Molecular Therapy
Oncolytics
Review

Immunotherapy by mesenchymal stromal cell delivery of oncolytic viruses for treating metastatic tumors

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Oncolytic viruses (OVs) have emerged as a very promising anti--cancer therapeutic strategy in the past decades. However, despite their pre-clinical promise, many OV clinical evaluations for cancer therapy have highlighted the continued need for their improved delivery and targeting. Mesenchymal stromal cells (MSCs) have emerged as excellent candidate vehicles for the delivery of OVs due to their tumor-homing properties and low immunogenicity. MSCs can enhance OV delivery by protecting viruses from rapid clearance following administration and also by more efficiently targeting tumor sites, consequently augmenting the therapeutic potential of OVs. MSCs can function as "biological factories," enabling OV amplification within these cells to promote tumor lysis following MSC-OV arrival at the tumor site. MSC-OVs can promote enhanced safety profiles and therapeutic effects relative to OVs alone. In this review we explore the general characteristics of MSCs as delivery tools for cancer therapeutic agents. Furthermore, we discuss the potential of OVs as immune therapeutics and highlight some of the promising applications stemming from combining MSCs to achieve enhanced delivery and anti-tumor effectiveness of OVs at different pre-clinical and clinical stages. We further provide potential pitfalls of the MSC-OV platform and the strategies under development for enhancing the efficacy of these emerging therapeutics.

INTRODUCTION

Cancer is one of the leading causes of mortality worldwide, accounting for almost 10 million deaths in 2020.[1] Whereas outstanding advancements in cancer treatment have been made in the past decades, stemming from novel and effective chemotherapeutics, targeted antibodies, and immunotherapeutics,[2] several tumor types still display resistance to available therapies or undergo recurrence following treatment. These challenges to treatment success, along with the late-stage diagnosis of many cancer types, result in limited treatment options and reduced survivability in afflicted patients.

The use of oncolytic viruses (OVs) represents an alternative strategy for the treatment of various cancers. OVs typically are replication-competent viruses that can infect and replicate within tumor but not normal cells.[3,4] This tumor selectivity can be naturally occurring or achieved by genetic engineering. These genetic manipulations can be performed to enhance the therapeutic efficacy of these viruses, for example, by addition of factors to disrupt cancer-specific pathways or overcome resistance mechanisms encountered at the tumor site. The selective cytopathic effects of these viruses in the tumors can also stimulate the establishment of anti-tumor immunity.[5]

Several OVs have shown promise in pre-clinical and clinical studies, including oncolytic herpes simplex type virus (oHSV), oncolytic adenovirus (oAd), and oncolytic measles virus (oMV). Tumor cell lysis by OVs can result in the release of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and tumor-associated antigens (TAAs) as well as elevated production of various cytokines and chemokines, such as type I interferons (IFNs).[6] All of these by-products of the oncolytic process can augment various aspects of the anti-tumor immune response (both innate and adaptive), including TAA presentation by antigen-presenting cells (APCs), induction of tumor-specific T cell responses, and immune activation in the tumor microenvironment.[7,8] Further, engineering OVs to express immunomodulatory genes may further augment the potential for these vectors to stimulate anti-tumor immunity. Several immune-stimulatory agents (and their combinations) that can alter the tumor microenvironment and ultimately promise to promote long-lasting clinical therapeutic benefit are under examination. Examples include exploring the expression of IFN-β to increase the immunogenicity of OV-treated tumor cells or delivering

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interleukin-12 (IL-12) and IL-18 via an OV, which promote potent anti-tumor effects of natural killer (NK) and cytotoxic T cell activity.[9] However, despite the highly promising features of OVs, a challenge to their use exists in the rapid clearance of the virions if administered without a vehicle, due to recognition by the immune system or sequestration in off-target sites, leading to poor accumulation at the tumor site and limited therapeutic efficacy.[10]

A promising strategy for the delivery of cancer therapeutic agents is the use of mesenchymal stromal cells (MSCs) as vehicles. MSCs are considered potential vehicles for therapeutic payloads (i.e., drugs, OVs, etc.) to solid tumors owing to their tumor tropism and limited immunogenicity. MSCs home to tumors because the tumor microenvironment resembles that of non-healing wounds.[11,12] These traits render them excellent candidates for the delivery of therapeutic cargo to the tumor site while protecting it from immune clearance.[13,14] In addition, in the case of OVs, cellular vehicles such as MSCs also can act as biological factories for these therapeutic agents, as this vehicle platform allows for replication of the OV cargo.[15] Importantly, the combination of all of these properties indicates that MSC delivery of OVs may enable high accumulation of OVs at tumors while maintaining a low toxicity profile to patients.

In this review, we explore the emergence and evolution of MSCs as a cellular vehicle for delivering various cancer therapeutics. Among the many possible MSC cargoes, we mainly focus on OVs and discuss the advances and present understanding of OV-mediated immunity and oncolysis. We review the promise of using MSCs as delivery vehicles for OVs in both the pre-clinical and the clinical landscape. This review also further discusses the key challenges to the clinical use of the MSC-OV platform, as well as groundbreaking innovations that have been made recently to further improve MSC-mediated therapy.

RATIONALE FOR USING MSCs AS A CELLULAR DELIVERY VEHICLE

MSC types and their characteristics

MSCs are a heterogeneous population of multipotent cells of mesenchymal origin that are of interest for several clinical applications, from tissue regeneration to cancer therapeutics, because of their ability to home toward sites of injury, differentiate into multiple lineages, and participate in tissue repair and immunomodulation.[16]Although the term "mesenchymal stem cell" was not adopted until 1991,[17] this population was first described as a subpopulation of bone marrow cells with osteogenic potential by Friedenstein and co-workers in their seminal studies conducted in the 1960s and 1970s.[18] Since then, MSCs have been isolated from several species and from many tissue sources, including the bone marrow, adipose tissue, dental pulp, birth-derived tissues, peripheral blood, synovium, endometrium, and others.[19] In addition, MSCs also have been effectively produced from induced pluripotent stem cells (iPSCs).[20]

While the terminology "mesenchymal stem cell" was the original denomination and is often used in the literature to describe these cells, the International Society for Cellular Therapy (ISCT) currently recommends the use of the term "mesenchymal stromal cells" to define them. As per the ISCT position statements,[21,22] the former term is recommended to be used to refer to a progenitor cell population with demonstrable functionality of self-renewal and differentiation. The latter is to be used to refer to a bulk population with notable secretory, homing, and immunomodulatory properties, although some mesenchymal stem cells may be present within the MSC population. Furthermore, because of inconsistent definition of the MSC characteristics among investigators, in 2006 the ISCT proposed a set of minimal criteria to distinguish MSCs or multipotent MSCs.[23] The first criterion is that MSCs must be plastic-adherent when maintained under standard culture conditions. Second, MSCs must meet specific surface-antigen expression profiles, as measured by flow cytometry. The MSC population must express ($\geq$95%) CD105 (endoglin), CD73 (ecto-5′-nucleotidase), and CD90 (THY-1) and lack expression of ($\leq$2%) CD45 (leukocyte common antigen), CD34 (hematopoietic progenitor cell antigen CD34), CD14 (monocyte differentiation antigen CD14) or CD11b (integrin subunit αM), CD79α (B cell antigen receptor complex-associated protein α) or CD19 (B lymphocyte surface antigen B4), and human leukocyte antigen (HLA) class II. Last, MSCs must be able to differentiate at a minimum into osteoblasts, adipocytes, and chondroblasts under standard *in vitro* differentiation conditions. Nevertheless, how to more thoroughly define MSCs remains an area of continued investigation, and additional phenotypical and functional properties, such as immune functionality, are being explored as alternative metrics to identify this population.[24] Of note, the established minimal identification criteria likely best fit *in vitro*-expanded MSCs and may need to be carefully revised for tissue-resident or freshly isolated MSCs, as recent evidence suggests some altered MSC characteristics may develop upon *in vitro* expansion. For example, whereas CD34 is typically included as a negative marker, its expression can be detected in tissue-resident MSCs, suggesting that its expression is lost during *in vitro* cultivation.[25,26]

MSCs have been extensively used in clinical trials in the past several decades.[27,28] Human MSCs derived from bone marrow and adipose tissue are the two most common and longest used sources in clinical trials, although in recent years there has been an increase in the use of perinatal tissue as a source of MSCs.[28,29] Clinical trials using MSCs range in application, with some of the most common treatment targets being neurological conditions, joint diseases, and cardiovascular diseases. While there remains much to be learned about the mechanism of MSC action in the clinical setting,[30] available reports from past trials appear to indicate that the systemic administration of these cells is safe.[16,31]

Benefits of MSCs as delivery agents for anti-tumor therapies

Several of the functional properties of MSCs have rendered them a potential candidate for use in the delivery of anti-cancer therapeutics.[14] Similar to the observed behavior of MSCs in response to signals produced at sites of injury, MSCs are recruited to tumor sites.[32] This, in conjunction with the immune-evasive status of these cells, renders

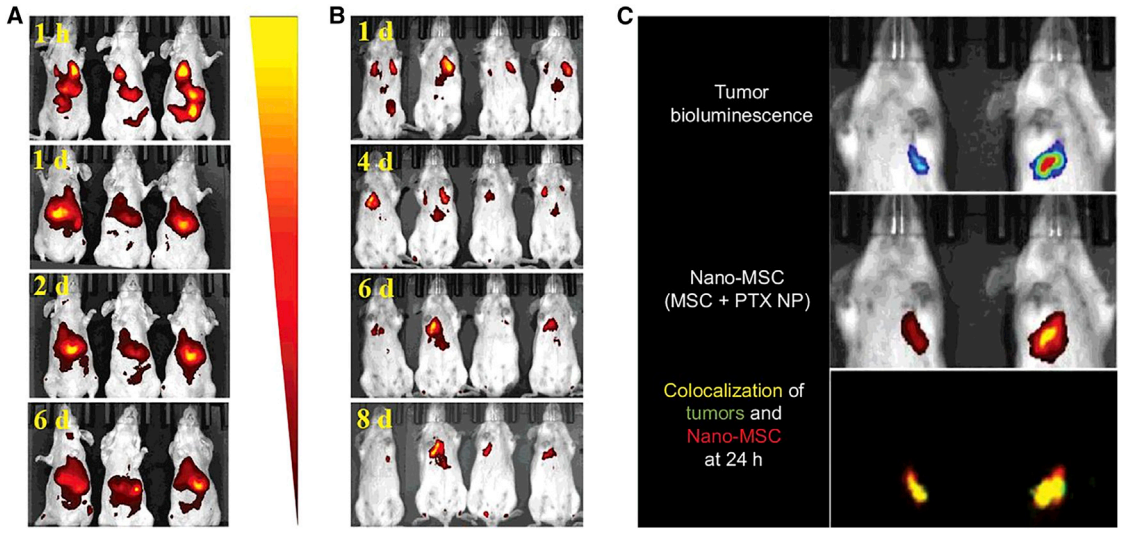

Figure 1. *In vivo* tumor-tropic properties of nanoengineered MSCs

MSCs engineered with near-infrared dye SDB 5491-labeled nanoparticles were injected in both (A) tumor-free and (B) A549-luc orthotopic lung tumor-bearing mice. Fluorescence images were captured at different time points. (C) MSCs engineered with SDB 5491-labeled nanoparticles were injected in A549-luc orthotopic lung tumor-bearing mice, and both fluorescence and bioluminescence images were captured at 24 h post injection. Reproduced from Layek et al.[33]

them a potential vehicle for the delivery of therapeutic payloads such as chemotherapeutics,[33] therapeutic antibodies,[34] and OVs.[35–38]

MSC tumor tropism

MSC homing is a multistep process modulated by several factors, including chemoattractant signals, surface receptors, and cell adhesion molecules.[39] Evidence of MSC tumor homing has been shown in many pre-clinical cancer models, including models of breast,[32,40] colon,[41] hepatocellular,[15] and lung[42] cancers and others (Figure 1). Although the mechanisms of MSC tumor homing are not fully understood, several molecules and receptors have been identified as implicated in this process.

In studies assessing MSC migration toward lung and breast cancer cell lines, macrophage migration inhibitory factor (MIF) was identified as a key chemoattractant during MSC recruitment to tumors.[43] MIF was shown to interact physically with the receptors C-X-C motif chemokine receptor (CXCR) 2, CXCR4, and CD74 (HLA class II histocompatibility antigen γ chain) in MSCs, yet MIF/CXCR4 was identified as the main axis driving MSC tumor homing. While the stromal cell-derived factor 1 (SDF-1)/CXCR4 axis is one of the most studied in MSC homing to injury sites, in this study, SDF-1 was not detected at significant levels within the molecules secreted by the cancer cell lines examined. Nonetheless, SDF-1 has been reported by others as being important in MSC migration toward tumors.[44,45] In these reports, SDF-1 was shown to be upregulated in MSCs exposed to tumor cell-conditioned medium,[34] and exposure of MSCs to recombinant SDF-1 led to activation of the Janus kinase 2/signal transducer and activator of transcription 3 (Jak2/STAT3) and mitogen-activated protein kinase/extracellular-signal-regulated kinase (MEK/ERK) signaling pathways, which in turn promoted MSC migration,[33] suggesting that SDF-1 acts in an autocrine manner to prepare MSCs to home toward the tumor microenvironment.

Other cytokines and their receptors also have been implicated in MSC tumor homing. The C-X-C motif chemokine ligand (CXCL) 16/CXCR6 axis has been shown to play an important role in the recruitment of MSCs to prostate tumors.[46] Signaling through the IL-6/IL-6 receptor axis has been identified as being important for the migration to hypoxic breast cancer tumor cells.[47] Monocyte chemotactic protein-1 has been reported to have a role in the recruitment of MSCs to primary breast tumors.[48] Additional examples of factors involved in MSC migration include IL-8,[49] fibroblast growth factor 2,[50] vascular endothelial growth factor,[50] cyclophilin B,[51] and hepatoma-derived growth factor.[51] CXCL1/CXCR1-2 signaling has been shown to drive adipose-derived MSC (ASC) migration toward prostate cancer cells.[52] Interestingly, observations from this study suggested that CXCL1 expression by prostate epithelium is obesity associated and further induced in the malignant prostate epithelial cells of obese patients. In contrast to ASCs, bone marrow-derived MSCs (BM-MSCs) have been reported to lack CXCR1 expression and their migration to be less influenced by CXCL1. In conjunction, this may imply that specific signals are involved in MSC recruitment to tumors, but at different contributing levels, depending on the patient co-morbidities and the MSC tissue(s) of origin. Thus, the MSC tissue of origin is of critical importance in the development of delivery strategies, as MSCs from some sources may be more efficacious in migrating toward tumors relative to others (i.e., ASCs may migrate more readily toward prostate tumors in obese patients relative to BM-MSCs due to CXCL1/CXCR1 signaling[52]). And whereas some studies have shown differences in the surface markers

expressed and some of the functional properties of MSCs depending on the tissue source, few reports directly compare MSCs from different sources regarding their tumor tropism ability. For example, a study assessing the tropism of ASCs and BM-MSCs toward glioma cell lines concluded that both of these had similar tumor tropism *in vitro*,[53] yet another study showed that perinatal MSCs had a higher migratory ability toward lung and prostate carcinoma cell lines relative to ASCs. In particular, umbilical cord and mixed (umbilical cord, placenta, amniotic sac) perinatal MSCs showed the highest migration potential compared with ASCs,[54] yet limitations of the study included a lack of assessment of the mechanisms driving migration. Thus, similarities or differences across MSC sources and in their tumor tropism need to be further explored in cell systems and *in vivo*.

While depletion or blocking of individual factors released from tumors decreases migration of MSCs toward tumor cells, this only incompletely inhibits MSC migration toward tumors.[43,47–49,52] An exception is inhibition or silencing of Jak2, which has been reported to completely abolish migration of human BM-MSCs induced by tumor conditioned medium.[44] This finding suggests that Jak2 signaling may be required for MSC migration, and its activation may be triggered by multiple factors released from tumor cells.

While there is much left to understand about MSC tumor homing, this process is envisioned as the means to target tumor sites for the delivery of therapeutic cargoes such as OVs. Further understanding the mechanisms of MSC tumor homing will be critical to the development of this field and the delivery of other therapeutic cargoes.

Payload encapsulation for protection from cell clearance and reduction of side effects

MSCs have been loaded with many different therapeutic cargoes and have delivered them successfully to tumors. Some anti-cancer therapeutic cargoes effective in pre-clinical models have included cytokines, tumor-suppressor genes, proteins, microRNAs, drugs, and OVs.[14] In addition to the targeting of tumors via MSC homing mechanisms, the loading of these therapeutic agents into MSCs is also thought to protect the therapeutic cargo from early clearance mediated by the immune system and blood-filtering organs. For example, MSCs have been shown to protect oMV from antibody neutralization in measles-immune mice, resulting in significantly enhanced survival of mice treated with oMV-infected MSCs compared with treatment with the naked virus or uninfected MSCs.[55] Similarly, in a clinical study assessing OV delivery by repeated administration of autologous MSCs, it was reported that most patients maintained adenoviral replication after initial detection, which suggests that the cells were able to target the tumors after repeated administration and likely shield the virus from immune recognition.[35]

Furthermore, encapsulation of therapeutic loads into MSCs has also been reported to prevent off-target cytotoxic effects after systemic administration. Studies have compared the tumor-targeted delivery of the anti-cancer drug paclitaxel (PTX) in nanoparticles (NP) by MSCs (MSC + NP PTX) versus NP alone for lung cancer.[33] When given in equivalent doses, MSC + NP PTX had no detrimental effect on white blood cell counts, whereas PTX NP caused leukopenia. The biodistribution of PTX within the lung tumors (μg.day/g tissue) was 9-fold higher when delivered by MSCs, with an accumulation ratio of PTX in the lung relative to the liver and spleen also being higher relative to nanoparticles alone. These observations translated into a higher therapeutic efficacy for MSC + NP PTX. Similarly, in studies evaluating the efficacy of MSCs as a delivery platform for an oAd in hepatocellular carcinoma, delivery by MSCs extended the blood circulation time, decreased off-target hepatic sequestration and hepatotoxicity, increased accumulation of the oAd in the tumor, and consequently increased its therapeutic efficacy, compared with naked virus administration.[15]

The protection conferred by MSCs to their cargo is attributed to the low immunogenicity displayed by these cells. Typically, MSCs express low levels of major histocompatibility complex (MHC) class I, and lack expression of MHC class II or co-stimulatory molecules (i.e., CD40, tumor necrosis factor receptor superfamily member 5). In addition, they are able to modulate innate and adaptive subsets of immune cells.[36] Thus, MSC immune evasiveness, and consequent protection of their therapeutic cargo, likely depends on a balance between low immunogenic factor expression and the production of immunosuppressive factors.[37]

MSCs can serve as biological factories for payloads

As a cellular delivery system, MSCs provide the additional advantage of being able to serve as biological factories for their therapeutic payloads. This can be harnessed by continuous production and release of the therapeutic agent by MSCs, such as is the case for genetically engineering MSCs to produce cytokines or secreted growth factors. For example, MSCs transduced to stably express IL-18 showed inhibition of breast cancer proliferation and metastasis *in vivo*. Alternatively, therapeutic cargoes such as OVs can undergo replication within MSCs[15,38] and be released upon replication-mediated cell lysis (Figure 2), leading to oncolytic action upon release.[15]

MSCs AND DELIVERY OF ONCOLYTIC VIRUSES: PRE-CLINICAL STUDIES IN THE CONTEXT OF IMMUNOTHERAPY DELIVERY

OVs as immune therapeutics

OVs have been highlighted as promising immune therapeutics for cancer patients due to their ability to enhance tumor-specific immune responses in three ways. First, OVs can increase the response of the host immune system, which would be suppressed otherwise in the tumor microenvironment.[3,4] Second, OV-mediated lysis of tumor cells can cause the release of TAAs and danger signal molecules, and type I IFN production, which eventually enhances tumor antigen presentation and induction of tumor-specific T cell responses.[6] Third, "armed" OVs can express high levels of immune-stimulatory therapeutic genes in tumor tissues, which further improves the anti-tumor immunity already mounted by OVs.[7,8] All of these attributes of OVs can promote inflammation within the tumor and

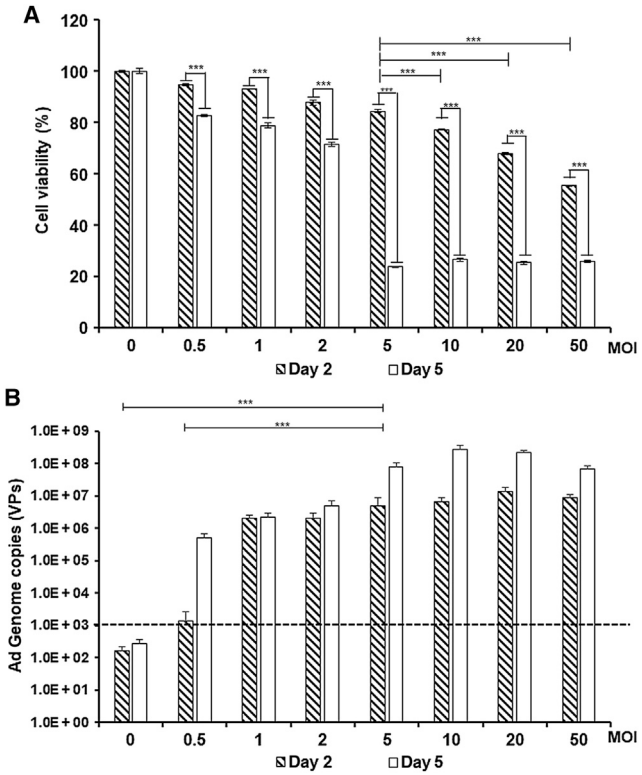

Figure 2. MSC killing effect of hepatocellular carcinoma (HCC)-targeting oAd and its viral production in MSCs

(A) MSC killing effect of HCC-targeting oAd. MSCs were infected with HCC-oAd-Luc at MOIs ranging from 0.5 to 50. At 2 or 5 days post infection, cell viability was assessed by the MTT assay. The data are representative of three independent experiments performed in triplicate. Bars, mean ± SD. (B) Viral production of HCC-oAd-Luc in MSCs. MSCs were infected with HCC-oAd-Luc at MOIs ranging from 0.5 to 50. At 2 or 5 days post infection, the total viral yield produced in MSCs was quantified by qPCR. Dashed line represents the detection limit of qPCR. The data are representative of three independent experiments performed in triplicate. Bars, mean ± SD. ***$p < 0.001$. Reproduced from Yoon et al.[15]

induce its microenvironment to be more immunologically favorable for other immunotherapeutics,[56–60] rendering OVs as powerful therapeutic adjuvants.

In this regard, many OVs are under development clinically as immune therapeutics for cancer. The first OV to be approved by the US Food and Drug Administration (FDA), IMLYGIC, is the only anti-tumor cytokine (granulocyte-macrophage colony-stimulating factor [GM-CSF])-expressing oncolytic HSV. However, numerous groups at present have reported that oHSVs expressing any anti-tumor immune transgene exert a more potent growth-inhibiting effect relative to cognate controls lacking any transgenes.[61–64] Notably, as there is a growing number of studies suggesting that potent anti-tumor cytokines such as IL-12 may exceed the tumor growth-inhibiting effects of GM-CSF,[65] the transgene payload of more recent oHSVs is tending toward expression of IL-12. IL-12 is known to facilitate Th1 differentiation and to augment the cytolytic effect of NK cells and cytotoxic T lymphocytes (CTLs), leading to enhanced anti-tumor immunity.[66,67] According to these trends, oHSV expressing IL-12 (oHSV-IL-12) has been evaluated in pre-clinical studies, where treatment with oHSV-IL-12 elicited a long-lasting immune response capable of halting the growth of brain tumor and its distant tumors, yet without adverse effects.[68] Further, one ongoing phase I clinical trial is evaluating the safety profile of an oHSV expressing IL-12 (M032-HSV-1) against recurrent malignant glioma (ClinicalTrials.gov: NCT02062827).[69] In fact, there are several oAds expressing GM-CSF or IL-12 under current clinical evaluation (GM-CSF, NCT01437280, NCT02143804, NCT02365818; IL-12, NCT02555397, NCT03281382, NCT02062827). In addition, oAds expressing anti-tumor immune transgenes other than cytokines have also begun to be evaluated clinically, and these include, for example, LoAd703 (oAd expressing CD40L and 4-BBL) and NG-350A (oAd expressing anti-CD40 antibody).

In addition to the clinically developed oAds, oAds expressing immune-stimulatory therapeutic genes have been also actively investigated and evaluated pre-clinically to overcome a tumor-mediated immunosuppressive microenvironment. For example, transforming growth factor β (TGF-β) attenuates cytokine-mediated anti-tumor immune responses in immunosuppressive tumor microenvironments. Therefore, with the rationale of enhancing the efficacy of IL-12-mediated cancer immunotherapy,[66,67] decorin (DCN), which is known to decrease the expression of TGF-β, was introduced to counteract the TGF-β-mediated immunosuppression.[57] Tumors treated with the oAd co-expressing IL-12 and DCN (RdB/IL-12/DCN) induced IFN-γ (and IFN-γ-expressing cell numbers), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) at levels significantly higher than control oAds expressing each of the therapeutic genes (RdB/DCN or RdB/IL-12). The RdB base vector in this work has a mutated Rb protein-binding site in E1A and lacks the E1B and E3 regions. Further, RdB/IL-12/DCN attenuated intra-tumoral TGF-β expression, which correlated positively with the reduction of regulatory T cells (Tregs) in draining lymph nodes and tumors. The virus was more proficient at spreading within tumor tissues in the RdB/IL-12/DCN-treated tissues, and higher infiltration by $CD8^+$ T cells was also observed. These results suggest that developing OVs expressing a suitable (synergistic) combination of cytokines is the appropriate way to find candidates for the next generation of OV clinical trials.

Vascular endothelial growth factor (VEGF) is pro-angiogenic and pro-metastatic[70,71] and also has been shown to interact with T cell precursor cells in the bone marrow, inhibiting proliferation and maturation of T cells and dendritic cells (DCs) and contributing to immunosuppression within the tumor milieu.[72,73] IL-12 is one of the representative candidates for immune gene therapy due to its ability to facilitate Th1 cell differentiation and augment the cytolytic effect of NK cells and CTLs, thus greatly promoting anti-tumor immunity.[66,67,74] Since IL-12 is effective at least in part due to its ability to attenuate VEGF expression,[75] an oAd co-expressing IL-12 and VEGF-specific short hairpin ribonucleic acid (shVEGF; RdB/IL-12/

shVEGF) was developed to enhance the potency of immunogene therapy.[76] The RdB/IL-12/shVEGF vector efficiently restored immune surveillance functions in tumor tissues and actively promoted immune cell recruitment by upregulating IL-12 and IFN-γ. This vector also was efficient at reducing VEGF expression, restoring an anti-tumor immune response, and preventing the thymic atrophy seen in VEGF-expressing tumor models due to inhibited thymocyte proliferation.[74] Delivery of RdB/IL-12/shVEGF directly to tumor tissues promoted very high infiltration of differentiated $CD4^+$ and $CD8^+$ T cells, NK cells, and DCs, particularly to the areas surrounding the necrotic regions of tumors. As a result, this therapeutic induced a more potent anti-tumor effect relative to the cognate control oAds expressing either of the single therapeutic genes (RdB/shVEGF or RdB/IL-12). Of note, the viral dose demonstrating anti-tumor effects in this study was ∼70% lower than the conventional doses needed in mice, indicating its superior potency as an immune therapeutic. With these promising results, this OV is currently under development for translational applications.

The intrinsic immunogenicity of OVs can further augment the therapeutic efficacy of any co-administered immune cells, by recruiting endogenous immune cells and leading to restoration of immune function in the tumor microenvironment.[3,4,6] These attributes of OVs make them prime candidates for combination with immune cell therapies. For example, an oAd co-expressing IL-12 and 4-1BBL (Ad-ΔB7/IL-12/4-1BBL) exhibited significantly enhanced IFN-γ expression and anti-tumor efficacy *in vivo*, suggesting that an anti-tumor type I immune response can be successfully activated by co-expression of these transgenes. More interestingly, when this vector was co-administered with DCs, the combination promoted greater anti-tumor and anti-metastatic effects, an enhanced type I anti-tumor immune response, and a higher DC migratory ability within tumors relative to the monotherapeutic regimen. This was a result that demonstrated the potential benefit of combining cytokine-expressing oAds with DCs.

MSCs as stealth carriers of systemically delivered OVs (MSC-OV)

Despite the many advantages of OVs, their therapeutic efficacy is insufficient when they are systemically delivered, i.e., into the bloodstream. Due to their native tropism, systemically administered viruses such as oAd typically accumulate in the liver, leading to hepatotoxicity.[77–79] Furthermore, the highly immunogenic viral capsid or the viral envelope typically activates innate and adaptive immune responses by the host against oAd.[80,81] Many different types of serum proteins and blood cells can adhere to the surface of viruses, reducing their blood circulation time due to complement activation.[82,83] As a result, viruses cannot appropriately accumulate at the target tumor site, ultimately leading to a low therapeutic efficacy. Due to these limitations, over 80% of current OV clinical trials utilize local injection as an administration route rather than systemic injection (https://beacon-intelligence.com/).

Currently, MSCs have been recognized as promising delivery tools for OVs, including oAd, oMV, oncolytic myxoma virus (oMyx), and oHSV (Table 1). MSCs can efficiently transport their therapeutic cargo toward the tumor site due to their low immunogenicity and tumor tropism.[44,84–87] Further, the high transduction efficacy of OV-mediated gene transfer allows MSCs to express the OV therapeutic genes and carry OVs to tumor sites, which later are released from the MSCs by viral replication-mediated lysis.[88–94] More importantly, MSCs loaded with OVs are lysed by viral replication after they reach the tumor site, thus preventing adverse side effects such as uncontrolled differentiation and any potential tumorigenesis associated with stem cell prolonged survival *in vivo*. These characteristics of OV-loaded MSCs strongly suggest that these cell carriers may enable more efficient and precise systemic delivery of OVs to tumor tissues relative to direct OV injection into inaccessible tumors, in particular, ones that are metastatic or distant from a primary site.

Based on this background, the first study to utilize MSCs as an OV carrier was conducted in 2009 using oMV.[55] In this study, oMV-infected MSCs were devised to protect oMV from antibody neutralization in measles-immune mice. Even though naked oMV was completely inactivated, intraperitoneally administered MSC-oMV reached target cells and induced the formation of syncytia in the presence of high titers of anti-measles antibody. MSC-oMV localized to peritoneal tumors and transferred viruses to tumors in both measles-naive and passively immunized mice. As a result, survival of the measles-immune mice was augmented by treating with oMV-infected MSCs relative to naked virus or uninfected MSCs.

The possibility of utilizing MSCs as a delivery tool for oHSV, the first OV to be approved by the US FDA, was assessed in melanoma brain metastasis pre-clinical models.[95] When naked oHSV was delivered via intracarotid administration, it did not reach tumors. In contrast, MSC-oHSV migrated to the tumor site in the brain and significantly prolonged the survival of the mice. Furthermore, in a syngeneic melanoma brain metastasis model, a combination of MSC-oHSV and PD-L1 blockade increased tumor-infiltrating $CD8^+$ and IFN-γ-producing T cells, promoting a significant increase in the survival of treated animals. This result provided an insight into MSC-based oHSV therapies that could overcome the hurdles of systemic delivery of OVs for treating brain-metastatic melanoma.

The therapeutic delivery of oMyx by MSCs has been assessed preclinically in a model of glioblastoma[38] utilizing ASC permissive for myxoma virus replication. When administered intracranially (∼1 mm anterior to the implanted tumor), delivery of oMyx by ASCs resulted in a significant survival increase in orthotopic studies *in vivo*.

The idea of combining MSCs and OVs to potentiate the therapeutic efficacy and safety profile of cancer treatment was most frequently assessed with oAd, among the many types of OVs. The feasibility of human MSCs as carriers of oAd was tested in an ovarian cancer model using D24RGD,[96] an adenovirus serotype 5 (Ad5) harboring a deletion in the Rb-binding domain and an insertion of the tumor-targeting RGD motif in the fiber region of the viral capsid to

Table 1. Examples of pre-clinical studies on the use of MSC-OV

Oncolytic virus	Cancer type	Results	Reference
MSCs as a carrier of systemically delivered OVs			
MV-CEA	ovarian cancer	- protection of oMV from antibody neutralization - localization of MSC to peritoneal tumors - enhancement of survival of measles-immune tumor-bearing mice	Mader et al.[55]
G47Δ-based recombinant oHSV	melanoma brain-metastaticcancer	- migration to the tumor site in the brain - increased anti-tumor immune response when combined with PD-L1 blockade	Du et al.[95]
D24RGD	ovarian cancer	- increased targeted delivery efficiency - reduced systemic toxicity	Dembinski et al.[96]
HCC-oAd	hepatocellular carcinoma	- homing to HCC tumors - cancer-specific killing effects through active viral replication within MSCs - reduction of overall toxicity	Yoon et al.[15]
ICOVIR-5	lung adenocarcinoma	- reduction of tumor growth and systemic activation of innate and adaptive immune response by MSCs (syngeneic or allogeneic) carrying the virus - increased infiltration of leukocytes into the core of the tumor	Morales-Molina et al.[97]
oAd d1E102	renal adenocarcinoma, melanoma	- reduction of tumor volumes - increased tumor immune infiltration by tumor-associated macrophages, NK cells, and tumor-infiltrating lymphocytes	Morales-Molina et al.[98]
Ad5/3	ovarian carcinoma	- increased survival - decreased tumor burden	Komarova et al.[99]
CRAdNTR	colorectal cancer	- protection of oAd from neutralization - oncolysis and tumor growth inhibition - MSC-mediated activation of co-administered pro-drug (CB1954)	Ho et al.[100]
vMyxgfp	glioblastoma multiforme	- reduction of brain tumor size - increased survival	Josiah et al.[38]
Immunotherapy applications of MSC-OVs			
ICOVIR-5 + CSF	osteosarcoma	- reduced tumor growth - higher tumor immune infiltration - reduced T cell exhaustion	Morales-Molina et al.[101]
oAd ICOVIR-15 + PBMC	lung adenocarcinoma	- increased anti-tumor efficacy	Moreno et al.[102]
oMyx + IL-15 (vMyx-IL15Rα-tdTr)	pulmonary melanoma	- reduction in the number of pulmonary foci - when administered three times, extension of survival was observed - elevated NK cells and $CD8^+$ cells and decreased $CD4^+$ cells in the lung tissues - elevated expression of immune-stimulatory genes in lung tissues	Jazowiecka-Rakus et al.[103]

improve its cancer specificity, which is currently being evaluated in clinical trials against glioblastoma under the name DNX-2401. The use of MSCs was efficacious in increasing the delivery of D24RGD to ovarian cancer cell lines. *In vivo*, intraperitoneal injection of naked D24RGD into mice resulted in strong hexon-positive spots and inclusion bodies in the tissues, including spleen, kidney, and liver, showing

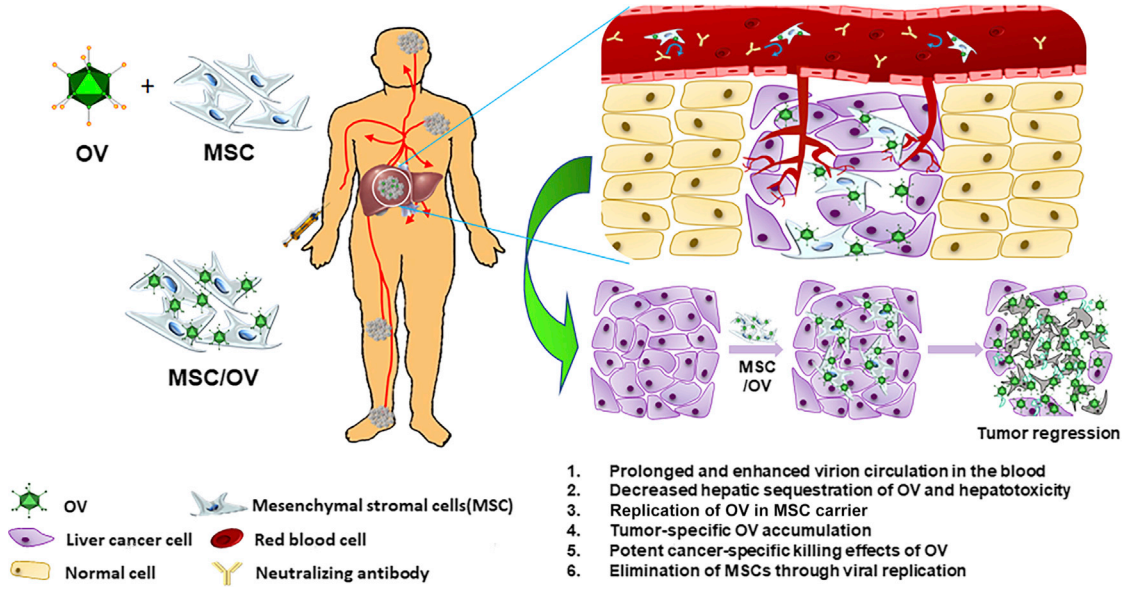

Figure 3. Potent therapeutic efficacy and safety profile of MSC loaded with OVs

MSC loaded with OVs can serve as "stealth carriers" in the clinical environment to preferentially transfer the virus to the tumor site while overcoming the limitations of naked virions (nos. 1–6) to achieve potent and safer therapeutic effects.

systemic distribution of virus. However, in contrast to the toxicity shown by D24RGD, the MSC-guided delivery of D24RGD reduced the overall systemic toxicity to negligible levels in the same mouse model. Intraperitoneal administration of MSC-D24RGD also increased the survival of mice bearing ovarian tumors. These results demonstrated that the tumor-homing ability of MSCs can improve oAd accumulation specifically in tumors and oAd-loaded MSCs can enhance the safety profile of oAd by significantly decreasing oAd hepatic sequestration.

In addition to reducing toxicity profiles, an additional innovative aspect of MSCs as carriers for OVs is that these cells can serve as a "biological factory" permissive to viral replication. To further maximize viral replication within MSCs, Hammer et al. engineered oAds either lacking the anti-apoptotic viral gene E1B19K or modified to express the TNF ligand superfamily member 10 (oAd-TRAIL).[104] These oAd modifications improved replication in both MSCs and cancer cells, indicating that even initial loading of a small viral titer into MSCs can subsequently efficiently deliver an effective (anti-tumor) viral dose. This higher viral dose could be achieved either from viral replication within the MSCs or within the tumor cells following delivery.[99,105] However, although viral replication within MSCs is a promising attribute, excessive viral replication might induce premature MSC lysis, which likely would reduce the efficacy of this system. Therefore, future approaches should continue to be examined to obtain an optimal balance between the increase in total viral yield and the impact on reduced MSC viability.

Yoon et al. developed hepatocellular carcinoma (HCC)-specific oAd (HCC-oAd) and intravenously delivered it to orthotopic tumors using MSCs.[15] The HCC-targeting oAd was loaded into MSCs (HCC-oAd/MSC) and effectively lysed HCC cells *in vitro*. Furthermore, systemically administered HCC-oAd/MSC homed to HCC tumors and showed a highly localized pattern of viral accumulation, ultimately leading to potent inhibition of tumor growth. Of note, the dose of HCC-oAd-loaded MSCs was 40-fold lower than the conventional oAd dose range ($\sim 2 \times 10^{10}$ viral particles) that frequently is utilized for systemic treatment of tumor-bearing mice,[106] indicating that (1) MSCs can function as a biological factory capable of viral production, (2) MSCs can substantially potentiate the anti-tumor effects of oAd, and (3) MSCs can enable a significant oAd dose reduction to improve the safety profile of this virotherapy modality. Moreover, systemically administered HCC-oAd/MSC prolonged the blood retention time of oAd, apparently by masking the surface of the oAd and reducing the virus immunogenicity. Other effects included reduced non-specific liver sequestration and thus less hepatotoxicity, suggesting that this oAd-loaded MSC can not only improve therapeutic efficacy but also increase the oAd safety profile. This study highlighted that (1) oAd loading into MSCs can elicit potent tumor-specific killing through viral replication within MSC carriers, (2) the tumor-homing tropism of MSCs helps improve oAd accumulation in a tumor-specific manner, and (3) the cargo-protective attributes of MSCs prolong and enhance virion circulation in the blood. Most interestingly, the loading of MSCs with oAd improves the safety profile of both the carrier cells and the viral payload, by decreasing the hepatotoxicity of oAd while promoting elimination of MSCs through viral replication (Figure 3). Taken together, MSCs loaded with OVs can serve as "stealth carriers" in the clinical environment, preferentially delivering OV to tumors while attenuating potential risks that may arise from systemically administering naked virions.

Immunotherapy applications of MSC-OV

Recently, the role of MSCs as antigen-presenting (and immune-effector) cells has been highlighted.[107,108] Further, MSCs possess a short-term memory for danger signals or environmental stimuli,[109] thereby allowing MSCs to facilitate anti-tumor immune therapeutics once they reach the tumor site. Therefore, these attributes of MSCs need to be critically considered in order to achieve the delicate balance between OV-MSC-mediated anti-tumor response and anti-viral immune responses. To meet this need, the immunological profiles of MSCs loaded with OVs have been characterized intensively. Morales-Molina et al. demonstrated that MSCs loaded with ICOVIR-5 (oAd-Δ24RGD) activate more potent NF-κB signaling for the release of cytokines such as IL-6, CXCL2, CXCL10, and C-C motif chemokine ligand (CCL) 5, which enhance recruitment of NK and T cells into the tumor microenvironment, relative to naked ICOVIR-5.[97]

Through understanding of the immunophenotypic changes occurring in MSCs after oAd infection, new approaches to improve the anti-tumor efficacy of infected MSCs can be developed. Combinations that increase immune cell infiltration into tumors such as ICOVIR-5-MSC with granulocyte-colony stimulating factor (G-CSF) have resulted in a maximized anti-tumor immune response.[101] ICOVIR-5-MSC/G-CSF showed significantly enhanced anti-tumor effects against osteosarcoma *in vivo*, with a higher proportion of tumor-infiltrating lymphocytes and reduced T cell exhaustion relative to controls. This study highlighted that immune-stimulatory cytokines such as G-CSF may be considered for the improvement of OV-MSC-mediated cancer therapy. Moreno et al. also demonstrated that oAd can work through other mechanisms, such as increased Toll-like receptor 9 expression, leading to activation of the NF-κB pathway in menstrual blood-derived MSCs.[102] Furthermore, oAd-loaded MSCs co-cultured with allogeneic peripheral blood mononuclear cells (PBMCs) could increase the expression of MIF, an upstream activator of innate immunity functions, promoting a pro-inflammatory tumor microenvironment. These phenomena were mainly mediated through monocyte activation, leading to the activation of both T and NK cells.

To enhance the anti-tumor immune response, immune-modulatory gene-expressing OVs were also utilized for a strategy of OV-loaded MSCs.[103] MSCs loaded with oMyx expressing IL-15, inducing the activation and proliferation of T cells and NK cells, demonstrated anti-tumor effects and anti-tumor immune response. The intravenous administration of MSC-shielded oMyx showed marked regression of lung melanoma and increased survival in mice. Elevated expression of genes (IFN-γ, TNF-α, $CD4^+$ T cell, $CD8^+$ T cell) that are involved in the adaptive immune response was confirmed. Further, lung infiltration of NK cells was observed, leading to inflow of $CD8^+$ T lymphocytes when MSC-shielded oMyx was delivered into melanoma lesions.

MSCs AND DELIVERY OF ONCOLYTIC VIRUSES: CLINICAL STUDIES

Clinical studies utilizing MSC-OV

There have been few clinical studies using MSC-OV for treatment of cancer patients (Table 2). The first-in-human trial for pediatric refractory metastatic neuroblastoma treatment (NCT01844661) used autologous MSCs loaded with oAd (ICOVIR), or CELYVIR.[35] CELYVIR was prepared from a bone marrow aspirate and delivered intravenously to the tumor site. In this phase I clinical trial, CELYVIR promoted only mild toxicity related to the Ad infusion (fever, chills, and discomfort). Therefore, this was a well-tolerated therapeutic with the potential to achieve clinical responses in patients with advanced tumors. Viral replication was detected in seven of the nine pediatric patients and was not detected in the adults. The absolute circulating number of leukocytes was virtually unchanged during therapy; however, some cell subsets were significantly different between the pediatric and the adult cohorts. Promising results included two neuroblastoma patients showing disease stabilization, with one continuing treatment for up to an additional 6 weeks. These results indicated the safety of CELYVIR, warranting its further evaluation in a phase II setting and illustrating how the use of MSCs might be a safe and effective strategy to increase the amount of OV administered to patients, while avoiding direct tumor injections and also toxicity. And even though data are currently lacking on the anti-tumor immune response with CELYVIR, this should be further investigated to enhance the future likelihood of therapeutic outcomes in the clinic. Additional clinical trials of the use of CELYVIR are underway for other patient groups (Table 2; EudraCT no. 2019-001154-26, NCT05047276).

Other trials are currently ongoing, and their results are yet to be posted. An example of this is a clinical trial where ASCs prepared from newly diagnosed and recurrent ovarian cancer patients (NTC02068794) are subsequently infected with oMV expressing thyroidal sodium iodide symporter (MV-NIS). The phase I/II trials assess the feasibility, safety, and clinical effects of MSC-oMV. This trial is the continuation of pre-clinical trials that already have been optimized for clinical trials and reported as the first study to use MSCs as a delivery tool for OVs.[15,38,55,97–99,101]

CHALLENGES TO CLINICAL TRANSLATION AND POTENTIAL SOLUTIONS

MSC heterogeneity

While MSCs are defined by a set of immunophenotypic markers, it is recognized that heterogeneity exists within the MSC population. Further exploration of the MSC subsets and the impact of any differences on their functional properties likely will play a key role in the optimization of MSC-based cell therapies for cancer and other diseases. Some factors that have been previously identified to account for differences in the therapeutic potential of these cells (i.e., multipotency, homing to injury/inflammation, immunomodulation, capability of forming a functional hematopoietic niche) have included donor health status and age, tissue source, *in vitro* culture conditions, and MSC passage number.[116]

One of the most studied aspects of MSC heterogeneity is that of the differences in cellular functional properties depending on the tissue source. The differences observed in phenotypical and functional features are likely a result of the various tissue environment niches

Table 2. Clinical trials on the use of MSC-OV in cancer patients

Identifier	Status	Description/results	Reference
EudraCT no. 2008-000364-16	ended prematurely	- trial to determine the toxicity and clinical outcome of infusion of autologous MSCs infected with the oncolytic adenovirus ICOVIR5 (CELYVIR) in children with refractory or recurrent metastatic solid tumors	ClinicalTrialsRegister.eu[110]
		- results not posted	
NCT01844661	completed	- phase I/II trial	Ruano et al.[35]
		- evaluation of the safety and clinical response of weekly (n = 6) infusions of CELYVIR in children and adults with metastatic and refractory solid tumors	
		- well-tolerated treatment, with only mild toxicity, with potential to achieve clinical responses in patients with advanced tumors	
NCT02068794	ongoing	- phase I/II trial	ClinicalTrials.gov. National Library of Medicine (USA)[111]
		- studies side effects and best dose of oncolytic measles virus encoding thyroidal sodium iodide symporter (MV-NIS)-infected MSCs in patients with ovarian, primary peritoneal, and fallopian tube cancer	
		- results not posted	
NCT03896568	ongoing	- phase I trial	ClinicalTrials.gov. National Library of Medicine (USA),[112] Chen et al.[113]
		- studies the best dose and side effects of the intra-arterial administration of oncolytic adenovirus DNX-2401-loaded hBM-MSCs in treating patients with recurrent high-grade glioma	
		- utilization of perfusion-guided endovascular super-selective intra-arterial injections enhances the targeting ability of therapeutic delivery to brain tumors	
		- trial results not posted	
EudraCT no. 2019-001154-26	ongoing	- studies the feasibility of the combination of AloCELYVIR with chemotherapy and radiotherapy for the treatment of children and adolescents with relapsed or refractory extracranial solid tumors	ClinicalTrialsRegister.eu[114]
		- results not posted	
NCT05047276	not yet recruiting	- phase I/II clinical trial	ClinicalTrials.gov. National Library of Medicine (USA)[115]
		- studies safety and efficacy parameters of AloCELYVIR in uveal melanoma patients with hepatic metastases	

and the many local functions of MSCs.[116] Although BM-MSCs remain the most studied cellular subtype, selection of MSC source might be important to tailor therapeutic results toward the most optimal clinical advantages provided by each subtype. For example, adipose tissue may yield higher numbers of cells and from less invasive protocols relative to BM-MSCs, allowing for a lesser need for cell expansion to achieve clinical doses.[117] Also, neonatal MSCs have been reported to have an improved lifespan and differentiation potential compared with BM-MSCs, rendering them interesting candidates for tissue regeneration applications.[118] Finally, BM-MSCs are capable of forming a functional hematopoietic niche important for applications where hematopoietic support is most beneficial.[116]

Donor-to-donor variability has also been reported by many groups. Some factors that might influence these differences include donor age and health status. Ruano et al. have evaluated the efficacy of autologous BM-MSCs infected with the oAd ICOVIR-5 in animal models, identifying intrinsic MSC characteristics that associate with better clinical outcomes.[35] Similarly, their group found differences in the profiles of gene expression of homing- and immune-related

genes in the MSC of responders versus non-responder patients in clinical trials,[35] which are to be disclosed in future publications. This suggests that pre-screening protocols could be implemented to identify more suitable MSC donors prior to clinical application depending on the desired therapeutic outcome.

Cell heterogeneity is also present even within MSC batches.[119] Data suggest that single cells may not possess all the properties of MSCs within a culture.[120] This observation has led to the idea that the functional attributes of MSCs are achieved by distinct subpopulations within the heterogeneous cell mix.[119] In line with this idea, efforts are underway to identify markers to define and enrich certain MSC subpopulations with specifically desired characteristics. For example, the mesenchymal stem cell markers Stro-1 and CD271 (low-affinity nerve growth factor receptor) have been explored as potential markers for the enrichment of higher proliferative cells.[120] In contrast, other markers or subsets of markers may be used to predict MSC multipotency and other functional properties.[119] These markers, however, cannot be used as the sole markers for MSC, as they are expressed by other cell subtypes present at MSC isolation sites, and some of these are not universally expressed in all types of MSC.[120] The enrichment of desired MSC subpopulations with more specific functions could provide a venue for improved reproducibility of therapeutic outcomes. Further, identification of better MSC markers may allow for their isolation without the need for culture expansion prior to their use in the clinic. As it stands, the majority of MSC products with regulatory approval are produced from culture-expanded MSCs.[121] One exception is Queencell, which, while including ASCs, is not limited to this population and may be best described as a stromal vascular fraction (SVF) product. At present, the differences in the therapeutic potential of freshly isolated MSCs in comparison with culture expanded remain highly unexplored. Studies on this topic primarily evaluate the therapeutic potential of culture-expanded ASCs versus SVF.[122–125] The field likely would benefit from the exploration of the therapeutic potential of sorted freshly isolated MSCs compared with their cultured counterparts, which in turn will depend on a more robust characterization of surface marker expression for these cells.

Last, although similar functional features of MSCs have been described across species, some differences have been noted in the mechanisms or molecules involved in these processes. Thus, it is important to account for species-specific variations when drawing conclusions regarding MSC functions across organisms.[118,126] One notable example are differences reported between human and murine MSCs in their soluble mediators of immunosuppression. Namely, under the same culture conditions, human MSC immunosuppression is mediated by indoleamine 2,3-dioxygenase, whereas nitric oxide mediates immunosuppression in murine MSCs.[127]

Because the functional properties of MSCs are dependent on many intrinsic and environmental factors,[116] it is important to consider these differences when developing protocols for the preparation of these cells for use in clinical applications to reduce their variability. Standardization of production and screening processes for MSCs would likely prove beneficial for the development of more robust and reproducible results. The development of non-destructive biomarker identification techniques for functional discrimination is discussed among the strategies to address the issue of MSC heterogeneity.[128] Interestingly, Boregowda et al.[129] proposed a clinical indications prediction scale (CLIP) for predicting functional properties of MSCs with respect to their proliferation, differentiation, and paracrine action, based on the expression of the transcription factor *TWIST1*. Further exploration of this scale, or development of similar tools for predicting the migration capabilities of MSCs, may be of interest for the use of these cells as therapeutic vehicles. In addition, the generation of MSCs from iPSCs has been discussed among strategies to address the issue of heterogeneity of MSCs, as iPSCs may have theoretically unlimited expansion capability from a single clone, thus limiting donor-to-donor variations.[128] Similarly, cell pooling from multiple donors also has been proposed to decrease variability in an MSC preparation without compromising cell function, as shown by studies assessing their proliferation and osteogenic differentiation potential.[130]

Inefficient tumor homing of MSCs

Despite MSCs having the ability to home to tumors and sites of inflammation, pre-clinical and clinical data suggest that this process is very inefficient, in some cases leading to the inability to detect MSCs (or detection of a very low proportion) at the target site.[131] Although targeted accumulation of MSCs occurs *in vivo*,[35] a significant amount of MSCs end up at off-target sites, one of the major concerns being lung entrapment following intravenous administration.[132,133]

Entrapment in the lungs is speculated to be caused by multiple factors, including cellular diameter and cellular attachment potential.[132,133] One of the strategies proposed for decreasing lung entrapment is the alteration of MSC cell-surface receptors. In a study with rats, in which quantification of cells reaching arterial circulation was performed after intravenous administration of MSCs, it was observed that the majority of MSCs were trapped inside the lungs following infusion. This entrapment in the lungs was reduced by antibody blocking integrin subunit α4 (CD49d), involved in MSC adhesion via vascular cell adhesion molecule 1 (VCAM-1) to lung endothelium.[132] Similarly, antibody blocking of integrin β1, integrin α5, or integrins αVβ3 also has been shown to result in decreased MSC entrapment in the lungs following intravenous administration. Interestingly, a decrease in expression of these integrins was also achieved by 3D culturing even after monolayer expansion, and these changes in culture conditions also resulted in reduced lung entrapment of MSCs upon infusion.[134]

Another example of cell-surface modification disrupting lung entrapment of MSCs following infusion was described by Kerkelä et al.,[135] where a comparison of lung clearance and targeting efficiency was performed using MSC from various species. In this study it was noted that cell detachment with Pronase, instead of trypsin, led to a decreased retention of MSCs in the lungs of mouse, rat, and porcine models, without compromising viability and functionality

of the cells. This group also reported a higher MSC migration toward the target tissue in a rat model of carrageenan-induced inflammation of Pronase-detached cells compared with those detached with trypsin, which they suggested may be due to increased bioavailability as the cells more rapidly transited through the lungs.[135]

On the other hand, Strategies to improve MSC targeting via pre-stimulation and modification of the expression of surface molecules are also explored by other researchers. For example, hyaluronic acid (HA) stimulation of MSCs by HA plate coating was shown in a murine model to lead to increased migration to inflammatory sites compared with untreated MSC controls, likely due to a transient induction of CD44 expression in MSCs.[136] Similarly, IL-3 pre-conditioning has been observed to increase CXCR4 expression on MSCs and enhance their migration *in vitro*.[137]

The choice of route of administration might also have an impact on the MSCs' ability to reach target inflammatory sites. For example, Mäkelä et al. reported in a porcine model that the biodistribution of BM-MSCs altered the therapeutic outcome, with significantly lower lung entrapment when the cells were administered intra-arterially relative to intravenously.[138] Avoiding lung entrapment likely enables more effective targeting of MSC therapies to other tissues. This is in line with the report by Walczak et al., where MSCs administered intravascularly in a rat model of transient cerebral ischemia were readily detected intracerebrally following intra-arterial but not intravenous injection.[139] All these route considerations are relevant to attempting to achieve more efficacious therapies that lack detrimental side effects, particularly from off-target accumulation.

While the effects of off-target accumulation may still need to be carefully considered in several clinical applications, pre-clinical studies suggest that for delivering OVs, the use of MSCs is generally safe. There are limited toxicity and off-site effects, however, attributed to several factors with MSC OV delivery. One of these factors is likely the lysis of MSCs following the increased oncolytic load due to replication of the viral cargo. This phenomenon has been reported *in vivo* in a study assessing the systemic delivery of a HCC-targeted oAd via MSCs in mice.[15] The MSCs were delivered systemically intravenously and could be detected at tumors following CD90 staining when they were given alone, but remained undetected when oAd-bearing MSCs were used. Taken together, these reports suggest that the delivery of OVs to tumor sites via MSCs could yet be improved by refinements in MSC culture, cell surface modifications, and cell administration protocols.

Concerns about potential tumorigenesis or promotion of inflammation by MSCs

As the interest in the use of MSCs in cancer therapeutics increases, so does the concern for the safety of their use, with particular interest in their potential for tumorigenesis. As reviewed by Lee and Hong,[140] MSCs can contribute to cancer pathogenesis via multiple mechanisms, including the release of factors involved in inflammation, angiogenesis, and immunosuppression of various immune subsets (i.e., B cells, NK cells, and T cells). Furthermore, MSCs have been demonstrated to transform into cancer-associated fibroblasts (CAFs) in response to signaling through the CXCL16/CXCR6 axis, which then stimulate cancer cell migration.[46]

Conversely, MSCs have also been reported to display tumor-suppressive effects, including induction of cell-cycle arrest and apoptosis, recruitment of inflammatory infiltrates, and through the regulation of cellular signaling (i.e., upregulation of phosphatase and tensin homolog [PTEN]).[141]

The delivery of OVs via MSCs leads to replication-mediated lysis of the cell vehicles, which then results in the release of the viral cargo at the target site.[15,142] This process may serve as a countermeasure to clear MSCs and prevent their potential contribution to disease progression in patients. In addition, limiting the expansion of MSCs for clinical use may also contribute to reducing risks associated with their use, as senescence resulting from extensive expansion may contribute to the pro-tumorigenic effects observed in these cells.[143,144]

Strategies to overcome the limitations of MSC delivery of oncolytic viruses

Modifications to MSCs as carriers

Increasing MSC availability. MSC availability can be a limiting factor in their potential use in clinical applications such as the delivery of OVs. Expansion of MSCs to achieve dose-relevant numbers can be a time-consuming and difficult process due to the limited availability of MSCs from different tissue sources, which may be hindered further by the patient's health status and the limited expansion capability of the cells. In a clinical trial evaluating the delivery of the OV ICOVIR-5 by autologous MSCs (CELYVIR) in adult and pediatric patients with advanced tumors, the use of autologous MSCs imposed a delay of 6 weeks on the manufacturing process. Further, the group experienced difficulties obtaining the target cell dose for adult patients, as the dosing is dependent on patient weight.[35]

Due to the immune-evasive status of MSCs, the use of allogeneic MSCs represents a potential avenue for the increased availability of these cells for therapeutic use. Pre-clinical studies on the use of allogeneic MSCs suggest that they exert a therapeutic efficacy similar to that of autologous MSCs and are safe to use. In a study comparing the efficacy of syngeneic and allogeneic mouse CELYVIR (mCelyvir) treatments, no significant differences were observed in homing capabilities, systemic immune response, anti-tumor efficacy, or intra-tumoral infiltration of leukocytes across the groups, with the exception of a significant increase in NK cells in the tumor noted in allogeneic but not syngeneic mCelyvir treatment groups.[97]

Although bone marrow remains the most commonly studied tissue source for MSCs, isolation from other tissues may be a promising alternative for a higher MSC yield acquisition. Calculations suggest that BM-MSCs comprise about 0.001%–0.01% of the total bone marrow nucleated cells. In contrast, ASCs are estimated to be present

at approximately 500-fold higher numbers when isolated from equivalent amounts of adipose tissue.[117] Alternative sources to BM-MSCs, such as ASCs and umbilical cord blood MSCs, also can have higher proliferation,[145] further aiding in reaching significantly higher numbers of cells for therapeutic use.

Evaluation of alternative sources of MSCs that can lead to an off-the-shelf option for MSCs to be used in clinical delivery of OVs may play a key role in the availability of this treatment option for patients with advanced disease.

Improving MSC persistence. Despite their very low immunogenicity, MSCs do not tend to persist for a very long time following administration.[146] Various mechanisms might play a role in this limited persistence, including detachment from the target site following localization or immune clearance.[37] Increasing MSC persistence may provide benefits for several therapeutic applications, including the delivery of OVs. Some of the strategies evaluated for improving the persistence of MSCs following transplantation have included encapsulation, cell-surface engineering, and pre-conditioning or co-administration strategies.[37]

Encapsulation strategies for MSCs have shown promise in increasing the MSC persistence at the target tissue. One example of this is MSC encapsulation with synthetic extracellular matrix (sECM). In a study assessing IFN-β secretion via locally delivered mouse MSC (MSC-IFN-β), it was shown that MSC encapsulation in sECM was necessary for the effective retention of the cells in the brain and consequent boost of therapeutic efficacy of the platform.[147] MSC encapsulation with sECM has also been evaluated pre-clinically for delivering the oAd ICOVIR-17 for glioblastoma.[148] In this study, the local administration of sECM-encapsulated human ASCs loaded with ICOVIR-17 resulted in a decrease in tumor regrowth and increased mouse survival. However, this report did not include a comparison of the efficacy of this platform with non-encapsulated MSC delivery of the OV. The use of other materials such as alginate for MSC encapsulation can also lead to a prolongation of MSC presence, as observed in a study evaluating allogeneic MSC local persistence following implantation in an immunocompetent rat model.[149] While these strategies appear to provide some therapeutic benefits following local administration, encapsulation strategies may affect the homing capabilities and immunogenicity of the cells[150] and have an impact on their functionality following systemic administration.

Because MSCs are immune evasive, but not immune privileged,[37,151] strategies that can improve immune evasion can be envisioned for augmenting persistence of allogeneic MSCs. These strategies may be of great importance to the field of OV cellular delivery, as cellular and humoral immune responses can hinder the localization of MSCs at the tumor site and the consequent cytolytic effects by promoting early cell clearance. Modification of surface molecules associated with immune recognition has been assessed as a means to reduce MSC immunogenicity. A study assessed the effects of downregulating MHC class I proteins on the MSC cell surface via retroviral transduction of the human cytomegalovirus immunoevasin US11. Interestingly, it was reported that the persistence of human MSCs following xenogeneic intrapinnal implantation could be improved, provided that the recipients' NK cells had been depleted.[152] However, this downregulation of MHC class I was observed to lead to faster clearance of transduced MSCs relative to unmodified MSCs when NK cells had not been depleted. This finding supports the notion that a complex balance of expression of immunogenic and immunosuppressive factors might need to be achieved,[37] and multiple targets for surface modification might be needed in order to achieve a less immunogenic status of MSCs following transplantation.[152]

Several reports in the field have indicated that immunosuppressive factors produced by MSCs have key roles in mediating the immune evasiveness of these cells. MSC are not inherently immunosuppressive, but rather acquire an immunosuppressive phenotype via interaction with stimulatory factors such as growth factors or cytokines.[153] The modification of MSCs into a more immunosuppressive phenotype is envisioned as a potential strategy to improve their persistence following administration by reducing their potential for immunogenicity.[37] Several strategies have been reported to promote an MSC immunosuppressive phenotype, including genetic engineering and priming or pre-conditioning strategies. In a study evaluating the anti-inflammatory potential of triple-mRNA-transfected (P-selectin glycoprotein ligand-1, Sialyl-Lewisx, and interleukin-10) MSCs, this platform promoted a transient increase in levels of IL-10 in the inflamed ear, consequently enhancing he anti-inflammatory effects of MSCs *in vivo*.[154] In this study, similar cell homing capabilities were observed for MSCs with or without IL-10 transfection, suggesting that while expression of this cytokine enhances their immunosuppressive potential at the site of inflammation, IL-10 alone does not reduce immunogenicity. Some pre-conditioning or priming strategies recently reported to improve the immunosuppressive potential of MSCs include the use of pro-inflammatory cytokines (i.e., IFN-γ), the use of pharmacological or chemical agents (i.e., rapamycin), changes in culture conditions (i.e., 3D culture), and Toll-like receptor stimulation (i.e., TLR3 stimulation with polyinosinic:polycytidylic acid) (reviewed in Noronha et al.).[155] In particular, MSCs can be polarized into what have been termed "MSC1" or "MSC2" phenotypes with TLR4 or TLR3 agonists at relatively low doses of agonists, to obtain either pro- or anti-inflammatory effects, respectively.[91] Although promising, several methods are still under investigation to achieve more precise stimulation of MSCs, since there are outcomes reported in the literature whereby MSC activation can lead to increased expression of MHC molecules,[118] thus potentially reducing the effectiveness of these cells in immune evasion.

Other strategies proposed to improve persistence of MSCs by immune avoidance rely upon temporary disruption of the immune response via administration of immunosuppressive drugs (i.e., rapamycin, mycophenolic acid).[37] Thus, these strategies are likely more suitable for other applications. In cancer patients, disruption of the immune response could have a detrimental impact on disease progression and on the efficacy of OV treatments, as part of their

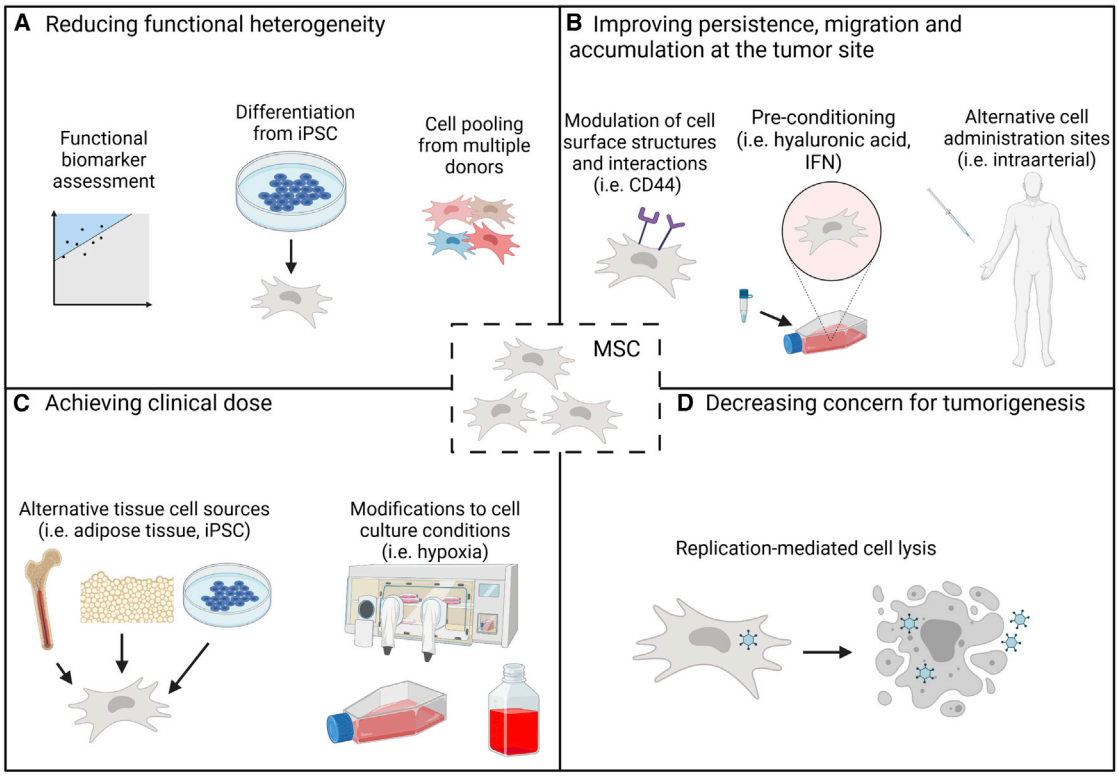

Figure 4. Strategies to improve the potential of MSCs as vehicles for the delivery of oncolytic viruses

(A) Different strategies can be used to enhance the functional properties of MSCs for delivering oncolytic viruses and decreasing their safety concerns. These strategies include screening or using MSCs from single clones or from pooled populations to reduce their functional heterogeneity. (B) Variations in cell culture and administration protocols may help limit the MSC sequestration at off-site tissues and by cells of the immune system, consequently leading to preferential accumulation of MSCs at tumors. (C) Obtaining cells from more abundant MSC sources and culturing them in a manner to enhance MSC proliferation can result in increasing the speed and likelihood of attaining cell doses needed for clinical use. (D) Permissive replication of the viral cargo within MSCs can reduce the risk of MSC persistence following delivery by promoting replication-mediated cell lysis. Created with Biorender.com.

mechanism of action relies on inducing an activated immune response against tumors.

While improving MSC persistence may enhance the therapeutic potential of these cells, the approach to achieve this likely will have to weigh many variables, including tumor type, the immune status of the patient, the best choice for the mode of cell administration, and other factors. The strategies discussed previously for enhancing the potential of MSCs as therapeutic vehicles for OVs (Figure 4) might have to be balanced so as not to come at the cost of other properties more critical to a desired platform's success (i.e., enhanced immunosuppressive action that may prolong MSC survival but may interfere with the OV-mediated immune response activation).

Modifications to the tumor microenvironment

Changes to the tumor microenvironment induced by other therapeutic approaches could be harnessed to improve the therapeutic efficacy of MSCs as carriers of OVs. In a study evaluating the effects of ionizing radiation on the tropism of MSCs toward gliomas, transwell migration assays and intravascular delivery both showed a significant increase in MSC migration toward irradiated glioma cells relative to non-irradiated controls.[156] In this study, CCL2 secretion by irradiated glioma cells was identified as having an important role in the ionizing radiation-induced tropism of MSCs toward gliomas. A similar effect on MSC migration following tumor irradiation pre-clinically was observed in colon and breast tumors, but not in head and neck squamous cell carcinoma, in a different study.[157] Although this study did not explore the mechanisms underlying MSC migration following tumor irradiation, a moderate increase in MCP-1 (CCL2) in irradiated tumors was noted. Expression of anti-angiogenic growth factors or anti-tumor cytokines such as the pigment-derived epithelium growth factor or IL-24[158] may further augment the efficacy of OV-mediated killing. Other treatment strategies such as chemotherapy can also induce changes to the secretory profile of tumor cells, including the stimulation of CCL2 production;[159] thus, it may be possible to harness these induced changes in the delivery of OVs via MSCs.

Modifications to viral payloads

Continuous enhancement of the MSC-OV platform will result in the development of new OVs and further modification of existing

ones. Several features of the viral payloads will likely be important considerations in these designs. Namely, MSC delivery of OVs could be improved by modifications to enhance transduction of the OVs in MSCs and improve their anti-tumorigenic effects.

Modifications to the structure of the OVs can be performed to increase the transduction efficacy of these payloads into the MSC carriers. Kuroki *et al.* reported that when they tested a panel of Ad5 fiber knob variants, Ad5pK7, a vector with a polylysine fiber knob modification, showed the highest transduction rates across a panel of 16 patient-derived ASC lines.[160] Using a similar approach, Yoon *et al.* demonstrated that by modifying the viral fiber knob from Ad5 to that of Ad serotype 35, the transduction efficiency efficacy into MSCs was enhanced.[15] Alternatively, the viral loading into MSCs could be facilitated by the addition of cell-penetrating peptides (CPPs) or other similar strategies. In experiments that evaluated the efficiency of adenovirus transduction into MSCs, Park *et al.* reported that MSC transduction could be further enhanced by using tetrameric relative to monomeric CPPs.[161]

Furthermore, strategies to overcome resistance mechanisms encountered at the tumor site can be employed to further enhance the anti-tumorigenic efficacy of this platform. For example, expression of soluble hyaluronidase by OVs can improve viral spreading and therapeutic efficacy in tumors expressing high levels of hyaluronic acid.[148] Also, the addition of hypoxia-responsive elements to the OVs can help overcome the hypoxia-mediated downregulation of viral replication experienced at the tumor site.[162] Finally, targeting of dysregulated signaling pathways involved in cancer growth and spread can enhance the cancer cell-killing effects of the OVs employed.[15]

CONCLUDING REMARKS

Pre-clinical data indicate that the use of MSCs as delivery vehicles for OVs significantly enhances their anti-cancer therapeutic potential by resulting in increased accumulation of the virus in the tumor following administration. Further development of these therapeutic tools will likely rely on a multifaceted approach where design parameters are selected to enhance the safety profile and potential of the carrier cells, enhance the activity of the viral payloads, and establish criteria for patient suitability, considering several aspects such as immunological status and treatment history.

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AUTHOR CONTRIBUTIONS

A.-R.Y. and C.R.-C. participated in conceptualization and writing of the original draft and review and editing. J.M.G. participated in editing, and C.-O.Y. and M.L.F participated in the conceptualization, writing, reviewing, editing, and supervision.

DECLARATION OF INTERESTS

C.-O.Y. is a founder and CEO of GeneMedicine Co., Ltd., which is developing an oncolytic virus for clinical application. J.M.G. is a co-founder, co-owner, member, and employee of Obatala Sciences (which includes the merged LaCell LLC), a for-profit company developing stromal-derived cells and products for clinical translational regenerative medical studies, as well as a co-founder of Talaria Antibodies. He is an inventor on multiple patents relating to adipose-derived cells and their utility.

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