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Gene therapy of neurological and non-neurological diseases using herpes simplex virus vectors

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ABSTRACT

Two main types of vectors can be derived from herpes simplex type 1 (HSV-1), non-replicative vectors and replication-selective oncolytic vectors. The main goal of this review is to describe the key features that make non-replicative HSV-1 vectors (nrHSV-1) extremely appealing for gene therapy of peripheral and central nerve diseases. This includes the many outstanding adaptations that this human neurotropic virus has developed to safely infect, persist, and perform long-term gene expression in neurons, their very large transgene cargo capacity, unique amongst nuclear mammalian viruses, and the very important fact that they can be readministered several times due to the extremely low levels of immunological responses they elicit. This review also describes several preclinical studies as well as the clinical trials that have been developed or are ongoing with nrHSV-1, including gene therapy of cancer-related intractable pain and of recessive dystrophic epidermolysis bullosa (RDEB), which has been recently approved by the FDA. In the second part of this review, we compare side-by-side, the advantages and the drawbacks of nrHSV-1 and adeno-associated vectors (AAV), the most currently used gene therapy vector. Lastly, we will very briefly describe oncolytic vectors and a second type of nrHSV-1 vectors known as amplicons.

Introduction

Several recent preclinical and clinical developments have confirmed the outstanding potential of gene therapy for the treatment of genetic and non-genetic diseases. Amongst the gene transfer instruments that are being used to reach these goals, non-replicative viral vectors occupy a prominent place. This review will focus on the main features of one of these systems, the recombinant non-replicative (nr) herpes simplex virus type 1 (HSV-1)-derived vectors. In the first and main part of this review article, following a brief introduction to relevant aspects of the molecular and cellular biology of HSV-1, we will address the current preclinical and clinical status of recombinant nrHSV-1 vectors. In the second part we will compare the relevant features of nrHSV-1 with those of the adeno-associated vectors (AAV), which are by far the most common viral vector currently used in gene therapy. In the last part, we will briefly tackle a different type of non-replicative HSV-1 vector (known as the amplicon vectors), which have not yet reached the clinic, but possesses some outstanding properties that make them potentially a vector system of choice for many gene therapy applications, provided that current limitations, linked mainly to the difficulty of producing these vectors in high amounts and GMP compliant procedures can be resolved.

Hsv-1 cell cycle and molecular biology

HSV-1 is a prevalent neurotropic enveloped human pathogen with worldwide prevalence of about 67%[1]. This virus normally induces immunity that prevents reinfections with the same serotype, but not with HSV-2[2]. Infection can be asymptomatic, mild, or life-threatening. Transmission of HSV-1 occurs through close contact and typically follows a three-phase biological cycle: primary infection of epithelial cells in the oral (HSV-1) or genital (HSV-2) mucosa, lifelong latency in the sensory neurons innervating the infected region, and reactivation, generally ending in reinfection at the site of entry in peripheral mucosa[2]. In immunocompetent individuals, HSV-1 causes only mild and self-resolving diseases, such as stomatitis, cold sores, or genital herpes[2]. In extremely rare situations, infection is associated with diseases with high morbidity (herpes stromal keratitis, meningitis) and mortality (herpes simplex encephalitis)[3]. Although the reasons underlying this increased morbidity are not completely elucidated, they seem to be related to subtle defaults in immunity, as genetic polymorphisms/mutations in innate defenses are often linked to a higher risk of encephalitis or disseminated disease[4-7]. Neonatal infection is more aggressive, leading to systemic dissemination with high morbidity if untreated[5]. In most cases, however, primary infection and reactivations are rapidly controlled by the immune system without significant inflammation or strong immune reactions[4,6]. In addition, effective anti-viral drugs, such as acyclovir, are available, as opposed to most other viruses[3]. HSV-1 has evolved numerous functions to evade, moderate, and subvert immune surveillance[2]. Engineering the virus to exploit and manipulate these counteracting functions is key to designing optimized HSV-1 vectors, allowing, for example, repeated vector administrations[8,9] in contrast to other vectors impeded by their immunogenicity.

The HSV-1 DNA genome is a linear molecule with a size of about 150-kbp and encodes more than 80 genes and more than a dozen miRNA[2]. This genome is contained into an icosahedral capsid, coated with tegument, a layer of at least 20 proteins important for gene expression and virion assembly, and a lipid envelope studded with 11 different virus-encoded glycoproteins[2]. The envelope glycoproteins are involved in viral entry, spread, and immune evasion. Several of them (gB, gD, gH, and gL), are necessary for infection, interacting with nectin-1, the main cellular receptor, and inducing fusion of the virus and cellular membranes[9,10]. Following fusion, the tegument proteins and capsid dissociate, with the capsid being transported to the nuclear membrane, from where the genome enters the nucleus[11]. Tegument proteins of incoming virus particles exert their effects immediately following entry. Many of them counteract cellular antiviral

innate defenses while others, such as the tegument VP16 (also known as α-TIF, or alpha-transinducing factor), promote gene expression[2]. Following arrival to the cell nuclei, the DNA genome, which is linear in virions, takes a circular configuration. Then, depending on the type of infected cell, the HSV-1 life cycle will establish either a lytic or a latent infection. During a lytic productive infection, which generally takes place in epithelial cells, a temporal cascade of gene expression occurs, characterized by the successive synthesis of immediate-early (IE or α), early (E or β), and late (L or γ) viral proteins[2]. Most IE proteins (ICP0, ICP4, ICP22, and ICP27), whose expression is induced by VP16, are regulatory proteins that counteract the innate cellular defenses, including genome silencing, and activate expression of the early and, after virus DNA replication, the late genes[2]. Another IE protein, ICP47, is an inhibitor of human transporter associated with antigen processing (TAP), blocking MHC I antigen presentation and facilitating escape from adaptive immune responses[12]. Early proteins include the virus DNA polymerase, helicase/primase, and other proteins required for replication and packaging of the virus genome, while the late proteins are structural proteins (capsid, tegument, envelope) involved in virion morphogenesis and cell exit, which is accompanied by cell death, generally between 12- and 20-hours post-infection, depending on the multiplicity of infection (MOI) and the susceptibility of the infected cells.

Following infection of sensory neurons through the neuron terminals and efficient retrograde transport along the axons to the neuron nucleus, the virus genome generally establishes a latent infection. In this case, no IE proteins are expressed, in part due to a retention of VP16 in the axons. In the absence of IE proteins the virus cannot counteract genome silencing nor induce expression of early and late genes, so that the transcription cascade is not triggered, the virus genome will be epigenetically repressed by heterochromatin, and will remain as a non-integrated silent nuclear extrachromosomal episome[2]. Only the LAT locus of the virus genome, which expresses a family of untranslated latency-associated transcripts (LATs), remains transcriptionally active since it is bordered by two DNA insulator motifs that protect this locus from DNA silencing[13]. Genome latency in the nuclei of sensory neurons in the dorsal root ganglia (DRG) or the trigeminal ganglia (TG) can be considered as a sort of compromise where the silent HSV-1 genome and infected cells are preserved from immune attack, while the host CNS is protected from virus spread. The virus genome can remain in a latent state for years, before being eventually reactivated by danger signals such as stress, fever, immunosuppression, or other stimuli, in which case the lytic gene expression cascade resumes, giving raise to progeny particles that are addressed via the axons in anterograde direction until reaching peripheral epithelial cells, where recurrent or secondary infection takes place[2,13].

Non-replicative hsv-1-based vectors

There are two types of vectors that can be derived from HSV-1: (a) non-replicative vectors for therapeutic transgene delivery to treat genetic and non-genetic diseases (gene therapy)[14] and (b) replication-selective oncolytic viruses for cancer treatment (virotherapy)[15]. Both vector types inherit many of the features of the wild-type virus, including the fact of being non-integrative, and leverage them to achieve their therapeutic goals. As above quoted, this review will focus mostly on recombinant nrHSV-1 vectors developed for gene therapy. These vectors are deficient in at least one essential function and can be efficiently produced only in complementing cells expressing the missing function(s). First-generation nrHSV-1 vectors were non-replicative due to the absence of the essential immediate-early ICP4 protein. However, they displayed cytotoxicity in non-neuronal cells due to the expression of the other IE functions, mainly ICP0 and ICP27[16]. These early studies introduced the notion that nrHSV-1 vectors were cytotoxic and immunogenic, an outdated view that has persisted despite further engineering that resulted in vectors functionally deficient in the expression of all IE

genes.[17] These last-generation vectors are fully non-toxic in infected cultured cells yet preserve robust and durable transgene expression when driven by adequate regulatory sequences and are protected from epigenetic silencing. Moreover, these vectors show no evidence of cell toxicity or induction of inflammatory cell infiltrates following injection in animal models in vivo, including the CNS[17,18].

Since HSV-1 is a natural neurotropic virus, it is normal that first attempts to perform gene therapies using recombinant nrHSV-1 vectors, mainly in preclinical settings, addressed diseases or aberrant gene expression of peripheral neurons, thereby exploiting the many outstanding adaptations of HSV-1 to these neurons. These features include:

- High density of cellular receptors (nectin1) in neurons[19], favoring vector infection and thus allowing for limited vector doses to reach efficacy,
- Tegument viral proteins that interact with microtubule-associated proteins[20], allowing the capsids to be very efficiently and rapidly retrogradely transported along the axons up to the neuron nucleus,
- The presence of several virus tegument proteins in the incoming particles, able to overcome cellular antiviral defenses from the beginning of infection[21], thereby reducing the multiplicity of infection required for efficient transgene transduction,
- Establishment of latent infection in sensory neurons[22], a condition in which, as above quoted, the vector genome is maintained as an extrachromosomal episome, with no insertional mutagenesis[23], and in which the full set of virus genes expressing lytic functions is epigenetically repressed. Only the short LAT region of the virus genome, which expresses no virus proteins, remains transcriptionally open and can be used to accommodate foreign transgenic DNA with long-term expression[24],
- The very large size of the virus genome, which allow to transport and deliver up to 30/40-kbp of foreign DNA to the nucleus of peripheral neurons[25],
- The fact that the immune reactions against the virus are low since they are efficiently counteracted by tegument proteins delivered to the cells or by viral proteins expressed immediately after infection[26], resulting in no rejection of virus particles or infected cells, no significant modification in the immune status of the receiving patient, no immune reaction precluding transgene expression and, above all, the possibility of redosing several times the vector at the same or different anatomic place[7,8],
- Lastly, owing to their large size and efficient entry, the vector displays limited tissular diffusion upon local administration, thus favoring targeted delivery.

Several nrHSV-1 vectors have intended to exploit one or more of these features, resulting in non-toxic vectors that can express transgenes in peripheral neurons in the medium or long-term. At the preclinical level, such vectors were used to treat experimental animal models of neurogenic bladder overactivity[27,28], neuropathic and inflammatory pain[29,30], or neuropathies.[31,32] Examples of transgenes used in these cases are (a) glutamic acid decarboxylase (GAD65 or 67)[27], to increase secretion of the inhibitory GABA neurotransmitter, or kynurenine aminotransferase (KAT)[28], to increase the expression of kynurenic acid, an inhibitor of glutamatergic receptors, in both cases resulting in reduction of neurotransmission and mitigation of chaotic bladder contractions in neurogenic detrusor overactivity, (b) human proenkephalin[29] or miRNA to inhibit sodium channel Nav subunits[30] to reduce pain, and (c) different neurotrophic factors such as NGF[31] or NT3[32] in different models of diabetic neuropathy. In all these cases the vector particles were inoculated in peripheral tissues, from where they were retrogradely transported to the innervating DRG followed by transgene expression, showing in all cases a clear alleviation of symptoms. Based in these and other observations, one vector prototype (termed NP2) was used for a first clinical trial, to treat intractable cancer-related pain[33].

Gene therapy for cancer-related pain

This first clinical trial exploited the ability of HSV-1 vectors to express transgenes after establishing a

latent infection in the DRG. A dose-escalating, phase 1 clinical trial of NP2 (NCT00804076), a non-replicative HSV-1 recombinant vector expressing human proenkephalin (PENK), was conducted in terminal cancer patients with intractable focal pain[33]. NP2 was injected into the affected dermatomes, with virus taken up by nerve terminals and transported to the DRG, where the vector institutes a persistent, quasi-latent state with stable PENK expression. No treatment-related serious adverse events (AE) were reported and no subject seroconverted[33]. Pain relief was reported for the middle and high doses (10E8-10E9 plaque forming units (pfu) per individual)[33]. Since NP2 establishes a silent latent infection in the DRG, no expression of toxic lytic functions took place. This clinical trial showed for the first time that intradermal delivery of a nrHSV-1 vector was safe and potentially efficacious, and that a nrHSV-1 recombinant vector expressing a therapeutic function from a latent genome in DRG could be used in humans, which were the primary endpoints of this trial. A phase 2 trial (NCT01291901) was unfortunately discontinued for financial reasons.

Gene therapy for severe skin diseases

More recently, a nrHSV-1 known as B-VEC (beremagene geperpavec, Vyjuvek™, or KB103) that expresses two copies of the COL7A1 gene driven by the strong HCMV promoter, was developed to treat recessive dystrophic epidermolysis bullosa (RDEB), a shattering skin disease resulting from collagen VII (C7) mutations impairing anchoring fibrils[34,35]. Repeated doses of B-VEC were topically administered to freshly renewed skin to treat the symptoms but not cure the disease. Unlike the NP2 study, this trial exploited the ability of B-VEC to strongly express transgenes in superficial layers of the skin and to be readministered as many times as required. This trial is unprecedent and innovative in that the vector does not address the peripheral nerve system. Latency is therefore irrelevant in this trial, as is the fact that B-VEC can express some toxic immediate-early functions, such as ICP0 and ICP27, since skin cells are constantly being renewed. B-VEC also expresses IE ICP47, which inhibits antigen presentation and reduce immune recognition[36], facilitating multiple administrations[34].

A randomized, placebo-controlled, phase 1/2 clinical trial (NCT03536143), matching wounds from RDEB patients receiving topical B-VEC and placebo repeatedly over 12 weeks, met primary and secondary objectives[34]. No grade 2 or above B-VEC-related AEs, vector shedding, or skin immune reactions were noted[34]. A phase 3, double-blind, intra-patient randomized, placebo-controlled clinical trial of weekly applications of B-VEC gel in RDEB patients (NCT04491604) was completed with similar results for safety and lack of significant immune responses[35]. The primary endpoint was complete wound healing at 6 months, reached in 67% of wounds and significantly better than the placebo 22%. Six of eight seronegative patients seroconverted, and 13 of 18 developed antibodies to C7 without significant immunologic reaction, and no association between HSV-1 serostatus or C7 seroconversion[35]. Based on this, B-VEC was the first nrHSV-1 vector and the first topical gene therapy approved in the US, in 2023[37]. The ease of topical application has advantages over invasive procedures, but more data is necessary to confirm long-term efficacy[38].

Following a similar approach, KB105, a non-replicative HSV-1 vector encoding human transglutaminase I (TGM1), was developed to treat autosomal recessive congenital ichthyosis (ARCI)[39]. Preclinical studies demonstrated that repeated topical administration induced TGM1 protein in the target epidermal layer only at the dose site, without fibrosis, necrosis, or acute inflammation[39]. A phase 1 clinical trial has recently begun (NCT04047732).

To summarize, these clinical trials and preclinical studies clearly demonstrate the absence of severe AEs thus confirming the safety of recombinant nrHSV-1 vectors, while displaying varying levels of efficacy. Importantly, in no case did vector administration or transgene expression result in

immune reactions that might prevent treatment efficacy, independently of pre-existing HSV-1 exposure.

nrHSV-1 and AAV vectors, A side-by-side comparison

As for now, eighteen in vivo viral based gene therapies have already obtained regulatory approval by FDA. Various non-replicative viral vectors are being used, including adenovirus (Ad), adeno-associated virus (AAV), lentivirus (LV), and herpes simplex virus (HSV). Of them, AAV is by far the preferred choice in clinical trials. AAV vectors have emerged as a potential cure for untreatable genetic diseases owing to their many distinctive appealing attributes, including the ease with which they can be built and the fact that they can be administered systemically via intravenous inoculation, and are being employed in ongoing clinical trials for ocular, neurological, hematological, metabolic, neuromuscular, and cardiovascular diseases as well as cancers. Seven AAV-based gene therapy products have received regulatory approval. For a recent and comprehensive review article on AAV vectors see[40].

However, despite some significative successes, such as Luxturna, an AAV vector developed to treat Leber congenital amaurosis type II[41], or Zolgensma, a vector used to treat spinal muscular atrophy (SMA)[42], numerous concerns and challenges remain that restrain the widespread applications of AAV, particularly for complex disorders. These limitations and drawbacks include inadequate tropism to many cell types, limited cargo capacity, inefficient transduction capability -thus requiring very high vector doses to obtain the desired therapeutic effect-, induction of immune responses to high systemic doses, coupled with significant sensitivity to neutralizing antibodies and, most important, several serious adverse effects such as genotoxicity, hepatotoxicity, thrombotic microangiopathy, and neurotoxicity, sometimes reaching lethality, have been reported following high-dose administration[40].

Although HSV-1 is currently less used as a gene therapy vector, we believe it is interesting and motivating to compare the main characteristics of AAV and HSV-1 side by side, in order to highlight the advantages of HSV-1 in many respects.

Cargo capacity. While the size of the AAV genome, and therefore its cargo capacity, does not exceed 4.7-kbp[40], recombinant nrHSV-1 can be used to deliver more than 30-kbp of foreign DNA, nearly one order of magnitude greater[14], and even higher amounts using HSV-1 amplicon vectors (see below). This is extremely important as many genes, including dystrophin, von Willebrand factor, type 1 neurofibromatosis (NF1), as well as genes involved in ophthalmic diseases such as Stargardt disease and Usher syndrome, amongst many others, are too long be incorporated into the AAV genome, even as a cDNA copy. Furthermore, most mammalian genes contain introns and use differential splicing as a mechanism allowing the generation of different protein isoforms from a single gene, which is of critical importance since different protein isoforms are often expressed in different tissues. This cannot be obtained with AAV as these vectors deliver a single cDNA, thus encoding a unique isoform. In addition, for safety and efficacy reasons, the therapeutic transgene should in many cases be driven by tissue-specific and physiologically controlled regulatory sequences, to ensure the precise quantitative, spatial, and temporal parameters defining efficient and safe therapeutic gene expression. These key sequences are generally quite long and, again, they cannot be included in the AAV vector genome. These requirements, in contrast, can be largely achieved with nrHSV-1 vectors, as illustrated by B-VEC, a recombinant vector which delivers some 20-kbp of foreign DNA to RDEB cells, even bringing 2 copies of the therapeutic transgene in a single particle[34], or by an HSV-1 amplicon vector delivering the 135-kbp human frataxin locus, including introns, to cells derived from Friedreich's ataxia patients[49].

DNA integration and genotoxicity. Wildtype AAV can integrate into the mammalian genome[43,44] and

data in animal models suggest that the chance for viral genome integration is similar between wildtype AAV and AAV vectors[45,46]. The liver appears to be a hotspot for AAV integration[47], but viral genome integration in other organs such as the heart has been found as well[45]. Although the frequency of AAV DNA integration is relatively limited, this clearly constitutes a critical safety issue as partial or complete genome integration is dangerous, it can be mutagenic, even oncogenic. At the opposite, wildtype HSV-1 never integrates[2,14] and no nrHSV-1 vectors, or even oncolytic HSV-1 vectors which are replication-competent, have ever been claimed to integrate into cellular DNA and are therefore safer than AAV in this regard.

Efficiency of transduction. Both in preclinical and clinical trials, the doses of nrHSV-1 vectors rarely exceed 10E9 plaque forming units (pfu)/animal or individual and are often lower. For example, in the case of B-VEC, the doses can vary from 10E8 to 8x10E8 pfu/wound, depending on the wound size, while demonstrating high efficacy of therapeutic action[34,35]. Furthermore, several preclinical studies demonstrate that doses as low as 10E6 pfu are enough to ensure high level transgenic expression and retrograde transport following stereotaxic or intrathecal inoculation of nrHSV-1 vectors in the rodent brain[18,48,50]. At the opposite, in many clinical trials with AAV, the inoculated doses often exceed 10E13 genome copies (gc)/kg and can be as high as 2X10E14 gc/kg[42,51], meaning for example that a 4-5-kg child could receive a dose in the order of 10E15 gc[42]. These astonishingly high quantities imply that the therapeutically efficient AAV doses are 10E4 to 10E5 times higher than those normally used with HSV-1. It is useful to highlight that AAV is not a neurotropic virus although some capsid types, such as AAV9, display superior neurotropism than others, and lack specific adaptations to neural tissues while, as above quoted, HSV-1 possess outstanding neural adaptations, such as very efficient penetration due to membrane glycoproteins interacting with high-density (nectin1) neuronal receptors, rapid and efficient retrograde axonal transport due to specific interactions with microtubule associated proteins, lifelong latency, and neural-specific DNA insulators preserving transgene expression from epigenetic silencing.

Several reasons can be advanced to explain why such high doses are required with AAV, including the absence of viral proteins in the incoming particles able to counteract cellular antiviral responses[40], the fact that AAV vectors are single-stranded and must be converted into double-stranded genomes before expression, which is a relatively inefficient event[52], or the fact that the vector genome does not have natural DNA insulators that could protect it from epigenetic gene silencing[53]. All these, and others, are important questions that cannot be developed further within the framework of this review. The central points that we wish to highlight here are the several negative outcomes that arise because of the high vector doses employed, which include (a) the much higher cost of production, (b) pronounced immune responses elicited by the huge amount of AAV particles following intravenous administration, or (c) the direct or indirect toxic effects often observed, particularly in the liver.

Immunogenicity. The immunogenicity of AAV poses a specific challenge, impacting the safety, durability, availability, and efficacy of gene therapy. AAV encounters numerous biological barriers instituted by the immune system, including pre-existing immunity, complement activation, innate pattern receptors, and adaptive B cell and T cell immunity[40]. Pre-existing immunity is the single most detrimental burden to AAV gene therapy because even low levels of seropositivity can markedly reduce transgene expression following intravenous administration[54]. While different strategies are being developed to mitigate immune responses, it must be emphasized that, as above quoted, AAV vectors are themselves defenseless particles in that they lack efficient biological weapons to counteract the immune barriers. One major consequence of the presence of neutralizing antibodies is the difficulty or the impossibility of redosing the vector. This is at the opposite of what happens with nrHSV-1 vectors, which contain in their tegument, or

express soon after infection, several functions able to counteract both innate and adaptive host immune responses from the beginning of the infection[2,26]. As above quoted, seropositivity and immune responses against nrHSV-1 vectors are low and do not eliminate vectors or infected cells, do not have a major impact on transgene expression, and the vectors can be re-administered several times, as illustrated by B-VEC gene therapy of RDEB[34,35].

Toxicity. Safety is a major concern for gene therapy development, and the use of AAV is not without risks. As above quoted, AAV could be genotoxic as AAV integration has been found even in humans. But still more challenging, several studies reported more direct forms of hepatotoxicity and neurotoxicity caused by AAV vectors[40,61]. Despite remarkable success in SMA and promising results in other diseases, fatality has been observed due to liver, kidney, heart, or lung failure. It has been reported that 4 boys treated for X-linked myotubular myopathy (XLMTM) died of liver failure following high-dose AAV vector administration[55], two patients with SMA have died of acute liver failure following treatment with Zolgensma[56], while a third patient with the same treatment died from thrombotic microangiopathy (TMA)[57], and a patient with Duchenne's muscular dystrophy (DMD) died after high-dose AAV9 gene therapy[58]. It is thought that innate and adaptive immune responses to the very high doses of vector administration, sometimes exceeding 10E15 total genome copies, play a critical role in the toxicity, and several articles have raised warnings and call for caution concerning the indiscriminate use of so large doses of AAV[59-61]. It is difficult however to compare AAV and nrHSV-1 induced toxicity as much less preclinical and clinical studies have been carried out with nrHSV-1 vectors, and most of them relate to the peripheral nerve system. It is noteworthy however that no major toxic effects have ever been described in studies with nrHSV-1 or even with oncolytic HSV-1.

So, a central question that can be asked is why nrHSV-1 vectors are less used than AAVs. To answer this question, it is important to realize that, at the opposite of simpler vectors such as AAV, HSV-1 vectors derive from a complex virus whose genome has a size of 153-kbp and encode some 80 genes and more than a dozen microRNA, and that can establish distinct interactions with different cell types. The molecular and biological complexity of HSV-1 has complicated the handling of the viral genome, which is why only a few teams, with a deep knowledge of the molecular biology of HSV-1, have devoted themselves to the design of these vectors. In addition, it was not until recently, with the advent of efficient cloning and engineering of large genomes in bacterial artificial chromosomes (BAC) together with inexpensive and timesaving sequencing technologies, that it was finally possible to advance more rapidly in vector development.

A second reason is that nrHSV-1 cannot be efficiently administered systemically via intravenous inoculation and does not cross the brain-blood barrier. This has restricted nrHSV-1 vector applications only to diseases that can be treated via focal administration. To date these vectors have been used either in the peripheral nerve system, where they establish latent infections[33], or in epithelial cells, where they are used for short-term but renewable therapeutic strategies[34,35].

The main technical difficulties in the development of non-replicative HSV-1 vectors that were encountered along the way were (a) the need to suppress or repress the full set of immediate-early proteins, which is the key for the stable silencing of the whole genome, (b) the need to generate producer cell lines able to express complementing amount of the missing viral functions, thereby producing large amounts of the defective vectors, and (c) the understanding of how to deal with the epigenetic repression of the virus genome that occurs when the full set of IE genes is silenced or deleted. These elements are currently better understood and being resolved[14,17,18,62], allowing now to produce and use these vectors in clinical trials.

Other HSV-1 based-vector types

In addition to recombinant nrHSV-1 there are two other vector types that can be derived from HSV-1, amplicon vectors and oncolytic vectors.

Amplicon vectors

HSV-1 amplicon vectors, or amplicons, also are non-replicative vectors. Although these vectors cannot yet be used clinically, it is important to briefly describe them because they have enormous therapeutic potential, provided that technical problems that currently hinder their production under GMP conditions could be resolved. These vectors are HSV-1 particles lacking the viral genome, allowing thus to deliver up to 150-kbp of foreign DNA, the full capacity of the virus particle, to mammalian cells. The amplicon vector genome derives from a plasmid (the amplicon plasmid) containing one origin of virus DNA replication (oriS) and one virus packaging signal (pac), that allow their replication/packaging into vector particles. The amplicon plasmids, and thus the amplicon vector particle, carry the desired transgenic expression cassette and frequently include a gene expressing a reporter protein to facilitate their counting. Since they lack the HSV-1 genes coding for virus proteins that are mandatory for the replication and packaging of its genome, they are helper-dependent vectors. The helper virus genome provides the genomic material required for replication and packaging of the amplicon genome, thus generating vector particles. Amplicons are extremely interesting and potentially very promising vectors since they do not express virus genes even at trace levels and are therefore absolutely safe, while being able to deliver very large amounts of foreign DNA to mammalian cells, both in vitro and in vivo. However, the presence of contaminating helper virus particles still hampers their use in clinical trials[63].

Some 20 years ago, critical improvements in amplicon vector technology permitted to reduce the contamination with helper particles. One of these methods[64] consists of co-transfecting eukaryotic cells with the amplicon plasmid together with a BAC containing a defective HSV-1 genome that cannot be packaged because the packaging sequences have been deleted. This strategy does produce helper-free amplicon vectors but only in very limited amounts, too low to be used in clinical trials at a reasonable cost. A different strategy[65] uses as helper a nrHSV-1 virus whose genome contains loxP1 sites surrounding the pac signals. In complementing eukaryotic cells expressing a Cre recombinase and the essential genes lacking in the helper, this system produces large quantities of amplicon vectors, while most of the helper genomes are not packaged into viral particles due to the deletion of the floxed pac sequences by the Cre recombinase. Although this method does produce large amounts of amplicon particles, the vector stocks remain however contaminated with about 1% of helper particles that have escaped Cre-loxP1 recombination, precluding their use in clinical trials even if the helper particles are defective. Therefore, there is yet no available production system that could simultaneously generate high amounts of amplicon vectors free from helper virus contaminants[63]. Other aspects of amplicon biology, such as the possibility to establish long-term latent infections in neurons have not been thoroughly investigated. Based on these shortcomings, more work needs to be done before HSV-1-derived amplicon vectors could be used in the clinic.

However, amplicon vectors remain interesting tools to investigate pathophysiology such as monosynaptic anterograde neural circuit tracing[66], to develop therapeutic proofs of concept, and to screen potential therapeutic transgenes. Indeed, amplicon vectors have many advantages, which are (i) reduced immunogenicity due to the lack of viral protein expression, (ii) very large cargo capacity, virtually up to 150 kb[49], and (iii) high versatility and ease of construction. In the last decade, amplicon vectors have been used in animal models as potential vectors to address cancer, neuropathic pain, and neurodegenerative diseases. Screening strategies can be illustrated by studies aiming to

use amplicons to screen potential transgene and promoter candidates to generate optimal expression cassettes. For instance, amplicons carrying expression cassettes coding for different botulinum toxin light chains (BoNT-LC) under the control of a strong promoter were generated to assess the inhibition of neuropeptide release (CGRP) in transduced primary cultures of embryonic DRG neurons[67]. This study discriminated the potency of different BoNT-LC to inhibit neurotransmission in this cellular model. In a complementary study the same group used amplicon vectors carrying two expression cassettes, one coding for firefly luciferase (FLuc) driven by different sensory neurons promoter candidates, and the other coding for renila luciferase (RLuc) driven by an invariable strong viral promoter[68]. This study allowed to assess the strength and selectivity of different promoter candidates in organotypic cultures of explanted adult DRG, or sympathetic and parasympathetic ganglia from control and spinal cord injured (SCI) rats. Other studies developed amplicons containing AT-rich and insulator-like DNA sequences to provide longer-term expression within the rodent central nervous system, up to at least 6 months[69]. Others have investigated retargeting strategies[70]. These studies are certainly stimulating innovation in this field and facilitate the potential use of amplicon vectors.

Oncolytic HSV-1 vectors

Although not the central focus of this article, we should not forget the oncolytic HSV-1 vectors (oHSV-1), which are attenuated but replication competent HSV-1 vectors that can selectively multiply only in cancer cells, thus sparing normal tissues[71]. They are a distinct class of cancer therapeutics, virotherapy, with two unique mechanisms of action: (i) oncolytic / cytotoxic activity directly killing cancer cells while sparing normal tissues and amplifying *in situ*, and (ii) inducing tumor inflammation and anti-tumor immune responses. Because cancer is often lethal with a large commercial market, there have been many more oHSV clinical trials investigating safety (phase 1) and efficacy (phase 2 and 3) than with nrHSV vectors. oHSV was the first genetically engineered oncolytic virus (OV) described and one of the most translated OVs to the clinic[72]. oHSVs can be engineered for oncolytic activity and safety by deleting/mutating viral genes necessary for pathogenicity, replication in normal post-mitotic cells, inhibiting anti-viral immune responses and/or apoptosis[31,73-75]. This targets dysregulated/ defective cell physiology common to most cancer cells, such as cell cycle control, cell proliferation, apoptosis, and innate immunity[71].

For oHSV, where immune responses are larger and a key component of efficacy, immunity does not seem to have a serious negative impact on safety and possibly has a positive impact on efficacy. Overall safety and tolerability in patients are much better than expected. The spectrum of AEs attributable to the virus has been relatively modest, predictable for a viral infection, and less than those seen with most cancer therapies.[76]

One oHSV-1 (T-Vec, Imlygic® or talimogene laherparepvec) a GM-CSF 'armed' virus, for the treatment of advanced melanoma[73] has been approved in the US. A second one (G47Δ, Delytact®) was recently approved in Japan for the treatment of GBM[77]. There have been an additional 22 different oHSVs in clinical trials, currently or previously, for a range of solid tumors: breast, colorectal, gallbladder, GBM, head and neck, liver, melanoma, neuroblastoma, prostate, soft tissue sarcoma, pancreatic, and metastatic (clinicaltrials.gov)[76,79].

Conclusion

The major conclusion stemming from preclinical and clinical studies is that all types of last-generation HSV-1 vectors are safe and potentially efficacious. nrHSV-1 vectors possess numerous salient features that make them very appealing for gene therapy. At the opposite of what happens with other vector systems, the evidence from preclinical and clinical studies indicates that

immune responses against HSV-1 vectors do not constitute a major impediment to efficacy or safety, and multiple dosing can be achieved[8,9,39], even with pre-existing HSV seropositivity. Moreover, nrHSV vectors elicit no or very low levels of immunity, too weak to impact therapeutic outcomes. However, nrHSV-1 vectors also have some limitations, and perhaps the most important is the difficulty to administer them via intravenous delivery due to their large size and membranous nature, thus precluding systemic administration. These vectors do not cross the brain-blood barrier.

Studies with non-replicative HSV-1 vectors have demonstrated that they can be used for stable transgene expression from latently infected DRG neurons[33] or for strong but transient expression in epithelial cells.[34,35] nrHSV-1 vectors have not yet been used in more mainstream gene therapy applications in non-neuronal cells, such as in diabetes, liver diseases, or muscular dystrophies. The next challenge therefore is to demonstrate that these vectors can produce safe and stable transgene expression in non-neuronal cells, by establishing a sort of latent-like infection. This will require not only the complete suppression of toxic functions but also to prevent long-term epigenetic silencing of the vector genome. While these goals seem ambitious, recent results suggest that we are not that far from reaching them.[14,17,18,62] Obtaining such results in preclinical models of non-neurologic or CNS disease would represent important progress toward uncovering the full therapeutic potential of non-replicative HSV-1 vectors.

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None.

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Declaration of Interests

ALE and CJ are cofounders and shareholders of EG 427. ALE is chief scientific officer (CSO) of EG 427. He is a co-inventor on patents related to the use of replication-incompetent herpes simplex virus-based vectors, owned by the University of Versailles Saint Quentin (France) and EG 427.

References:

1. James, C. *et al.* (2020) Herpes simplex virus: global infection prevalence and incidence estimates, 2016. *Bull World Health Organ* 98, 315-329. 10.2471/blt.19.237149

2. Knipe, D. *et al.* (2021) Herpes simplex viruses: Mechanisms of lytic and latent infection. In *Fields Virology* (7th edn) (Howley, P.M. *et al.*, eds), pp. 235-296, Wolters Kluwer

3. Whitley, R. and Baines, J. (2018) Clinical management of herpes simplex virus infections: past, present, and future. *F1000Research* 7. 10.12688/f1000research.16157.1

4. Zhu, H. and Zheng, C. (2020) The Race between Host Antiviral Innate Immunity and the Immune Evasion Strategies of Herpes Simplex Virus 1. *Microbiol Mol Biol Rev* 84. 10.1128/mmbr.00099-20

5. Pinninti, S.G. and Kimberlin, D.W. (2018) Neonatal herpes simplex virus infections. *Semin Perinatol* 42, 168-175. 10.1053/j.semperi.2018.02.004

6. Danastas, K. *et al.* (2020) Herpes Simplex Virus Type 1 Interactions with the Interferon System. *International journal of molecular sciences* 21. 10.3390/ijms21145150

7. Skouboe, M.K. *et al.* (2023) Inborn Errors of Immunity Predisposing to Herpes Simplex Virus Infections of the Central Nervous System. *Pathogens* 12. 10.3390/pathogens12020310

8. Goss, J.R. *et al.* (2002) Herpes simplex-mediated gene transfer of nerve growth factor protects against peripheral neuropathy in streptozotocin-induced diabetes in the mouse. *Diabetes* 51, 2227-2232. 10.2337/diabetes.51.7.2227

9. Chahlavi, A. *et al.* (1999) Effect of prior exposure to herpes simplex virus 1 on viral vector-mediated tumor therapy in immunocompetent mice. *Gene Ther* 6, 1751-1758

10. Madavaraju, K. *et al.* (2020) Herpes Simplex Virus Cell Entry Mechanisms: An Update. *Front Cell Infect Microbiol* 10, 617578. 10.3389/fcimb.2020.617578

11. Jambunathan, N. *et al.* (2021) Two Sides to Every Story: Herpes Simplex Type-1 Viral Glycoproteins gB, gD, gH/gL, gK, and Cellular Receptors Function as Key Players in Membrane Fusion. *Viruses* 13. 10.3390/v13091849

12. Smith, G.A. (2021) Navigating the Cytoplasm: Delivery of the Alphaherpesvirus Genome to the Nucleus. *Curr Issues Mol Biol* 41, 171-220. 10.21775/cimb.041.171

13. Bauer, D. and Tampe, R. (2002) Herpes viral proteins blocking the transporter associated with antigen processing TAP--from genes to function and structure. *Curr Top Microbiol Immunol* 269, 87-99.

14. Cohen, J.I. (2020) Herpesvirus latency. *J Clin Invest* 130, 3361-3369. 10.1172/jci136225

15. Artusi, S. *et al.* (2018) Herpes Simplex Virus Vectors for Gene Transfer to the Central Nervous System. *Diseases (Basel, Switzerland)* 6. 10.3390/diseases6030074

16. Aldrak, N. *et al.* (2021) Oncolytic Herpes Simplex Virus-Based Therapies for Cancer. *Cells* 10. 10.3390/cells10061541.

17. Krisky, D.M. *et al.* (1998) Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther* 5, 1593-1603. 10.1038/sj.gt.3300766

18. Miyagawa, Y. *et al.* (2015) Herpes simplex viral-vector design for efficient transduction of nonneuronal cells without cytotoxicity. *Proc Natl Acad Sci U S A* 112, E1632-1641. 10.1073/pnas.1423556112

19. Verlengia, G. *et al.* (2017) Engineered HSV vector achieves safe long-term transgene expression in the central nervous system. *Sci Rep* 7, 1507. 10.1038/s41598-017-01635-1.

20. Richart SM, Simpson SA, Krummenacher C, Whitbeck JC, Pizer LI, Cohen GH, Eisenberg RJ, Wilcox CL. Entry of herpes simplex virus type 1 into primary sensory neurons in vitro is mediated by Nectin-1/HveC. J Virol. 2003 Mar;77(5):3307-11. doi: 10.1128/jvi.77.5.3307-3311.2003. PMID: 12584355; PMCID: PMC149788.

21. Miranda-Saksena M, Denes CE, Diefenbach RJ, Cunningham AL. Infection and Transport of

Herpes Simplex Virus Type 1 in Neurons: Role of the Cytoskeleton. Viruses. 2018 Feb 23;10(2):92. doi: 10.3390/v10020092. PMID: 29473915; PMCID: PMC5850399.

22. Kurt-Jones EA, Orzalli MH, Knipe DM. Innate Immune Mechanisms and Herpes Simplex Virus Infection and Disease. Adv Anat Embryol Cell Biol. 2017;223:49-75. doi: 10.1007/978-3-319-53168-7_3. PMID: 28528439; PMCID: PMC7254490.

23. Cohen JI. Herpesvirus latency. J Clin Invest. 2020 Jul 1;130(7):3361-3369. doi: 10.1172/JCI136225. PMID: 32364538; PMCID: PMC7324166.

24. Nicoll MP, Proença JT, Efstathiou S. The molecular basis of herpes simplex virus latency. FEMS Microbiol Rev. 2012 May;36(3):684-705. doi: 10.1111/j.1574-6976.2011.00320.x. Epub 2012 Jan 10. PMID: 22150699; PMCID: PMC3492847.

25. Bloom DC, Giordani NV, Kwiatkowski DL. Epigenetic regulation of latent HSV-1 gene expression. Biochim Biophys Acta. 2010 Mar-Apr;1799(3-4):246-56. doi: 10.1016/j.bbagrm.2009.12.001. Epub 2010 Jan 4. PMID: 20045093; PMCID: PMC2838971.

26. Lachmann R. Herpes simplex virus-based vectors. Int J Exp Pathol. 2004 Oct;85(4):177-90. doi: 10.1111/j.0959-9673.2004.00383.x. PMID: 15312123; PMCID: PMC2517519.

27. Chew T, Taylor KE, Mossman KL. Innate and adaptive immune responses to herpes simplex virus. Viruses. 2009 Dec;1(3):979-1002. doi: 10.3390/v1030979. Epub 2009 Nov 18. PMID: 21994578; PMCID: PMC3185534.

28. Miyazato M, Sugaya K, Goins WF, Wolfe D, Goss JR, Chancellor MB, de Groat WC, Glorioso JC, Yoshimura N. Herpes simplex virus vector-mediated gene delivery of glutamic acid decarboxylase reduces detrusor overactivity in spinal cord-injured rats. Gene Ther. 2009 May;16(5):660-8. doi: 10.1038/gt.2009.5. Epub 2009 Feb 19. PMID: 19225548; PMCID: PMC2881227.

29. Jia C, Yoshimura N, Liao L. Herpes simplex virus vector-mediated gene transfer of kynurenine aminotransferase improves detrusor overactivity in spinal cord-injured rats. Gene Ther. 2014 May;21(5):484-9. doi: 10.1038/gt.2014.19. Epub 2014 Mar 6. PMID: 24598891.

30. Goss JR, Mata M, Goins WF, Wu HH, Glorioso JC, Fink DJ. Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion. Gene Ther. 2001 Apr;8(7):551-6. doi: 10.1038/sj.gt.3301430. PMID: 11319622.

31. Chattopadhyay M, Zhou Z, Hao S, Mata M, Fink DJ. Reduction of voltage gated sodium channel protein in DRG by vector mediated miRNA reduces pain in rats with painful diabetic neuropathy. Mol Pain. 2012 Mar 22;8:17. doi: 10.1186/1744-8069-8-17. PMID: 22439790; PMCID: PMC3388457.

32. Walwyn WM, Matsuka Y, Arai D, Bloom DC, Lam H, Tran C, Spigelman I, Maidment NT. HSV-1-mediated NGF delivery delays nociceptive deficits in a genetic model of diabetic neuropathy. Exp Neurol. 2006 Mar;198(1):260-70. doi: 10.1016/j.expneurol.2005.12.006. Epub 2006 Jan 20. PMID: 16427624.

33. Chattopadhyay M, Mata M, Goss J, Wolfe D, Huang S, Glorioso JC, Fink DJ. Prolonged preservation of nerve function in diabetic neuropathy in mice by herpes simplex virus-mediated gene transfer. Diabetologia. 2007 Jul;50(7):1550-8. doi: 10.1007/s00125-007-0702-4. Epub 2007 May 17. PMID: 17508196.

34. Fink D. J., Wechuck, J., Mata, M. & Glorioso J.C. Gene therapy for pain: results of a phase I clinical trial. *Ann. Neurol.* **70**, 207–212 (2011).

35. Gurevich, I. *et al.* (2022) In vivo topical gene therapy for recessive dystrophic epidermolysis bullosa: a phase 1 and 2 trial. *Nat Med* 28, 780-788. 10.1038/s41591-022-01737-y

36. Guide, S.V. *et al.* (2022) Trial of Beremagene Geperpavec (B-VEC) for Dystrophic Epidermolysis Bullosa. *N Engl J Med* 387, 2211-2219. 10.1056/NEJMoa2206663

37. Bauer, D. and Tampe, R. (2002) Herpes viral proteins blocking the transporter associated with

antigen processing TAP--from genes to function and structure. *Curr Top Microbiol Immunol* 269, 87-99.

38. Dhillon, S. (2023) Beremagene Geperpavec: First Approval. *Drugs*. 10.1007/s40265-023-01921-5

39. De Rosa, L. and De Luca, M. (2022) The joint battle to tackle epidermolysis bullosa through gene therapy. *Trends Mol Med* 28, 533-535. 10.1016/j.molmed.2022.05.001

40. Freedman, J.C. *et al.* (2021) Preclinical Evaluation of a Modified Herpes Simplex Virus Type 1 Vector Encoding Human TGM1 for the Treatment of Autosomal Recessive Congenital Ichthyosis. *J Invest Dermatol* 141, 874-882.e876. 10.1016/j.jid.2020.07.035

41. Wang, JH., Gessler, D.J., Zhan, W. *et al.* Adeno-associated virus as a delivery vector for gene therapy of human diseases. *Sig Transduct Target Ther* 9, 78 (2024). https://doi.org/10.1038/s41392-024-01780-w

42. Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A, et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet. 2017 Aug 26;390(10097):849-860. doi: 10.1016/S0140-6736(17)31868-8. Epub 2017 Jul 14. Erratum in: Lancet. 2017 Aug 26;390(10097):848. PMID: 28712537; PMCID: PMC5726391.

43. Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. N Engl J Med. 2017 Nov 2;377(18):1713-1722. doi: 10.1056/NEJMoa1706198. PMID: 29091557.

44. Im DS, Muzyczka N. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. Cell. 1990 May 4;61(3):447-57. doi: 10.1016/0092-8674(90)90526-k. PMID: 2159383.

45. Rosas LE, Grieves JL, Zaraspe K, La Perle KM, Fu H, McCarty DM. Patterns of scAAV vector insertion associated with oncogenic events in a mouse model for genotoxicity. Mol Ther. 2012 Nov;20(11):2098-110. doi: 10.1038/mt.2012.197. Epub 2012 Sep 18. PMID: 22990674; PMCID: PMC3498805.

46. Martins KM, Breton C, Zheng Q, Zhang Z, Latshaw C, Greig JA, Wilson JM. Prevalent and Disseminated Recombinant and Wild-Type Adeno-Associated Virus Integration in Macaques and Humans. Hum Gene Ther. 2023 Nov;34(21-22):1081-1094. doi: 10.1089/hum.2023.134. Epub 2023 Nov 6. PMID: 37930949; PMCID: PMC10659022.

47. Nault JC, Datta S, Imbeaud S, Franconi A, Mallet M, Couchy G, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. Nat Genet. 2015 Oct;47(10):1187-93. doi: 10.1038/ng.3389. Epub 2015 Aug 24. PMID: 26301494.

48. Greig, J.A., Martins, K.M., Breton, C. *et al.* Integrated vector genomes may contribute to long-term expression in primate liver after AAV administration. *Nat Biotechnol* (2023). https://doi.org/10.1038/s41587-023-01974-7

49. Lilley CE, Groutsi F, Han Z, Palmer JA, Anderson PN, Latchman DS, Coffin RS. Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system in vivo. J Virol. 2001 May;75(9):4343-56. doi: 10.1128/JVI.75.9.4343-4356.2001. PMID: 11287583; PMCID: PMC114179.

50. Gimenez-Cassina A, Wade-Martins R, Gomez-Sebastian S, Corona JC, Lim F, Diaz-Nido J. Infectious delivery and long-term persistence of transgene expression in the brain by a 135-kb iBAC-FXN genomic DNA expression vector. Gene Ther. 2011 Oct;18(10):1015-9. doi: 10.1038/gt.2011.45. Epub 2011 Apr 14. PMID: 21490681.

51. Zhu J, Kang W, Wolfe JH, Fraser NW. Significantly increased expression of beta-glucuronidase in the central nervous system of mucopolysaccharidosis type VII mice from the latency-associated transcript promoter in a nonpathogenic herpes simplex virus type 1 vector. Mol Ther. 2000 Jul;2(1):82-94. doi: 10.1006/mthe.2000.0093. PMID: 10899831.

52. Zsebo K, Yaroshinsky A, Rudy JJ, Wagner K, ET AL. Long-term effects of AAV1/SERCA2a gene transfer in patients with severe heart failure: analysis of recurrent cardiovascular events and mortality. Circ Res. 2014 Jan 3;114(1):101-8. doi: 10.1161/CIRCRESAHA.113.302421. Epub 2013 Sep 24. PMID: 24065463.

53. Wang Z, Ma HI, Li J, Sun L, Zhang J, Xiao X. Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo. Gene Ther. 2003 Dec;10(26):2105-11. doi: 10.1038/sj.gt.3302133. PMID: 14625564.

54. Recillas-Targa F, Valadez-Graham V, Farrell CM. Prospects and implications of using chromatin insulators in gene therapy and transgenesis. Bioessays. 2004 Jul;26(7):796-807. doi: 10.1002/bies.20059. PMID: 15221861.

55. Gross DA, Tedesco N, Leborgne C, Ronzitti G. Overcoming the Challenges Imposed by Humoral Immunity to AAV Vectors to Achieve Safe and Efficient Gene Transfer in Seropositive Patients. Front Immunol. 2022 Apr 7;13:857276. doi: 10.3389/fimmu.2022.857276. PMID: 35464422; PMCID: PMC9022790.

56. Philippidis A. Fourth Boy Dies in Clinical Trial of Astellas' AT132. Hum Gene Ther. 2021 Oct;32(19-20):1008-1010. doi: 10.1089/hum.2021.29182.bfs. PMID: 34662231.

57. Philippidis A. Novartis Confirms Deaths of Two Patients Treated with Gene Therapy Zolgensma. Hum Gene Ther. 2022 Sep;33(17-18):842-844. doi: 10.1089/hum.2022.29216.bfs. PMID: 36125439.

58. Guillou J, de Pellegars A, Porcheret F, Frémeaux-Bacchi V, et al. Fatal thrombotic microangiopathy case following adeno-associated viral SMN gene therapy. Blood Adv. 2022 Jul 26;6(14):4266-4270. doi: 10.1182/bloodadvances.2021006419. PMID: 35584395; PMCID: PMC9327533.

59. Lek A, Wong B, Keeler A, Blackwood M, Ma K, Huang S, et al. Death after High-Dose rAAV9 Gene Therapy in a Patient with Duchenne's Muscular Dystrophy. N Engl J Med. 2023 Sep 28;389(13):1203-1210. doi: 10.1056/NEJMoa2307798. PMID: 37754285.

60. Kishimoto TK, Samulski RJ. Addressing high dose AAV toxicity - 'one and done' or 'slower and lower'? Expert Opin Biol Ther. 2022 Sep;22(9):1067-1071. doi: 10.1080/14712598.2022.2060737. Epub 2022 Apr 3. PMID: 35373689.

61. High-dose AAV gene therapy deaths. *Nat Biotechnol* 38, 910 (2020). https://doi.org/10.1038/s41587-020-0642-9

62. Stone, D., Aubert, M. & Jerome, K.R. Adeno-associated virus vectors and neurotoxicity—lessons from preclinical and human studies. *Gene Ther* (2023). https://doi.org/10.1038/s41434-023-00405-1

63. Han, F. *et al.* (2018) Cellular Antisilencing Elements Support Transgene Expression from Herpes Simplex Virus Vectors in the Absence of Immediate Early Gene Expression. *J Virol* 92. 10.1128/jvi.00536-18.

64. Epstein AL. HSV-1-derived amplicon vectors: Recent technological improvements and remaining difficulties – A review. Mem Inst Oswaldo Cruz. 2009;104(3):399-410.

65 Saeki Y, Fraefel C, Ichikawa T, Breakefield XO, Chiocca E.A. Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. Mol Ther. 2001 3: 591-601.

66. Zaupa, C.; Revol-Guyot, V.; Epstein, A.L. Improved packaging system for generation of high levels non-cytotoxic HSV-1 amplicon vectors using Cre-loxP site-specific recombination to delete the packaging signals of defective helper genomes. *Hum Gene Ther.* 2003, *14*, 1049-1063.

67. Xiong, F., Yang, H., Song, YG. *et al.* An HSV-1-H129 amplicon tracer system for rapid and efficient monosynaptic anterograde neural circuit tracing. *Nat Commun* 13, 7645 (2022). https://doi.org/10.1038/s41467-022-35355-6

68. Joussain C, Le Coz O, Pichugin A, Marconi P, Lim F, Sicurella M, Salonia A, Montorsi F, Wandosell F, Foster K, Giuliano F, Epstein AL, Aranda Muñoz A. Botulinum Neurotoxin Light Chains Expressed by Defective Herpes Simplex Virus Type-1 Vectors Cleave SNARE Proteins and

Inhibit CGRP Release in Rat Sensory Neurons. Toxins (Basel). 2019 Feb 19;11(2):123. doi: 10.3390/toxins11020123.

69. Joussain C, Le Coz O, Pichugin A, Marconi P, Lim F, Sicurella M, Foster K, Giuliano F, Epstein AL, Aranda Muñoz A. Development and Assessment of Herpes Simplex Virus Type 1 (HSV-1) Amplicon Vectors with Sensory Neuron-Selective Promoters. Int J Mol Sci. 2022 Jul 30;23(15):8474. doi: 10.3390/ijms23158474.

70. Soukupová M, Zucchini S, Trempat P, Ingusci S, Perrier-Biollay C, Barbieri M, Cattaneo S, Bettegazzi B, Falzoni S, Berthommé H, Simonato M. Improvement of HSV-1 based amplicon vectors for a safe and long-lasting gene therapy in non-replicating cells. Mol Ther Methods Clin Dev. 2021 Mar 29;21:399-412. doi: 10.1016/j.omtm.2021.03.020.

71. Ho, I., Miao, L., Sia, K. et al. Targeting human glioma cells using HSV-1 amplicon peptide display vector. Gene Ther 17, 250–260 (2010). https://doi.org/10.1038/gt.2009.128

72. Peters, C. and Rabkin, S.D. (2015) Designing Herpes Viruses as Oncolytics. *Mol Ther Oncolytics* 2, 15010. 10.1038/mto.2015.10

73. Zhang, S. and Rabkin, S.D. (2021) The discovery and development of oncolytic viruses: are they the future of cancer immunotherapy? *Expert Opin Drug Discov* 16, 391-410. 10.1080/17460441.2021.1850689

74. Bommareddy, P.K. *et al.* (2018) Oncolytic Herpes Simplex Viruses as a Paradigm for the Treatment of Cancer. *Annual Review of Cancer Biology* 2, 155-173. 10.1146/annurev-cancerbio-030617-050254

75. Aldrak, N. *et al.* (2021) Oncolytic Herpes Simplex Virus-Based Therapies for Cancer. *Cells* 10. 10.3390/cells10061541

76. Jahan, N. *et al.* (2021) In Situ Cancer Vaccination and Immunovirotherapy Using Oncolytic HSV. *Viruses* 13. 10.3390/v13091740.

77. Koch, M.S. *et al.* (2020) HSV-1 Oncolytic Viruses from Bench to Bedside: An Overview of Current Clinical Trials. *Cancers (Basel)* 12. 10.3390/cancers12123514

78. Todo, T. *et al.* (2022) Intratumoral oncolytic herpes virus G47Δ for residual or recurrent glioblastoma: a phase 2 trial. *Nat Med.* 10.1038/s41591-022-01897-x

79. Scanlan, H. *et al.* (2022) Herpes simplex virus 1 as an oncolytic viral therapy for refractory cancers. *Front Oncol* 12, 940019. 10.3389/fonc.2022.940019

