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Chapter 11

Oncolytic HSV Vectors and Anti-Tumor Immunity

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Abstract

The therapeutic promise of oncolytic viruses (OVs) rests on their ability to both selectively kill tumor cells and induce anti-tumor immunity. The potential of tumors to be recognized and eliminated by an effective anti-tumor immune

response has been spurred on by the discovery that immune checkpoint inhibition can overcome tumor-specific cytotoxic T cell (CTL) exhaustion and provide durable responses in multiple tumor indications. OV-mediated tumor destruction is now recognized as a powerful means to assist in the development of anti-tumor immunity for two important reasons: (i) OVs, through the elicitation of an anti-viral response and the production of type I interferon, are potent stimulators of inflammation and can be armed with transgenes to further enhance anti-tumor immune responses; and (ii) lytic activity can promote the release of tumor-associated antigens (TAAs) and tumor neoantigens that function as *in situ* tumor-specific vaccines to elicit adaptive immunity. Oncolytic herpes simplex viruses (oHSVs) are among the most widely studied OVs for the treatment of solid malignancies, and Amgen's oHSV Imlygic® for the treatment of melanoma is the only OV approved in major markets. Here we describe important biological features of HSV that make it an attractive OV, clinical experience with HSV-based vectors, and strategies to increase applicability to cancer treatment.

A. Introduction

Recent advances in cancer therapeutics include the use of tumor-specific lytic (oncolytic) viruses that both debulk tumors - reduce their size by eliminating cancer cells - and break down barriers to the induction of anti-tumor immunity. Oncolytic viruses have been derived from a wide variety of both DNA and RNA virus families, each having unique host ranges and replication mechanisms that make them attractive for attacking different tumor types. Oncolytic virus-mediated destruction of tumor cells leads to the production of danger signals by infected cells, a process referred to as immunogenic cell death, and to the release and presentation by antigen presenting cells (APCs) of virus and tumor-derived antigens. Tumor antigenic peptides are created by either an accumulation of mutations in cellular proteins, re-emergence of fetal/testis proteins without complete host tolerance, or by misfolded proteins to create novel processed peptides (Coulie et al., 2014; Kamran et al., 2016; Wang and Wang, 2017; Yarchoan et al., 2017). Robust immune responses to viral antigens can attract APCs to the tumor site wherein viral and tumor antigenic

peptides can participate in cross-priming and the development of both anti-viral and anti-tumor immunity.

Oncolytic herpes simplex viruses (oHSVs) are among the most widely pursued for treatment of solid malignancies and have been validated with the commercial approval of Imlygic® (Talimogene laherparepvec, Amgen, Inc.) for the treatment of metastatic melanoma (Andtbacka et al., 2015; Pol et al., 2016). HSVs have several important features that contribute to their utility as tumor virolytics. These include a broad host cell range, which renders multiple types of tumors permissive for viral growth, and an ability to accommodate large or multiple transgene expression cassettes (referred to as large payload capacity) that enables oHSV-mediated delivery of multiple non-viral immunomodulatory products. Arming oHSVs with combinations of transgenes may increase oncolytic activity, help promote an immune-responsive tumor microenvironment and enhance the induction of innate and adaptive anti-tumor immune responses (Martuza et al., 1991; Andreansky et al., 1998; Chase et al., 1998). Advances in HSV engineering over the past decade and a half have produced oHSVs that have the full complement of viral genes, including virulence functions that resist cellular anti-viral responses and enable aggressive infection and destruction of tumors (Kambara et al., 2005; Gambini et al., 2012; Uchida et al., 2013). Strategies to enable uncompromised viral infection of tumor cells while preserving safety, to increase intratumoral vector spread, and to resist rapid elimination of replicating virus by counteracting anti-viral host responses should enhance oHSV potency and utility as anti-cancer agents. In this review, a brief history of the development of oHSVs, clinical outcomes in monotherapy and in combination with checkpoint inhibitors, and prospects for improved vector design are described.

B. oHSV design: curbing neurovirulence

1. Relevant biology of HSV

HSV-1 and HSV-2 are closely related neurotropic viruses that infect humans, their natural hosts. However, the virus can infect a wide variety of species, which has enabled the evaluation of oHSV vector designs in xenogeneic and

syngeneic rodent tumor models. Some 70% of the adult human population is seropositive for HSV-1 (Kaufman et al., 2005), the most commonly used oncolytic HSV, and is likely to harbor latent virus in sensory neurons that innervate the site of initial infection. Latent virus reactivation can lead to virus spread to other susceptible individuals by direct contact with a virus-containing lesion. With the exception of herpes keratitis and encephalitis, HSV-1 infections of adults are mostly self-limiting, and latency is held in check largely by local anti-viral immunity (St Leger and Hendricks, 2011; St Leger et al., 2013).

The HSV genome is a double-stranded 152-kb DNA that encodes at least 84 gene products, the majority of which have multiple functions (Roizman, 2001; Roizman and Zhou, 2015). The genome consists of large and small unique segments flanked by repeat elements containing duplicate genes that contribute to virus replication, neurovirulence and latency. The repeat elements are responsible for genome isomerization such that 4 isomers are produced in infected cells. Approximately half of the viral genes encode essential products to complete the replication cycle in permissive cell cultures while the remaining genes contribute to the complex life cycle in the host. The viral genome is surrounded by an icosahedral capsid; a tegument composed of viral proteins involved in processes such as regulation of viral and host gene expression, host immune response, capsid trafficking and viral egress; and a host-derived lipid envelope studded with viral glycoproteins, many of which are involved in host-cell entry and virus spread to neighboring cells.

The basic biology of the virus has been exploited and modified in multiple ways in the design of oHSV. The 14-kb internal repeat region (IRR) can be removed without preventing virus replication since the full array of gene functions remains (Poffenberger et al., 1983; Meignier et al., 1988; Mazzacurati et al., 2015). IRR removal prevents genome isomerization, locking the viral genome into a single species and enhancing recombinant virus stability during production. With IRR removal, the HSV genome can accept ~25-kb of foreign DNA without straining the capsid packaging limits, allowing virus-mediated expression of one or more transgenes that can assist in the induction of anti-

tumor immunity. In first-generation oHSVs, nonessential genes that overcome innate anti-viral responses, such as type I interferon (IFN) production, were deleted or modified to attenuate the virus in non-tumor cells and limit neurovirulence. More recent modifications have included the addition of microRNA response elements in the 3' UTR of essential genes to restrict replication to tumor cells (Lee et al., 2009; Mazzacurati et al., 2015) and modification of several viral envelope glycoproteins to allow only tumor-specific virus infection (Campadelli-Fiume et al., 2016). Inhibition of neuronal retrograde transport through specific mutation in the R2 domain of the tegument protein UL37 is a recently described alternative to prevent HSV-1 infection of the brain, enabling the design of HSV vaccines (Richards et al., 2017). Because HSV-1 is neurotropic, the initial work in developing oHSV vectors centered on the treatment of brain tumors by direct intratumoral injection (Markert et al., 2000; Rampling et al., 2000; Papanastassiou et al., 2002). Tumor cells of neuronal origin, such as glioma, are generally permissive for HSV mutants that are attenuated for replication in normal brain and do not elicit encephalitis. Although early clinical trials have thus far documented few complete responses using attenuated HSV, recent data from Todo and colleagues have shown promising activity and tolerability for G47Δ, an unarmed oHSV, in patients with recurrent or residual GBM (Todo, 2019), spurring a renewed interest into the treatment of GBM by oHSV.

2. oHSV vectors and treatment of neuronal tumors

a. Glioblastoma multiforme (GBM)

GBM (grade IV glioma) is the most common type of primary brain tumor in adults and is almost universally fatal despite aggressive therapies, including combinations of surgery, radiotherapy, and chemotherapy. The incidence of GBM is approximately 3.2 per 100,000 persons in the United States (Tamimi and Juweid, 2017), and GBM patients have a median survival of 12-18 months from initial diagnosis and 6-9 months after recurrence, with only 5% of patients surviving for 5 years (Wen and Kesari, 2008). The Standard of Care for management of these tumors includes tumor resection, followed by external beam radiotherapy with treatment doses of approximately 60 Gy (Desjardins et

al., 2009; Thaker and Pollack, 2009). Partial responses to chemotherapy [e.g., temozolomide (Temodar)] are seen in approximately 30% of patients (Gilbert et al., 2013; Norden et al., 2013; Blumenthal et al., 2017), but overall survival is only improved by 2.5 months in newly diagnosed GBM in combination with radiotherapy (Stupp et al., 2005; Stupp et al., 2009; Grossman et al., 2010; Delgado-Lopez and Corrales-Garcia, 2016; Feng et al., 2017). Because tumor cells infiltrate the brain, tumor resection is not always complete. Even removal of the entire tumor-containing hemisphere (hemispherectomy) has been found to be non-curative (Chaichana et al., 2014). This is further compounded by the fact that the tumors are genetically and phenotypically heterogeneous, are poorly immunogenic, with scarce CD8+ T-cell infiltration (few T cells in mesenchymal and classical glioblastomas, essentially none in proneural GBM), and do not appear to respond to immune checkpoint inhibitors without induction of an inflammatory tumor microenvironment (Preusser et al., 2015; Reardon et al., 2016; Hodges et al., 2017). Due to the limited benefit of these therapeutic approaches, efforts to develop treatments that improve survival have included various biologic therapies, such as different types of oncolytic viruses, cell-based immunotherapy including chimeric antigen receptor (CAR)-T cells (Bagley et al., 2018), CAR-NK cells (Han et al., 2015), and gene therapy (Chiocca et al., 2019). Below is a brief history of the development of oHSVs for treatment of brain tumors.

b. Preclinical development

The first oncolytic HSV-1 described was dlsptk, a U_L23 [thymidine kinase (TK)] deletion mutant that displayed a promising therapeutic potential in the treatment of glioma in animal studies (Martuza et al., 1991). Replication of this mutant HSV-1 virus occurs only in mitotic tumor cells that upregulate their endogenous tk gene. Despite demonstrated tumor-specific replication in mouse models, there were serious concerns about vector safety because this strain of virus is insensitive to the most potent anti-herpetic agents, such as acyclovir, and because high titers of dlsptk caused neurotoxicity in mouse brain tumor models (Martuza et al., 1991). Hence, further development of HSV-1

vectors involved a search for other genes, the inactivation of which blocked virus replication in normal neurons but not in dividing cells.

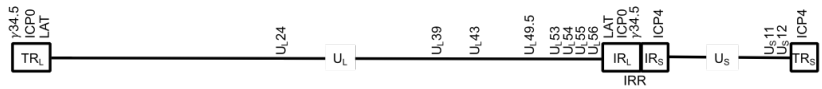
One resulting vector was hrR3, an HSV-1 mutant with an in-frame insertion of the *lacZ* gene into the U_L39 (ICP6) gene encoding the large subunit of ribonucleotide reductase (RR), expressed as an ICP6-LacZ fusion protein that retained no RR activity. RR is a key enzyme for viral DNA synthesis in non-dividing cells but not in dividing cells where elevated levels of the host-cell RR can provide the deoxynucleotides needed as substrates for viral DNA synthesis. In tumor cells that are defective for p16 or the retinoblastoma (Rb) gene product, the transcription factor E2F is activated, resulting in increased endogenous RR activity (Hanson et al., 1994; DeGregori et al., 1995) that enables replication of HSV-1 mutants lacking virally encoded RR. Therefore, HSV-1 ICP6 mutants are relatively selective for cells with defects in the p16 tumor suppressor pathway (Aghi et al., 2008). Intratumoral inoculation of hrR3 into rats bearing malignant gliosarcomas improved animal survival (Fulci et al., 2007; Aghi et al., 2008), and this was further improved by ganciclovir (GCV) administration (Boviatsis et al., 1994). Viral TK converts GCV to a toxic product that acts as a chain terminator during DNA synthesis (Fulci and Chiocca, 2007), and the RR-defective phenotype conferred tumor-cell hypersensitivity to GCV (Luo et al., 2007).

Another approach to achieve tumor-restricted vector replication targets viral proteins involved in counteracting the innate immune response to HSV. Incoming HSV is sensed by a variety of pattern recognition receptors (PRRs), including cGAS, melanoma differentiation-associated protein 5 (MDA5), and Toll-like receptors (TLR-2, -3 and -7) (Mossman and Smiley, 2002; Leib et al., 2009; Paludan et al., 2011). These sensors, via multiple signaling pathways, induce the production of type 1 IFNs and the expression of over 2,000 IFN-stimulated gene products (ISGs) that mediate viral clearance and increase the expression of MHC class I molecules and the recruitment of innate lymphoid cells. Unsurprisingly, HSV expresses several gene products that counteract the IFN response, including ICP34.5 encoded by the γ34.5 gene. ICP34.5 is an

inhibitor of NF-κB and STING (Pan et al., 2018), key mediators in the IFN pathway needed for induction of the ISGs. One well-characterized ISG, protein kinase R (PKR), shuts off host and viral protein synthesis by phosphorylating the translation initiation factor eIF2α. PKR is activated by binding to double-stranded RNA generated during viral infection and subsequent autophosphorylation and dimerization. While protein phosphatase-1α (PP1α) can inactivate PKR by dephosphorylation (Cheng et al., 2003), viral ICP34.5 selectively activates PP1α to mediate eIF2α dephosphorylation, allowing viral protein synthesis to proceed (Li et al., 2011). In addition to these functions, ICP34.5 can directly bind to and inhibit Beclin-1 to block cell death by autophagy (Orvedahl et al., 2007). Another viral protein, encoded by the U_S11 gene, inhibits sensing of viral dsRNA by direct interaction with RIG-I and MDA-5 and works together with ICP34.5 to inhibit host shut-down of viral protein synthesis by the PKR pathway (Poppers et al., 2000; Xing et al., 2012).

While the type I IFN response induced upon virus infection represents an essential first line of defense against incoming virus in normal cells, tumors are often defective for components of the IFN response pathway and this can be exploited in the design of oHSV vectors. Eliminating HSV-1 gene products, such as ICP34.5, compromises virus growth in normal cells with an intact IFN response but allows growth in tumor cells. For example, HSV-1 strains expressing a mutant form of ICP34.5 fail to replicate in normal brain tissue and are non-pathogenic for highly HSV-susceptible primates such as *Aotus* monkeys following intracranial inoculation (Hunter et al., 1999; Todo et al., 2000; Varghese et al., 2001). Replication of HSV1716, a γ34.5 deleted oHSV, in ependymal cells in BALB/c mice is a notable exception (Kesari et al., 1998). In tumor cells with increased signaling through epidermal growth-factor receptor 1 (EGFR) or 2 (HER2/Neu) or platelet-derived growth-factor receptor (PDGFR), persistent Ras activation inhibits PKR activity. Many glioblastomas express a constitutively active mutant form of EGFR as part of the tumor phenotype that may contribute to effective replication of γ34.5 mutant HSV-1 vectors.

HSV-1 vectors deleted for γ34.5 function have thus been highly touted for utility as oncolytic vectors. R3616, a replication competent mutant of HSV-1 strain F, lacks most of the coding regions of the two γ34.5 genes (Kramm et al., 1997) (Figure 1A). R3616 is capable of replicating in glioma xenografts in nude mice, and tumor killing is enhanced by ionizing radiation (Advani et al., 1998; Bradley et al., 1999; Huszthy et al., 2008). R3616 has also been shown to be effective for treatment of chemotherapy-resistant tumors (Chahlavi et al., 1999). HSV1716, a mutant of HSV-1 strain 17, also lacks both γ34.5 genes (Figure 1B) (MacLean et al., 1991; Valyi-Nagy et al., 1994; Kesari et al., 1995) and shows a similar anti-tumor and safety profile (Randazzo et al., 1995; Rampling et al., 2000).



	Virus	Strain	γ34.5	U_L23 (TK)	U_L24	U_L39 (ICP6)	U_L43	U_L49.5 (gN)	U_L55/ U_L56	IRR	U_S11	U_S12 (ICP47)	Transgene(s)
A	R3616	F	Δγ34.5										
B	HSV1716	17	Δγ34.5										
C	G207	F	Δγ34.5			ΔICP6							ICP6p-LacZ [ΔICP6]
D	G47Δ	F	Δγ34.5			ΔICP6					U_S12p	ΔICP47	ICP6p-LacZ [ΔICP6]
E	T-VEC	JS1	Δγ34.5								U_S12p	ΔICP47	CMVp-GMCSF [Δγ34.5]
F	rQNestin34.5	F	Δγ34.5			ΔICP6							NestinEp-γ34.5 [ΔICP6]
G	C134	F	Δγ34.5										CMVp-CMVIRS1 [U_L3/4]
H	M032	F	Δγ34.5			ΔICP6							Egr1p-hIL12 [Δγ34.5] ICP6p-LacZ [ΔICP6]
J	NV1020[1]	F		ICP4p-TK	ΔU_L24				ΔU_L56	ΔIRR			
K	HF10[2]	HF					ΔU_L43	ΔgN	ΔU_L55/56	ΔLAT			
L	rRp450	KOS				ΔICP6							ICP6p-rCYP2B1 [ΔICP6]

[1]NV1020 is deleted for a 700-bp region encompassing the thymidine kinase (tk) locus and the promoter of the U_L24 gene, as well as a region encompassing U_L56 and the internal repeat region (IRR, containing a single copy of ICP0, LAT, γ34.5, and ICP4); the U_L/U_S junction carries a 3.7-kb duplication of U_L5/6, a 5.2-kb fragment of HSV-2 DNA, and a copy of the tk gene under the ICP4 promoter.
[2]HF10 naturally overexpresses U_L53 (gK) and U_L54 (ICP27).

Figure 1. Oncolytic HSV vectors employed in human clinical trials. Schematic of the wild-type HSV genome (top) with relevant genes indicated above. The unique long (U_L) and unique short (U_S) segments contain single copies of each gene, while the terminal (TR_S and TR_L) and internal repeats (IR_L and IR_S, together comprising the IRR) contain duplicate copies of ICP4, ICP0, γ34.5, and LAT. The genome modifications used to attenuate the oHSV vectors described in this review are summarized in the table underneath (descriptions in the text refer to the letters labeling each row). Transgenes expressed from the vectors, their promoters (p), and [insertion sites] in the viral genome are listed in the last column.

HSV1716 was among the first oHSVs to be taken into human clinical trials, along with G207, another γ34.5-deleted virus that additionally contains a *lacZ*-disrupted U_L39 RR (ICP6) gene (Figure 1C). In preclinical experiments, G207 extended the survival of mice with intracranial GBM tumors, and more recent studies have shown that cisplatin enhanced the effect of G207 without inhibiting viral replication (Chahlavi et al., 1999). Radiation has also been shown to be effective in combination with oncolytic vector therapy (Jorgensen et al., 2001; Blank et al., 2002; Stanziale et al., 2002). Additional viral genome modifications have been considered to improve oncolytic activity of HSV-1. γ34.5 mutants have been combined with deletion of the immediate-early U_S12 gene encoding ICP47, a protein that blocks the loading of HSV-1 antigenic peptides onto MHC class I surface molecules (York et al., 1994; Fruh et al., 1995; Goldsmith et al., 1998). In the absence of ICP47, both viral and tumor-related antigenic peptides can be presented to cytotoxic T cells (CTLs) to eliminate virus-infected and potentially uninfected tumor cells (York et al., 1994; Fruh et al., 1995; Goldsmith et al., 1998). Deletion of the U_S12 gene and the U_S11 gene promoter in G207-derivative G47Δ (Figure 1D) (Todo et al., 2001; Zeng et al., 2013) places the U_S11 gene, normally a true late gene, under the control of the U_S12 immediate-early gene promoter, resulting in compensation for γ34.5 deletion, increased virus yield and killing of GBM and other tumor lines in culture (Cassady et al., 1998; Mohr et al., 2001; Todo et al., 2001; Liu et al., 2003).

c. Clinical trials

The tumor-restricted replication and efficacy of γ34.5 mutant oHSVs in preclinical experiments led to multiple clinical trials with various HSV-1 strain backgrounds and mutation combinations (Figure 1). In a Phase-I clinical trial of 21 patients with non-histologically proven recurrent GBM, G207 (Figure 1C) was delivered by intra-tumoral injection at three-fold increasing doses from 1 x 10^6 to 3 x 10^9 pfu (Markert et al., 2000). Patients only experienced mild adverse events such as fever, malaise and other signs of viral infection that resolved after 3-4 days. This trial showed that inoculation of oHSV was relatively safe in human brain but did not provide an effective treatment. This

was followed by a Phase-Ib trial in 6 patients with recurrent GBM using a dose of 1.15 x 10^9 pfu, showing radiographic evidence of tumor regression and the presence of an immune infiltrate (Markert et al., 2009). Another trial employing G207 at a dose of 3 x 10^9 pfu in combination with 5-Gy radiotherapy is currently recruiting children with recurrent GBM (ClinicalTrials.gov NCT0257845) (Waters et al., 2017; Friedman et al., 2018). In a Phase-I trial of the related G47Δ virus (Figure 1D) (UMIN000015995), doses of up to 1 x 10^9 pfu/injection for a total of 6 injections were found to be safe, and efficacy -- 92.3% of recurrent GBM patients remaining alive at 12 months -- was deemed sufficient to prompt the Japanese Ministry of Health, Labor and Welfare to designate G47Δ for fast-track approval (Fukuhara et al., 2016; Todo, 2019).

HSV1716, the strain 17 γ34.5 mutant (Figure 1B), was well tolerated at doses of up to 10^5 infectious units after stereotactic injection into recurrent malignant gliomas (Papanastassiou et al., 2002; Harrow et al., 2004), a dose much lower than the safe doses observed with G207 (Markert et al., 2000; Markert et al., 2009), which may be due to strain differences and the retention of ICP6 in HSV1716. Analysis of HSV1716-infected tumor explants revealed viral replication at 4-9 days after injection, and the amount of recovered virus exceeded the input dose in at least some patient samples. Radiographic evidence supported a reduction in tumor mass in some patients; yet, long-term survival was not altered, suggesting that more robust oHSV replication may be required.

Strategies employed to maintain vector safety while increasing efficacy include the expression of γ34.5 from a hybrid nestin enhancer-HSP68 minimal promoter in the ICP6-deleted oHSV mutant rQNestin34.5 (Figure 1F), an oHSV that was first shown to be highly active in a GBM model in nude mice (Kambara et al., 2005). A Phase-I trial safety/dose-escalation study using rQNestin34.5 alone or in combination with cyclophosphamide (CPA) for treatment of recurrent GBM patients is underway (NCT03152318). Another strategy is employed in the strain F-based vector C134 that expresses the human cytomegalovirus (CMV) IRS1 gene (Figure 1G) to enhance the

oncolytic activity of γ34.5 mutant oHSV (Cassady et al., 2017). A Phase-I dose-escalation trial (NCT03657576) using C134 began enrolling patients in November 2018.

A new wave of pre-clinical studies, ultimately leading to further human clinical trials for GBM, now employs these and other oHSV backbones for expression of various transgenes to increase viral spread and penetration within the tumor mass (Dmitrieva et al., 2011; Kim et al., 2014; Jaime-Ramirez et al., 2017; Sette et al., 2019), block neo-vascularization of the tumor (Goodwin et al., 2012; Yoo et al., 2012; Fujii et al., 2013) or modify the immune response in the host tumor microenvironment via the expression of cytokines or chemokines (Liu et al., 2003; Roth et al., 2014; Meisen et al., 2015; Patel et al., 2016; Leoni et al., 2018b). For example, G207 was armed with human IL-12 expressed from the Egr1 promoter (Figure 1H). The resulting oHSV, M032, has been shown to be safe in non-human primates and only induce a transient neutrophil and white blood cell host immune response (Roth et al., 2014). It is currently being tested in a Phase-I dose-escalation trial (NCT02062827) in 36 patients with recurrent GBM (Patel et al., 2016). The results of this trial await publication and are likely to influence further human trials for treating GBM with armed oHSV (see section D below for a discussion of arming genes). Together, these Phase-I oHSV trials for GBM demonstrated that the vectors are safe both in HSV-naïve patients and in patients with pre-existing anti-HSV immunity, but they were not designed to examine long-term improvement in overall survival (OS) or progression free survival (PFS).

3. oHSV vectors and treatment of non-neuronal tumors

Despite the vast preclinical and clinical effort to develop oHSVs to treat GBM, the initial success of using oHSVs to treat patients has come about by treating a tumor of non-neuronal origin. Talimogene laherparepvec (T-VEC; Imlygic®), an oHSV developed by BioVex (under the name Oncovex$^{GM\text{-}CSF}$) and Amgen, has achieved regulatory approval for the treatment of metastatic melanoma (Pol et al., 2016). T-VEC is an intratumorally injected product; its clinical development for melanoma was facilitated by the accessibility of the malignant

lesions and the possibility to perform repeated injections. Lesions distant to the sites of injection or visceral metastases, left uninjected, allow for the observation of an abscopal effect, i.e., the induction of anti-tumor activity affecting untreated tumor masses, likely through the generation of a systemic anti-tumor immune response. In addition, melanoma is well-known for its high degree of antigenicity and immunogenicity, a notably 'hot' tumor with expression of several prominent tumor antigens, such as MART-1, gp100, MAGE-1.A1, MAGE-3.A1, Melan-1, Mel-3 and tyrosinase, and a high degree of response to checkpoint inhibitors (Gujar et al., 2018a; Gujar et al., 2018b; Kamran et al., 2018). T-VEC (JS1/γ34.5^{-}:HCMVp-GM-CSF/ICP47^{-}; Figure 1E) was derived from an HSV-1 clinical isolate (JS1) and is deleted for both copies of the γ34.5 gene (Liu et al., 2003). The human GM-CSF gene is expressed from both of the deleted γ34.5 loci. In addition, the U_S12/ICP47 gene is deleted to release interference with transporter associated with antigen presentation (TAP)-dependent MHC class I antigen presentation. As in G47Δ discussed above, the deletion of ICP47 changes the temporal expression of the late gene U_S11 to that of an immediate early gene, at which time the U_S11 product can block PKR activation (Cassady et al., 1998; Mohr et al., 2001; Todo et al., 2001; Liu et al., 2003), thereby enhancing viral replication in tumor cells. The T-VEC Phase-I study in 30 patients established dose and regimen and showed that the intratumoral injection was well tolerated, with minor treatment-associated adverse events (AEs), including flu-like symptoms such as fatigue, pyrexia, chills, nausea, and focal cellulitis at the injection site. Notably, these events were more pronounced in previously HSV-seronegative patients, leading to the adoption of an initial dose of 1-4 x 10^6 pfu to seroconvert all patients before full therapeutic doses of 1-4 x 10^8 pfu every 2 weeks (Hu et al., 2006). T-VEC DNA was detected in injected tumors and in some neighboring uninjected lesions, but not in distal lesions, indicating that T-VEC did not spread to distal tumor sites.

The favorable results of this Phase-I trial supported a Phase-II trial with 50 melanoma patients, in which a similar safety profile was observed as in the Phase-I trial, an overall response rate (ORR; i.e., arrested tumor growth) of

26%, and a complete response rate (CR; tumor no longer detectable) of 16.9% (Senzer et al., 2009; Kaufman et al., 2010; Andtbacka et al., 2019). Regression of both injected local tumors as well as more distal tumors was observed, with overall survival (OS) rates of >50% at 1-2 years post-therapy. The immune response was evaluated and showed an increase in MART-1-specific T cells and a corresponding decrease in regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) within the tumor microenvironment (TME) (Senzer et al., 2009; Kaufman et al., 2010). This prompted a large Phase-III OPTiM (Oncovex$^{GM\text{-}CSF}$ Pivotal Trial in Melanoma) study of 436 patients, in which T-VEC was compared to GM-CSF protein as a standard control therapy for melanoma. T-VEC decreased by >50% the size of 64% of the injected tumors, 34% of non-visceral tumors, and 15% of visceral tumors (Andtbacka et al., 2015). The durable response rate (DRR; no evidence of recurrent disease) was 16.3% for T-VEC, but only 2.1% for GM-CSF alone, while the ORR for T-VEC was 26.4% versus 5.7% for GM-CSF alone (Andtbacka et al., 2015; Harrington et al., 2016). These numbers were even better for the earlier-stage subgroup (57.1% of evaluated patients) with DRRs of 25.2% versus 1.2% and ORRs of 40.5% versus 2.3% (Andtbacka et al., 2015; Harrington et al., 2016). Together, these results warranted the first FDA approval of an oncolytic virus and gene therapy product in the U.S. for the treatment of a solid tumor, melanoma, in October of 2015.

Currently there are 37 active clinical trials employing T-VEC under its commercial name (Imlygic®), targeting melanoma, sarcomas, breast cancer, pancreatic cancer, colorectal cancer, head and neck squamous cell carcinoma (HNSCC), lymphoma, liver cancer, pleural and intraperitoneal cancer, and bladder cancer. Since its original success in melanoma treatment, T-VEC has been evaluated in combination with chemo- and radiotherapies. A Phase-I/II trial using T-VEC in HNSCC, in combination with 70 Gy radiotherapy and cisplatin, in 14 patients showed an 82.3% tumor response and OS of 82.4% at 2-years post therapy (Harrington et al., 2010). HSV-1 DNA was detected not only within the injected tumor but also in adjacent tumors, and the number of detected genomes was significantly higher than the injected dose, suggesting

active virus replication in the TME. The fact that T-VEC treatment alone resulted in regression of local tumors but not distal and visceral tumors suggested that the immunosuppressive TME may be inhibiting the cytotoxic T lymphocyte (CTL) response required to effectively eliminate untreated distal and visceral lesions, pointing to a need for combination trials with immune checkpoint-inhibitory antibodies such as anti-CTLA-4 ipilimumab (Yervoy) and anti-PD-1 pembrolizumab (Keytruda). The combination of T-VEC with anti-CTLA-4 (Puzanov et al., 2016) showed no added dose-limiting toxicities (DLTs) of T-VEC and raised the ORR from 26% seen in the Phase-III OPTiM trial to greater than 50%. A greater increase in CD8+ T-cells was detected in patients that were considered to be complete responders (CR) compared to partial responders (PR) or patients with progressive disease. T-VEC combination with pembrolizumab in a Phase-Ib/II trial (Ribas et al., 2017) was found to increase the frequency of complete responses to 62% compared to 24% for T-VEC alone, and a dramatic increase in circulating CD3+/CD8+ T-cells was observed. The field is eagerly awaiting the outcome of the large phase III clinical trial Keynote 034 (NCT02263508) combining T-VEC and Keytruda to hopefully confirm the promising phase Ib/II data. Additional combination studies with T-VEC, checkpoint-inhibitory antibodies and chemo- or radio-therapy are underway. Radiotherapy can increase the levels of tumor antigens and, in combination with T-VEC, may increase the number of tumor-specific targets for activated CTLs.

In addition to T-VEC, other oHSVs have been tested in non-CNS tumor clinical trials. HSV1716 has been evaluated in Phase-I trials for melanoma (MacKie et al., 2001), HNSCC (Mace et al., 2007; Mace et al., 2008) and pediatric non-CNS tumors (Streby et al., 2017). In these trials, doses of $1x10^5$ - $2x10^6$ pfu proved safe and led to virus replication in the tumor, yet failed to provide compelling evidence of efficacy. NV1020 (Figure 1J) contains deletions of the γ34.5 genes, the U_L24 promoter, the complete internal repeat region, and the U_L56 gene that encodes a protein likely involved in Golgi vesicular trafficking of virions (Koshizuka et al., 2005). These deletions resulted in a severely attenuated recombinant virus that can still replicate in tumor cells. *In-vivo*

analysis with this vector demonstrated tolerability and efficacy in a number of experimental models of bladder carcinoma (Cozzi et al., 2001), HNSCC (Wong et al., 2001), and non-small cell lung cancer (Ebright et al., 2002). These strong efficacy and safety data in mice led to Phase-I trials conducted by hepatic artery infusion of the virus for colorectal cancer (CRC) metastatic to the liver. The results demonstrated safety of NV1020 with only mild to moderate AEs, such as fever and transient lymphopenia, and no patients displayed evidence of virus shedding (Kemeny et al., 2006; Geevarghese et al., 2010; Sze et al., 2012). Antitumor efficacy was limited in this refractory setting, with 50% of patients showing stable disease. Another oHSV, HF10 (Figure 1K), is a natural HSV-1 variant defective for expression of U_L43, $U_L49.5$, U_L55, U_L56 and LAT (Ushijima et al., 2007). HF10 replicates to high efficiency in tumor cells while demonstrating substantially reduced toxicity compared to wild-type HSV-1. Takara-sponsored Phase-I trials have been conducted in Japan for solid tumors with superficial lesions (Ferris et al., 2014), breast cancer (Nakao et al., 2004; Kimata et al., 2006; Nakao et al., 2007; Sahin et al., 2012), unresectable pancreatic cancer (Nakao et al., 2011; Kasuya et al., 2014; Hirooka et al., 2018), and resectable HNSCC (Fujimoto et al., 2006). In these Phase-I trials, HF10 was well-tolerated without virus-attributable serious adverse events (SAEs). While viral replication was detected in the tumor mass, no evidence of virus shedding from the injection sites was observed. Some evidence was obtained of moderate tumor cell killing along with CD4+ and CD8+ T-cell infiltrates within the TME. These results led to a Phase-II combination trial (NCT02272855) of HF10 with ipilimumab in unresectable stage III-IV malignant melanoma that demonstrated an immune-related response criteria (irRC) best overall response rate (BORR) at 24 weeks of 41% [18% complete disappearance of all lesions (irCR) and 23% decrease in tumor burden by ≥50% (irPR)] (Andtbacka et al., 2018). G47Δ (Figure 1D) has been evaluated in a Phase-I dose-escalation study for the treatment of castration-resistant prostate cancer (UMIN000010463) and in a Phase-I trial (UMIN000011636) for recurrent olfactory neuroblastoma (Fukuhara et al., 2016). Finally, oHSV rRp450, containing a rat cytochrome P450 gene in place of the U_L39 RR gene (Figure 1L) (Chase et al., 1998), is under study in a

Phase-I trial (NCT01071941) for primary liver cancer and liver metastases in 40 patients, comparing single and repeated infusions into the hepatic artery.

4. Summary

To date, clinical trials using oHSVs have demonstrated that all of the viral recombinants (i) were well-tolerated; (ii) had limited risk of shedding; (iii) tended to induce some level of T-cell recruitment; (iv) caused sero-conversion of patients who were HSV-seronegative at the time of oHSV administration; and (v) displayed evidence of intra-tumoral virus replication. When evaluated as monotherapy, clinical efficacy of most oHSVs was limited for indications other than melanoma. Currently, the data evaluating clinical benefit of oHSVs in combination with chemo-, radio- or immunotherapy is limited by the absence of controlled Phase-III studies. Still, Phase-II clinical data evaluating T-VEC and HF10 in combination with ipilimumab or pembrolizumab appear very promising, with roughly a doubling of the response rate achieved by these checkpoint inhibitors alone. More importantly, in the case of T-VEC combined with pembrolizumab, complete responses were achieved with patients presenting with low intratumoral CD8+ T-cell counts and low expression of PD-L1 and IFN-responsive genes, parameters that are indicative of immunologically "cold" tumors that are not expected to respond to immunotherapy (Ribas et al., 2017). These data, on the heels of T-VEC/ Imlygic's approval by the FDA, are fueling the current enthusiasm for oncolytic viruses and the efforts to improve upon the first-generation oHSV vectors.

C. Advances in oHSV design for increased specificity and efficacy

1. Selective HSV attenuation by microRNAs

As described above, the primary method of attenuating oHSV has been to remove or inactivate genes that contribute to innate immune evasion in order to render the virus susceptible to the innate cellular defenses that are active in normal cells but compromised in tumor cells. However, tumors can vary widely in their loss of type I IFN responses (e.g., (Alain et al., 2010)) and IFN can be produced by myeloid cell types within the TME. Therefore, the maintenance of the complete set of viral defense functions is thought to be beneficial to retain

full vector replication activity *in vivo*, thus prompting the development of novel approaches for virus attenuation. The maintenance of normal cellular function by microRNAs (miRNAs) is frequently disrupted in tumors, and this dysregulation of miRNA expression represents a distinct signature of the tumor that has been exploited to attenuate oncolytic vector replication in normal cells. The use of differential miRNA expression to limit oHSV replication to tumor cells was first described in a prostate cancer model using an amplicon vector with recognition sequences for miR-143 and miR-145 engineered into the 3'UTR of the ICP4 gene; both miRNAs are expressed in normal tissues but are significantly down-regulated in prostate cancer cells (Lee et al., 2009). The promise of this strategy was confirmed in models of other tumors, including liver tumors where miR-222 expression is highly reduced compared to hepatocytes (Fu et al., 2012), non-small-cell lung cancer (miR-145) (Li et al., 2013), and urothelial bladder cancer (miR-143 + miR-124) (Zhang et al., 2016). In GBM, tumor cells have low or undetectable expression of miR-124, an miRNA that is highly expressed in normal neurons (Sempere et al., 2004; Gaur et al., 2007; Silber et al., 2008). Incorporation of miR-124 target sites into the 3'UTR of the ICP4 gene blocked virus replication in miR-124 expressing cells in culture and virus replication in normal brain, preventing HSV-induced encephalitis (Mazzacurati et al., 2015). The field is only beginning to harness the power of miRNAs to control off-target oHSV replication, which can be combined with additional safety measures, such as the use of tumor-specific promoters to control the expression of virulence genes (Kambara et al., 2005; Nakashima et al., 2018) and, as described below, retargeting of the virus attachment and entry machinery to tumor-related cell surface receptors (Campadelli-Fiume et al., 2016). These combinatorial approaches will be particularly important in the development of vectors for systemic treatment of metastatic cancer.

2. Retargeting HSV infection to tumor-cell receptors.

Vector retargeting is a promising strategy to achieve both oHSV safety and efficacy by restricting virus infection to cells expressing tumor-associated cell surface receptors. By limiting off-target infection, this approach provides

opportunities for the use of unattenuated oHSVs in cancer therapy. HSV vectors have been retargeted to a number of cell surface receptors, including the epidermal growth factor receptor (EGFR/EGFRvIII) (Uchida et al., 2013), epithelial cell adhesion molecule (EpCAM) (Shibata et al., 2016), and human epidermal growth factor receptor 2 (HER2) (Menotti et al., 2008) (Figure 2), and several of these retargeted viruses have demonstrated considerable efficacy in *in-vivo* mouse models of solid tumors. Although efficacy following systemic virus administration has yet to be reported, preferential virus homing to tumor sites has been observed (Uchida et al., 2013). Retargeting of HSV is thus emerging as a promising strategy to accomplish unimpaired tumor-specific lytic activity.

a. Virus attachment and entry into host cells

To accomplish vector retargeting, the virus must be modified to eliminate entry through its cognate receptors. In our current understanding, HSV adsorption and entry involves the coordinated activities of glycoproteins gB, gC, gD and the gH/gL heterodimer (see Chapter 2). Virus attachment is initially mediated by binding of gC and gB to cell surface glycosaminoglycans (GAGs), primarily heparan sulfate (HS) (WuDunn and Spear, 1989; Herold et al., 1991; Shieh et al., 1992; Spear et al., 1992; Gruenheid et al., 1993; Herold et al., 1994; Tal-Singer et al., 1995; Trybala et al., 2002). Deletion of gC and the HS binding domain of gB in a single mutant virus impairs virus adsorption and reduces but does not eliminate entry, and wild-type HSV can infect HS-deficient cells (Gruenheid et al., 1993; Laquerre et al., 1998b). Retargeting of gC to a model receptor (erythropoietin receptor) combined with disruption of the HS-binding elements of gC and gB was first reported by Laquerre and colleagues (Laquerre et al., 1998a) and has since been demonstrated by others for additional receptors (Argnani et al., 2004; Grandi et al., 2004; Wang et al., 2005; Kouvatsis et al., 2007; Cao et al., 2010; Grandi et al., 2010). However, while these viruses may preferentially attach to their target cells, their entry specificity is determined by another envelope glycoprotein, gD. In a typical HSV infection, virion attachment to HS is followed by gD-mediated binding to one of several entry receptors: (1) Herpesvirus entry mediator (HVEM/HveA or

TNFRSF14), a member of the TNFα receptor superfamily (Montgomery et al., 1996); (2) nectin-1 (CD111/HveC), a cell adhesion molecule and a member of the immunoglobulin superfamily (Geraghty et al., 1998; Krummenacher et al., 1998); (3) nectin-2 (CD112/HveB), a receptor for HSV-2 and variant HSV-1 strains that carry a specific amino acid change at position 27 of gD (Q27P or Q27R) (Warner et al., 1998); and (4) 3-*O*-sulfated HS (3-*OS* HS), a carbohydrate receptor for HSV-1 (Shukla et al., 1999). Interaction with any of these receptors causes a conformational change in gD that is currently thought to activate the gH/gL heterodimer, which, in turn, activates the fusogenic activity of gB (Atanasiu et al., 2010; Eisenberg et al., 2012). Depending on the cell type, HSV entry takes place either by envelope fusion with the plasma membrane (Fuller and Spear, 1987; Fuller et al., 1989) or by virion endocytosis followed by envelope fusion with the endosomal membrane (Nicola et al., 2003; Nicola and Straus, 2004).

b. HSV retargeting via modification of glycoprotein D

As the receptor-dependent initiator of virus entry, gD is the key determinant of HSV tropism. Retargeting, therefore, requires both disruption of the ability of gD to interact with its cognate receptors and establishment of entry-initiating interactions with a desired target receptor through incorporation of a cognate ligand into gD. Substantial insight into the structure-function relationship of gD has come from extensive mutagenesis studies, mapping of monoclonal antibody binding sites, and the crystal structures of gD alone and complexed with HVEM or nectin-1 (Feenstra et al., 1990; Krummenacher et al., 1998; Whitbeck et al., 1999; Carfi et al., 2001; Connolly et al., 2003; Milne et al., 2003; Yoon et al., 2003; Jogger et al., 2004; Manoj et al., 2004; Krummenacher et al., 2005; Di Giovine et al., 2011). Pertinent to the goal of altering the binding specificity of gD without disrupting its entry-initiating response to receptor binding are the following. First, the membrane-proximal (C-terminal) region of the ectodomain, roughly between residues 260 and 310 and referred to as the profusion domain (Cocchi et al., 2004; Fusco et al., 2005), is required for receptor-dependent signal transduction to gH/gL and gB. Second, the N-terminal region contains multiple residues required for binding to HVEM

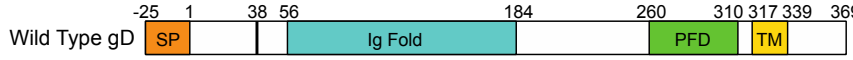
(Connolly et al., 2003) and 3-*OS* HS (Yoon et al., 2003), and complete disruption of this binding can be accomplished by mutations, insertions, and small or large deletions. Third, certain substitutions or deletion of residue 38 abolishes nectin-1 binding. Fourth, the central core of the gD ectodomain (residues 61-218) that includes much of the immunoglobulin (Ig) fold of the ectodomain [residues 56-184 (Carfi et al., 2001)], is dispensable for gD activity (Zhou and Roizman, 2007). Accordingly, HSV retargeting by the insertion of a novel ligand into gD has thus far relied almost exclusively on disruption of natural receptor binding by mutations and ligand insertion at the amino terminus, or on replacement of the central Ig domain, which also eliminates natural receptor binding. Recent studies have additionally shown that while short insertions (≤ 20 amino acids) C-terminal to the Ig fold are readily tolerated, longer insertions [62 aa or a single-chain antibody (scFv) of nearly the same size as the gD ectodomain] typically curtail cell surface expression and, consequently, membrane fusion (Fan et al., 2017).

Early attempts at HSV retargeting succeeded in ablating HVEM recognition and facilitating gD engagement of non-HSV receptors through insertion of cognate foreign ligands, including 247- and 256-amino-acid long scFvs. However, elimination of the interaction of gD with nectin-1 proved to be more challenging as the nectin-1-binding site was poorly defined and mutations that selectively eliminated nectin-1 binding were elusive (Zhou et al., 2002; Kamiyama et al., 2006; Menotti et al., 2006). The first fully retargeted HSV recombinant, R5141 (Figure 2), was identified in a stepwise process that included a screen of point mutations in a targeted gD construct for loss of virus entry through nectin-1 (Zhou and Roizman, 2006). The ligand in this construct, IL-13, was genetically fused to gD residue 33 to promote gD interaction with the IL-13 receptor α2 (IL-13Rα2), which is specifically overexpressed in malignant brain tumors. The chimeric IL-13-gD protein lacked residues 1-32 of gD, ablating HVEM binding, and used the IL-13 signal peptide instead of the native signal peptide. The screen identified V34S as a substitution that abolished nectin-1 binding while preserving the ability of the virus to use IL-13Rα2 as an entry receptor. The authors were able to grow this fully

retargeted virus on HSV receptor-deficient J1.1-2 cells transduced with IL-13Rα2 (J-13R), thereby eliminating selective pressure for reversion or complementation of the V34S substitution during propagation, although presumably not during replication in nectin-1-positive tumors. Additionally, R5141 could be grown on IL-13Rα2-transduced Vero cells (Vero-13R), albeit to lower titers than parental wild-type HSV-1, but not on standard Vero cells. Interestingly, while a variety of HSV gene products, including other glycoproteins, were abundantly visible on Western blots of R5141-infected Vero-13R cells, the retargeted gD protein was undetectable, suggesting that very low levels of gD were sufficient for infection, but also that the protein was improperly processed, undermining robust production of progeny virus.

A number of HSV-1 recombinants, fully retargeted to HER2 using a HER2-specific scFv, have since been described (Menotti et al., 2008; Menotti et al., 2009) (Figure 2). In recombinant R-LM113, the anti-HER2 scFv replaced gD residues 6-38, eliminating both HVEM and nectin-1 binding, after attempts to do so by ligand insertion between residues 24 and 25, combined with the V34S mutation, failed (Menotti et al., 2008). Replacement of the entire Ig-like core of the gD ectodomain (residues 61-218) with the anti-HER2 scFv, comprising two Ig domains, also yielded a fully retargeted virus, referred to as R-LM249 (Menotti et al., 2009), in agreement with the evidence that the gD core is dispensable for retargeted HSV entry (Zhou and Roizman, 2007). The authors implied that the deletion of the core ensured resistance to reacquisition of natural receptor binding ability, but no specific evidence supporting this claim was presented.

An orthogonal approach to HSV retargeting involved "bridging" gD with non-cognate tumor-specific receptors by using soluble bi-specific adapters (Nakano et al., 2005; Baek et al., 2011). One such adapter, comprising the N-terminal gD-binding Ig domain of nectin-1 and an EGFR-specific scFv that also recognizes the tumor cell-specific EGFR variant III (EGFRvIII), mediated EGFR-dependent HSV-1 infection (Nakano et al., 2005). Since complete detargeting from cellular nectin-1 proved problematic and since extracellular



Name	Targeting Ligand	Insertion Site	Signal Peptide	Additional gD Detargeting Mutations	Strain	Reference
R5141[1]	IL13	gD Δ1-32	IL13	gD V34S	F	Zhou and Roizman 2006
R-LM113	scFv HER2	gD Δ6-38	gD	NA	F	Menotti et al, 2008
R-LM249	scFv HER2	gD Δ61-218	gD	NA	F	Menotti et al, 2009
KNE[2]	scFv EGFR/EGFRvIII	gD Δ2-24	gD	gD Y38C	KOS	Uchida et al, 2013
KGNEp[2]	scFv EpCAM	gD Δ2-24	gD	gD Y38C	KOS	Shibata et al, 2016
R-593	scFv PSMA	gD Δ6-38	gD	NA	F	Menotti et al, 2018
R-611	scFv EGFR	gD Δ6-38	gD	NA	F	Menotti et al, 2018
R-613	scFv EGFRvIII	gD Δ6-38	gD	NA	F	Menotti et al, 2018

[1]R5141 is also retargeted via gC with IL13 replacing gC residues 1-140.
[2] The viral backbone contains two point mutations in gB, referred to as gB:NT, that enhance virus entry.

Figure 2. oHSV retargeting via modification of gD-receptor interactions. (top) Schematic representation of gD, illustrating the domains described in the text: signal peptide (SP), Ig fold, profusion domain (PFD), transmembrane domain (TM). The amino acid positions defining each domain are indicated above the schematic; mature gD starts at amino acid 1. The table underneath describes viruses bearing fully retargeted recombinant gD molecules.

adapters cannot redirect lateral virus spread, the scFv was inserted into gD between residues 24 and 25, as in the early gD constructs described above. gD detargeting from HVEM was enforced by deletion of gD residues 7-11, while detargeting from nectin-1 was achieved by changing positions 3 and 38 to cysteines [A3C/Y38C (Connolly et al., 2005)]. Phenotypic complementation assays of a gD-null virus confirmed uniquely EGFR-dependent viral entry that was enhanced by a pair of entry-accelerating mutations in gB (gB:NT) (Uchida et al., 2010). In contrast, replacement of residues 7-39 or 61-218 of gD with the same scFv, similar to the anti-HER2 scFv position in the R-LM113 and R-LM249 viruses, respectively, failed to enable entry through EGFR engagement. Indeed, it was recently reported that replacing the 61-218 region of gD with a

different EGFR-specific scFv or with scFvs recognizing EGFRvIII or prostate-specific membrane antigen (PSMA), failed to enable virus entry through the respective cognate receptors although all three scFvs were effective when replacing gD residues 6-38 (R-593, R-611, R-613, Figure 2) (Menotti et al., 2018). The authors of this study proposed that the failure of these new scFvs to function within the 61-218 deletion region might be due to the arrangement of each of these scFvs as V_H-spacer-V_L, as opposed to the V_L-spacer-V_H arrangement of the successful HER2-specific scFv. However, the earlier anti-EGFR/EGFRvIII scFv had the V_L-spacer-V_H arrangement (Uchida et al., 2010), suggesting that the relative positioning of the two antibody V domains alone cannot account for the unique ability of the HER2-specific scFv to function from within the 61-218 deletion. More recently, another effective, fully retargeted virus (KNE, Figure 2) was obtained by substitution of gD residues 2-24 with the anti-EGFR/EGFRvIII scFv, in combination with the Y38C mutation (Uchida et al., 2013). This virus could be grown on standard Vero cells without noticeable reversion of the nectin-1-detargeted phenotype (Uchida et al., 2013). Nevertheless, reversion was observed for other retargeted viruses grown on cognate receptor-transduced nectin-1+ cell lines, suggesting that while entry of the EGFR/EGFRvIII-retargeted virus through endogenous (simian) Vero-cell EGFR may be sufficiently efficient to minimize selective pressure for reversion, avoidance of nectin-1+ cell lines for virus production and rigorous detargeting from nectin-1 are critical. It was recently reported that deletion of gD residue 38 from the reversion-sensitive viruses provides reliable resistance to reversion (Uchida et al., 2018).

c. Retargeting virus infection by modification of other glycoproteins

In recent years, both gH/gL and gB have been found to interact with specific cellular receptors (Parry et al., 2005; Satoh et al., 2008; Arii et al., 2010; Suenaga et al., 2010; Arii et al., 2015), and these interactions may facilitate HSV entry. To clarify the role of gH/gL in HSV-1 entry and to explore whether the receptor specificity of this heterodimer affects HSV tropism, the anti-HER2 scFv was inserted into the amino terminus of gH (gH-HER2) in a wild-type gD virus that could be grown on nectin-1+ cells (Gatta et al., 2015). Remarkably,

this virus could both infect and spread on J-HER2 cells that expressed HER2 but lacked functional gD receptors. In addition, a derivative detargeted from HVEM/nectin-1 by deletion of gD residues 6-38 infected a HER2+/nectin-1+ cancer cell line but not HER2-negative cell lines that were permissive for wild-type HSV-1. Similarly, insertion of the anti-HER2 scFv near the amino terminus of gB (gB-HER2) enabled infection of J-HER2 cells and gD detargeting of this virus did not substantially diminish infection of HER2+ cancer cell lines (Petrovic et al., 2017). While it might be concluded from this result that gB-retargeted virus would no longer require gD and, perhaps, gH/gL for entry, neutralizing antibodies to gD or gH were found to block HER2-dependent entry of gD-detargeted gB-HER2 virus. This observation suggested that gD performs a required function in entry that is independent of its binding to a receptor. In support of this, fusion of glycoprotein-transfected cells with J-HER2 cells was equally efficient when gB-HER2 and gH/gL were transfected in the presence of wt gD or detargeted gD but was abolished in the absence of gD (Petrovic et al., 2017). The same results were obtained when gH-HER2 was used with gB and gD (Petrovic et al., 2017). Together, these results not only demonstrated that HSV can be fully retargeted by insertion of a foreign ligand into gD, gH or gB in viruses that cannot utilize the natural gD receptors, but also indicated that none of the four essential entry glycoproteins are dispensable, with the implication that these viruses remain susceptible to neutralization by anti-glycoprotein antibodies in HSV-seropositive cancer patients (Cairns et al., 2015).

d. Production of retargeted viruses

Until recently, retargeted HSV was preferably generated and propagated on gD-receptor-deficient cells transgenic for the target receptor to avoid selective pressure for mutations that could restore gD binding to one of its natural receptors. To obviate the laborious generation of new cell lines for each new target receptor, a standardized system to produce retargeted viruses without dependence on expression of the target receptor on the producer cells was developed (Leoni et al., 2017). This system is analogous to the peptide tag/ anti-tag pseudoreceptor systems previously used to grow fully retargeted adenoviruses and measles viruses (Douglas et al., 1999; Nakamura et al.,

2005). A yeast GCN4 peptide was inserted into the N-terminal region of gH while Vero cells (nectin-1+), which are FDA-approved for clinical-grade virus preparation, were engineered to express a high-affinity scFv to the GCN4 peptide as an artificial GCN4 peptide receptor (GCN4R). This tag/pseudoreceptor system was shown to enable nectin-1-detargeted virus entry into Vero cells (Leoni et al., 2017). In a subsequent study, the same group reported that gD could simultaneously accommodate the HER2 scFv and the GCN4 peptide for dual virus retargeting by a single recombinant glycoprotein, and described smaller and novel deletions suitable for nectin-1 detargeting and foreign ligand insertion (gD residues 35-39, 214-223 and 219-223) (Leoni et al., 2018a). Moreover, they showed that double retargeting to HER2 and GCN4R could also be accomplished by insertion of the GCN4 peptide at any of four different positions within the N-terminal 100 residues of gB, in combination with either the 6-38 deleted version of HER2-retargeted gD or a novel retargeted gD design combining a deletion of residue 30 with insertion of the anti-HER2 scFv in place of residue 38 (Petrovic et al., 2018). Although differences in replication and spread efficiencies were noted on either or both Vero-GCN4R and HER2+/nectin-1+ cancer cells between different doubly retargeted viruses, all were detargeted from the natural gD receptors and cytotoxic for both cell lines (Leoni et al., 2018a; Petrovic et al., 2018).

e. Retargeted virus efficacy in preclinical models

Ultimately, retargeted viruses are intended to increase tumor cell specificity and decrease off-target cell infection and toxicity, thereby improving vector safety while retaining efficient vector replication in tumor cells *in vivo*. The EGFR- and HER2-retargeted viruses have both been tested in preclinical mouse models of human tumors. The retargeted virus bearing an scFv against EGFR (KNE, Figure 2) recognizes both wild-type EGFR and its uniquely tumor-associated variant EGFRvIII prevalent on human GBM cells. This virus was effective in an orthotopic human-tumor model of GBM, i.e., a model where the tumor is located at its normal place (brain for GBM), with approximately 75% of oHSV-treated mice surviving beyond 90 days following a single intratumoral virus injection. No toxicity was observed upon direct injection of the virus into mouse

brain, and systemic injection demonstrated preferential homing of the virus in nude mice to GBM flank tumors over non-tumor tissues (Uchida et al., 2013). The HER2-retargeted virus R-LM113 (Figure 2) was also found to be safe upon direct brain injection. In murine GBM tumors engineered to express human HER2, a single injection of R-LM113 lengthened median survival time of treated animals by 21 days compared to controls (Gambini et al., 2012). Both of these studies illustrated that retargeted oHSVs abolished neuro-invasiveness in the brain. However, to further ensure tumor specificity, the EGFR-retargeted gD was coupled with insertion of miR-124 binding sites in the 3' UTR of the essential ICP4 gene, abolishing virus (KGE-4:T124) replication in miR-124-expressing neurons while permitting virus replication in miR-124 deficient GBM tumor cells (Mazzacurati et al., 2015). KGE-4:T124 was as efficacious in the treatment of orthotopic human tumors as its retargeted parent virus, lacking the miR-124 binding sites (KGE), while providing increased safety (Mazzacurati et al., 2015).

The HER2-retargeted virus R-LM249, bearing a different insertion site for the anti-HER2 scFv than R-LM113 (Figure 2), readily infected a HER2+ human ovarian carcinoma cell line, SK-OV-3. R-LM249 demonstrated a dose-dependent reduction in tumor growth following a single administration of virus directly into subcutaneous SK-OV-3 flank tumors, while repeat administration of the virus (4 doses) led to a significant reduction in tumor burden, with 60% of mice being tumor-free past 200 days (Menotti et al., 2009). Treatment of intraperitoneal (i.p.) SK-OV-3 tumors with weekly i.p. delivery of R-LM249 (5 doses) also reduced tumor burden and increased median survival from 103 days to 440 days (Nanni et al., 2013). HER2 is also expressed in a subset of human breast cancers, and weekly intratumoral administration of R-LM249 was effective against subcutaneous flank tumors formed by HER2+ human breast cancer cells (Nanni et al., 2013). Moreover, weekly i.p. injection of R-LM249 in a multiorgan metastasis model induced by intravenous injection of the same cells significantly reduced ovarian and brain metastatic burden (Nanni et al., 2013). These studies demonstrated that R-LM249 in repeated

doses was able to impair the growth of both local and disseminated HER2-expressing tumors.

While the work described above has shown that treatment with retargeted viruses can effectively reduce tumor burden and extend the survival of tumor-bearing immune-deficient mice, the current approach to oncolytic virotherapy seeks to expand immune-system engagement by arming vectors with immune modulatory transgenes. Recently, HER2-targeted R-LM113 armed with IL-12 (R-LM115) was tested in immune-competent mice in a flank tumor model induced by implantation of HER2-transduced murine Lewis lung carcinoma (LLC) cells (Leoni et al., 2018b). The results demonstrated that 4 doses of either R-LM113 or R-LM115 extended animal survival compared to controls, particularly when treatment was given at the early time point of 3 days post tumor cell injection. Of note, while the R-LM115 virus expressing IL-12 was significantly more effective than the unarmed virus at extending animal survival and reducing tumor volume, both viruses inhibited formation of tumors after rechallenge of animals that survived the primary tumor. Analysis of an array of immune cell markers and cytokines illustrated key changes in the tumor microenvironment in response to virus treatment and, in particular, to IL-12 expression, suggesting increased immune-stimulatory factors and tumor infiltration by activated immune cells in responder mice. These data highlight the importance of the immune system in tumor treatment, stimulated, in part, by the virus and its lytic activity, and, in part, by the expression of immune-stimulatory transgenes as discussed in detail below.

D. oHSVs as the next wave of immuno-oncology therapies

1. oHSV and immunogenic tumor cell killing

HSV replication causes tumor cell death resulting in the release of tumor-associated antigens/neoantigens (Bommareddy et al., 2018; Thomas et al., 2019) (Fig 3), viral pathogen-associated molecular patterns (PAMPs), and cell-derived damage-associated molecular patterns (DAMPs). Induction of innate immunity, assessed by the production of type I IFN and other proinflammatory cytokines, is triggered by these DAMPs, PAMPs, and various sensing

mechanisms that recognize viral proteins, DNA and RNA [also see section B. 2b; reviewed in (Paludan et al., 2011)]. For example, viral dsDNA is sensed by the cGAS/STING pathway (Ishikawa et al., 2009; Sun et al., 2013; Wu et al., 2013; Zhang et al., 2013) and the PYHIN domain-containing protein IFI16 (Unterholzner et al., 2010; Almine et al., 2017), which induce type I IFN and the expression of proinflammatory cytokines such as CXCL9 and CXCL10. HSV-1 infection also leads to the production of prototypic DAMPs, including the extracellular release of high mobility group protein B1 (HMGB1) and translocation of the ER chaperone calreticulin to the surface of infected cells, which, in conjunction with IFN and cytokines, results in recruitment and activation of NK cells, phagocytic cells and APCs to the site of infection (Obeid et al., 2007; Paolini et al., 2015; Takasu et al., 2016; Muller et al., 2017).

NK cells are innate immune cells recruited to the site of viral infection that are cytotoxic and can produce cytokines without prior antigen stimulation (Orr and Lanier, 2010; Abel et al., 2018). In part, NK cell cytotoxicity is regulated by binding of NK cell activation receptors, such as NKG2D and the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, to various cell surface ligands expressed on target cells (Sauer et al., 2017). Humans express eight NKG2D ligands [U_L16 binding proteins (ULBPs) 1 to 6 and MHC class I-related chains A and B (MICA, MICB)] while mice express 5-6 different ligands among the group of RAE-1(α-ε), H60(a-c), and MULT1, depending on the mouse strain (Vivier et al., 2002; Abel et al., 2018). Healthy cells express MHC class I and typically do not display NK cell-activating ligands on their surface and are shielded from NK cell-mediated lysis. However, cellular stresses associated with transformation, viral infection or other danger signals to the host cause an up-regulation of NKG2D/NCR ligand expression and NK cell activation. The anti-tumor innate immune response mediated by NK cells can be directly inhibited by tumor-expressed metalloproteinases that cleave NKG2D and NKp30 ligands on the cell surface or by tumor-mediated suppression of the expression of NK cell-activating ligands (Raulet et al., 2013; Schlecker et al., 2014; Pogge von Strandmann et al., 2015). However, these effects may be tempered by oHSV infection, which causes upregulation of NCR ligand

expression (Chisholm et al., 2007). In addition, NK cells can eliminate HSV-infected tumor cells by HSV-antibody-dependent cell-mediated cytotoxicity (ADCC) involving simultaneous binding of anti-HSV IgG to viral proteins on the

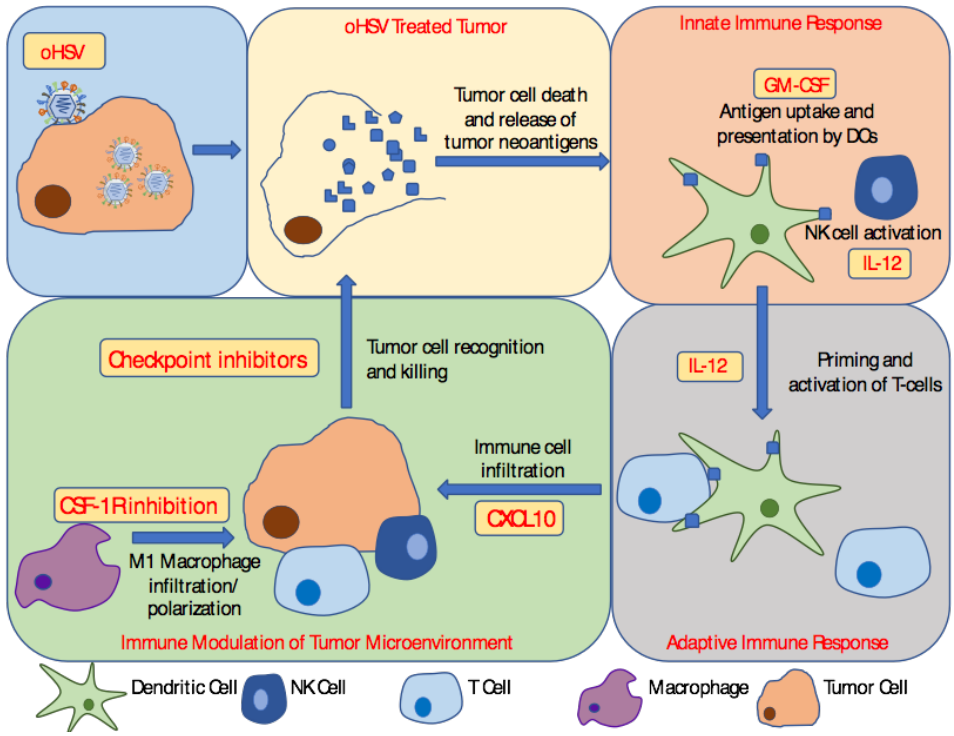


Figure 3. Armed oHSV treatment of GBM. Illustration of the immune response to oHSV infection of GBM tumor cells and examples of arming genes or gene functions (yellow boxes) for oHSV to augment the immune response to tumor antigens. Tumor and immune cell types are depicted at the bottom. Clockwise from the top left, oHSV infection of tumor cells results in virus replication and cell lysis, releasing tumor and viral antigens. Viral infection/cell death elicits an innate immune response, recruiting denditic cells (DCs) and natural killer (NK) cells to the site of infection. The adaptive immune response is stimulated via antigen uptake and presentation by DCs and results in priming and activation of T cells that infiltrate the TME. Tumor infiltration by immune cells results in further tumor cell killing by NK cells, T cells, or macrophages. Arming of oHSV with immune modulatory transgenes can influence different steps of the process: recruitment of DCs (GM-CSF), activation of NK and T cells (IL-12), recruitment of activated immune cells to the tumor (CXCL10), polarization and/or recruitment of pro-inflammatory macrophages (CSF-1R inhibition), and increased tumor cell recognition and killing within the TME (checkpoint inhibitors).

tumor cell surface and the Fc receptor CD16a on NK cells (Dai and Caligiuri, 2018). Furthermore, recent evidence suggests that NK cells can recognize and destroy oHSV-infected GBM in the absence of specific anti-viral antibodies in a process referred to as Fc-bridging cellular cytotoxicity (FcBCC) (Dai et al., 2017a; Dai and Caligiuri, 2018). Although NK cells can lead to premature virus clearance in GBM xenograft models (Alvarez-Breckenridge et al., 2012; Alvarez-Breckenridge et al., 2013), the combination of oHSV and NK cells can be exploited to induce tumor cell killing (Chen et al., 2016; Yoo et al., 2016). The outcome of this complex dual role of NK cells in response to virotherapy may be a function of the ratio of effector to target cells in the tumor microenvironment (Kim et al., 2018).

A key feature of oncolytic viral therapies is the capacity to induce both anti-viral and anti-tumor adaptive immune responses (Figure 3). The initial infection of tumor cells by oHSV causes tumor cell virolysis and an innate immune response to virus infection mediated by NK cells and DCs, ultimately creating a pro-inflammatory, antiviral state. Activated NK cells are a primary source of cytokines (e.g., FLT3L) and chemokines (e.g., CCL5) that can recruit and activate cross-presenting cDC1 cells (Barry et al., 2018; Bottcher et al., 2018), which prime an adaptive immune response by activating antigen-specific CD8+ T cells. cDC1 cells, also referred to as conventional type 1 DCs, are a subset of migratory DCs that are CD103+ (mice)/CD141+ (human), depend on the basic leucine zipper ATF-like transcription factor 3 (BATF3), and have been identified as key players in the cross presentation of both viral and tumor antigens (Hildner et al., 2008; Schlitzer and Ginhoux, 2014; Schlitzer et al., 2015; Roberts et al., 2016; Dai et al., 2017b). This subset has been associated with enhanced therapeutic responses in animal models (Roberts et al., 2016; Salmon et al., 2016; Spranger et al., 2017; Bottcher et al., 2018), and the prevalence of its gene expression signature in human tumors is positively correlated with T cell infiltration and clinical outcome (Broz et al., 2014; Spranger et al., 2016). Together, these innate and adaptive immune responses to viral infection offer a mechanism to explain how oHSVs can promote the generation of a 'hot' tumor environment and a clinical response in combination

with immune checkpoint inhibitors, as observed by Ribas and colleagues (Ribas et al., 2017).

2. Immunotherapy and oHSV arming with immune-modulatory genes

Current immunotherapy approaches fall into two broad categories. In the first, specific antigens are targeted while the second is agnostic in relation to tumor antigens (Kamran et al., 2016). Antigen-targeted therapies such as CAR-T therapy, T-cell receptor (TCR)-transduced T-cell therapy, and peptide/RNA based-vaccine immunotherapy generate T cells recognizing known or predicted tumor antigens or antigens that would not be normally expressed in adult diploid tissue (Coulie et al., 2014). While these strategies may be effective, they require advance knowledge of the tumor antigen, autologous immune cells, complex and individualized manufacturing processes, and are often restricted to a specific MHC haplotype. They also depend on functioning active T cells, and T-cell exhaustion within the TME is an important problem so far limiting the clinical efficacy of these agents to hematological malignancies (Kalos et al., 2011; Grupp et al., 2013; Kamran et al., 2016; Bagley et al., 2018). Conversely, therapies such as checkpoint pathway inhibitors, cytokine gene therapy, and oncolytic viral therapies can counter the immunosuppression created in the TME and enhance T-cell recognition of tumor cells without the knowledge of specific antigens.

Checkpoint inhibition therapies have demonstrated remarkable success in recent years. These therapies block T-cell inhibitory receptors that function to turn off inflammation, in an attempt to maintain CD8+ T-cell anti-tumor activity. The interaction between PD-L1 on tumor cells and PD-1 on T cells results in T cell exhaustion (Zhang et al., 2018), and CTLA-4 binding to B7-1 on antigen-presenting cells (APCs) blocks the expansion of T cells and interferes with activation of the T-cell co-stimulatory protein CD28 (Chikuma, 2017). Targeting the PD-1/PD-L1 axis and CTLA-4 has received FDA approval in multiple cancer indications (Hargadon et al., 2018). Several factors have been considered in determining whether a patient will be responsive to checkpoint therapy, including expression of PD-L1 on tumor cells and the presence of

intra-tumoral CD8+ T cells (Tumeh et al., 2014; Shields et al., 2017; Zhang et al., 2018). In the TME, CD8+ T cells may be tumor-reactive, but exhausted by checkpoint ligand-receptor interactions. Blocking of these interactions can lead to CTL reactivation and tumor-cell killing. In addition, increased tumor mutational burden has been linked to response to immunotherapy (Rizvi et al., 2015; Colli et al., 2016; Hellmann et al., 2018). In a first-of-its-kind decision, the FDA approved the anti-PD-1 antibody pembrolizumab (Keytruda) for use in any cancer that is high in microsatellite instability or deficient in mismatch repair, has progressed following prior treatment, and has no suitable alternative treatment options (Prasad et al., 2018). These tumors typically have an extremely high mutation load (Le et al., 2015; Le et al., 2017). While checkpoint inhibitors have mostly failed in brain tumor patients, reported cases of response have included patients with mismatch-repair deficiency (Bouffet et al., 2016). The concepts of immunosuppression, mutational burden, and immune evasion can help explain why a tumor may be immunologically "cold" and refractory to immunotherapy or immunologically "hot", and thereby responsiveness to immunotherapy. Cold tumors typically contain large numbers of immunosuppressive monocytes and few tumor-specific cytotoxic T cells whereas hot tumors are seeded with immunologically active macrophages and lymphocytes.

The promise of OVs turning up the heat in cold tumors and enabling a response to immune checkpoint inhibitors is under evaluation in multiple large clinical studies (Chen et al., 2018; Gujar et al., 2018b). The MASTERKEY-265/ KEYNOTE-034 trial (NCT02263508) is sponsored by Merck Sharp and Dohme Corp. and Amgen, Inc. and will report the outcome of the combination of Keytruda with and without T-VEC/Imlygic in 713 patients with unresected melanoma (Dummer et al., 2017; Ribas et al., 2017; Sun et al., 2018). This combination is also being tested in recurrent metastatic head and neck squamous cell carcinoma (MASTERKEY232/KEYNOTE-137 (NCT02626000), in sarcoma (NCT03069378), and in liver cancer (NCT02509507). T-VEC is also under evaluation in smaller studies in combination with anti-PD-1 antibody nivolumab (Opdivo) in lymphoma and pleural effusion and with anti-PD-L1

antibody atezolizumab (Tecentriq) in triple-negative breast cancer (TNBC) and colorectal cancer (CRC) with liver metastasis. T-VEC in combination with ipilimumab (Yervoy) displayed slightly improved efficacy over T-VEC or ipilimumab monotherapies for stage II-IV melanoma (Puzanov et al., 2016). HF10 in combination with nivolumab is being tested in the neoadjuvant setting in resectable melanoma (NCT03259425).

Preclinically, many groups are actively engaged in establishing the potential benefit of adding checkpoint blockade to HSV therapy for GBM because checkpoint inhibition monotherapies have provided convincing evidence of increased survival and immune activation in GBM mouse models (Reardon et al., 2016; Garg et al., 2017; Garzon-Muvdi et al., 2018; Hung et al., 2018). In two different syngeneic models of murine glioma, using G47Δ expressing murine IL-12 (G47Δ-mIL12) in combination with anti-PD-1 and anti-CTLA-4 antibody administration, long term survival was only observed in mice that received the triple combination therapy for CT-2A tumors (50% long-term survival) or glioblastoma stem cell line-derived tumors (89% long-term survival) (Saha et al., 2017b). In this model, CD4+ T cells, CD8+ T cells and macrophages were required for the efficacy of the triple combination therapy. Additionally, triple combination therapy resulted in an influx of pro-inflammatory and anti-tumor M1-polarized macrophages (Saha et al., 2017b). While clinically little therapeutic benefit was afforded by anti-PD-1 monotherapy or the combination of PD-1 and CLTLA4 blockade for GBM (Filley et al., 2017; Omuro et al., 2018), these preclinical data suggest that a greater benefit may be provided by a combination of intratumoral injection of oHSV and systemic treatment with anti-checkpoint monoclonal antibody.

Alternative checkpoint pathways exist that can also be targeted for antibody-mediated inhibition to help maintain CD8+ T-cell anti-tumor activity (Anderson et al., 2016). These include (i) LAG-3 (lymphocyte activation gene 3) on T cells and NK cells that can inhibit T cell expansion (He et al., 2016), (ii) TIGIT (T cell immunoreceptor with Ig and ITIM domains) on T cells and NK cells that can bind CD155 [PVR (poliovirus receptor) or necl-5] and CD112 (nectin-2) to

suppress immune activation (Solomon and Garrido-Laguna, 2018), and (iii) TIM-3 (T cell immunoglobulin and mucin domain 3) on multiple immune cell types that can trigger T cell and NK cell exhaustion and immune suppression through different pathways involving DCs, MDSCs and Tregs (He et al., 2018). Tim-3, TIGIT and Lag-3 expression have been associated with glioblastoma in mouse tumor models or in human tumor samples (Li et al., 2017; Harris-Bookman et al., 2018; Hung et al., 2018), and preclinical studies in a GL261 mouse model of GBM demonstrated that combining PD-1 and TIGIT checkpoint blockade resulted in improved survival over each monotherapy (Hung et al., 2018). Future studies will likely engage antibodies that antagonize these interactions in combination with oHSV therapy. While systemic use of anti-checkpoint antibodies could provoke autoimmunity, vector-mediated local expression of antibodies will likely be effective and minimize potential side effects. Attempts are currently underway to locally deliver an anti-PD1 single-chain variable fragment antibody (scFv) by expression from an oHSV (oHSVscFvPD-1). In early reports, oHSVscFvPD-1 was able to express the scFv in multiple glioma models and delayed tumor growth in mice bearing orthotopic gliomas (Passaro et al., 2018). Other efforts in this direction include the exploitation of PTEN to block oHSV infection-induced upregulation of PD-L1 expression on tumor cells to augment anti-tumor immunotherapy in conjunction with oncolysis (Russell et al., 2018). As described above, the effectiveness of checkpoint inhibitors relies on the reactivation of CD8+ T cells that recognize tumor antigens but are exhausted by checkpoint ligand-receptor interactions. In the event that these cells are not available or the number of TAAs is limited, the use of oHSV to stimulate a T-cell response to tumor antigens could potentiate the effect of checkpoint antibodies, whether delivered systemically or as oHSV cargo.

When considering potential targets within the TME for further oHSV vector arming (Figure 3), it is important to recognize that the composition of the TME differs between tumor types such that arming strategies will likely require type-specific optimization. The immune stimulatory cytokine IL-12 plays an important role in activating both adaptive (NK cell) and acquired (T cell)

immune responses and, as already referred to, IL-12-expressing oHSV alone or in combination with other therapies has delivered promising results in preclinical studies (Toda et al., 1998; Parker et al., 2000; Chiu et al., 2012; Cody et al., 2012; Markert et al., 2012; Cheema et al., 2013; Roth et al., 2014; Thaci et al., 2014; Bauer et al., 2016; Patel et al., 2016; Ring et al., 2017; Saha et al., 2017a; Friedman et al., 2018; Leoni et al., 2018b), including an increase of pro-inflammatory M1-polarized macrophages. Multiple studies have highlighted the immunosuppressive and tumor-supportive activities of M2-like macrophages as opposed to the anti-tumor activities of M1-like macrophages and their association with poor and improved prognosis, respectively (Lisi et al., 2017; Saha et al., 2017b; van den Bossche et al., 2018). In GBM, tumor associated macrophages, in addition to CNS-resident microglia, are particularly prevalent, representing more than half of the cell content (Darmanis et al., 2017). Macrophages in GBM express transcripts that are associated with both M1 and M2 phenotypes, suggestive of a wound healing and tolerogenic program (Bowman et al., 2016; Takenaka et al., 2019). The chemokine CCL2, through its receptor CCR2, and the growth factor CSF-1 via binding to the macrophage CSF-1 receptor (CSF-1R), are thought to play major roles in macrophage recruitment and maintenance, respectively. Small-molecule inhibitors as well as monoclonal antibodies are under development to block these interactions [reviewed in (Ries et al., 2015)]. In a mouse GBM model, the small molecule CCR2 inhibitor CCX872 reduced myeloid cells recruitment and enhanced the survival benefit of a PD-1 inhibitor antibody (Flores-Toro et al., 2020). CSF-1R inhibition using the receptor tyrosine kinase inhibitor BLZ945 counteracted M2 polarization while increasing phagocytosis, consistent with macrophage/microglia "re-education" (Pyonteck et al., 2013) (Figure 3). Unfortunately, IGF1-driven activation of the PI3K pathway led to resistance to CSF-1R inhibitor and the evaluation of this pathway in the clinic using PLX3397 (NCT01349036) did not yield a significant therapeutic benefit despite reaching pharmacologically active CNS concentration (Butowski et al., 2016; Quail et al., 2016). The investigation of BLZ945 is ongoing in solid tumors in combination with spartalizumab, Novartis' anti-PD-1 antibody (NCT02829723). Expression of indoleamine-2,3-dioxygenase (IDO) in the myeloid cell line

THP-1 promotes adoption of the immunosuppressive M2 phenotype (Wang et al., 2014). oHSV infection can downregulate IDO synthesis in IFNγ-treated glioma cells (Reinhart et al., 2012), raising the possibility that it can promote macrophage M2-to-M1 repolarization. The failure of the IDO1 inhibitor epacadostat to show a benefit in combination with pembrolizumab in the ECHO301/Keynote 252 randomized phase III melanoma trial led to a global demise of the investigation of this axis (Long et al., 2019). Although the CSF-1 and IDO axes may represent viable targets for combining with oHSV to repolarize M2 macrophages to an M1-like tumoricidal phenotype, clearly other therapeutic approaches are warranted in light of the lackluster activity of the drugs targeting these pathways.

Recently, the publication of an armed HSV-1 highlighted the potential for the KLRK1 ligand ULBP3 to modulate tumor associated macrophages and prolong survival in a genetically engineered model of glioblastoma (Wirsching et al., 2019b). oHSV expressing ULBP3, but not the empty oHSV, stimulated abscopal activity in a dual GBM tumor model and its activity was enhanced by the administration of a PD-1 antagonist antibody. Surprisingly, while the cognate receptor for ULBP3 is not expressed in myeloid cells and ULBP3 has no mouse ortholog, oHSV-ULBP3 upregulated pathways linked to antigen processing and presentation in tumor myeloid cells. Although much remains to be understood regarding the mode of action of oHSV-ULBP3, this data suggest a different route for macrophage modulation.

In addition to tumor associated macrophages, the immunosuppressive TME is supported by myeloid-derived suppressor cells (MDSCs) (Ostrand-Rosenberg and Sinha, 2009; Youn and Gabrilovich, 2010), a poorly defined myeloid population in humans, which have been shown to suppress anti-tumor immunity while stimulating tumor growth, angiogenesis and metastasis (Kohanbash and Okada, 2012; Gabrilovich, 2017). In mice, MDSCs are defined as a diverse population of CD11b+/Gr1+ immature myeloid cells that include precursors of granulocytes, macrophages and dendritic cells (Fujita et al., 2011; Zhu et al., 2011). MDSC-mediated immunosuppression involves the

production of IL-10 and IL-4 (Kohanbash et al., 2013), which promotes M2 macrophage polarization and inhibits interferon production. MDSC, through high expression of arginase, may lead to the local depletion of arginine, an essential amino acid for T-cell activation and proliferation (Lisi et al., 2017). Additionally, MDSCs can inhibit anti-tumor immunity through production of iNOS, reactive oxygen species, and TGFβ, and can recruit M2 macrophages through the secretion of chemokines that act via CCR2 (Chang et al., 2016; Kamran et al., 2018). Three primary ways can be considered to target human MDSCs: (i) depletion by antibodies targeting MDSC, (ii) maturing the cells to an M1 macrophage phenotype, and (iii) targeting the specific immunosuppressive pathways, such as arginase activity. Antibody-mediated depletion of MDSCs is effective in mice (Fujita et al., 2011), although as yet not fully specific (Kamran et al., 2018). However, so far no antibodies have been described for selective human MDSC depletion as the definition of human MDSC remains unsettled (Elliott et al., 2017). It is unclear which of these strategies may ultimately provide the most therapeutic value but each may potentially be accomplished through the selection of suitable oHSV-arming genes (Kamran et al., 2017; Lowenstein and Castro, 2018).

oHSV infection must recruit APCs to traffic tumor antigens to regional lymph nodes where they may be presented by DCs for T-cell activation (Alvarez-Breckenridge et al., 2015; Roberts et al., 2016). Therefore, arming vectors with cytokines/chemokines such as FLT3L, XCL1 or CXCL10 could enhance APC recruitment (Figure 3) while inhibition of CD47, a "don't-eat-me" protein expressed on various tumor cells, may improve antigen presentation (Chao et al., 2011; Willingham et al., 2012; Wang et al., 2013; Cioffi et al., 2015). Toll-like receptor agonists may be useful as direct tumor immune-therapeutics and can act as adjuvants for T-cell activation (Kaczanowska et al., 2013; Sato-Kaneko et al., 2017). The large payload capacity of some oHSVs (Mazzacurati et al., 2015), approximately 25 kb, allows combinations of arming genes to be introduced into a single vector, but the most effective combinations have yet to be determined. Replimune, Inc., a company funded by the T-VEC developers, is leading the way with the progression in clinical trials of a first oHSV

expressing the fusogenic protein GALV (Fu et al., 2003; Price et al., 2010; Thomas et al., 2019), in addition to GM-CSF, and a second oHSV candidate in addition expressing a CTLA-4 antagonist antibody.

3. GBM models for armed oHSV evaluation

The initial studies of oncolytic vectors employed orthotopic implantation of various genetically distinct human tumor cell lines in immune-deficient mice. While appropriate for the evaluation of oncolytic activity, the lack of an adaptive immune response in these mice does not allow for an assessment of the impact of the immune system on both virus replication and tumor cell destruction. Preclinical studies of the ability of armed oHSVs to induce anti-tumor immunity are essential for success in treatment of highly aggressive GBM and necessitate the use of immunocompetent model systems. To achieve this goal, models have been established using murine cells implanted in syngeneic backgrounds [reviewed by (Oh et al., 2014)]. For example, models of GBM have been created by intracranial injection of murine glioma tumor cell lines grown *in vitro* and by using genetic systems where tumors arise locally from neuronal precursor cells within the brain parenchyma.

The simplest model systems rely on the injection of well-characterized murine tumor cell lines. The CT-2A cell line was derived by chemical mutagenesis and has been shown to consistently form heterogenous, invasive tumors that demonstrate multiple characteristics of GBM in the C57BL/6 mouse strain background. The GL261 model, widely used in preclinical testing of GBM therapeutics, is another C57BL/6-based, chemically induced model of GBM that is more immunogenic than most human GBM. GL261 lacks MHC class II molecules while displaying low levels of B7-1 and B7-2, and is sensitive to radiotherapy and to immune checkpoint inhibitor treatment (Szatmari et al., 2006; Wu et al., 2016). More recently, a glioma stem cell (GSC)-based model was developed by Inder Verma (Salk Institute, San Diego) and co-authors from GBM tumors induced by Cre-mediated expression of activated H-Ras and Akt into the stem cell niche of GFAP-Cre $Tp53^{+/-}$ immunocompetent C57BL/6 mice (Marumoto et al., 2009). This model possesses such characteristics of GBM as

invasiveness and genetic heterogeneity as well as an immunosuppressive TME and moderate response to G47Δ-IL-12 oHSV (Cheema et al., 2013).

One genetic model system, developed by Eric Holland (Fred Hutchinson Cancer Research Center), uses an HSV-permissive transgenic mouse strain with genotype N/t-va;Ink4A/Arf-/-;PTENfl/fl that forms brain tumors on intracranial administration of a pair of engineered avian retroviruses (RCAS) expressing Cre recombinase and PDGF B-chain, respectively. The mice express the RCAS receptor (TVA) from the nestin promoter, which restricts Cre and PDGFB expression to neuronal precursor cells. Cre expression results in deletion of the loxP-flanked ("floxed", fl/fl) PTEN tumor-suppressor gene, and the resulting tumors resemble the proneural variant of glioblastoma with few, if any, T cells. In mice with a N/t-va;Ink4A/Arf-/-;PTENfl/fl;LSL-EGFRvIII/vIII background, Cre expression additionally induces EGFRvIII expression by removal of a transcription termination element (lox-stop-lox, LSL) between the promoter and coding sequence. EGFRvIII leads to a more classical or mesenchymal phenotype that includes the presence of a few T cells primarily in the perivascular region. Both models give rise to rapidly developing, predominantly mono-focal murine GBM with a TME that closely resembles that in human patients. Tumors arising in these models mimic many other aspects of human GBM, including tumor invasion of the brain parenchyma. This and similar systems with varying genetic mutations have been used to model GBM for oncolytic-vector and other therapeutic treatments (Huse and Holland, 2009, 2010; Pyonteck et al., 2013; Quail et al., 2016).

Another genetic model system that has been utilized to study gliomas, including GBM, is the Sleeping Beauty (SB) glioma model (Wiesner et al., 2009). In this model, neonatal mice receive intracerebroventricular injections of an SB transposase together with SB-compatible vectors containing glioma-relevant oncogenes of the investigator preference, such as EGFRvIII, NRAS(V12), PDGFB, shP53 and many others. In time following the injection, the animals develop tumors and, depending on the gene combination and site of injection, different glioma types can be generated. Additionally, a SB-

compatible luciferase vector can be included to allow for non-invasive bioluminescence imaging. The benefits of this model are that it is compatible with any strain or background of mice, and that cell lines can be derived from resulting tumors to create genetically relevant cell-line injection models (Fujita et al., 2010; Fujita et al., 2011; Kohanbash et al., 2013). The pathobiology of the tumors arising in these models is highly reminiscent of human GBM, with a high frequency of Iba-1$^+$ myeloid cells and a low frequency of TILs (Wirsching et al., 2019a).

These models of GBM have all been used to characterize the efficacy of either armed or unarmed oHSV vectors, and comparisons among these models will hopefully shed light on the ability of oHSV to alter the TME in ways to promote an anti-tumor immune environment that can also contribute to the prevention of secondary tumor formation.

E. Conclusions and future directions

HSV has several important attributes that make it a desirable oncolytic vector, including broad tumor cell permissivity, the ability to engineer variants that specifically lyse tumors and a large transgene capacity that allows for *in situ* delivery of multiple payload products by a single vector. With the approval of Imlygic® (T-VEC) in the US for the treatment of malignant melanoma, oHSVs are gaining prominence as an addition to standard-of-care, i.e., current clinical protocols including immune therapy and radiation therapy of cancer (Harrington et al., 2010; Andtbacka et al., 2015; Pol et al., 2016; Blake et al., 2018). Combined with checkpoint inhibitors, further improvement of tumor treatment outcome may be achieved, a result pending the completion of phase 3 randomized clinical trails such as Keynote034/Masterkey265. A current focus in the development of oHSVs for the treatment of solid tumors is to demonstrate that arming genes expressed from a lytic vector can overcome immunosuppression and enhance innate and adaptive anti-tumor immunity. Ultimately, the aim is to identify the most effective genes and combinations that create a potent immunostimulatory OV vector. By combining virus-mediated tumor destruction with the activities of specific arming-gene products, oHSVs

have considerable potential to create tumor vaccines that rely on the ability of the immune system to reject tumors. Ideally, payloads that are tailored to the TME of specific tumors should be developed. At present, several clinical trials are investigating the TME in biopsy material by RNA or proteomics profiling prior to treatment. This information could be very important for selection of arming genes.

Increasing preclinical evidence suggests that armed oHSVs can not only promote the conversion of a tumor-supportive environment in the injected primary tumor to a tumor-hostile environment, but can also bring about rejection of untreated secondary distal or visceral tumors in what has been termed an abscopal effect, the essence of an effective tumor vaccine. While much remains to be learned about the timing, reach and potency of these effects, they raise hope for the treatment of metastatic disease. As an additional strategy to attack metastatic tumor foci, there is also increasing interest in the development of oncolytic vectors for systemic application. These vectors would be (i) engineered for selective infection and replication in tumor cells and (ii) antigenically "stealthed" and thus resistant to neutralization by pre-existing or acquired virus-neutralizing antibodies. As described above, oHSV vectors have been developed that are uniquely retargeted to tumor-associated cell surface markers for attachment and entry in combination with miRNA-dependent attenuation to block virus replication in off-target tissue. While these "retargeted" oHSVs can home in on tumors bearing the target receptor in immune-compromised mice (Uchida et al., 2013), a large fraction of the human population is HSV-seropositive, and systemic treatment of metastatic cancer with even tumor-targeted oHSV will likely be impaired in these patients. Thus, another important future goal is to create retargeted oHSVs that are concealed from pre-existing immunity. Successful development of this novel class of oHSVs would represent a next-generation OV applicable to the systemic treatment of metastatic cancer.

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G. Web resources

1. http://www.abta.org/about-us/news/brain-tumor-statistics/
2. https://clinicaltrials.gov/

H. References

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