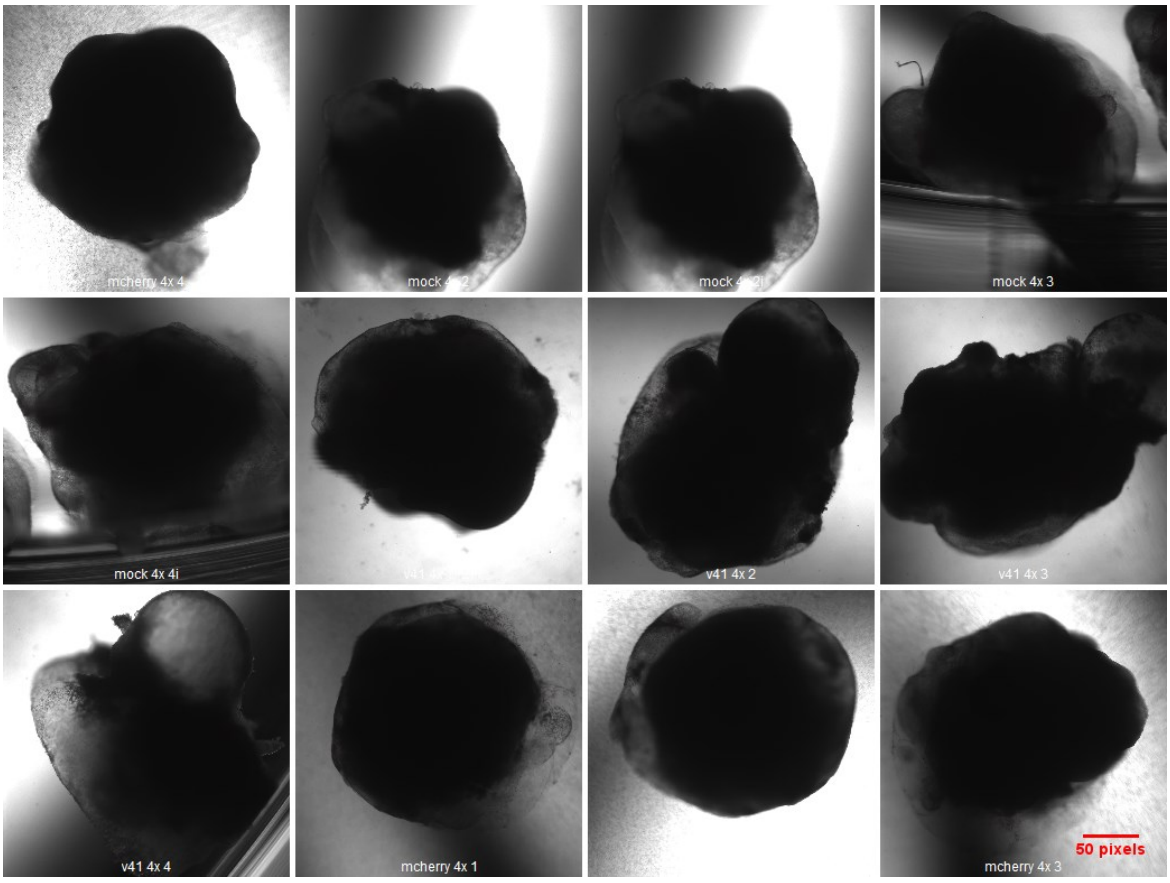
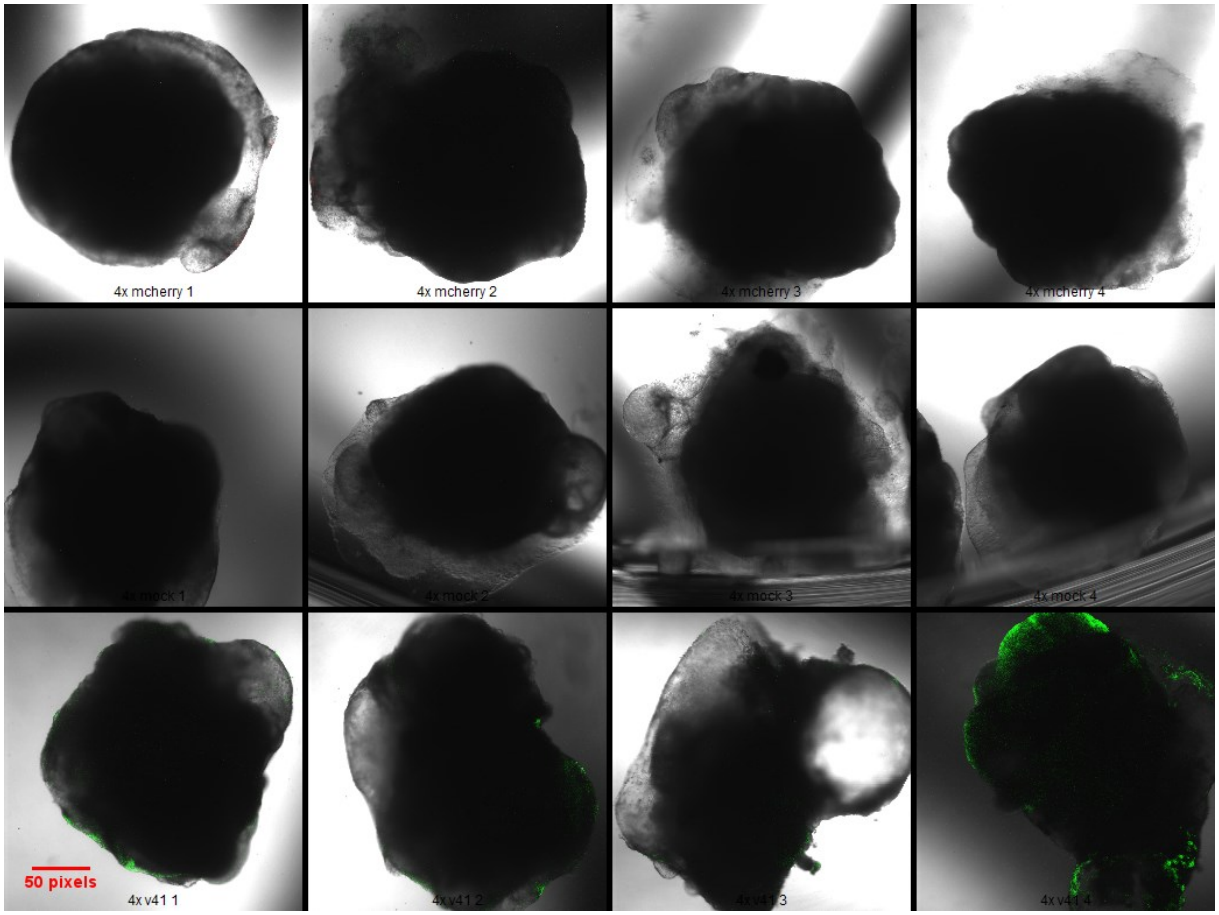


Figure 6. Panel A: Images representing again the three different conditions (four images per condition), but at day 1. Panel B: There are some zoomed places for better visualization of the fluorescence of the three conditions (one image per condition). Adopted with Imagej software.

Panel A:




Panel B:

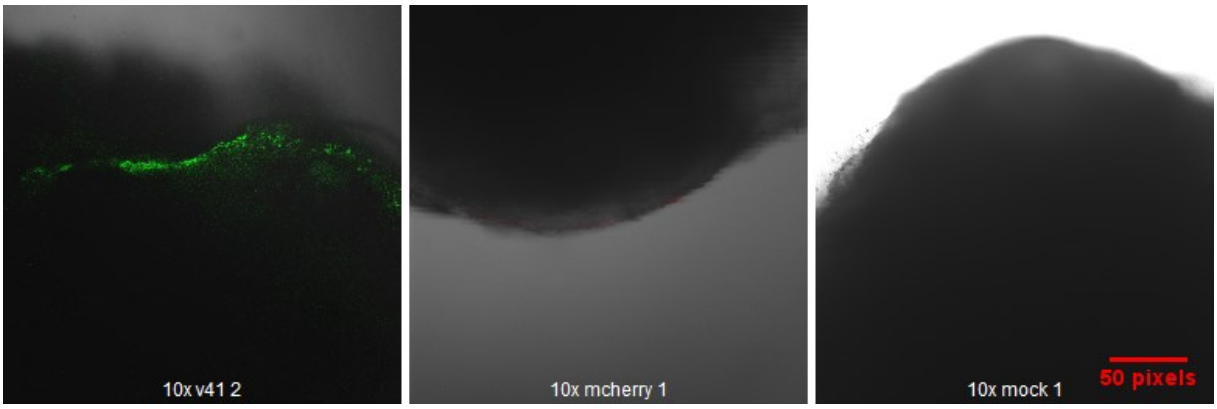
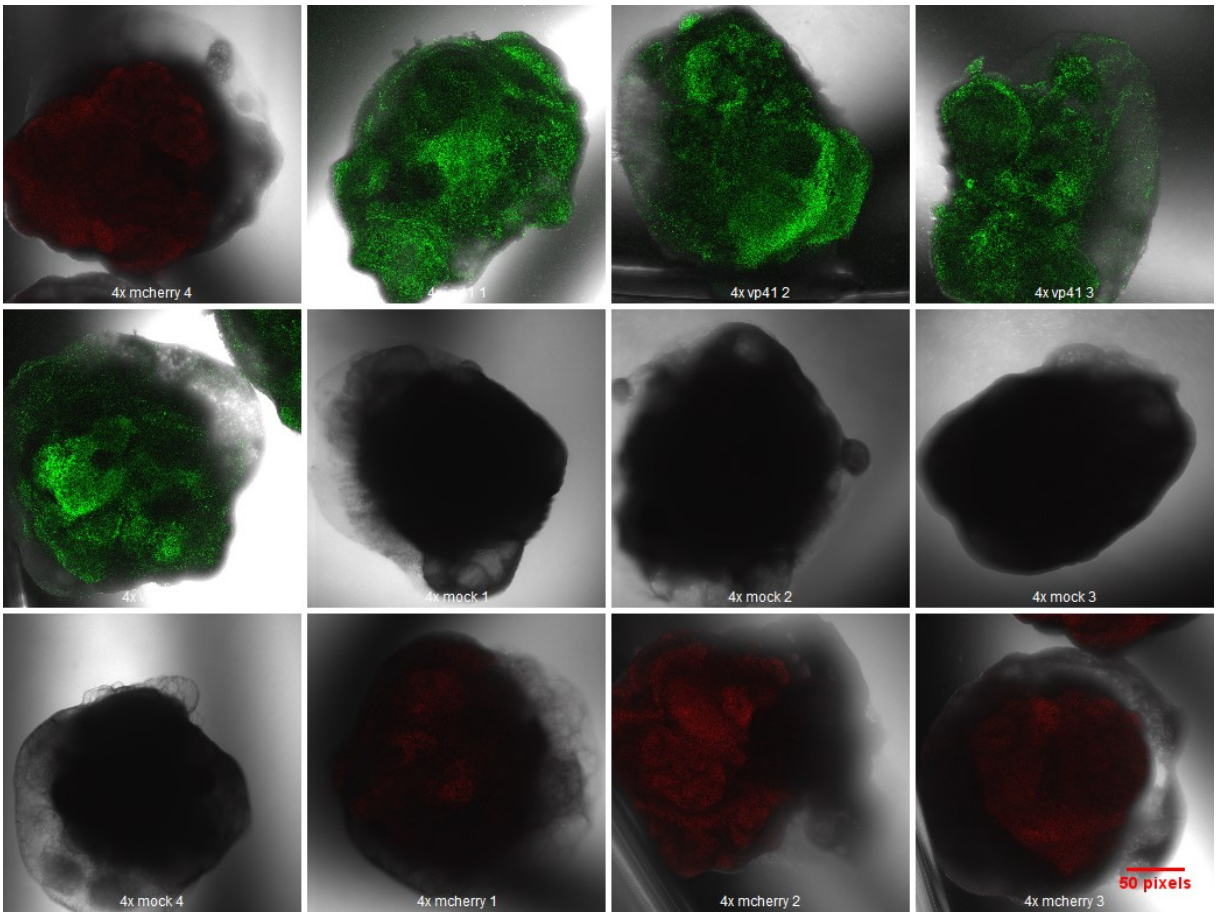


Figure 7. Images representing again the three different conditions (four images per condition), but at day 3. Adopted with Imagej software.




## 3. The oHSV1-mCherry viral titer is lower than HSV1-V41

In order to evaluate how much viral load from each type of virus is present in each condition, we performed a viral titration assay through plaque assay method. Upon following the final steps of the viral titration procedure, we observed the plates under the fluorescent microscope and counted how many plaques were present. From the number of plaques, we proceeded to determine the number of Plaque forming units (PFU). Particularly, for the calculation, we considered the following formula:

$$\frac{\text{PFU}}{\text{ml}} = \frac{\text{Number of Plaques}}{\text{Diluition Factor x Volume Plated}}.$$

For HSV1-V41 virus we observed a viral titer of $2.9 * 10^3$ pfu/ml while the viral titer for oHSV1-mCherry was $1.2 * 10^3$ pfu/ml. So, the viral load of oHSV1-mCherry was more than twice less than

the wild-type virus, which justifies our hypothesis that the oncolytic virus is less cytotoxic and has more sparing capability than the wild type virus, confirming the previous results obtained by confocal microscopy.

# Discussion and conclusion with future perspectives

Oncolytic viruses are clearly very potential way to treat cancer, but also to spare healthy cells. As we have seen in this experiment, oncolytic HSV-1 virus has the potential to effectively spare healthy neurons; particularly, the analysis conducted on cerebral organoids confirmed the neuro-attenuated phenotype of the virus, highlighting a favorable safety profile toward healthy tissue, a condition essential for future clinical application. In parallel, further studies, conducted in Prof.ssa Arianna Calistri laboratory, carried out on cerebral glioblastoma (GBM) tumoroids demonstrated a marked cytotoxic effect on tumor cells, confirming the virus's ability to target the neoplastic compartment. Finally, the analysis of cerebral assembloids, a combination between cerebral organoids and tumor GBM spheroids, provided further evidence of the virus's antitumor specificity, revealing a clear tropism toward tumor cells and the capacity to progressively disrupt the neoplastic component within complex three dimensional environments.

Despite these promising data for a future use of OVs in GBM treatment, different challenges are still ongoing. [5]

## 1. Systemic OVs administration

Systemic administration of OVs faces several challenges, including rapid clearance by the immune system, meaning that the viruses encounter neutralizing antibodies, complement system activation, antiviral cytokines (type I IFN), and sequestration by liver/spleen, which requires more often administration of the therapeutics. [5] Another problem is the nonspecific accumulation in off-target tissues (like the liver, spleen, lungs, or vascular endothelium). This happens because OVs often lack strong tumor specificity after systemic injection. [5] The limited ability to reach the brain due to the blood-brain barrier (BBB) poses a further issue; [4], [6] it blocks most OVs from reaching the CNS parenchyma, the high-molecular-weight virions can't easily extravasate, due to poor penetration capabilities of the barrier. As a result, efficient delivery of OVs to central nervous system malignancies such as glioblastoma remains a major obstacle. A previous study conducted in Prof.ssa Arianna Calistri laboratory, demonstrated that monocytes could be great carrier cells for OVs delivery. [4] Particularly, these cells are capable of crossing the blood-brain barrier, protect OVs from

antibody neutralization and transfer viral payload to GBM spheroids, negatively affecting their viability. [4]

## 2. Combinational therapies

Despite OVs represent a promising approach for GBM treatment, the tumor heterogeneity and the immunosuppressive microenvironment can reduce overall efficacy and complicate clinical translation. For this reason, combinational approaches, which integrate oncolytic virotherapy with radiotherapy, chemotherapy, and/or immunotherapy, represent a promising strategy to overcome these limitations, thereby improving therapeutic efficacy and patient outcomes. [5]

Among other immunotherapies, the Chimeric Antigens Receptors (CAR)-T therapy is one of the most innovative methods for treating cancer. It emerged in the 50s/60s and was developing from that time on, until great success was made in the early 2000s. This sort of immunotherapy works in this way: a patient's own T cells are harvested from blood, genetically engineered in a lab to express a synthetic CAR receptor – fusing tumor antigen recognition (scFv from antibodies) with T-cell signaling domains – expanded massively, then reinfused to search for and destroy cancer expressing specific antigens. This cellular immunotherapy is functioning in a similar way to other therapies of this kind like TCR-T (engineered T cell receptor therapy), TILs (tumor-infiltrating lymphocyte therapies), and NK cells. All of them are successful against blood cancers but struggle with solid tumor infiltration, so they can be boosted by combining them with oncolytic viruses (OVs). Engineered OVs expressing transgenes (e.g., IL-7 from oncolytic adenovirus paired with B7H3 CAR-T) dramatically enhance T cell proliferation, reduce apoptosis, and improve tumor control compared to monotherapies. Multi-armed OVs delivering cytokines, checkpoint inhibitors, and BiTEs alongside HER2 CAR-T cells further amplify efficacy, promoting better survival through synergistic immune activation and viral oncolysis. [5] The same therapies can be applied with oHSV-1, making it valuable tool for future analysis, studying, and integration into the scientific field.

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