UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Medicina Molecolare

CORSO DI DOTTORATO DI RICERCA IN: MOLECULAR MEDICINE
CURRICOLO: BIOMEDICINE
CICLO XXXIV

Pre-clinical optimization of oncolytic Herpes Simplex Virus type 1 vectors

Tesi redatta con il contributo finanziario della Fondazione Cariparo

Coordinatore: Ch.mo Prof. Riccardo Manganelli
Supervisore: Prof.ssa Arianna Calistri

Dottorando: Alberto Reale

Index

Abstract

Oncolytic viruses (OVs) are emerging therapeutics that selectively replicate in cancer cells, either naturally or following genetic engineering. OVs also elicit an immune response against cancer and are therefore an immunotherapeutic tool. Furthermore, OVs can be modified to express therapeutic genes. An OV based on an attenuated herpes simplex virus type 1 (HSV-1), talimogene laherparepvec (T-VEC), has been approved in the US in 2015 and in the EU in 2016 for the treatment of advanced-stage malignant melanoma. T-VEC has deletions in the neurovirulence γ34.5 gene and Us12 gene (Δγ34.5/ΔUs12) and is further armed with human granulocyte-monocyte colony stimulating factor (hGM-CSF) gene. Our research group developed several oncolytic HSV-1 (oHSV1s) with a Δγ34.5/ΔUs12 backbone, armed with an array of immunotherapeutic genes other than GM-CSF. During this PhD project, we focused on developing a systemic delivery system by means of carrier cells, to achieve a pre-clinical optimization of oncolytic HSV-1. Monocytes were chosen because 1)they have an inherent tropism for tumors, being the precursors of tumor associated macrophages (TAMs), 2)they are capable of migrating into most compartments of the body, including the central nervous system, 3)autologous monocytes can be easily recovered in large amount from peripheral blood. Using the human monocytic cell line THP-1, we demonstrated that monocytic cells can migrate towards human breast cancer cells and transmit oHSV1 infection. These findings were confirmed with primary human monocytes. THP-1 cells also delivered oHSV1 to human head-and-neck UM-SC-11B cancer cells growing on the chorioallantoic membrane (CAM) of embryonated chicken eggs, following intravascular injection. Finally, we developed a new miRNA-based neuroattenuation system for oHSV1 to enhance safety following intravenous injection.

I virus oncolitici (OV) sono agenti terapeutici emergenti che si replicano selettivamente nelle cellule tumorali, o naturalmente o in seguito a modificazioni genetiche. Gli OV provocano anche una risposta immunitaria antitumorale e pertanto hanno anche un effetto immunoterapeutico. Inoltre, i virus oncolitici possono anche essere modificati per esprimere geni terapeutici. Un OV basato sul virus herpes simplex di tipo 1 (HSV1), il talimogene laherparepvec (T-VEC), è stato approvato negli Stati Uniti nel 2015 e nell'Unione Europea nel 2016 per il trattamento del melanoma in stadio avanzato. T-VEC ha una delezione del gene della neurovirulenza γ34.5 e del gene Us12 (Δγ34.5/ΔUs12), inoltre è armato col gene dello human granulocyte-monocyte colony stimulating factor (hGM-CSF). Il nostro gruppo di ricerca ha sviluppato diversi HSV-1 oncolitici (oHSV1) basati su un backbone Δγ34.5/ΔUs12, armati con diversi geni immunoterapeutici diversi da GM-CSF. Durante questo progetto di dottorato, il focus è stato spostato sullo sviluppo di un sistema di somministrazione sistemico basato su cellule carrier al fine di raggiungere un'ottimizzazione preclinica di oHSV1. I monociti sono stati scelti perchè 1)hanno un tropismo intrinseco per i tumori, essendo i precursori dei macrofagi associati a tumore (TAMs), 2)sono in grado di migrare nella maggior parte dei distretti corporei, incluso il sistema nervoso centrale, 3)i monociti autologhi possono essere recuperati in grande quantità con un semplice prelievo di sangue periferico.

Usando la linea cellulare monocitaria umana THP-1, abbiamo dimostrato che i monociti possono migrare verso cellule di carcinoma mammario umano e trasmettere l'infezione da oHSV1. Queste osservazioni sono state confermate usando monociti umani primari. Le cellule THP-1 infettate sono state in grado anche di trasmettere l'infezione a cellule umane di carcinoma squamoso testa-collo (UM-SC-11B) che crescono sulla membrana corioallantoica di uova embrionate di pollo, in seguito a iniezione intravascolare. Infine, abbiamo sviluppato un nuovo sistema di neuroattenuazione basato su miRNA per oHSV1 per aumentare ulteriormente la sicurezza in seguito a somministrazione sistemica.

105

106

107

108

109

110

111

112

113

114

115

116

117

118

119

120

121

Summary

Oncolytic viruses (OVs) are attenuated viruses that exploit defects in antiviral pathways in cancer cells, and they are promising tools for the treatment of tumors poorly responsive to current therapies[1]. An oncolytic herpes simplex virus type 1 (oHSV-1), talimogene laherparepvec (T-Vec), has been approved in the US and the EU for the treatment of unresectable melanoma[2]. It has been acknowledged that, besides causing the death of cancer cells, OVs also elicit an antitumoral immune response and modulate the immunologic features of the tumor microenvironment (TME)[3]. The most investigated delivery method for OVs is intratumoral injection, to avoid neutralization of the viruses by the immune system in the bloodstream

Our research group previously developed several oncolytic HSV-1 vectors with immunotherapeutic genes which were inserted in the UL55-UL56 intergenic by bacterial artificial chromosome (BAC) mutagenesis. Genes included interleukin 12 (IL-12) and a single chain antibody against chemokine receptor CCR4. During this PhD project, more oHSV-1s with genes FMS-like tyrosine kinase 3 ligand (Flt3L) and soluble programmed cell death 1 (sPD1) were generated.

However, even intratumoral injection of an oncolytic virus armed with multiple therapeutic genes is only partially effective to achieve a systemic antitumoral immunotherapeutic effect [4]. Therefore, a refinement of the approach towards a clinically appealing strategy should involve a form of systemic delivery that allows targeting of both the primary tumor and metastases.

In this setting, the possibility of using carrier cells has emerged as a promising method to achieve a systemic delivery of OVs[5]. Carrier cells can be infected *ex vivo* and then injected intravenously, and it has been demonstrated in animal models that they can effectively shield OVs from antibody-

mediated neutralization. Still, the research in this specific field is in a relatively early phase compared to intratumorally delivered OVs, both in basic and clinical research.

In particular, most researchers working with OVs delivered by carrier cells focused on mesenchymal stem cells (MSCs), due to their tropism for tumors and interestingly, also for their immunosuppressive properties [6]. This feature protects the OVs from being cleared by the immune system, however it can easily become a double-edged sword considering that therapeutic efficacy of OVs is also due to the immune system. Furthermore, MSCs have biodistribution problems following intravenous injection, probably because of their relatively large size [7] (Krutzke et al, unpublished observations).

Until now, some studies considered other cells as candidate carriers, including neural stem cells, T lymphocytes [8], and different myeloid cell lineages. We became particularly interested in monocytes, due to several favorable characteristics:

1)Circulating monocytes are the precursors of tumor associated macrophages (TAMs), at least in several clinically important cancers [9] [10]. TAMs are fundamental for the survival and growth of many tumors and also for the establishment of metastases, thus they are actively recruited by cancer cells, providing monocytes a strong tropism for malignant tissues.

2)Monocytes make up approximately 10% of circulating leukocytes in healthy humans, which means that recovery of autologous monocytes from cancer patients potentially involves only peripheral blood sampling, without the biopsy and *in vitro* amplification steps required by MSCs.

3)Monocytes are cells that naturally evolved to migrate from the bloodstream into tissues to differentiate into macrophages, also in body compartments which are difficult to reach, such as the central nervous system (CNS) [11]

Therefore, we hypothesized that autologous monocytes are ideal carrier cells for OVs, due to their inherent tropism for the microenvironment of solid tumors and to the possibility of easily recovering large numbers of autologous cells from peripheral venipuncture. We initially employed a human monocytic cell line (THP-1), which was susceptible to infection with our backbone oHSV-1 expressing enhanced green fluorescent protein (oHSV1-GFP) but less permissive than epithelial cell lines, as indicated by reduced viral titres and very high cell viability 24 hours post-infection. THP-1 cells infected with a multiplicity of infection (MOI) of 3 plaque forming units (PFU)/cell could transmit oHSV-1 infection to human breast cancer MDA-MB-231 cells in a coculture assay and could migrate towards breast cancer cell supernatants in Boyden chambers. Importantly, these findings could be confirmed with primary human monocytes purified from buffy coats of blood donors.

We then sought a more biologically relevant model to evaluate trafficking of monocytes towards human tumors, before moving to an *in vivo* model. For this purpose, we started a collaboration with Dr. Lea Krutzke and Prof. Stefan Kochanek from the Department of Gene Therapy of the University of Ulm (Germany), who established a model in which human head-and-neck squamous cancer cells (UM-SC-11B) grow on the chorioallantoic membrane (CAM) of embryonated chicken eggs[12]. Chicken embryos at a relatively early stage of development are easy to handle and inexpensive. At the same time they have a defined circulatory system and internal organs, and it is possible to treat them by intravascular injection. Following injection of oHSV-1-infected THP-1 cells in this CAM model, monocytes and most importantly oHSV-1 could be detected in UM-SC-11B tumors, but not in the liver and kidneys of chicken embryos, by immunohistochemistry (IHC) and real time PCR. UM-SC-11B cells were highly susceptible to infection and lysis by oHSV1-GFP *in vitro.* Thus, human monocytes infected with oHSV1-GFP could migrate throughout the complex vasculature of a developing organism to specifically reach a tumor.

Foreseeing the use of immunocompetent mice as an optimal *in vivo* model, we also assessed the behavior of oHSV-1 in a murine monocyte/macrophage cell line with Balb/c genetic background (J774a.1). Interestingly, while these cells are indeed permissive to oHSV1-GFP infection, virion production and reporter gene expression were high 24 hours post infection but rapidly declined to become non-detectable 72 hours post infection. We hypothesize this may be due to a combination of a strong interferon-mediated response by J744a.1 cells and a cross-species barrier effect. Therefore, we will also test if *in vitro* treatment with inhibitors of the interferon pathway extends the timespan during which mouse monocytes allow oHSV-1 replication. Further investigation, propedeutical to the use of these carrier cells in the mouse model, will include testing of viral replication in primary mouse monocytes.

A very interesting characteristic of monocytes is their capability of migrating into body compartments that are notoriously difficult to reach for intravenously administered drugs, such as the central nervous system (CNS). On the one hand, this feature is very attractive, as it enables the intravenous treatment of intracranial tumors by oncolytic virotherapy. On the other hand, it raises safety concerns, considering that herpetic encephalitis is the most severe infection caused by HSV-1 in immunocompetent patients. While our oHSV-1 has a deletion of the so-called "neurovirulence gene" γ34.5, we noticed that there are no reports of use of this specific backbone (Δγ34.5/ΔUs12) to treat intracranial tumors in humans. Furthermore, in our hands (Vitiello 2018, PhD thesis) Δγ34.5/ΔUs12-oHSV-1 could replicate and show cytopathic effect in human induced pluripotent stem cells (iPSCs)-derived neurons. Therefore, we added a further layer of neuroattenuation by performing BAC mutagenesis to insert several copies of the target sequence of a neuron-specific microRNA, mir124, at the 3' of UL29, a viral gene essential for the HSV-1 life cycle due to its role in viral DNA replication. oHSV1-UL29mir124 was severely attenuated in 293T cells which exogenously express mir124, compared to the parental oncolytic virus. To further validate this finding, we

infected human embryonic stem cells (hESCs)-derived brain organoids which were kindly provided by Dr. Veronica Krenn from the Knoblich Lab of the University of Vienna (Austria). Brain organoids expressed mir124 as evaluated by reverse transcriptase real time PCR. We confirmed reduced replication of oHSV1-UL29mir124 compared to parental virus and attenuation of both compared to wild-type strain 17+ HSV-1. Finally, both in 293T cells and brain organoids, the selective downregulation of UL29 mRNA was measured by reverse transcriptase real time PCR.

In conclusion, our data obtained *in vitro* and in a more relevant *in ovo* model show that human monocytes can act as effective carriers for the systemic administration of an oncolytic HSV-1 expressing a reporter gene (EGFP), which can be considered as a proxy for the other oHSV-1 viruses encoding therapeutic genes already produced by our research group. Preliminary data indicate that potentially, mouse monocytes can also be used as carrier cells to develop an *in vivo* model.

Finally, we also developed a novel neuroattenuation system by downregulation of an essential HSV-1 gene with neuron-specific miRNA target sequences, thus paving the way for an intravenous oncolytic virotherapy strategy with clinical potential.

I virus oncolitici (OV) sono virus attenuati che sfruttano difetti nelle vie di segnalazioni antivirali presenti nelle cellule tumorali, e sono strumenti promettenti per il trattamento di tumori con una scarsa risposta alle attuali terapie[1]. Un virus herpes simplex di tipo 1 oncolitico (oHSV-1), il talimogene laherparepvec (T-VEC), è stato approvato negli Stati Uniti e nell'Unione Europea per il trattamento del melanoma non resecabile chirurgicamente[2]. E' ormai riconosciuto che, oltre a causare la morte delle cellule trasformate, i virus oncolitici favoriscono anche una risposta immunitaria antitumorale e modulano le caratteristiche immunologiche del microambiente[3]. La via di somministrazione che è stata maggiormente studiata è l'iniezione intratumorale, per evitare che i virus siano neutralizzati dal sistema immunitario nel torrente circolatorio.

Il nostro gruppo di ricerca ha già sviluppato diversi vettori HSV-1 oncolitici con geni immunoterapeutici inseriti nella regione intergenica UL55-UL56 tramite mutagenesi in cromosoma batterico artificiale (BAC). I geni includevano interleuchina 12 (IL-12) umana e murina e un anticorpo single chain contro il recettore delle chemochine CCR4, espresso sui linfociti T regolatori. Durante questo progetto di dottorato, sono stati generati ulteriori HSV-1 oncolitici armati coi geni Flt3L e sPD1.

In ogni caso, anche l'iniezione intratumorale di un virus oncolitico armato con molteplici geni terapeutici è solo parzialmente efficace per ottenere una risposta antitumorale sistemica[4]. Pertanto, un miglioramento dell'approccio nella direzione di una strategia clinicamente efficace deve coinvolgere una forma di somministrazione sistemica, che permetta di colpire sia il tumore primario che le metastasi. In questo contesto, la possibilità di usare cellule carrier è emersa come un metodo promettente. Le cellule carrier possono essere infettate ex vivo e poi iniettate in vena, ed è stato dimostrato in modelli animali che possono proteggere efficacemente gli OV dalla neutralizzazione mediata da anticorpi. Tuttavia, la ricerca in questo specifico settore è ancora in una fase precoce rispetto alla somministrazione intratumorale, sia nella ricerca di base che clinica.

In particolare, la maggior parte dei ricercatori che studiano l'associazione tra cellule carrier e virus oncolitici si sono concentrati sulle cellule staminali mesenchimali (MSC), a causa del loro tropismo per i tumori e delle loro proprietà immunosoppressive. Questa caratteristica protegge gli OV dalla rimozione da parte del sistema immunitario, ma può diventare facilmente un'arma a doppio taglio, considerando che l'efficacia terapeutica dei virus oncolitici è anche dovuta al sistema immunitario stesso. Inoltre, le MSC hanno dei problemi di biodistribuzione in seguito all'iniezione intravenosa, probabilmente a causa delle loro dimensioni.

Finora, alcuni studi hanno considerato altre cellule come carrier, incluse le cellule staminali neurali, i linfociti T e diverse classi di cellule mieloidi. Ci siamo interessati particolarmente ai monociti a causa delle loro caratteristiche favorevoli:

1)I monociti circolanti sono i precursori dei macrofagi associati a tumore (TAMs) in diverse neoplasie clinicamente importanti. I TAM sono fondamentali per la sopravvivenza e la crescita di molti tumori e anche delle metastasi. Per questo motivo sono attivamente reclutati dalle cellule tumorali, conferendo ai monociti un forte tropismo per i tessuti neoplastici.

2)I monociti costituiscono circa il 10% dei leucociti circolanti negli umani sani, il che significa che potenzialmente, l'isolamento di monociti autologhi da pazienti malati di cancro comporta solo un prelievo di sangue periferico, senza i prelievi bioptici e i passaggi di amplificazione in vitro richiesti dalle MSC.

3)I monociti si sono evoluti naturalmente per migrare dal torrente circolatorio ai tessuti per differenziarsi a macrofagi, anche in compartimenti del corpo che sono difficili da raggiungere, come il sistema nervoso centrale (CNS).

Pertanto, abbiamo ipotizzato che i monociti autologhi siano cellule carrier ideali per gli OV, per via del loro tropismo intrinseco per il microambiente dei tumori solidi e della possibilità di recuperare

grandi quantità di cellule da sangue periferico. Inizialmente abbiamo impiegato una linea cellulare monocitaria umana (THP-1), che si è dimostrata suscettibile all'infezione da parte di un virus oHSV-1 "backbone" che esprime il gene reporter enhanced green fluorescent protein (oHSV1-GFP), sebbene sia meno permissiva delle linee cellulari epiteliali, come indicato da titoli virali ridotti e vitalità cellulare molto elevata 24 ore post infezione. Le cellule THP-1 infettate con una molteplicità di infezione (MOI) di 3 unità formanti placca (PFU)/cellula sono state in grado di trasmettere oHSV-1 a cellule umane di carcinoma mammario (MDA-MB-231) in un saggio di cocoltura e di migrare verso surnatanti di cellule di carcinoma mammario in camere di Boyden. Questi dati sono stati confermati con monociti primari purificati da buffy coat di donatori di sangue.

Abbiamo poi cercato un modello con maggiore rilevanza biologica per valutare la migrazione dei monociti verso tumori umani, prima di utilizzare un modello in vivo. A questo scopo, abbiamo avviato una collaborazione con la dott.ssa Lea Krutzke e il prof Stefan Kochanek del Department of Gene Therapy dell'Università di Ulm (Germania), che hanno creato un modello sperimentale in cui cellule umane di carcinoma squamoso testa-collo (UM-SC-11B) crescono sulla membrana corioallantoica (CAM) di uova embrionate di pollo.

Gli embrioni di pollo a uno stadio di sviluppo relativamente precoce sono semplici da manipolare e poco costosi. Allo stesso tempo hanno un sistema circolatorio ben definito e organi interni, ed è possibile trattarli tramite iniezioni intravascolari. In seguito all'iniezione di cellule THP-1 infettate con oHSV-1, i monociti e soprattutto il virus oncolitico erano rilevabili nei tumori ma non nel fegato e nei reni degli embrioni, tramite immunoistochimica e real time PCR. Le cellule UM-SC-11B sono molto suscettibili a infezione e lisi da parte di oHSV1-GFP in vitro. Pertanto, monociti umani infettati da un HSV-1 oncolitico sono in grado di migrare attraverso il sistema vascolare complesso di un organismo in via di sviluppo per raggiungere un tumore in modo specifico.

Prevedendo l'uso di topi immunocompetenti come modello in vivo, abbiamo anche valutato il comportamento di oHSV-1 in una linea murina di monociti/macrofagi originata da topi di ceppo Balb/c (J774a.1). Tali cellule possono essere infettate da oHSV1-GFP, tuttavia la produzione di particelle virali e l'espressione del gene reporter sono alti 24 ore dopo l'infezione ma calano rapidamente fino a diventare non rilevabili a 72 ore dall'infezione. Ipotizziamo che questo comportamento possa essere dovuto alla combinazione di una forte risposta interferonica da parte delle cellule J774a.1 e di un effetto di barriera di specie. Pertanto, controlleremo anche se il trattamento in vitro con inibitori della via di segnalazione dell'interferone estenda la finestra temporale durante la quale i monociti di topo permettono la replicazione di oHSV-1. Un'ulteriore indagine propedeutica all'uso delle cellule carrier nel modello animale, includerà lo studio della replicazione virale in monociti primari di topo.

Una caratteristica molto interessante dei monociti è la loro capacità di migrare in compartimenti che sono notoriamente difficili da raggiungere per i farmaci somministrati per via endovenosa, come ad esempio il sistema nervoso centrale. Da un lato questa proprietà può essere molto utile, poichè permette il trattamento intravenoso di tumori intracranici con viroterapia oncolitica. D'altra parte, solleva dei timori riguardo alla sicurezza, considerando che l'encefalite erpetica è la patologia più grave causata da HSV-1 nei pazienti immunocompetenti. Anche se il nostro oHSV-1 ha una delezione del cosiddetto "gene della neurovirulenza" γ34.5, abbiamo notato che non ci sono dati riguardo all'uso di questo specifico backbone virale (Δγ34.5/ΔUs12) per trattare tumori intracranici nell'uomo. Inoltre, nella nostra esperienza (Vitiello 2018, tesi di dottorato) Δγ34.5/ΔUs12-oHSV-1 è in grado di replicarsi e causare effetto citopatico in neuroni derivati da cellule staminali pluripotenti indotte umane (iPS). Perciò abbiamo aggiunto un ulteriore livello di neuroattenuazione inserendo, tramite BAC mutagenesi, diverse copie della sequenza target di un microRNA neurone-specifico (mir124) al 3' di UL29, un gene essenziale per il ciclo vitale di HSV-1 per il suo ruolo nella replicazione

del genoma virale. oHSV1-UL29mir124 era fortemente attenuato in cellule 293T con espressione esogena di mir124, se paragonato al virus oncolitico parentale. Per validare questo risultato, abbiamo infettato organoidi di cervello umani derivati da cellule staminali embrionali (hESC) che ci sono stati gentilmente forniti dalla dott.ssa Veronica Krenn del Knoblich Lab dell'Università di Vienna (Austria). Gli organoidi cerebrali esprimono mir124, come dimostrato tramite reverse transcriptase real time PCR. Abbiamo confermato una ridotta replicazione di oHSV1-UL29mir124 rispetto al virus parentale e attenuazione di entrambi rispetto a HSV-1 wild type ceppo 17+. Infine, abbiamo misurato la soppressione selettiva del mRNA UL29 tramite reverse transcriptase real time PCR sia nelle 293T che negli organoidi cerebrali.

In conclusione, i nostri dati, ottenuti sia in vitro che in un modello *in ovo*, mostrano che i monociti umani possono fungere efficacemente da cellule carrier per la somministrazione sistemica di un HSV-1 oncolitico esprimente un gene reporter (EGFP), che può essere considerato un modello anche per gli altri HSV-1 oncolitici esprimenti geni terapeutici, già prodotti dal nostro gruppo di ricerca. Dati preliminari indicano che potenzialmente, anche i monociti di topo possono essere utilizzati nello sviluppo di un modello in vivo.

Infine, abbiamo anche sviluppato un nuovo modello di neuroattenuazione, basato sulla soppressione di un gene essenziale di HSV-1 per mezzo di sequenze target di un miRNA neurone-specifico, aprendo la strada per un approccio viroterapeutico per via endovenosa con potenziale di applicazione clinica.

Introduction

1.Epidemiology and prognosis of the most frequent solid tumors

According to data collected in 2020 for the GLOBOCAN estimates produced by the International Agency for Research on Cancer, lung and prostate carcinomas were the most frequent tumors in males, whereas breast cancer was the most frequent tumor in females [13]. Lung cancer also caused the highest number of deaths in males while it was the second cause of cancer-associated death in women, after breast cancer. [Figure 1]

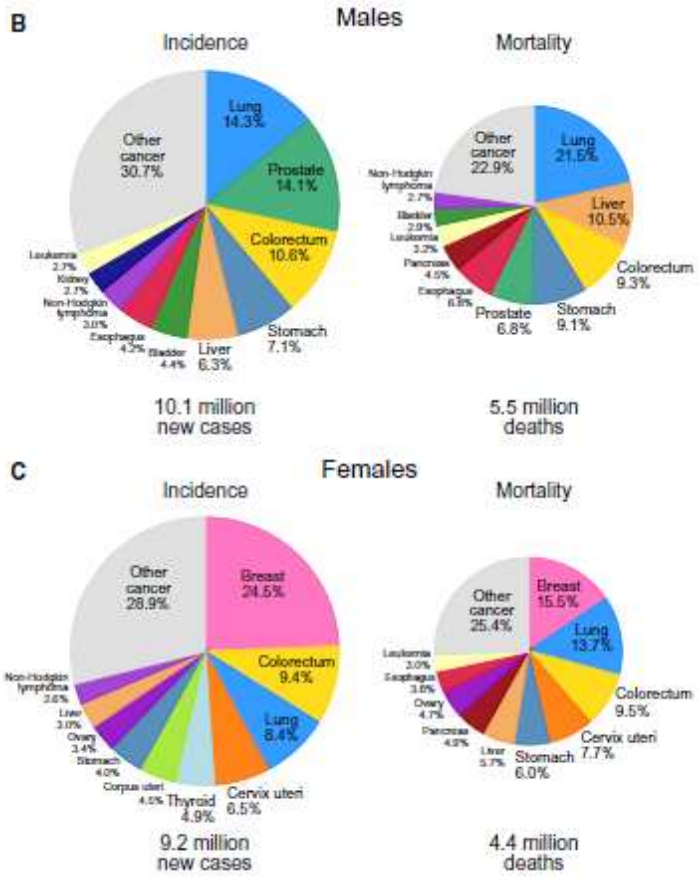


Figure 1 Incidence and mortality of different cancers by gender. Modified from Sung et al (2021)

The situation, however, is quite complex and the epidemiologic pattern is different in so-called economically “transitioned” countries compared to “transitioning” ones. In general, for most types of cancer, incidence is higher in wealthier countries but mortality is lower [13].

However, some tumors, particularly those associated with infectious diseases, are more prevalent in “transitioning” countries. For example, cervix uteri carcinoma, which is caused by high-risk human papillomaviruses (HPV) declined steeply in countries which employ cancer screening and HPV vaccines, but remains the most frequent female tumor throughout Subsaharan Africa[14]. Kaposi sarcoma, which is caused by human herpesvirus 8 (HHV8) and is often associated with uncontrolled human immunodeficiency virus (HIV) infection and AIDS, has a very low incidence in many countries, but is the most frequent male tumor in Mozambico, Malawi and Uganda[15].

On the whole, the fifteen most common types of cancer in men are: lung, prostate, colorectal, stomach, liver, esophagus, bladder, lip and oral cavity carcinoma, non-Hodgkin lymphoma, leukemia, kidney, pancreas, larynx carcinoma, brain and nervous system tumors, and melanoma.

The picture is different in women due to the presence of gender-specific tumors, which produce the following list: breast, cervix uteri, colorectal, lung, thyroid, corpus uteri, ovary, stomach, liver carcinoma, leukemia, non-Hodgkin lymphoma, esophagus, pancreas carcinoma, brain and nervous system tumors, lip and oral cavity carcinoma (Figure 2).

Some tumors can be effective targets for primary (vaccines, lifestyle modifications) or secondary prevention (screening programs). This is usually reflected by decreased mortality, especially in high income countries, as can be seen in the case of breast carcinoma, colorectum, cervix uteri and prostate cancer.

As mentioned before, cervix uteri cancer is “special” because of its association with a viral infection, and thus it is preventable by a vaccine[16]. In the case of breast and prostate cancer, one should

not mistakenly conclude that prevention will completely solve the therapeutic problem. In fact, prognosis varies, depending on the different subtypes of tumors and the stage at the moment of diagnosis [17][18]. For example, triple negative breast cancer (TNBC) doesn't express the molecules enabling targeted therapies in breast cancer (estrogen or progesterone receptors, HER2 overexpression) [19]. Therefore, it has a significantly worse prognosis than other subtypes, especially in case of relapse after surgery and/or dissemination. Investigational drugs are being tested against TNBC, while a small subset of TNBC patients may also be responsive to immunotherapy with immune checkpoint inhibitors (see following paragraph)[20]. The anti-PD-L1 antibody atezolizumab in combination with nab-paclitaxel was granted an emergency approval by the FDA for the treatment of advanced or metastatic TNBC, but in August 2021 it was withdrawn by the producer itself for this specific indication, after the drug combination did not meet its primary endpoint criteria in PD-L1 positive TNBC in the IMpassion131 Trial[21].

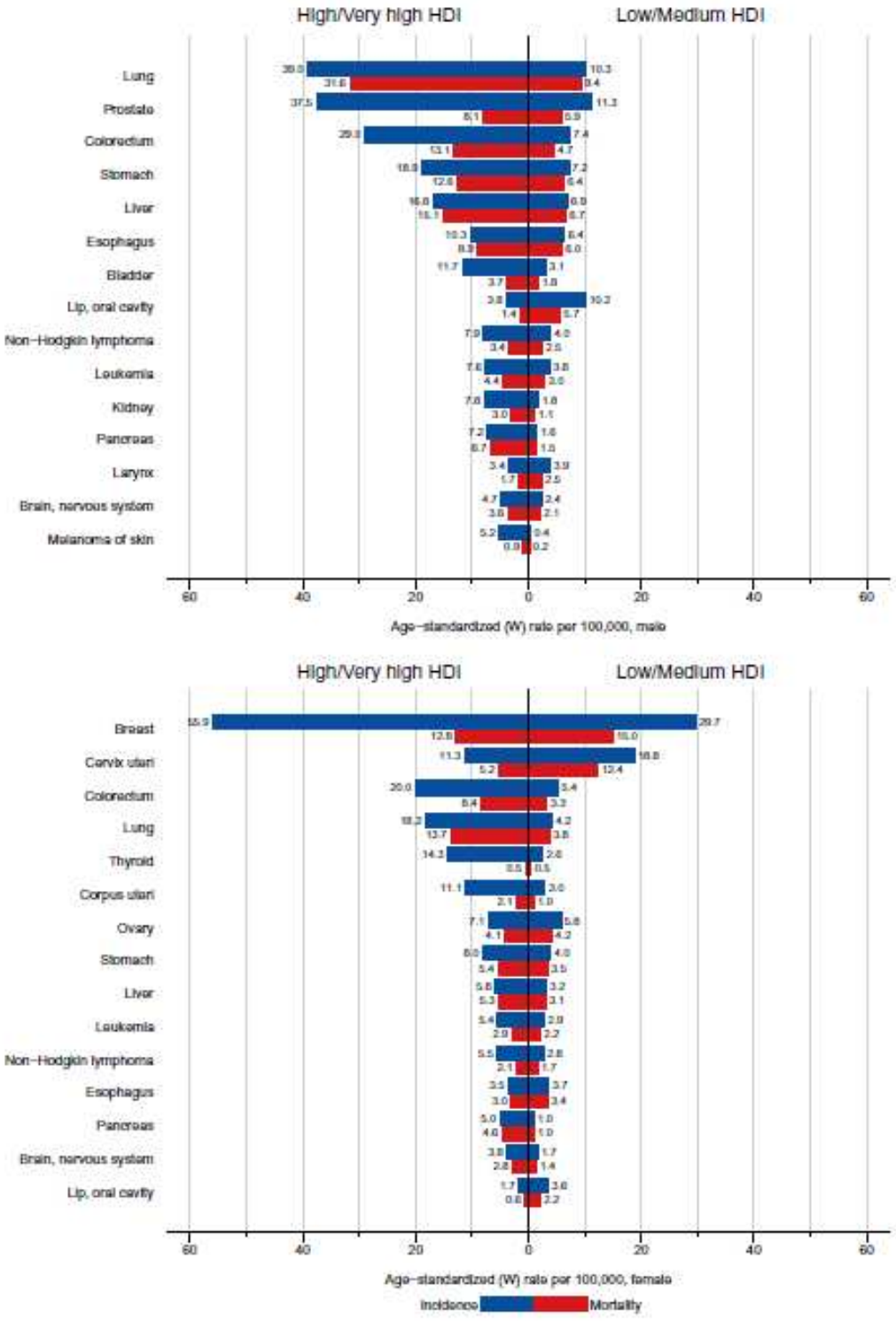


Figure 2 Incidence and mortality of the most common types of cancer, divided by High (HDI) and Low (LDI) Development Index of countries. Above: male tumors Below: female tumors. Modified from Sung et al (2021)

As it can be seen in Figure 2, there are also tumors for which incidence and mortality are almost identical, for example in the case of liver, esophagus, pancreas and brain tumors. This feature is explained by different characteristics: these malignancies are usually diagnosed when surgical resection is not possible, due to absence of early symptoms and early metastatization (pancreas) or local invasion (glioblastoma). Furthermore, resistance to chemo- and radiotherapy contributes to the unfavorable prognosis [22][23]. As a result, 5-year survival rate of patients with a diagnosis of glioblastoma or pancreatic adenocarcinoma is <5% [24] [24]. In pancreatic adenocarcinoma, the final figure (around 4%) mainly results from longer survival in surgically resected patients.

Overall, these data underline the necessity of finding innovative therapeutic approaches for different types of solid tumors.

2.Innovative therapeutic approaches

2.1 Signaling pathway inhibitors

Tumor cells depend on aberrant signaling pathways for growth and survival. Some crucial signaling proteins were discovered because of their role in oncogenesis, for example Ras[26]. Therefore, pharmaceutical researchers looking for cancer-specific therapeutics focused on finding small-molecule inhibitors of these pathways.

Tyrosine kinase proteins are the targets of some of the most successful, clinically approved molecules, such as gefitinib and erlotinib for mutated epidermal growth factor receptors (EGFR) in lung cancer, or lapatinib and neratinib for HER2 in breast cancer [27]. Hematologic malignancies, like chronic myeloid leukemia (CML), were the first to be treated with the tyrosine kinase inhibitor imatinib, an early “success story” due to the dependence of CML on the Philadelphia chromosome and aberrant fusion BCR-ABL1 tyrosine kinase[28].

Other pathways and proteins which were targeted include vascular endothelial growth factor (VEGF)[29] , Fibroblast Growth Factor (FGF), Wnt/beta-catenin [29], PI3K/Akt/mTOR[31], Hippo [32] and RAS itself, which was once considered “undruggable” [33].

Many of these pathways are intertwined. However, cascades consist of many different steps and actors (Figure 3), which allow cancer cells to exploit multiple possibilities of escaping the therapeutic action of inhibitors.

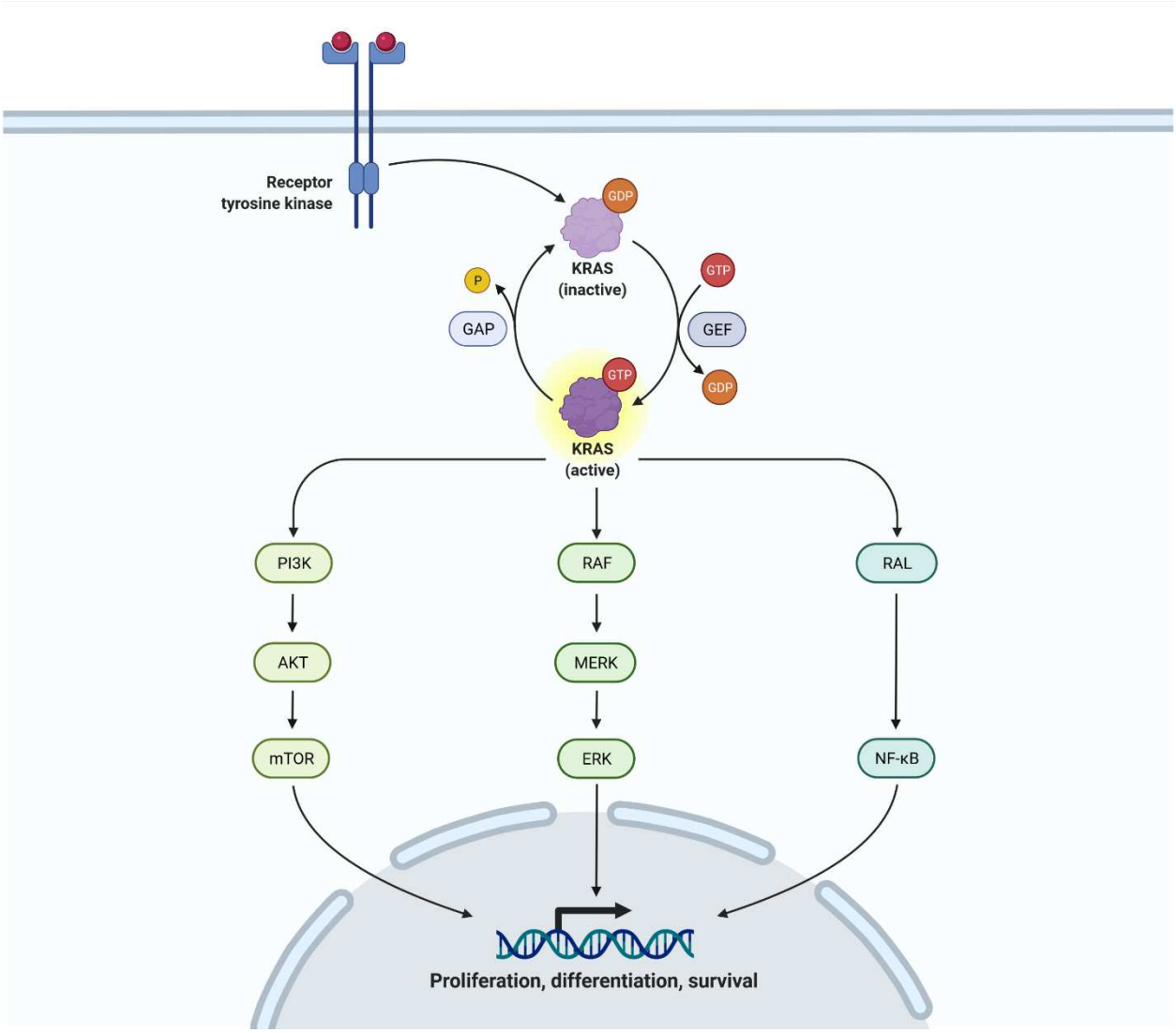


Figure 3 KRAS signaling pathway. Created with biorender.com

In clinical practice, tumors usually develop resistance to tyrosine kinase inhibitors (TKIs) after an initial response. This happens in the treatment of lung cancer [34][35], cholangiocarcinoma [36] and breast cancer [37], among the others.

In the past decades, angiogenesis inhibitors, including both monoclonal antibodies and small molecules, were investigated in several in vivo studies and clinical trials, which also resulted in

approval for the treatment of some tumors in association with other therapeutics [38]. Nevertheless, tumors usually develop resistance and in some cases become more aggressive after angiogenesis blockade[39].

"Classical" protumoral pathways do not only play a role in aberrant cell growth and survival, but are also involved in other crucial characteristics, such as the formation of an immunosuppressive TME. For example, activation of Epidermal Growth Factor family receptors like ErbB dampen the efficacy of immunotherapy[40], and Yes associated Protein (YAP), which is part of the Hippo signaling pathway, also has an immunosuppressive role [41]. Therefore, inhibitors of signaling pathways could be used not just in association with chemotherapy, but also with immunotherapy[42].

2.2 Immunotherapy

Cancer immunotherapy takes advantage of the capacity of the immune system to recognize antigens that are either specific for or preferentially associated with tumor cells [43]. Tumor associated antigens (TAAs) are self-proteins that are overexpressed in cancer cells, due to genetic or posttranslational mechanisms. They can be further divided into overexpressed antigens, differentiation/ lineage-specific antigens and cancer-testis (germline) antigens [44].

Traditionally, cancer immunotherapy focused on the use of cytokines that activate the immune system, such as interleukin 2 (IL-2)[45], or therapeutic cancer vaccines to elicit an antitumoral immune response[46]. High-dose, recombinant IL-2 was mainly effective in a subset of melanoma and kidney cancer patients. Despite limited efficacy and safety issues, it marked a milestone in the

history of cancer immunotherapy, since it provided the first demonstration that it could be clinically effective[45].

Many different cancer vaccines have been devised and tested, both in animal models and in clinical trials. Overexpressed TAAs have been a popular choice and include mucin1 (MUC1), a glycoprotein overexpressed in cancers originating from glandular tissues (breast and pancreas for example) [47], HER2/neu (EGFR family receptor, mainly overexpressed in a subset of breast cancer but also in other tumors)[48] and human Telomerase Reverse Transcriptase (hTERT, overexpressed by many tumors)[49].

Differentiation and germline antigens have higher specificity for neoplastic tissues compared to overexpressed ones. Differentiation antigens include gp100 or melanocyte protein PMEL (often expressed in malignant melanoma) and prostatic acid phosphatase (PAP) (prostate carcinoma)[50][51]. Germline or cancer-testis antigens are expressed in some tumors and in germline tissues, for example Melanoma-Associated Antigen 3 (MAGE-3) and New York Esophageal Squamous Cell Carcinoma 1 (NY-ESO1)[52][53].

Neoantigens, instead, result from mutations in proteins that generate new epitopes and are thus also called Tumor Specific Antigens (TSA). Theoretically, targeting neoantigens offers many advantages, including specificity and the absence of previous immune tolerance against self proteins. Unfortunately, only a limited amount of TSA have immunogenic potential and a personalized validation process is necessary [54]. In spite of many clinical trials with different vaccine formulations, the efficacy of antitumoral therapeutic vaccines is limited, and the only product that has been approved for clinical use is Sipuleucel-T, which targets PAP [55].

A turning point in cancer immunotherapy arrived with the acknowledgement that previous strategies were not effective not because of defective stimulation, but because of highly efficient,

in situ immunosuppression by cancer cells or other cells in the TME. A mechanism recent research focused on is centered on immune checkpoint molecules, especially Cytotoxic T Lymphocyte Antigen 4 (CTLA-4) and Programmed cell Death 1 (PD-1)[56] [57]. These molecules are negative regulators of the activity of T lymphocytes and are part of the mechanisms of peripheral immune tolerance (Figure 4).

Subsequently, monoclonal antibodies were developed to block the interaction of CTLA-4 and PD-1 with their ligands, which are respectively CD80/CD86 and PD ligand 1 (PD-L1) and less frequently PD-L2. Blocking these inhibitory pathways exploited by cancer cells to evade the immune response results in objective therapeutic responses in different tumors including melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma among others. Other checkpoint molecules have also been studied (e.g TIGIT [58] or Tim-3 [59]).

The discovery of immune checkpoint molecules and their inhibitors resulted in the Nobel prize for medicine and physiology which was awarded to Tasuku Honjo and James Allison in 2018[60].

Tumors, however, also exploit other immunosuppressive mechanisms. The tumor microenvironment (TME) is a complex system including several types of cells, many of which (cancer associated fibroblasts, tumor associated macrophages, myeloid derived suppressor cells, regulatory T cells) can modulate the adaptive immune response. In some solid tumors with a particularly negative prognosis, such as pancreatic adenocarcinoma and glioblastoma (see §Epidemiology and prognosis of the most frequent solid tumors), the TME excludes cytotoxic T lymphocytes, creating a so-called “immunologic desert” and making checkpoint inhibitors intrinsically ineffective[61].

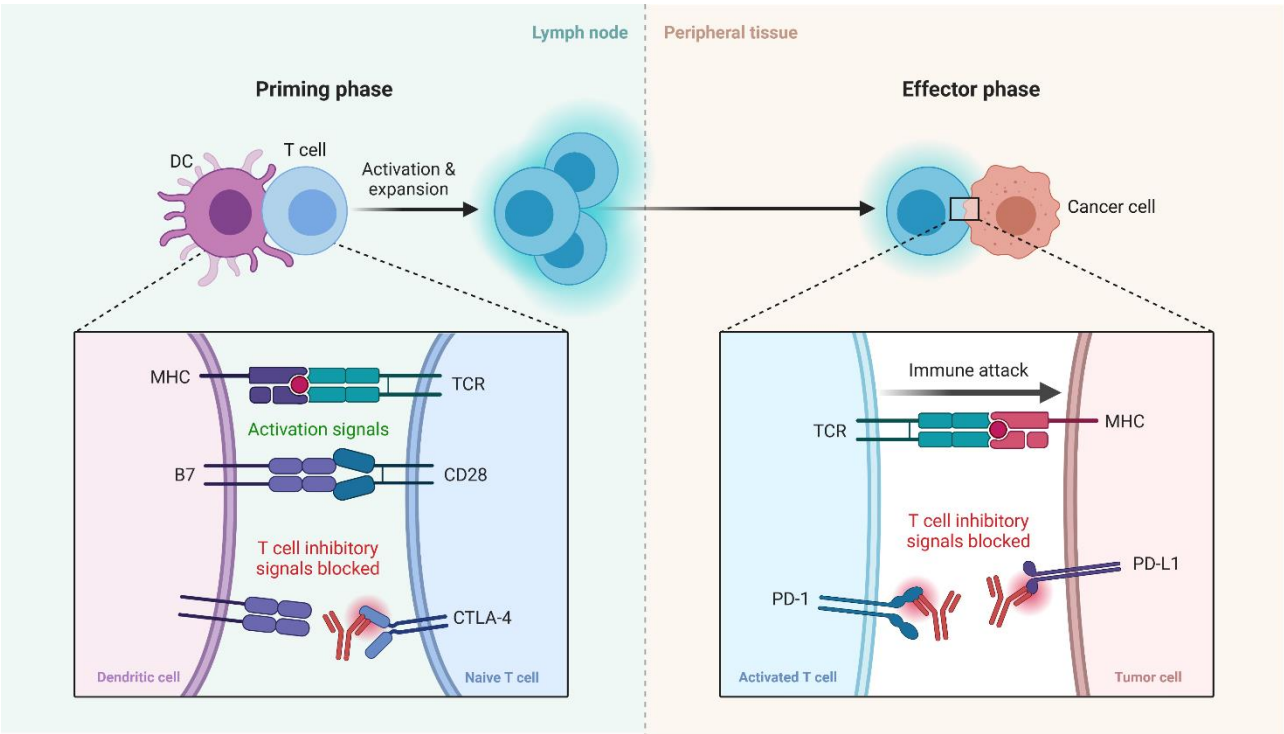


Figure 4 The mechanism of action of immune checkpoint inhibitors, in lymph nodes (priming phase) and in peripheral tissue (effector phase). Created with biorender.com

Finally, acquired resistance to checkpoint inhibitors has been described. This phenomenon can be due to loss of neoantigens, mutations in the interferon pathway or changes in the TME [62].

To enhance the infiltration of T lymphocytes in immunologically “cold” tumors different strategies have been proposed, which are based on the introduction of proinflammatory stimuli in the TME, such as agonists of the innate immunity, bacterial toxins, bacterial cells, or oncolytic viruses (see below).

3.Herpes Simplex Virus type 1

Herpes simplex virus type 1 (HSV-1) is a member of the Alphaherpesvirus subfamily of the *Herpesviridae* family, and it is one of the most ancient and well-adapted human viruses. It causes an acute infection of skin and mucosae of the orolabial region (herpes labialis) or more rarely of the genital mucosa (herpes genitalis). [63]

Traditionally, the closely related HSV-2 was responsible for most cases of genital herpes, but the epidemiology is changing[64]. The seroprevalence of HSV-1 in the general population was traditionally very high, however it changes according to the socioeconomical condition, ranging from less than 50% in high-income countries [65] to more than 90% in low-income countries [62,63].

The most striking biological characteristic of HSV-1 is its capacity to undergo latency in neurons from peripheral sensory ganglia, which enables the viral genome to persist in episomal form for the entire lifespan of the host and spread by periodic reactivation following stimuli such as physical/ emotional stress, UV ray exposure and immunosuppression. The biology of HSV-1 latency is still a matter of intense investigation[68].

In most individuals, primary HSV-1 infection involves the orolabial region. This self-limited infection can be followed by localized reactivations (cold sores) [63].

However, different severe infections are also possible. The most severe clinical manifestation in immunocompetent patient is herpetic encephalitis, which is the most frequent cause of sporadic viral encephalitis and can also trigger autoimmune encephalitis [69]. Although its prognosis became significantly better after the introduction of inhibitors of viral DNA polymerase (mainly acyclovir), herpetic encephalitis remains a severe disease with possible long-term sequelae[70] .

552 The virus can produce disseminated infections in individuals with a defective immune system, such
553 as the newborn (neonatal herpes) or immunocompromised patients. Many organs are potentially,
554 albeit rarely, susceptible to HSV-1 infection, including lungs (herpetic pneumonia) [71] and liver
555 (herpetic hepatitis) [72].

556

557

558 ## 3.1 Virion structure and viral genome

559

560 HSV-1 virions are roughly spherical particles with an average diameter ~185nm without considering
561 spike proteins. Structurally, the HSV-1 virion can be divided in 4 parts: a lipidic envelope with proteic
562 spikes, a loosely structured proteinaceous layer called the tegument, an icosahedral capsid, and an
563 electron-opaque core, which contains the viral genome.

564 The envelope is a lipid bilayer with as many as 13 distinct embedded viral glycoproteins. The virion
565 envelope glycoproteins include gB (UL27 gene), gC (UL44), gD (US6), gE (US8), gG (US4), gH (UL22),
566 gI (US7), gK (UL53), gL (UL1) and gM (UL11). The presence of gJ (US5) and gN (UL49.5) in virions has
567 not been demonstrated. Envelopes also contain at least two nonglycosylated intrinsic membrane
568 proteins (UL20 and US9)[73].

569

570 The most notable of the proteins associated with the tegument are the VP16 virion transactivator
571 protein (also known as α-*trans*–inducing factor or αTIF, encoded by the *UL48* ORF), the virion host
572 shutoff (VHS) protein (*UL41*), VP22 (*UL49*), which was reported to have the ability to spread cell to

cell**,** and a very large protein (VP1–2; *UL36*), which plays a role in DNA release at the nuclear pore during viral entry [74].

The capsid has 162 capsomers, including 140 hexons, 11 pentons, and one portal, arranged in a T = 16 icosahedral symmetry. The outer shell of the capsid is composed of four viral proteins, VP5 (*UL19*), VP26 (*UL35*), VP23 (*UL18*), and VP19C (*UL38*). VP5, the major capsid protein, is present in five copies in each penton capsomere and six copies in each hexon capsomere in this icosahedral shell. VP26 is present in a ring of six copies on each hexon, on top of the VP5 subunits.

Adjacent capsomeres are linked by triplexes made up of one VP19C molecule and two VP23 molecules. The capsid also contains the UL6 protein, which forms a dodecamer thought to form a portal through which viral DNA is packaged**,** and VP24 (*UL26*), a protease that aids in processing the scaffolding during DNA encapsidation [75].

The core contains the double-strand (ds) DNA genome wrapped as a toroid in a liquid crystalline state. A small fraction of the virion DNA may be circular[76].

The HSV-1 genome is approximately 150kb long, depending on the viral strain. It is composed of two unique long and short regions (U_L and U_S) and inverted terminal and internal, long and short repeated regions (TR_L, TR_S, IR_L, and IR_S respectively) (Figure 5). As a result, genes encoded by the repeated regions are present in two copies in the HSV-1 genome [76]

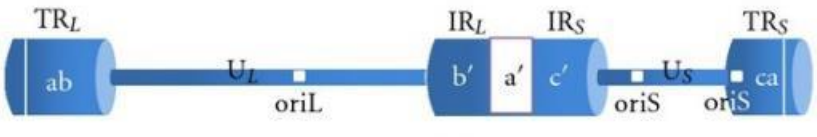


Figure 5 Representation of the HSV-1 genome in linear form, from Elbadawy et al (2012)

3.2 Viral life cycle

To initiate infection, the virus must attach to cell surface receptors. Fusion of the envelope with the plasma membrane or an internal membrane (following endocytosis) rapidly follows the initial attachment [77,78].

The HSV-1 entry is a complex process that involves at least five glycoproteins. Glycoproteins C (gC) and gB interact with glucosaminoglycanes (GAGs) on the cell surface, whereas gD is fundamental for interaction with the specific viral receptors, which are nectin-1, herpes virus entry mediator (HVEM, a member of the tumor necrosis factor receptor family) and 3-O-sulfated heparansulfate (3-OS HS). Binding of gD to one of its receptors triggers fusion by gB with the mediation of heterodimeric gH/gL proteins [79].

The de-enveloped tegument-capsid structure is then transported to the nuclear pores, where DNA is released into the nucleus. Transcription of the viral genome, replication of viral DNA, and assembly of new capsids take place in the nucleus. Viral DNA is transcribed throughout productive infection by host RNA pol II, but with the participation of viral factors at all stages of infection. The synthesis of viral gene products is tightly regulated: Viral gene expression is regulated and sequentially ordered in a cascade fashion [80].

The gene products studied to date form at least five kinetic groups (α, $\beta1$, $\beta2$, $\gamma1$, and $\gamma2$) as a result of both transcriptional and posttranscriptional regulation. The α or immediate-early genes are expressed first and are, by definition, transcribed in the absence of de novo viral protein synthesis. The α gene products are involved in activating expression of the β or delayed early genes. Several of these genes products are enzymes and DNA-binding proteins involved in viral DNA replication in nuclear replication compartments (e.g the viral DNA polymerase). [80]

Viral DNA is synthesized by a rolling circle mechanism, producing concatemers that are cleaved into monomers during the process of nucleocapsid assembly. The γ or late genes are then transcribed efficiently following viral DNA replication, and these gene products are often involved in the assembly of progeny virions[81].

Assembly occurs in several stages. After packaging of DNA into preassembled capsids, the filled capsid or nucleocapsid matures into a virion and acquires infectivity by budding through the inner lamella of the nuclear membrane [82].

The transit of virions from the space between the inner and outer nuclear membranes to the subcellular space is less well defined. It has been suggested that the virion envelope is processed by transit through Golgi stacks, by being de-enveloped and then re-enveloped at the trans Golgi network, or by the nucleocapsid exiting the nucleus through nuclear pores and then budding into the Golgi apparatus. In fully permissive tissue culture cells, the entire process takes approximately 18 to 20 hours. HSV-1 causes an extensive reorganization of cell structure, with nuclear changes including margination of chromatin, enlargement of the cell nucleus, formation of replication compartments, disruption of the nuclear lamina and nucleoli, and cytoplasmic changes including disruption of the Golgi apparatus and microtubules. It was recently discovered that HSV infection alters cellular metabolism, in part by diverting the central carbon metabolism toward the production of pyrimidine nucleotide components [83].

3.3 Functions of ICP34.5, ICP47 and ICP8

The HSV-1 γ34.5 gene encodes the Infected Cell Protein 34.5 (ICP34.5), known mainly as a neurovirulence factor, although it plays a critical role in contrasting the host antiviral response in a broader way . As depicted in Figure 6, ICP34.5 interferes with several host defense mechanisms by binding to proteins that are involved in cell autophagy, translational-arrest, and type I interferon response to viral infection. The γ34.5 gene is classified as a late gene and it is present in two copies mapping within the inverted repeat sequences ab and b' a' which flank the Unique Long segment of the viral genome.

ICP34.5 is able to block the shutoff of protein synthesis induced by the protein kinase activated by dsRNA (PKR), interacting with the protein phosphatase 1 (PP1) and redirecting its activity to mediate de-phosphorylation of Ser51 in the α-subunit of eIF-2. This results in a continued viral protein synthesis even in the presence of an active PKR. [84]

Interestingly, the conserved carboxyl (C)-terminal domain of ICP34.5, which is responsible for blocking accumulation of phosphorylated eIF-2α, shares sequence similarity with the cellular Growth Arrest and DNA Damage34 (GADD34) protein [85]. Another function of ICP34.5 involves inhibition of autophagy by binding Beclin1 with its aminoterminal region[86]. This portion of the viral protein also interacts with TANK-binding kinase 1 (TBK1), thus potentially interfering with the activation of the interferon-regulatory factor 3 (IRF-3), which is a TBK1 substrate [87].

Us12 is an immediate early gene and encodes the ICP47 protein, which prevents MHC-I-mediated antigen presentation by binding the cellular TAP (transporter associated with antigen presentation)[88]. The deletion of Us12 found in talimogene laherparepvec does not only enhance antigen presentation on infected cells, but also shifts the kinetics of expression of the Us11 gene

from late to immediate early. Us11 encodes a RNA-binding protein which partially complements the activity of ICP34.5, but does not compromise safety[89].

Infected cell protein (ICP)8 is the product of UL29, an early (β) gene. It is the main single-stranded DNA binding protein and is essential for the replication of the HSV-1 genome [90], though several other activities have been ascribed to it [91,92]. It has also been used as a therapeutic target for short interfering RNA antiviral therapies [93].

4.Oncolytic virotherapy

4.1 General overview

Oncolytic viruses (OVs) are defined as viruses able to selectively replicate in and kill cancer cells[1]. The history of OVs is quite long, since already at the beginning of the twentieth century physicians observed that cancer patients experienced partial disease remissions after natural infections[94]. It was, therefore, hypothesized that cancer cells were somehow more vulnerable to viral infections, and that attenuated viral strains could be used in cancer therapy. However, many factors, including safety concerns, the development of cytotoxic chemotherapy, and the lack of tools to manipulate viruses, hindered research in this field. In recent years OV studies were revived by better knowledge of viral gene function and advancements in molecular biology, which allow precise modifications of viral genomes to maximize both efficacy and safety.

Over the last years, a new paradigm emerged according to which OVs might also function as a form of immunotherapy [95]. Indeed, it has been shown that the proinflammatory stimuli provided by viruses can overcome the TME immunosuppression and, thereby, elicit a systemic antitumoral immune response. Such a response was observed also when OVs were injected locally (intratumoral injection), rather than systemically[96]. It was demonstrated that the first OV approved for cancer treatment in North America and Europe, the HSV-1 based talimogene laherparepvec (T-VEC), has an immunological mechanism of action, which also causes the regression of uninjected and uninfected metastases [97].

Nevertheless, OVs are still not powerful enough, especially for scarcely immunogenic or immunosuppressive solid tumors, which unfortunately are quite frequent in the population, like pancreatic adenocarcinoma, triple negative breast cancer, hepatocellular carcinoma [98–100].

This lack of efficacy is somehow unexpected, as OVs should make the TME significantly more immunogenic due to inflammation and the presence of viral antigens. Such a consideration fuels the feeling that major improvements in the OV therapy field are at hand. [3]

4.2 Oncolytic viruses and gene therapy

Thanks to advanced molecular biology techniques, the genome of most OVs can be manipulated to "arm" viruses with therapeutic genes, apart from few exceptions, such as the H-1 rat parvovirus which has a 5kb genome and does not allow insertion of sequences longer than ~200bp[101].

Many OVs, including RNA viruses, can accommodate single therapeutic genes, such as measles virus [102] and vesicular stomatitis virus (VSV) [103]. However, large dsDNA viruses such as HSV-1 can contain much larger cassettes[4].

In general, therapeutic genes expressed by OVs can be roughly divided into two major categories:

1)Genes that enhance cancer cell death. Those are either oncosuppressor genes, such as wild-type p53 and PTEN, which were included in the genome of an oncolytic VSV and HSV-1 vector, respectively[104,105], or genes that make infected cells susceptible to an approved drug, for example an enzyme which confers susceptibility to 5-fluorocytosine, as in the case of the oncolytic vaccinia virus TG6002[106]. The most important objection to this strategy is that, when using replication-competent, cytopathic viruses, the emphasis should not be placed on killing infected cells, but rather other cells that have not been reached by the virus.

2)Immunotherapeutic genes. This class includes numerous cytokines, such as IL-2, TNF-α, IL-12 and others, with the general idea of activating the immune system in close proximity to the infected cancer cells[107,108]. Other possibilities are genes encoding chemokines to recruit a "desirable" (i.e antitumoral) type of immune infiltrate into the TME, but most importantly immune checkpoint inhibitors, usually in the form of single-chain antibodies.

4.3 Carrier cells for the delivery of OVs

Despite advancements in OV development over the years[109], the most appropriate delivery mode for OVs is still a matter of debate. In theory, intravenous injection of antitumoral drugs seems to be ideal in order to target the primary tumor, metastases and micrometastases which are below the limit of detection of current diagnostic techniques. However, apart from safety issues, there are many drawbacks to systemic injection of OVs, the most important being the effect of the immune system which threatens to remove attenuated OVs before they reach the tumor[110].

This effect was observed with different viruses including HSV-1[111] and adenoviruses [112] in preclinical models, in which most of the injected virus was sequestered in the liver and spleen[113]. While this pattern of accumulation does not exclude intravenous treatment of hepatic tumors [114] , it negatively affects the treatment of other deep-seated tumors.

Neutralization of virions in the bloodstream is particularly relevant in the case of viruses with a high seroprevalence in the population, such as HSV-1[115]. Therefore, intratumoral injection has become the method of choice for OV delivery, especially since the immunologic mechanism of action was widely accepted. The problem of targeting metastases was addressed relying on the “in situ vaccine” effect[116].

According to this hypothesis, the lytic effect of the virus is limited to the primary tumor in which it is injected, but the immune response against tumor associated antigens (TAAs) will also be effective against uninjected masses. Indeed, such a response was observed in clinical trials of T-Vec against melanoma but only in a limited number of cases (9% response in visceral metastases in the Optim trial)[117], which led to the investigation of synergism with ICIs.

In this setting, the possibility of using carrier cells has emerged as a promising method to achieve systemic delivery of OVs[6].

Carrier cells can be infected *ex vivo* and then injected intravenously, and it has been demonstrated in animal models that they can effectively shield OVs from antibody-mediated neutralization and nonspecific uptake. This would greatly improve the biodistribution and potentially enhance safety, since lower systemic doses would be needed in order to achieve a sufficient amount of virus delivered to the tumor. Still, the research in this specific field is in a relatively early phase compared to intratumorally delivered OVs, both in basic and clinical research. This review will focus on the different cell types that have been proposed as OV carriers and the many unresolved issues in the intricate interplay between carrier cells, the host immune system, and the tumor microenvironment. Until now, the cells that were by far the most investigated as carriers for OVs were mesenchymal stem cells (MSCs)[7,118].

Without neglecting the potential of MSCs, we think that there are good reasons to also consider other candidates. In an earlier phase of OV research, it was assumed that an antiviral immune response should be considered as a negative factor because it induces viral clearance before all cancer cells within the mass have been killed[3]. This is also a factor in the choice of MSCs, which have immunosuppressive properties, in many studies on carrier cells and OVs. However, as a consensus was reached that oncolytic virotherapy is actually a form of immunotherapy, this approach may need to be revisited.

MSCs were also shown to have pharmacokinetic problems, accumulating mainly in the lungs of experimental animals following intravenous injection, probably due to their dimensions. These problems resulted in some research groups trying to use MSCs for intratumoral delivery, which is

768 feasible in some particular instances but mostly seems to ignore the full potential of the use of
769 carrier cells.
770
771
772
773
774
775
776
777
778
779
780
781
782

Materials and methods

Cell lines

THP-1 (ATCC TIB-202™) is a human monocytic cell line originally derived from a patient with acute monocytic leukemia. It was already available in our laboratory. THP-1 cells are maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS, Gibco #10270106) and 1% penicillin/streptomycin (Gibco, #15140122).

J774A.1 (ATCC TIB-67™) is a murine monocyte/macrophage cell line with Balb/c genetic background, derived from ascites in a female animal with sarcoma. It was kindly provided by Dr.Lea Krutzke from the Department of Gene Therapy, University of Ulm. J774a.1 cells are maintained in Dulbecco's modified Eagle Medium (DMEM, high glucose, pyruvate, Gibco #41966029) supplemented with 10% FBS and 1% penicillin/streptomycin.

MDA-MB-231 (ATCC HTB-26 ™) is a human triple negative breast cancer (TNBC) cell line, originally isolated from a 51-year-old patient with neoplastic pleural effusion. MDA-MB-231 cells form poorly differentiated adenocarcinoma in nude mice. These cells were kindly provided by prof. Stefano Piccolo from the University of Padova. MDA-MB-231 cells are maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

MCF-7 (ATCC HTB-22 ™) is a human, estrogen receptor-positive breast cancer cell line, originally isolated from a 69-year-old patient with pleural effusion. These cells were kindly provided by prof. Stefano Piccolo from the University of Padova. MCF-7 cells are maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

UM-SC-11B (RRID:CVCL_7716) is a human head-and-neck squamous cell (larynx) carcinoma. It was kindly provided by Dr.Lea Krutzke from the Department of Gene Therapy, University of Ulm. UM-SC-11B cells are maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

293T (ATCC CRL-3216^{TM}) is a human embryonic kidney cell line, a highly transfectable derivative of the 293 cell line. It was already available in our laboratory. 293T cells are maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Vero (ATCC CCL-81^{TM}) is an African green monkey kidney cell line. It was already available in our laboratory. Vero cells are maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin.

Viral infections and plaque titration

The protocol for cell infection was different for cells growing in adhesion and in suspension.

In the case of adherent cells, complete medium was removed, cells were washed once with phosphate buffer saline (PBS) 1X and infected with the desired amount of virus in serum-free medium for 1h at 37°C in a 5% CO_2 atmosphere. Serum-free medium was then replaced with complete medium and cells were incubated for further analysis.

Cells growing in suspension were pelleted down, washed once with PBS1X and resuspended in 1 or 2mL of serum-free medium containing the desired amount of virus, for 1h at 37°C in a 5% CO_2 atmosphere. Thereafter, cells were pelleted, resuspended in complete medium and incubated for further analysis.

Viral plaque titration was performed on Vero cells in 24-well plates (organoid supernatants) or in 48-well plates (other supernatants). Briefly, supernatants of infected cells were serially diluted in serum-free medium (DMEM) and used to infect Vero cells for 1h at 37°C in 5% CO_2 atmosphere in a volume of 250µL (24-well plates) or 125µL (48-well plates). Dilutions were adjusted according to the order of magnitude of produced virions. In each titration, two wells were infected with each dilution. After incubation, the supernatant dilutions were removed, Vero cells were washed with PBS1X and overlaid with DMEM, 2% FBS, 0.75% carboxymethylcellulose to allow formation of discrete plaques.

Plaques were counted 72 hours post infection following fixation with 5% formaldehyde (10 minutes, room temperature) and staining with a 0.1% crystal violet solution. To determine the titer using the number of plaques, the following formula was employed:

$$Titer\left(\frac{PFU}{mL}\right) = \frac{average\ plaques\ *\ dilution}{Infection\ volume}$$

Dilutions which resulted in an average number of plaques 20<n<100 were considered suitable for counting.

Immunofluorescence

Immunofluorescence was performed on primary human monocytes to assess their purity after purification by adhesion and removal of cells remaining in suspension, and to evaluate the infection rate with oHSV-1.

In both cases, cells were cultivated on round tissue culture-treated (TC-treated) coverslips with a 12mm diameter inserted in a 24-well plate. Cell culture medium was removed and after washing with cold (4°C) PBS 1X cells were fixed and permeabilized with -20°C 100% methanol for 5 minutes.

Aspecific sites were blocked with a 30 minutes incubation in PBS-5% BSA (Bovine Serum Albumin) at room temperature, followed by washing with cold PBS and incubation with primary antibodies.

The primary antibodies were:

- Mouse anti-CD14 (reactivity: human), Abcam (ab181470). CD14 is part of the lipopolysaccharide (LPS) receptor and is strongly expressed on the surface of primary monocytes. Ab181470 was diluted 1:100 in PBS-0.1% BSA prior to use.

- Mouse anti-ICP4, Abcam (ab6514). ICP4 (Infected cell protein 4) is an immediate early gene of HSV-1, which is essential for the activation of viral gene expression and is itself often expressed even during abortive infections[119]. Ab6514 was diluted 1:100 in PBS-0.1% BSA prior to use.

Cells were incubated overnight with primary antibodies at 4°C.

They were then washed three times with cold (4°C) PBS 1X (5 minutes for each washing) and incubated at room temperature for 1 hour in the dark with the following secondary antibody:

- Goat anti-mouse IgG H&L, conjugated with Alexa Fluor® 488, Abcam (ab150113). Alexa Fluor® 488 has an excitation wavelength of 495nm and an emission wavelength of 519nm. The antibody was diluted 1:500 in PBS-0.1% BSA prior to use.

Cells were washed three times with cold PBS1X in the dark (5 minutes for each washing). Finally, cells stained with the anti-ICP4 antibody were counterstained with DRAQ5™ Fluorescent Probe Solution (ThermoFisher Scientific) for 20 minutes at room temperature in the dark.

Imaging was performed with a Nikon Ti Eclipse confocal microscope.

Immunohistochemistry

Immunohistochemistry (IHC) was performed by Dr. Massimiliano Cadamuro using the aforementioned primary antibodies (anti-CD14 ab181470 and anti-ICP4 ab6514) and a secondary goat anti-mouse antibody conjugated with horseradish peroxidase.

Embryonated chicken eggs

All experiments on the CAM model were performed by Dr.Lea Krutzke at the Department of Gene Therapy of the University of Ulm (Germany), using THP-1 cells and the EGFP-oHSV1 virus provided by us. UM-SC-11B tumors were established as previously described [12].

11 embryonated eggs were treated by intravascular injection, while 1 embryonated egg with UM-SC-11B tumor was used as a negative control. On day 11, $5x10^5$ THP-1 cells were infected with EGFP-oHSV1 (MOI 3) for 1h, washed three times in PBS 1X, resuspended in PBS1X and injected intravascularly. On day 15, tumors, livers and kidneys were harvested, fixed in a paraformaldehyde (PFA) 4% solution, embedded in Tissue-Tek© and frozen prior to shipping in dry ice.

Primary monocytes isolation

Buffy coats (50 mL) from HIV, HBV and HCV-negative blood donors were provided by the Padova University Hospital. Concentrated blood was diluted 1:2 with sterile PBS. 25mL of PBS diluted blood were overlaid with 20 mL of Ficoll® Paque Plus solution (Sigma-Aldrich) and centrifuged at room

903 temperature in a Heraeus Megafuge 1.0R centrifuge at 760g (1900rpm) for 20 minutes without
904 brakes.

905 The PBMC layer was collected with a disposable transfer pipet and moved to a new 50 mL tube,
906 which was filled with PBS 1X. PBMCs were centrifuged at 350g (1300rpm) for 8 minutes with brakes.
907 This washing step was repeated for three more times. The last washing was performed at 200g for
908 10 minutes to remove platelets.

909 Finally, PBMCs were counted in a Bürker chamber and diluted in RPMI medium supplemented with
910 10% FBS to achieve a final concentration of about $1x10^7$ cells/mL (of which approximately 10% are
911 expected to be monocytes).

912 PBMCs were allowed to adhere overnight to tissue culture vessels. Floating cells were removed and
913 adherent cells (including monocytes) were washed once with PBS 1x and incubated with RPMI 10%
914 FBS. A small aliquot of PBMCs was allowed to adhere by the same procedure on 12mm round
915 coverslips for immunofluorescence (as described above) to evaluate the amount of CD14+ cells.

916 The average fraction of CD14+ cells (monocytic) counted over 4 representative 10x microscopic
917 fields was 0.67 (sample standard deviation 0.04).

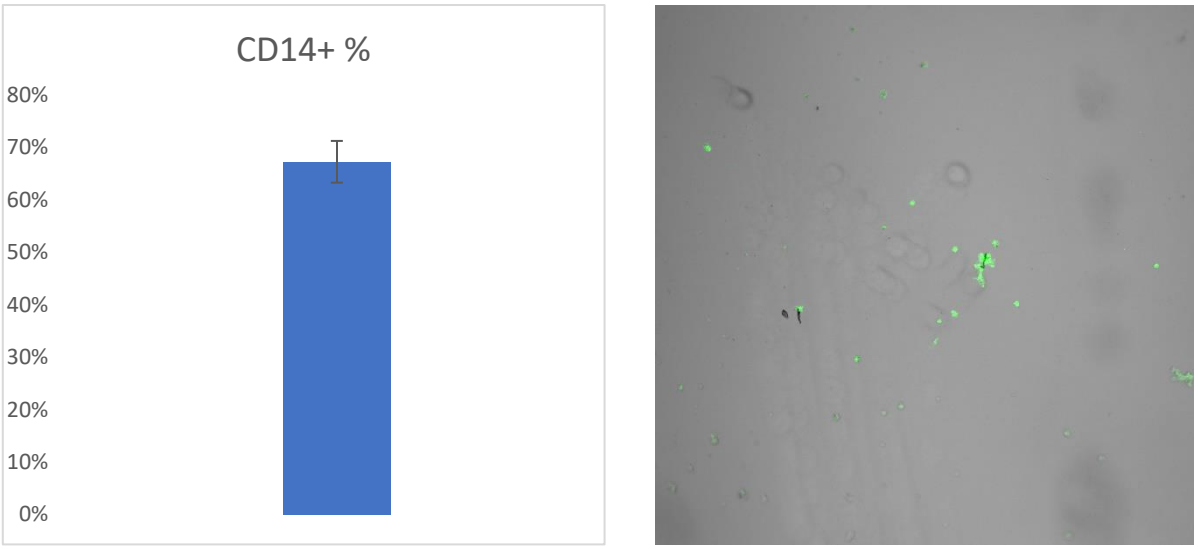


Figure 6 Left: percentage of CD14+ adherent PBMCs. Right: representative 10x brightfield and fluorescence microscopy picture of adherent PBMCs stained with primary mouse anti-CD14 antibody and secondary goat anti-mouse Alexa Fluor 488-conjugated antibody

Oligonucleotides

We employed the following oligonucleotides:

mir124For_NEW

TTACACACATTCCCCGCCCCGCCCTAGGTTCCCCCACCCCCCAACCCCTATACACCTTCGCACATAACAGGATGACGACGATAAGTA

GGG

mir124_Rev

GGGAGGGGCCCCCCGACAAAAAGGGAGACCTGACGTTGGATATGCTGTGAATAGGGCCCTCTAGATGCAT

UL29RealTimeFor

AAGAGCCGCGTGTTGTTC

UL29 RealTimeRev

GTCCGAGGAGGATGTCCA

UL29 Taqman Probe (5’ FAM dye, 3’ NFQ quencher)

CCTACCAGAAGCCCGACAAGC

Bacterial strains

DH5α (chromosomal genotype: fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) were the standard E.Coli strain employed for cloning of non-BAC plasmids.

GS1783 (chromosomal genotype: DH10B l cI857 Δ(cro-bioA)<>araC-PBADIsceI) is an E.Coli strain, characterized by a chromosomal encoded inducible Red and I-SceI-expression and was employed for the Lambda Red *en passant* mutagenesis, as already described [120]. Briefly, it derives from the DH10B strain of *E. coli* and it is characterized by a temperature inducible promoter, driving the expression of the Red recombinase genes, while an arabinose-inducible promoter drives the expression of the homing endonuclease I-SceI from *Saccharomyces cerevisiae*. The enzyme has an 18 bp recognition site that is rarely present in other bacterial sequences. It was used for BAC propagation and BAC mutagenesis.

Brain organoids

Brain organoids (40 days old) were kindly provided by Dr. Veronica Krenn from the Knoblich Laboratory of the University of Vienna[121]. Organoids were maintained in sterile, non tissue culture-treated 90mm dishes, in 10 mL organoid medium and were divided in two batches, one of approximately 50 and one of approximately 200 organoids.

The first batch was divided in 5 sterile, non tissue culture-treated 90mm dishes (around 10 organoids per plate). These organoids were infected with multiplicity of infection (MOI) 0.1 and 1 Plaque Forming Units (PFU)/cell EGFP-oHSV1 in order to determine the optimal MOI and the spatial pattern of infection, thanks to the fluorescent reporter gene.

According to previous calculations by Dr. Krenn on a large number of organoids, MOI 0.1 corresponded to $4.5x10^4$ PFU/organoid while MOI 1 corresponded to $4.5x10^5$ PFU/organoid.

The second batch was divided in 10 dishes (around 20 organoids per plate). These organoids were infected with MOI 0.1 FLuc-oHSV1 or Mir124-oHSV1. Supernatants were collected 1,2 and 3 weeks post infection, as well as some organoids for RNA extraction.

All organoids were maintained in 10mL organoid medium supplied by Dr. Veronica Krenn, with the following composition for 1 liter of medium:

- 500 ml DMEM/F12 (Invitrogen cat#11330-032)
- 500 ml Neurobasal (Invitrogen cat# 21103049)
- 5 ml N2 supplement (Invitrogen cat# 17502048)
- 20 ml B27 + vitamin A supplement (Invitrogen cat# 17504044)
- 250 ul insulin (Sigma cat# I9278-5ML)
- 350 ul 2-Mercaptoethanol 1:100 solution

- 10 ml Glutamax supplement (Invitrogen cat#35050-038)
- 5 ml MEM-NEAA (Sigma cat#M7145)
- 1g NaHCO3 (Sigma-Aldrich, Cat#S5761)
- 10 ml Vitamin C solution 40 mM (ascorbic acid, Sigma-Aldrich A4544-25G):
    - 350 mg in 50 ml of DMEM/F12– store at +4C, light-protected
- 10 ml P/S (Sigma, cat#P0781)
- Filtered using 0.22 µm filter bottle

. Medium was regularly changed at least once a week. Infections were performed by removing approximately 9mL of medium, adding 9mL of medium containing the desired amount of virions and incubating organoids overnight at 37°C in a 5% CO_2 atmosphere. The following day, as much medium as possible was removed and replaced with fresh one.

Plasmids and bacterial artificial chromosomes (BACs)

- pSMPUW-miR-124-GFP-Puro is a plasmid which drives overexpression of mir-124 in eukaryotic cells, encoded in a human beta-globin intron. It was purchased from the non-profit repository Addgene (Addgene plasmid #117321 ; http://n2t.net/addgene:117321; RRID:Addgene_117321) and originally developed by Volker Busskamp.
- pUC57-3P+5P is a plasmid containing three repetitions of the target sequences of two isoforms of mir124 (3P and 5P), flanked by an EcoRV and a NotI restriction site. It was synthesized by BioFab Research S.r.L (Rome, Italy) following our instructions.
- pEPkan-S2 (Addgene plasmid #61601) is a plasmid containing a kanamycin resistance selection marker and a I-SceI restriction site and it is commonly used as a PCR template for BAC mutagenesis. It was kindly provided by Prof. Jens von Einem (University of Ulm, Germany)
- pEP-KanS2-3P+5P was obtained by subcloning the 3P+5P sequence in pEPkan-S2 by EcoRV and NotI digestion, followed by T4 ligase-mediated ligation. It was used as a PCR template to amplify the cassette which was then used for the first recombination of the BAC mutagenesis to generate pHSV1-Δγ34.5/ΔUs12/FLucUL29mir124 (Figure 7).

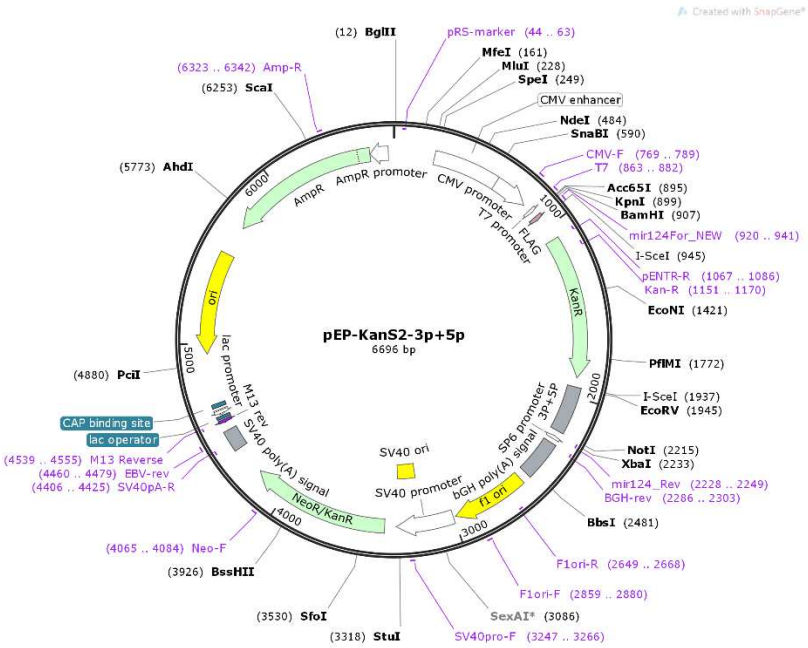


Figure 7 Map of the pEP-KanS2-3P+5P plasmid

- pHSV1-Δγ34.5/FLuc is a BAC containing the entire genome of strain 17+ HSV-1, with a deletion of both copies of the γ34.5 virulence gene, and a cassette encoding Firefly Luciferase in the UL55-UL56 intergenic region. The BAC cassette includes a cloramphenicol resistance gene and a Cre recombinase under the control of an eukaryotic promoter. Since the BAC cassette is flanked by LoxP sites, it self-excides once the BAC is transfected in eukaryotic cells to obtain the resulting virus. This BAC was kindly provided by Prof. Beate Sodeik (Hannover University, Germany) and it was used as a starting backbone for all our subsequent mutageneses (Figure 8).

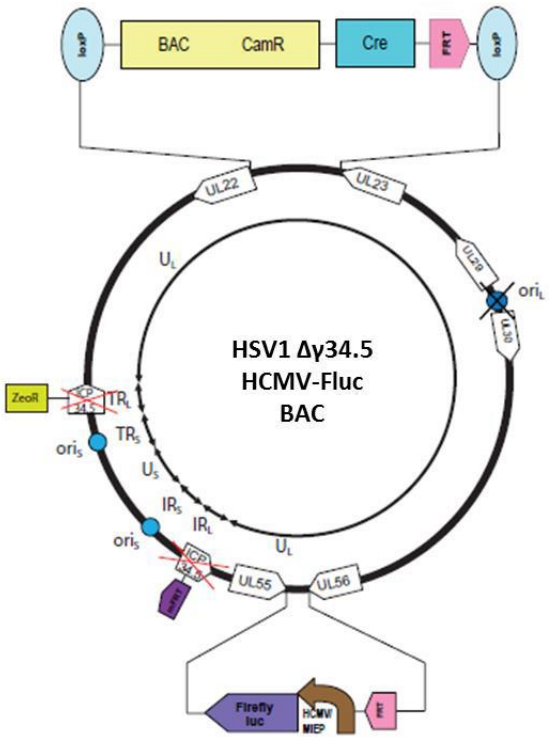


Figure 8 Schematic representation of pHSV1-Δγ34.5/FLuc, with the BAC cassette (intergenic region UL22-UL23), γ34.5 deletions, and an expression cassette for the Firefly Luciferase gene in the UL55-UL56 intergenic region.

- pHSV1-Δγ34.5/ΔUs12/FLuc was previously obtained by BAC mutagenesis of pHSV1-Δγ34.5/FLuc, by a technique described elsewhere[120]. This BAC contains a deletion of the Us12 gene analogous to the one found in talimogene laherparepvec (T-Vec).
- pHSV1-Δγ34.5/ΔUs12/EGFP was previously obtained by BAC mutagenesis of pHSV1-Δγ34.5/ΔUs12/FLuc, by substitution of the firefly luciferase gene with the enhanced green fluorescent protein (EGFP) gene in the UL55-UL56 intergenic region.
- pHSV1-Δγ34.5/ΔUs12/FLucUL29mir124 was obtained by BAC mutagenesis of pHSV1-Δγ34.5/ΔUs12/FLuc. Briefly, a cassette containing the kanamycin resistance gene aminoglucoside phosphotransferase (KanR) and the 3P+5P target sequence in the correct orientation, was amplified by polymerase chain reaction (PCR) using the mir124For_NEW and mir124_Rev primers at a 200nM final concentration, and the Phusion Hot Start II DNA

polymerase (Thermo Fisher Scientific), using pEP-KanS2-3P+5P as a template. 50 nucleotide-long homology sequences for the desired insertion site in the HSV-1 genome were included in the primers. The PCR product was purified by extraction from an agarose gel (QiaQuick Gel Extraction Kit, Qiagen), eluted in MilliQ water, and electroporated into electrocompetent bacteria containing the Lambda-Red recombination system [120] and the pHSV1-Δγ34.5/ΔUs12/FLuc BAC. Clones containing the integrated cassette were selected on Luria Bertani (LB) agar plates supplemented with kanamycin (50µg/mL) and chloramphenicol (34µg/mL). Second recombination was then performed to remove the KanR gene. The final BAC construct was verified by Sanger sequencing (performed by BMR genomics, Padova, Italy).

Transfection

Transfections were carried out in the highly transfectable 293T cell line, using the Invitrogen™ Lipofectamine™ 2000 reagent (ThermoFisher Scientific) following the manufacturer's instructions. Briefly, plasmid DNA was diluted in Opti-MEM™ reduced-serum medium (Gibco #31985070), mixed 1:1 with Lipofectamine reagent diluted in Opti-MEM medium and incubated for 5 minutes at room temperature before being put in contact with the cells.

Transfections of the pSMPUW-miR-124-GFP-Puro plasmid were carried out in 6-well plates. For each well, we mixed 1 mL of Opti-MEM medium containing approximately 2.5µg of plasmid DNA with 1mL of Opti-MEM medium containing 10µL of Lipofectamine reagent.

Successful transfection was assessed by fluorescence microscopy since pSMPUW-miR-124-GFP-Puro also drives the expression of EGFP (data not shown).

Virus reconstitution and amplification

Once a correct BAC clone was validated, high-scale DNA purification was obtained using the Qiagen MIDIPrep Kit (Qiagen). Approximately 1µg of BAC DNA in Opti-MEM medium was transfected in semi-confluent 293T cells in a 25cm^2 tissue culture flask, using lipofectamine 2000 (ThermoFisher Scientific). After 5 hours of incubation, Opti-MEM™ was removed and 293T cells were incubated with DMEM supplemented with 10% FBS.

After 72 hours, viral cytopathic effect could be seen. Infected cells were harvested by pipetting and were transferred together with their supernatant into a 75cm^2 tissue culture flask in which confluent Vero CCL81 cells were seeded the previous day and incubated for 72 hours.

After checking cells for visible cytopathic effects, the supernatant was used to infect five more 75cm^2 flasks seeded with confluent Vero cells. After 1 hour of infection, the supernatant was replaced with DMEM 2% FBS 1% penicillin/streptomycin.

After three days, cells were harvested by scraping and pelleted. Intracellular virions were recovered by three freeze-and-thaw cycles followed by a 3 minutes treatment in a sonicated bath. Cellular debris was pelleted at >12000g in a tabletop microcentrifuge and the supernatant containing concentrated virions was aliquoted for subsequent titration and infections.

Viruses

Δγ34.5/ΔUs12/FLuc-oHSV1 (FLuc-oHSV1) was previously obtained by reconstitution of pHSV1-Δγ34.5/ΔUs12/FLuc and leads to the expression of Firefly Luciferase under the control of the immediate early cytomegalovirus (CMV) promoter.

Δγ34.5/ΔUs12/EGFP-oHSV1 (EGFP-oHSV1) was obtained by reconstitution of pHSV1-Δγ34.5/ΔUs12/EGFP and leads to the expression of the Enhanced Green Fluorescent Protein (EGFP) under the control of the immediate early cytomegalovirus (CMV) promoter

Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 (Mir124-oHSV1) results from the reconstitution of pHSV1-Δγ34.5/ΔUs12/FLucUL29mir124 and contains three repeats of the target sequences of two isoforms of the neuron-specific mir124 (3P and 5P) downstream of the UL29 gene.

Real time PCR

Real time PCR was performed on DNA extracted from 20µm-thick sections of tumors grown on CAM using the DNEasy Blood&Tissue Kit (Qiagen), using the TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific), the UL29RealtimeFor and UL29RealtimeRev primers and the Taqman probe UL29 (5' FAM dye and 3' NFQ quencher). Results were analyzed with the QuantStudio™ Design&Analysis Software v1.5.2.

Cell viability assay

The employed cell viability assay was the Cell Proliferation Kit I (MTT) (Roche #11465007001) used according to the manufacturer's instructions.

Monocyte migration assays

The assays employed to evaluate the migration of monocytic cells towards cancer cell supernatants *in vitro* were the following:

THP-1 cells: we employed the InnoCyte™ Monocyte Cell Migration Assay (CBA098, Calbiochem), in which 10^5 cells, resuspended in 100µL of Opti-MEM medium, were allowed to migrate overnight. Briefly, the insert was removed and the migrated cells were transferred into a black 96-well plate and labeled with a calcein solution. Each supernatant was tested in six replicates. Emission was measured using a fluorescence plate reader set at an excitation wavelength of ~485 nm and an emission wavelength of ~520 nm.

Primary monocytes: infected cells were stained with CellTracker™ Red CMTPX Dye 1:2000 for 30 minutes at room temperature, washed in PBS1x, then 10^5 cells, resuspended in 100µL of Opti-MEM medium, were allowed to migrate in Corning® Transwell® polycarbonate membrane cell culture inserts with 5.0 µm pore polycarbonate membrane insert (Corning) for 3hours at 37°C in a 5% CO_2 atmosphere. 600µL of supernatant of cancer cells cultured in Opti-MEM medium or Opti-MEM medium alone were left in the lower chamber. Each supernatant was tested in three replicates. The inserts were removed and migrated cells were imaged with the help of a Nikon Ti Eclipse confocal microscope.

Cell cocultures

THP-1 cells were infected with an MOI of 1 PFU/cell, whereas primary monocytes were infected with an MOI of 5 PFU/cell. Following 1hour of infection, cells were washed three times in PBS1x before being resuspended in RPMI 10% FBS, 1% penicillin streptomycin and being cocultured with confluent MDA-MB-231 cells seeded in a 6 well-plate. The carrier cell to cancer cell ratio was 1:1 ($5x10^5$ cells). PBS 1x from the last washing was titrated on Vero cells to ensure that no free virions were present. Primary monocytes were also stained with CellTracker™ Red CMTPX Dye 1:2000 for 30 minutes at room temperature (as above), before coculture.

Results

1.THP-1 monocytes are permissive to Δγ34.5/ΔUs12/EGFP-oHSV1 replication

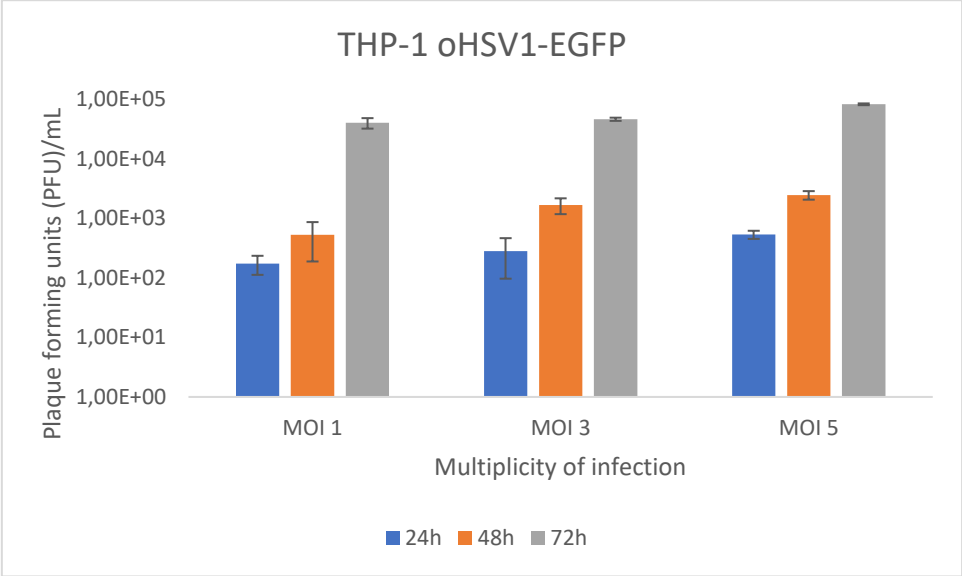


Figure 9 Plaque titration assay on the supernatants of human monocytic THP-1 cells infected with oHSV1-Δγ34.5/ΔUs12/EGFP at MOI 1, 3 and 5. Logarithmic scale. Experiments were performed in three biological replicates. Error bars reflect the sample standard deviation.

Following infection with oHSV1-Δγ34.5/ΔUs12/EGFP at three different MOIs, infectious virions could be detected in the supernatant of THP-1 cells 24 hours post infection. Titres steadily increased at 48hours and at 72hours post infection, with all the MOIs. Since THP-1 cells grow partially in adhesion and partially in suspension, at each time point the supernatant was completely removed, cells in suspension were spinned down, resuspended in fresh medium and added back to the corresponding well. Thus, the measured titres reflect newly produced viral particles.

2.THP-1 monocytes maintain high viability after Δγ34.5/ΔUs12/EGFP-oHSV1 infection

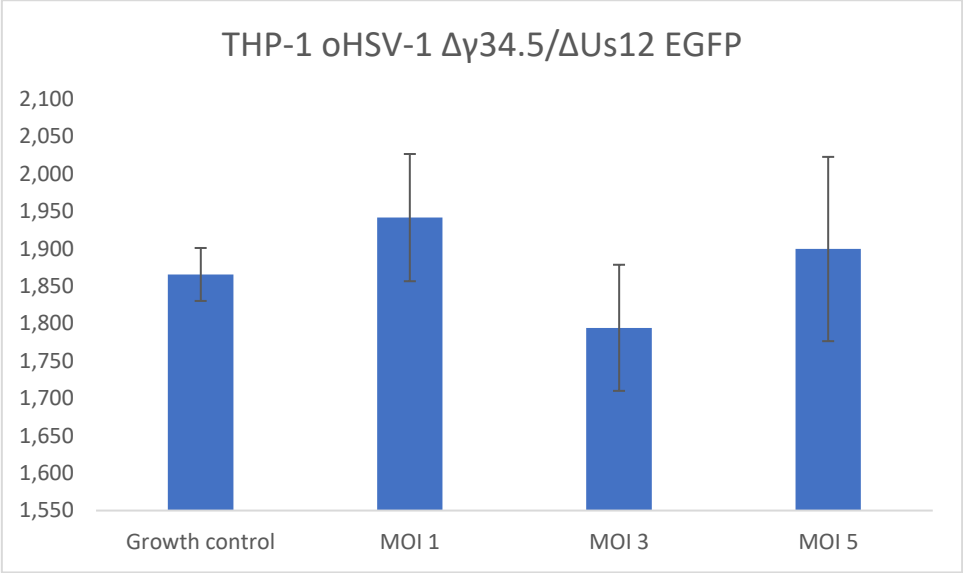
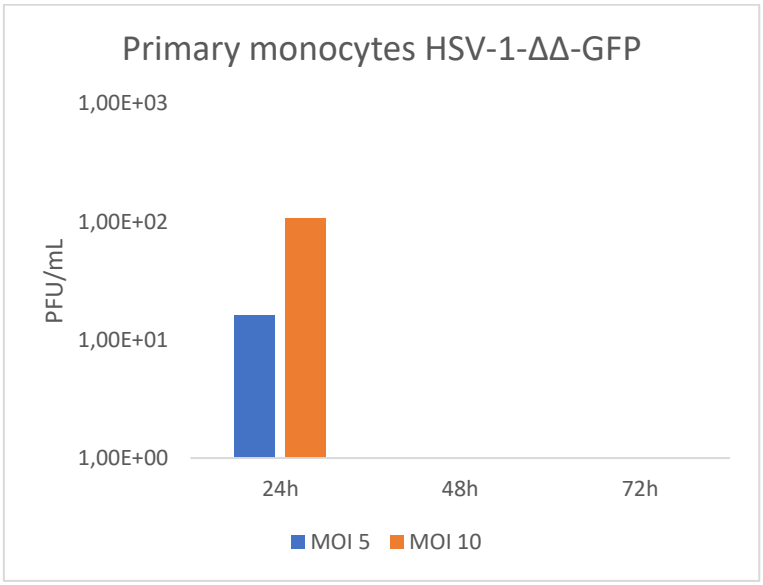


Figure 10 MTT assay of THP-1 cells infected with EGFP-oHSV1 24 hours post infection. Each experiment was performed in three replicates.

There was no statistically significant difference between the growth control and each of the employed MOIs ($p>0.05$), as assessed by the Mann-Whitney U test.

Primary human monocytes allow infection and limited replication of Δγ34.5/ΔUs12/EGFP-oHSV1



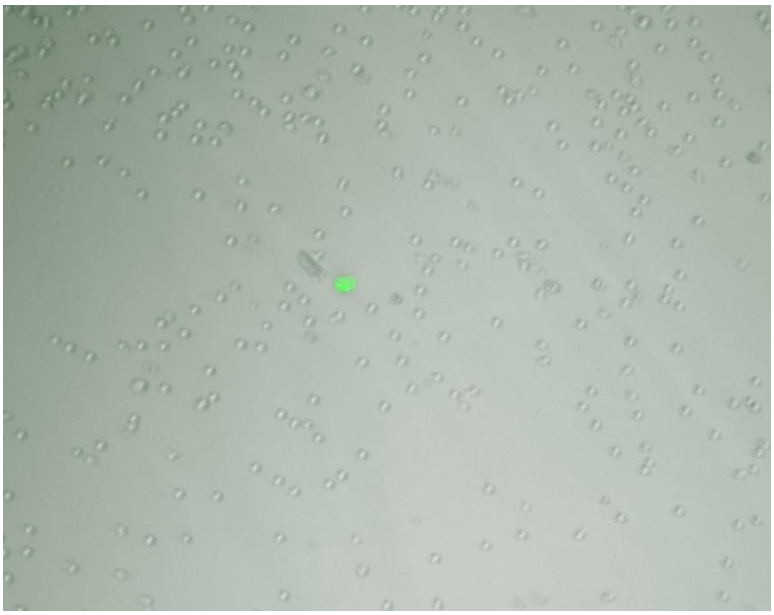


Figure 11 Above: Plaque titration assay on the supernatants of primary human monocytes, infected with MOI 5 and MOI 10 Δγ34.5/ΔUs12/EGFP-oHSV1. Y axis in logarithmic scale. Below: brightfield and fluorescence microscopic image of primary human monocytes infected with MOI 10 Δγ34.5/ΔUs12/EGFP-oHSV1. Image representative of multiple 10x fields.

Considering that infectious virions were detectable only 24 hours post infection, when also very limited GFP-positivity could be observed (1-4 positive cells in a well in which 10^5 MOI 10-infected cells were seeded), we infected primary monocytes with FLuc-oHSV1 (which does not express any fluorescent green protein) and performed immunofluorescence with a primary anti-ICP4 antibody. ICP4 is an immediate early viral gene which is usually expressed also in abortive infections[119].

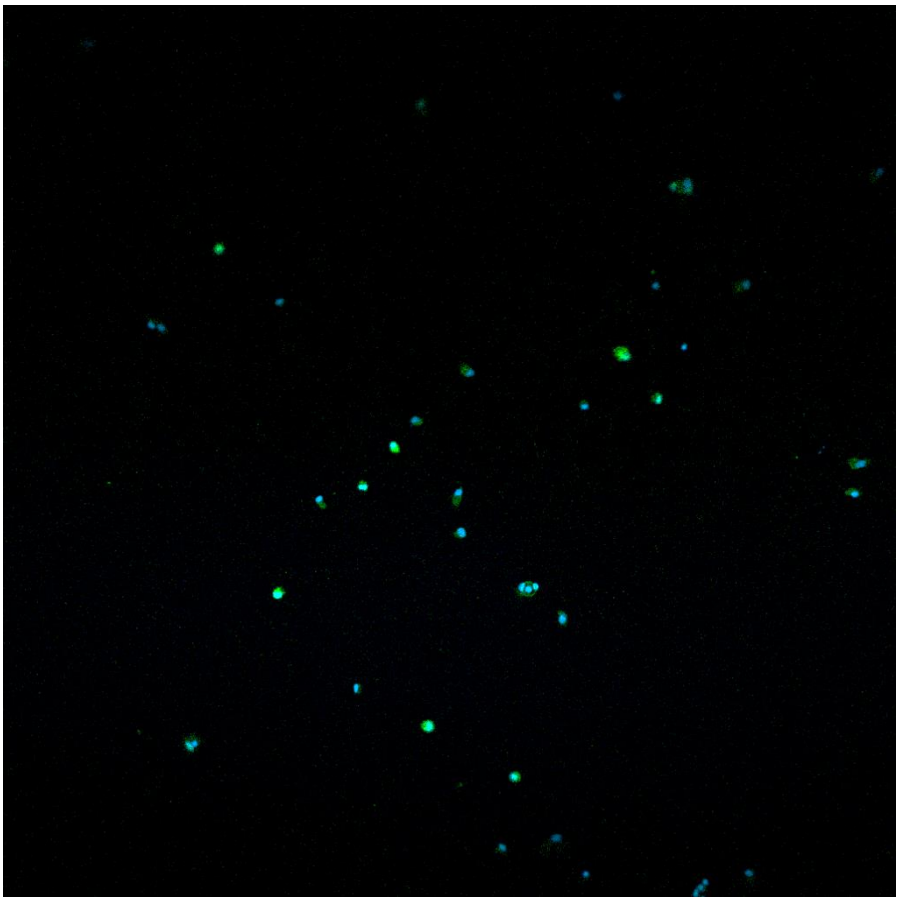


Figure 12 Immunofluorescence of primary human monocytes infected with FLuc-oHSV1, MOI 5. 24 hours post infection cells were stained with a mouse primary anti-ICP4 antibody and a secondary goat anti-mouse Alexa Fluor 488-conjugated secondary antibody. Cells were also counterstained with DRAQ5 (here artificially visualized as blue)

Even after infection with MOI 5, ICP4 was present in ~50% of primary monocytes, indicating that probably the viral life cycle is restricted at a later stage. It is possible to hypothesize that an activation of the interferon pathway contributes to this effect.

3. Mouse monocytic J774A.1 cells are permissive to Δγ34.5/ΔUs12/EGFP-oHSV1 replication

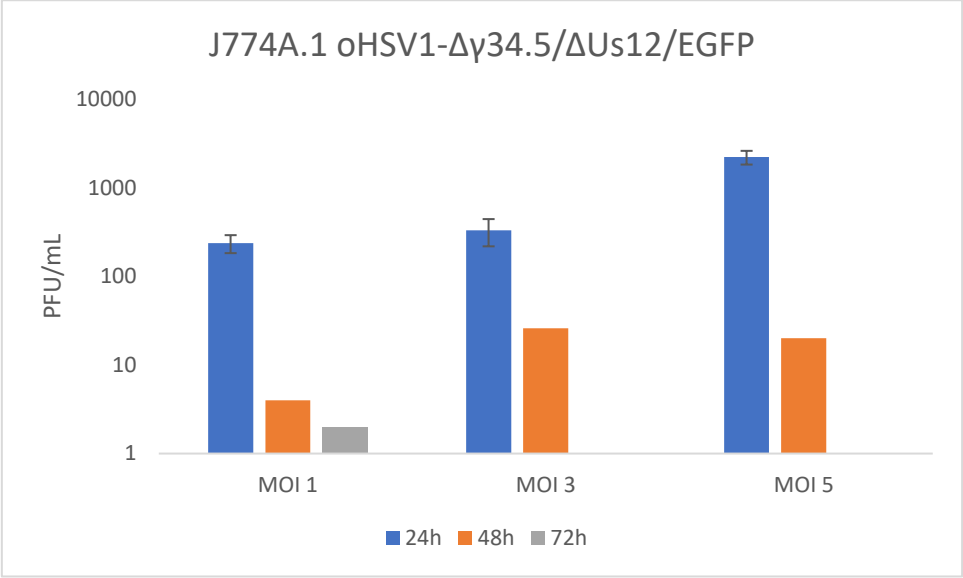


Figure 13 Plaque titration assay on the supernatants of mouse monocytic J774A.1 cells infected with oHSV1-Δγ34.5/ΔUs12/EGFP (EGFP-oHSV1) at MOI 1, 3 and 5. Logarithmic scale. Experiments were performed in three biological replicates. Error bars reflect the sample standard deviation.

Following infection with oHSV1-Δγ34.5/ΔUs12/EGFP at three different MOIs, infectious virions could be detected in the supernatant of J774A.1 cells 24 hours post infection. Titres rapidly declined at 48hours and at 72hours were totally undetectable in MOI 3 and 5-infected cells. The supernatant was completely removed and replaced with fresh medium at each time point, thus the measured titres reflect newly produced viral particles.

Titration data correspond to expression of the EGFP reporter gene (present at 24hours post infection but not at 72 hours post infection), assessed by fluorescence microscopy (data not shown).

4.THP-1 cells and primary human monocytes migrate towards breast cancer cell supernatants

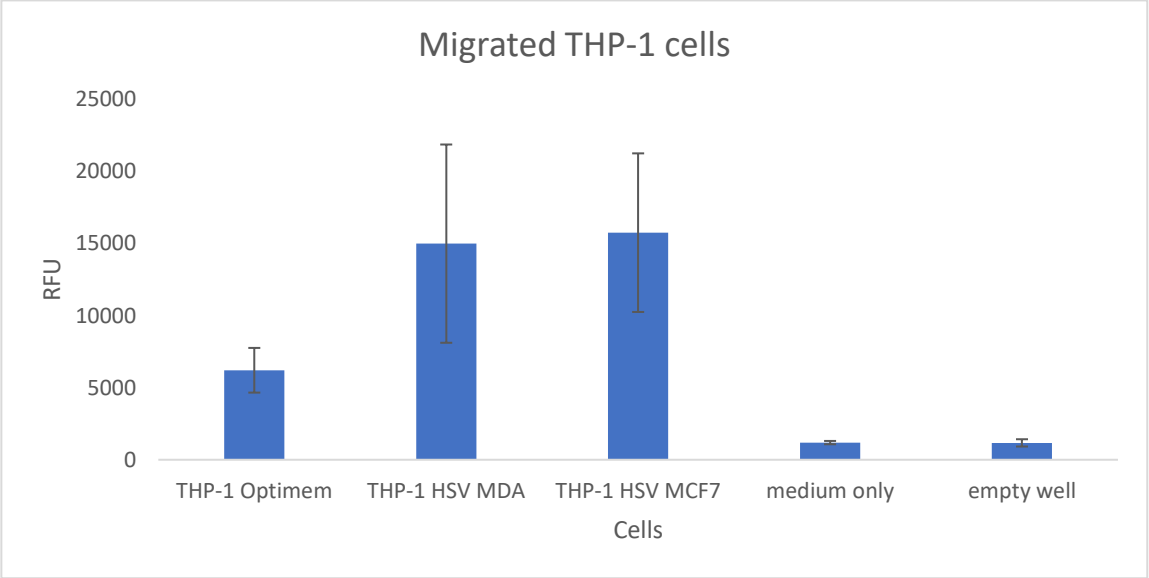


Figure 14 Fluorescence of calcein-labeled, migrated THP-1 cells after overnight incubation in a 96-well plate. THP-1 Opti-MEM™: migration towards serum-free medium alone THP-1 HSV MDA: migration of oHSV1-infected THP-1 cells towards MDA-MB-231 supernatants. THP-1 HSV MCF7: migration of oHSV1-infected THP-1 cells towards MCF7 cell supernatants. Each migration assay was repeated in six different wells with the InnoCyte™ Monocyte Cell Migration Assay

.

As assessed by the InnoCyte™ Monocyte Cell Migration Assay (see "Methods" section), THP-1 cells infected with oHSV1-EGFP migrated towards serum-free medium supernatants of breast cancer cell lines (MDA-MB-231 and MCF7) more than towards serum-free medium alone. This suggests that indeed breast cancer cells release soluble factors that are chemotactic for monocytes.

To confirm this finding with primary cells, we performed a similar Boyden chamber migration assay with primary monocytes, marked with CellTracker™ Red CMTPX Dye (see "Methods" section) migrating towards the same cancer cell supernatants or Opti-MEM™ medium alone.

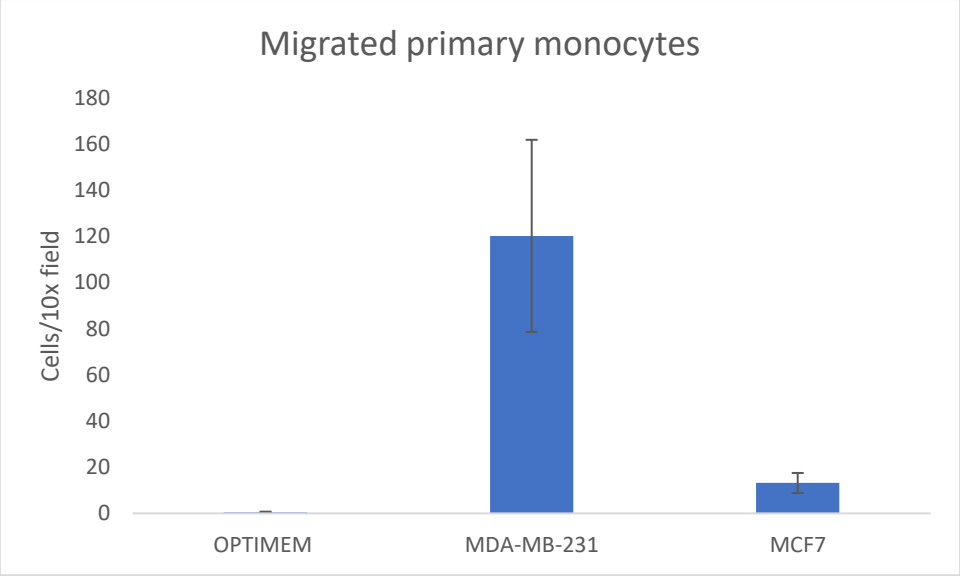


Figure 15 Average cell number in four 10x microscopic fields in a Boyden chamber after 3 hours of migration of oHSV-1-infected primary human monocytes towards supernatants of cancer cells cultivated in serum-free medium or towards serum-free medium (Opti-MEM™)

Despite a remarkable difference in the amount of primary monocytes that migrated towards MDA-MB-231 and MCF7 supernatants (which was not observed with THP-1 cells), in both cases there was a significant increase in chemotaxis compared to serum-free medium alone.

Δγ34.5/ΔUs12/EGFP-oHSV1-infected THP-1 cells and primary human monocytes transmit the infection to MDA-MB-231 breast cancer cells in coculture

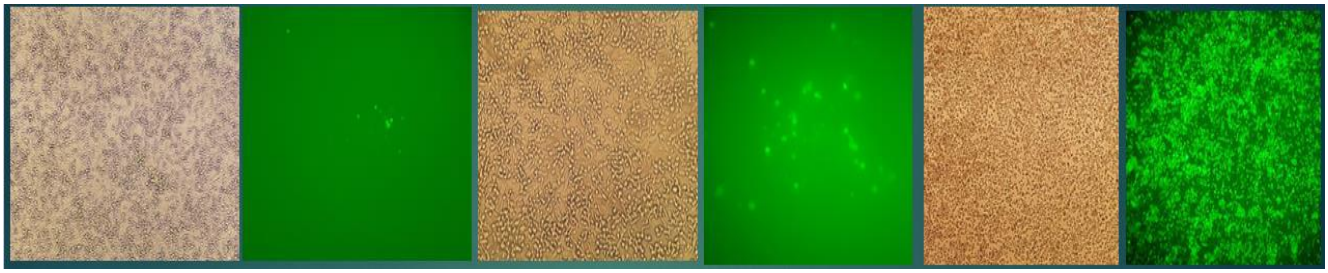


Figure 16 Infected THP-1 cells and MDA-MB-231 cells at 24 (left), 48 (middle) and 96 hours (right) post-infection. 10x brightfield and fluorescence microscopy pictures. Images are representative of multiple 10x fields.

In infected-THP-1/MDA-MB-231 cell cocultures (MOI 1, cell ratio 1:1) only some foci of EGFP+ cells are visible 24 hours post infection, however green fluorescence spreads progressively and involves most cells by 96 hours (5 days) post infection. At 5 days post infection, rounding of spindle-shaped cancer cells indicating cytopathic effect is visible (Figure 16).

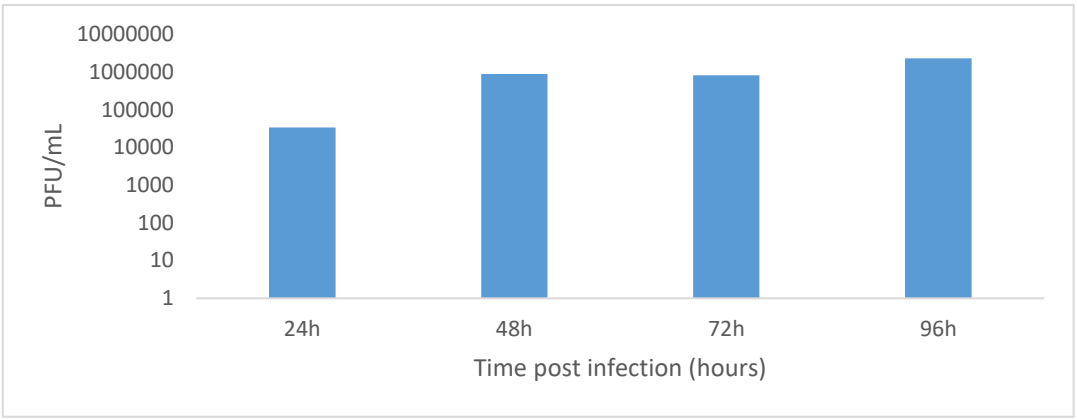


Figure 17 Plaque titration assay of 1:1 cocultures of oHSV1-infected THP-1 cells (MOI 3) and MDA-MB-231 cells. Y axis in logarithmic scale.

Plaque titration assays indicated a remarkably constant viral production (Figure 17), with higher titres than those observed in the infection of THP-1 cells alone.

These experiments were carried out either once or in two replicates. The second and third replicates will be performed to allow a statistical analysis.

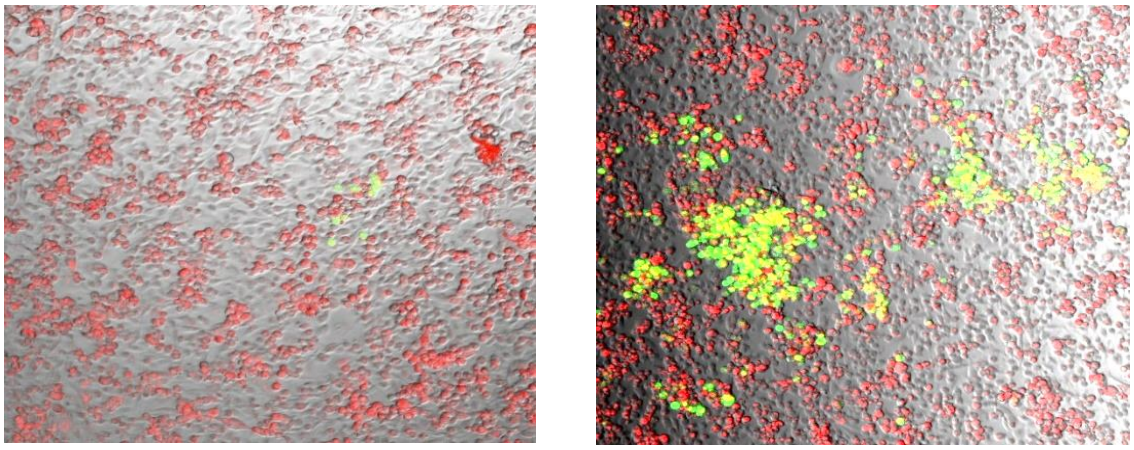


Figure 18 1:1 coculture of infected primary monocytes (MOI 5) marked with cell tracker Red and MDA-MB-231 cells 24 hours (left) and 48 hours (right) post infection. 10x brightfield and multi-channel fluorescence microscopy pictures, representative of multiple 10x fields.

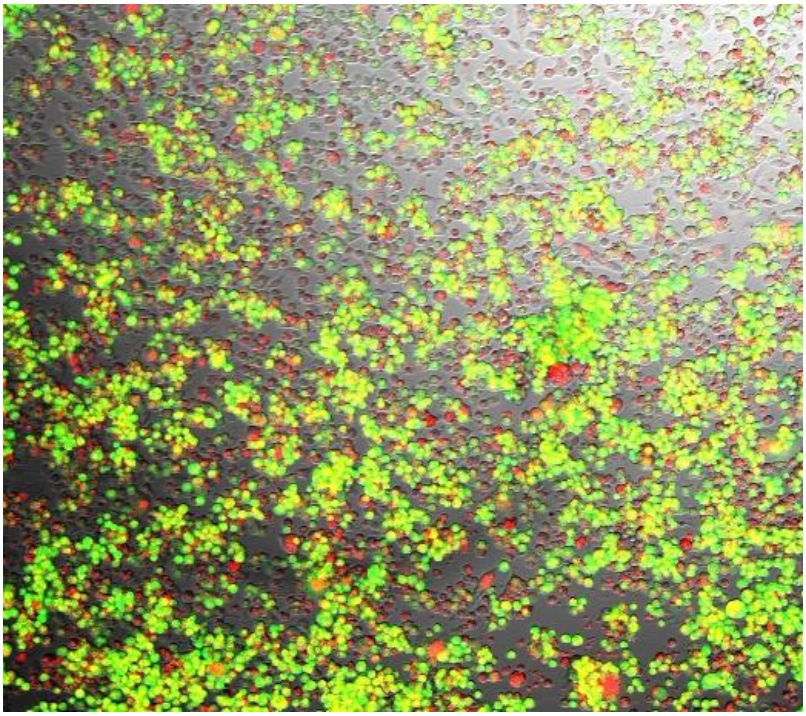


Figure 19 1:1 coculture of infected primary monocytes (MOI 5) marked with cell tracker Red and MDA-MB-231 cells 6 days post infection. 10x brightfield and multi-channel fluorescence microscopy picture, representative of multiple 10x fields.

Thanks to the red fluorescent cell tracker applied to primary monocytes, in these pictures it is possible to distinguish monocytes not expressing viral EGFP (red), monocytes expressing viral EGFP (yellow) and infected cancer cells (green).

At the early time points (24 and 48 hours post infection, Figure 18) clusters of green cells can be observed in close contact with red or yellow cells, indicating transmission of the infection from carrier monocytes. At the later time point (6 days post infection, Figure 19), widespread green fluorescence and rounding of normally spindle-shaped MDA-MB-231 cells is seen.

It is interesting to notice that in these pictures, yellow cells (EGFP-expressing monocytes) are much more represented than EGFP+ cells in infected primary monocytes monoculture (Figure 11), in spite of a lower MOI (MOI 5 in the coculture assay vs MOI 10 in Figure 11). This observation has fascinating implications that will be further described in the "Discussion" section, however, it has to be confirmed employing different and possibly more quantitative methods.

Since plaque titration assay in infected-THP-1/MDA-MB-231 cocultures had shown that viral titres remained stable in the time range 48h-96h, we decided to shift the time points for supernatant collection to 48hours, 5 days (96h) and 8 days post infection in infected primary monocytes/MDA-MB-231 cocultures.

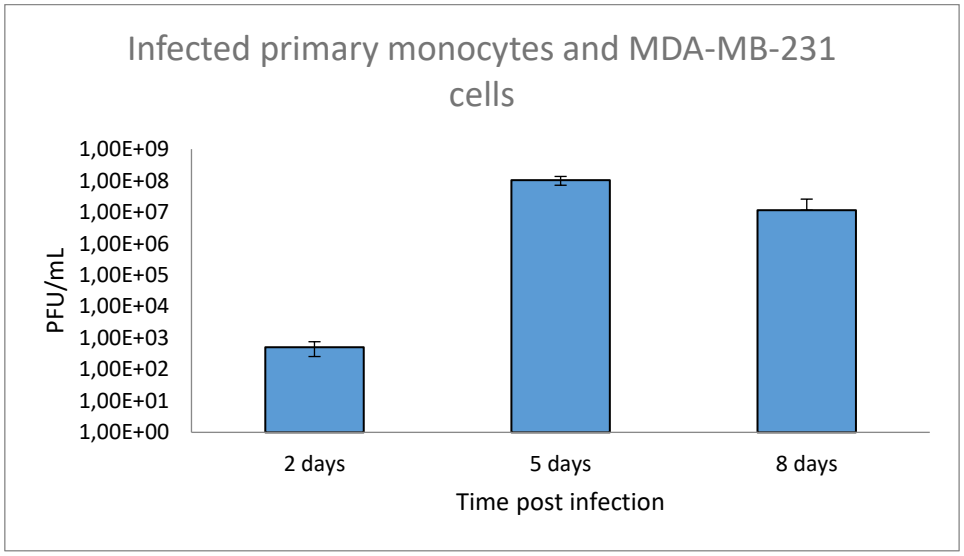


Figure 20 Plaque titration assay of 1:1 cocultures of oHSV1-infected primary human monocytes (MOI 5) and MDA-MB-231 cells. Y axis in logarithmic scale. Experiments were performed in three biological replicates. Error bars represent sample standard deviation.

Titration assays confirm that indeed there is prolonged production of infectious virions and also a striking difference in comparison with infected primary monocytes monoculture (Figure 11). The amount of infectious viral particles peaked 5 days post infection and declined slightly 8 days post infection, probably due to viral-induced death of cancer cells.

As reported in the “Methods” section, in all coculture assays we were especially careful to exclude a carryover of free virions by washing monocytes 3 times in PBS 1x and titrating undiluted PBS from the last washing.

5.THP-1 cells infected with Δγ34.5/ΔUs12/EGFP-oHSV1 migrate selectively into human tumors growing in chicken embryos

12 embryonated eggs were inoculated intravascularly with $5x10^5$ THP-1 cells infected with Δγ34.5/ΔUs12/EGFP-oHSV1. Tumors and livers were harvested for further analysis. By immunohistochemistry (IHC) CD14+ and ICP4+ (virally infected) cells could be detected in the 5 embryos analyzed so far but not in the negative (uninjected) control (Figure 21)

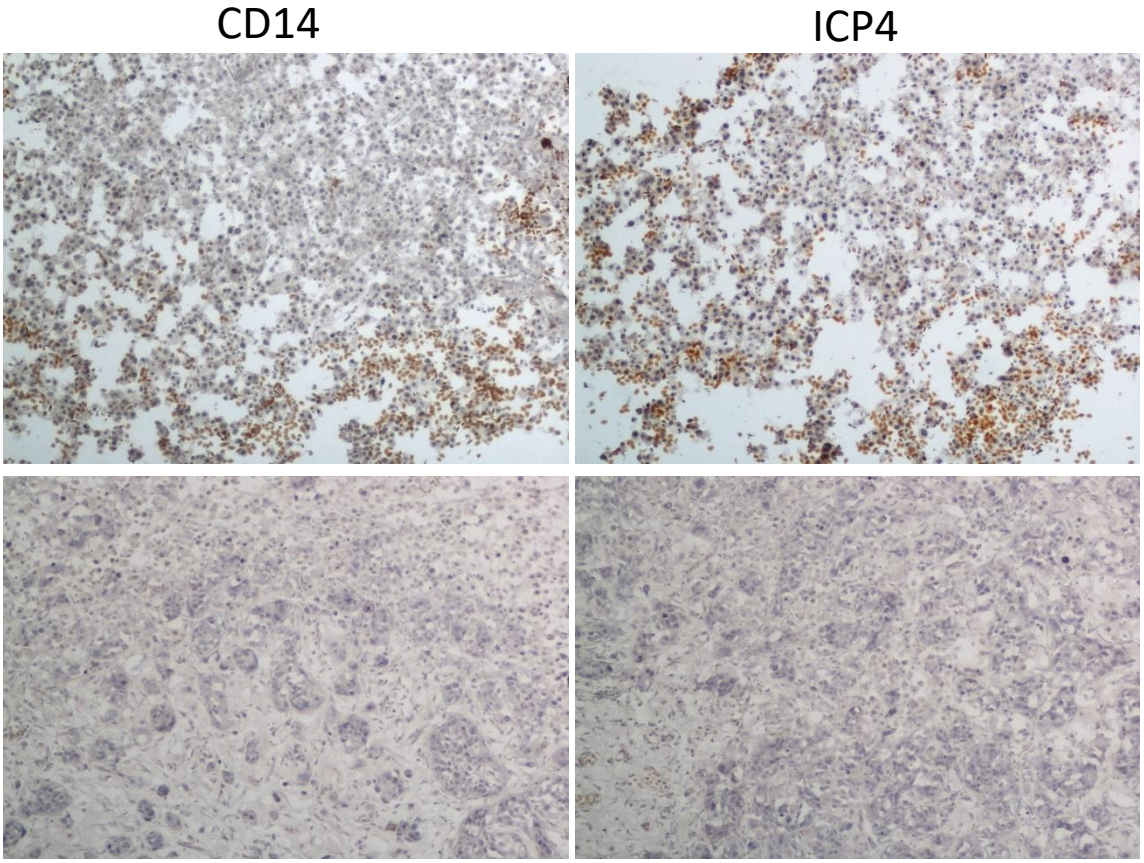


Figure 21 10x immunohistochemistry pictures of UM-SC-11B tumors growing on the CAM of embryonated chicken eggs, stained with anti-CD14 primary antibody (left) and anti-ICP4 primary antibody (right). Top row: egg injected with oHSV1-infected THP-1 cells. Bottom row: uninjected egg (negative control)

On the other hand, only few CD14 and ICP4 positive cells could be found in the liver of treated eggs, and those appeared to be circulating in blood vessels (Figure 22)

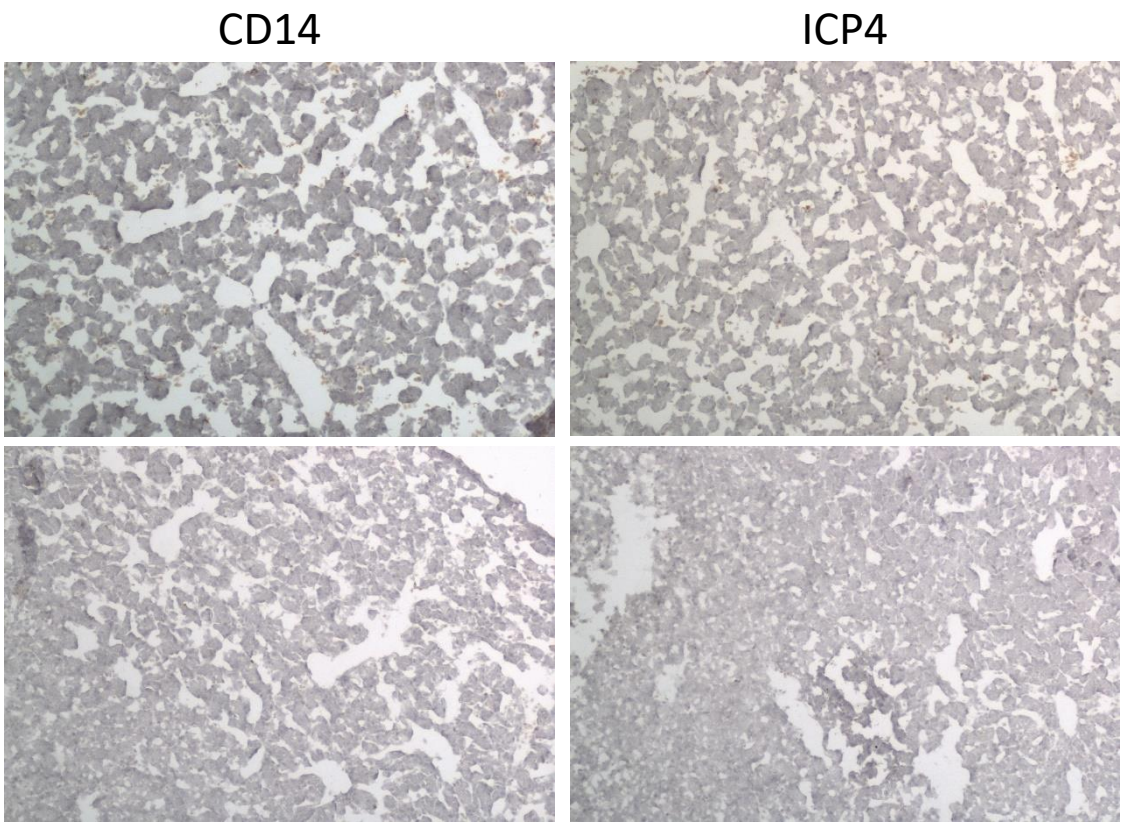
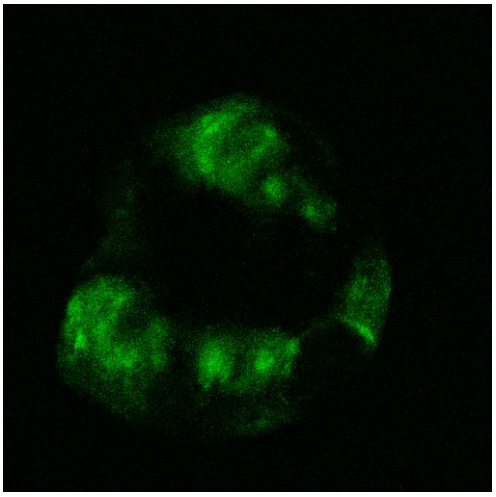


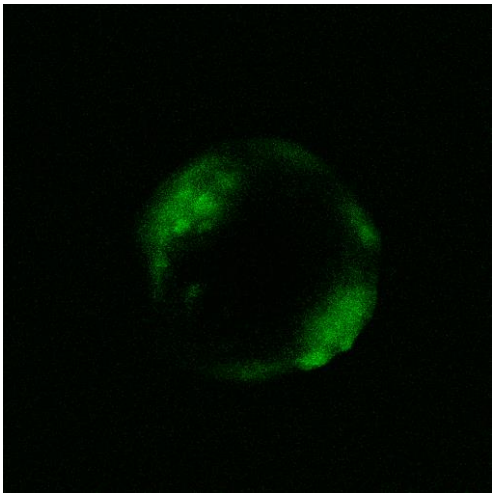
Figure 22 10x immunohistochemistry pictures of livers of chicken embryos with UM-SC-11B tumors growing on the CAM, stained with anti-CD14 primary antibody (left) and anti-ICP4 primary antibody (right). Top row: egg injected with oHSV1-infected THP-1 cells. Bottom row: uninjected egg (negative control)

We further confirmed the presence of the HSV-1 genome in 4 out of 11 tumors by real time PCR using published primers and probes specific for the viral UL29 gene[122].

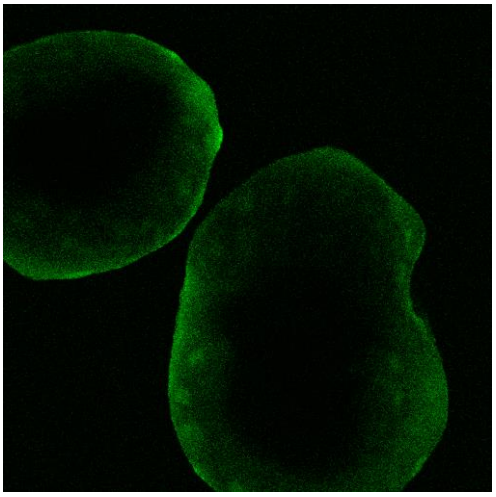
5. Human brain organoids are permissive to Δγ34.5/ΔUs12/EGFP-oHSV1 infection

a





b



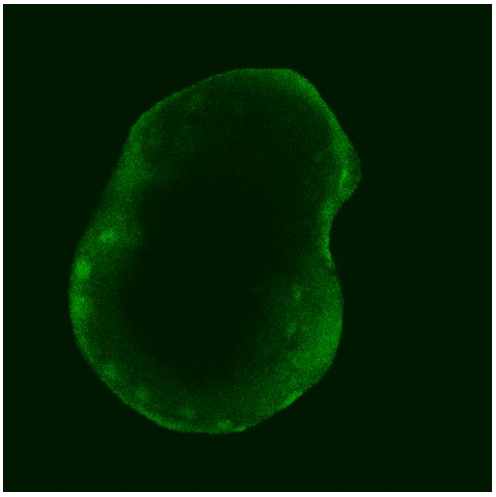
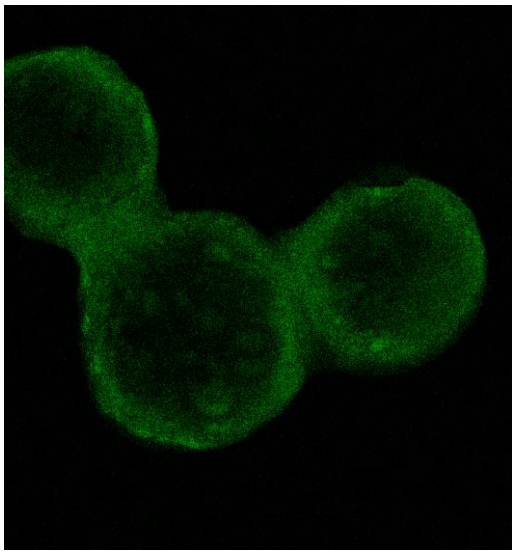
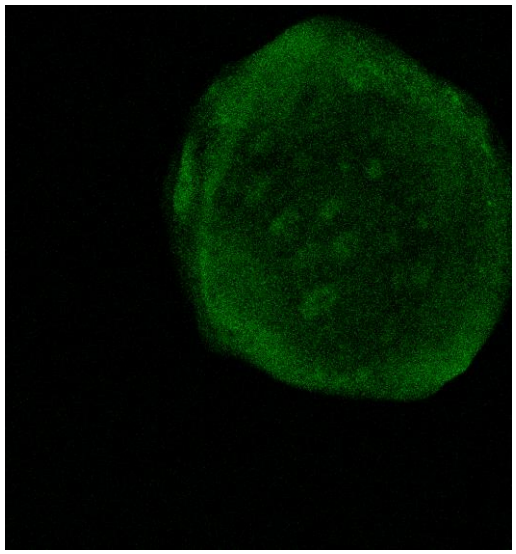


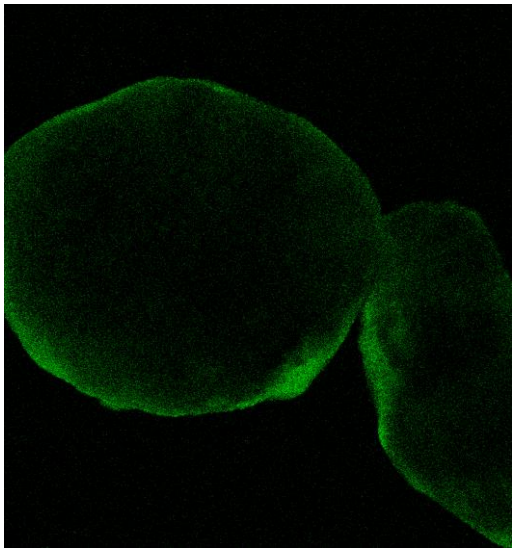
Figure 23 Fluorescence microscopy pictures of brain organoids 2 days after infection with Panel a)MOI 0.1 Panel b)MOI 1

c





d



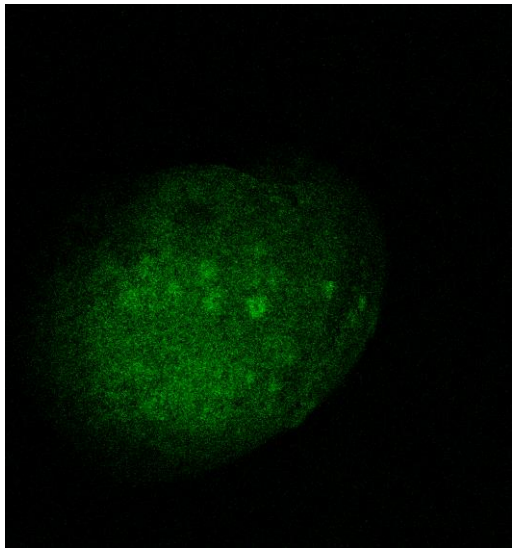
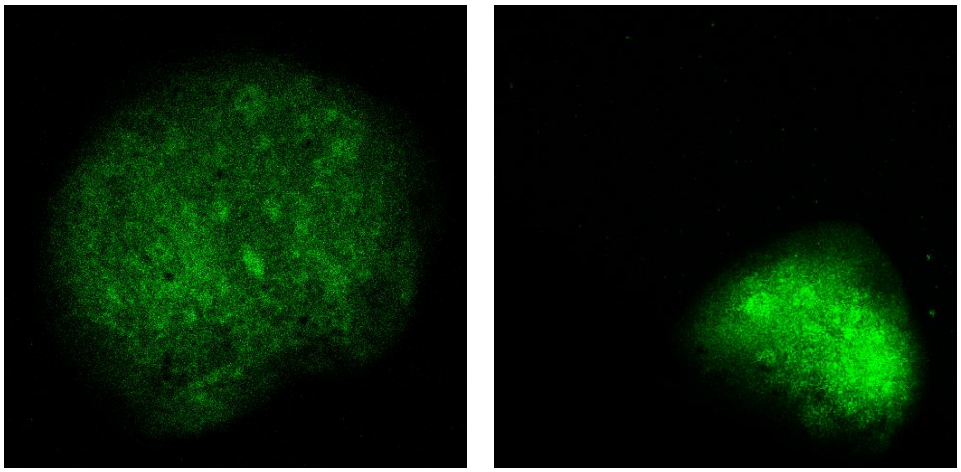


Figure 24 Fluorescence microscopy pictures of brain organoids 5 days after infection with Panel c)MOI 0.1 Panel d)MOI 1

Viral infection, as indicated by GFP fluorescence, spread progressively from the superficial layers of organoids (48 hours post infection) (Figure 23) to the inner layers 5 days post infection (Figure 24). The organoids were completely infected 7 days post infection (Figure 25).

e



f

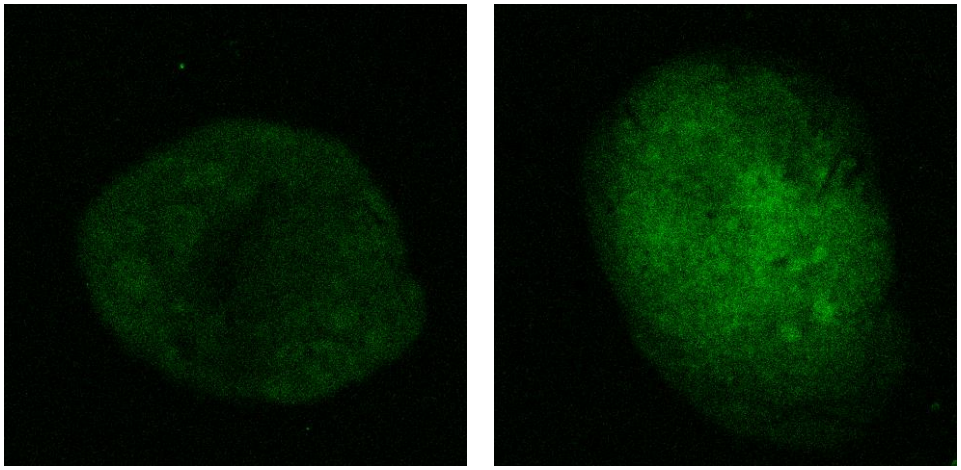
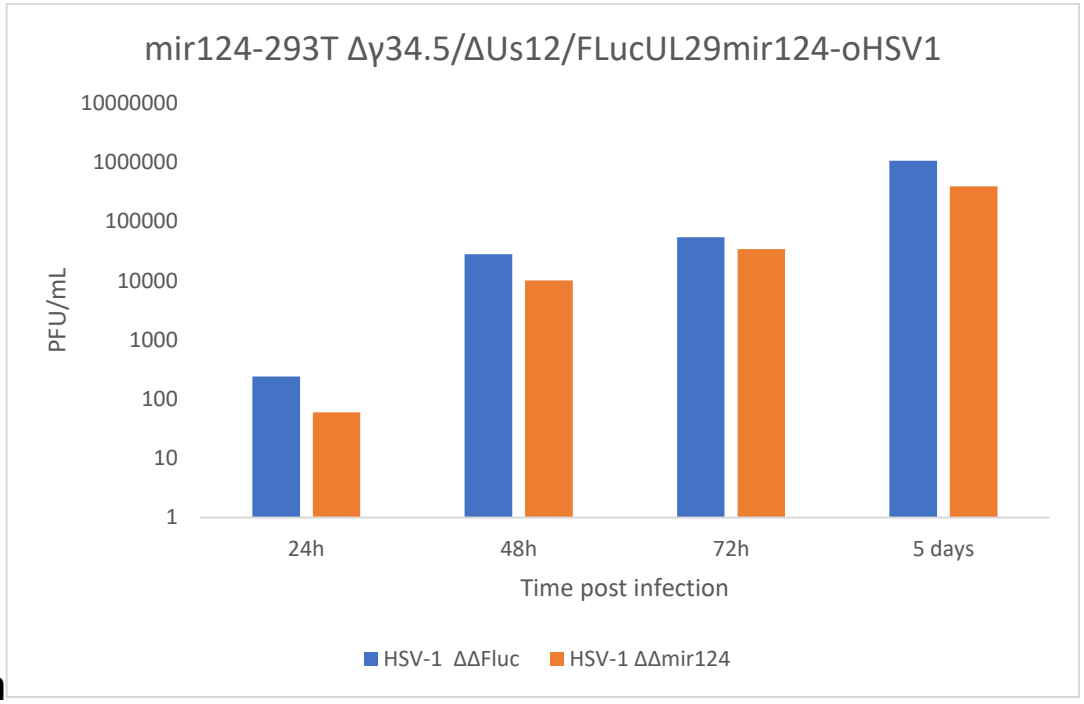


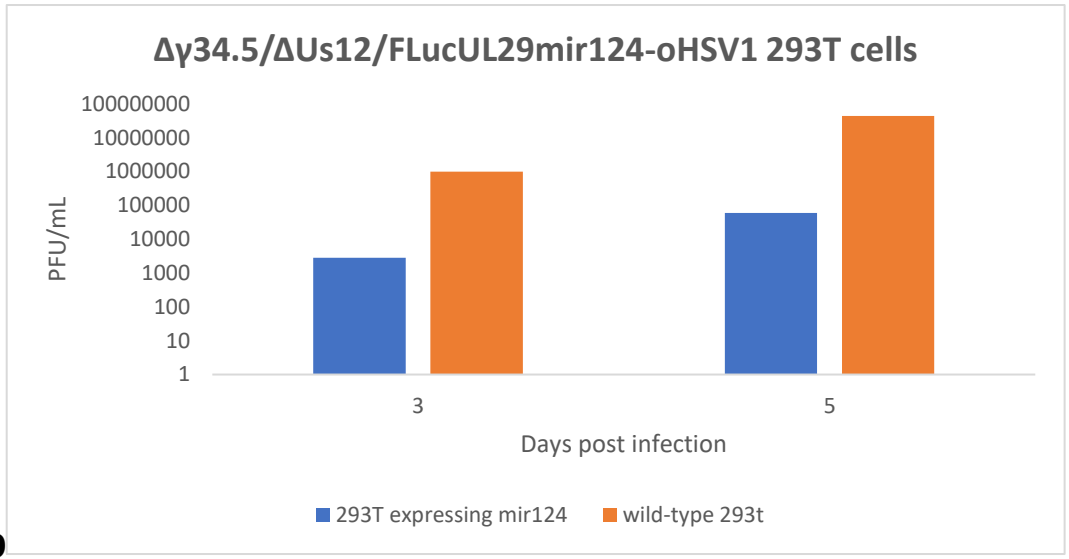
Figure 25 Fluorescence microscopy pictures of brain organoids 7 days after infection with Panel e)MOI 0.1 Panel f)MOI 1

Since in this first round of experiments we did not observe striking differences in the pattern of infection employing MOI 0.1 and MOI 1, we chose to perform the subsequent infections with the lower MOI (0.1), which in our opinion mimics a more realistic *in vivo* scenario.

7. Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 is attenuated in 293T cells overexpressing mir124



a



b

Figure 26 Replication of Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 (MOI 0.1) compared to parental Δγ34.5/ΔUs12/FLuc-oHSV1 in 293T cells transfected with pSMPUW-miR-124-GFP-Puro plasmid (a) and replication of Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 in transfected vs non-transfected 293T cells

Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 (ΔΔmir124) virus replicates to lower titres than its parental Δγ34.5/ΔUs12/FLuc-oHSV1 virus in 293T cells transfected with a plasmid causing the expression of human mir124 (Figure 26a).

To confirm that this finding was not due to an aspecific attenuation of ΔΔmir124 we tested its replicative capacity in non-transfected 293T cells and found that infectious virions strongly increased 3 and 5 days post infection (Figure 26b).

These experiments were carried out either once or in two replicates. The second and third replicates will be performed to allow a statistical analysis.

8.Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 is attenuated in human brain organoids compared to Δγ34.5/ΔUs12/FLuc-oHSV1

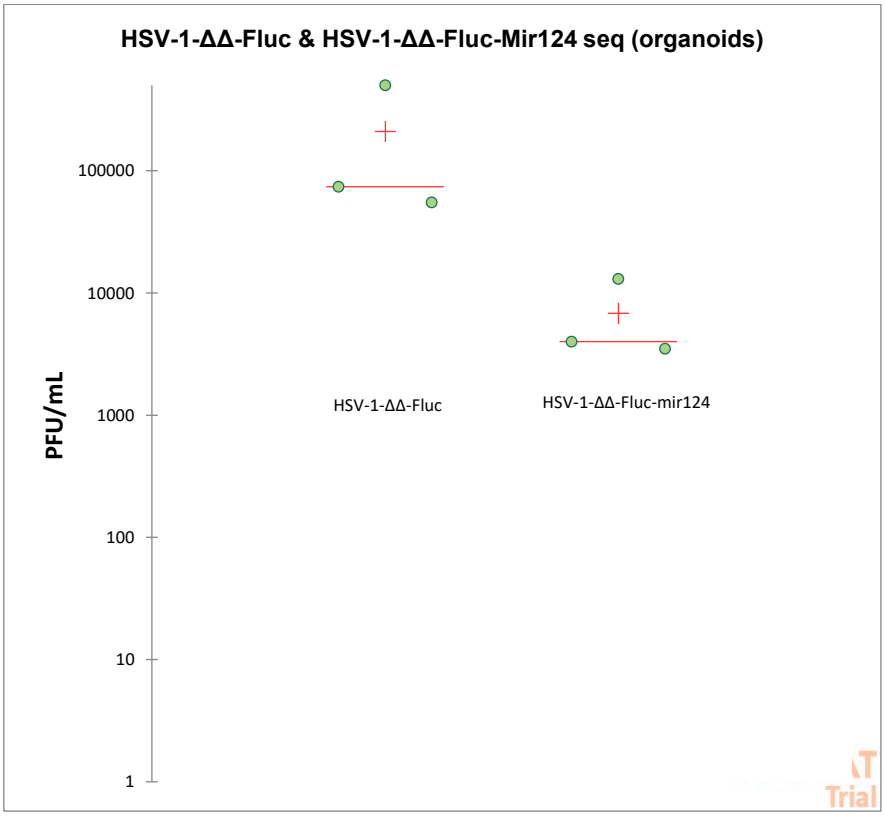


Figure 27 Plaque titration assay on the supernatants of organoid dishes (approximately 20 organoids/dish) 1 week after infection with the two indicated viruses (MOI 0.1 PFU/cell). Y axis in logarithmic scale. Experiments performed in three replicates.

Δγ34.5/ΔUs12/FLucUL29mir124-oHSV1 replication was tested in a more relevant biological model, i.e. in human brain organoids. In this setting, HSV1-ΔΔmir124 replicated to lower titres than HSV1-ΔΔFLuc.

Discussion

This study contributes to devising a strategy for the systemic delivery of oncolytic viruses (OVs). Preclinical and clinical studies tested several strategies, including intravenous viral injection and use of carrier cells, which are infected *ex vivo* with the OV and have a tropism for tumors. The most frequently employed carrier cells for OV delivery have been mesenchymal stem cells (MSCs), however MSCs face two main problems that hinder their clinical development: 1)autologous MSCs need to be recovered by biopsy and cultured *ex vivo,* which makes the manufacturing process more time-consuming and difficult, 2)most importantly, MSCs have biodistribution problems, probably due to their large dimensions.

Other studies focused on autologous immune cells that naturally infiltrate the tumor microenvironment (TME). Circulating monocytes are the precursors of tumor associated macrophages (TAMs), and thus they are natural candidates to be used as carrier cells, as detailed in the introduction. Nevertheless, so far only few studies investigated circulating monocytes or monocyte-derived macrophages as carrier cells for an oncolytic adenovirus and oncolytic measles virus [123][124].

The presented results indicate for the first time that blood monocytes can be used as carriers for the delivery of Δγ34.5/ΔUs12 oncolytic HSV-1 (oHSV-1), which has been the most successful oncolytic virus so far, gaining clinical approval in the USA and the EU for malignant melanoma, althoguh using intratumoral injection as a route of administration. Finding an efficient way of delivering oHSV-1 systemically is the key to use this powerful OV for the treatment of deep-seated or metastatic tumors, which are not as easily accessible as melanoma.

Interestingly, during natural infection, circulating monocytes are not a primary target for HSV-1, which can replicate in more differentiated myeloid cells, such as macrophages and dendritic cells

[125]. However, we could demonstrate that our oHSV-1 infects both monocytic cell lines and primary monocytes *in vitro*, both by detecting viral replication (much more prominent in cell lines) and expression of the immediate early ICP4 viral protein. Although it was possible to expect that this oncolytic virus would have been severely restricted in monocytes, this was not the case, raising interesting questions about the role of the HSV-1 virulence factor γ34.5 in the infection of myeloid cells.

Furthermore, the fluorescence microscopy images we collected using CellTracker™ Red CMTPX Dye-marked primary human monocytes infected with oHSV1-EGFP, in a coculture with human breast cancer MDA-MB-231 cells, compared with the microscopic images of oHSV1-EGFP-infected primary monocytes alone, suggest that coculture conditions enhance viral gene expression in carrier cells.

In fact, after infection of primary monocytes in monoculture with the oncolytic virus, we could demonstrate that >50% of cells were actually infected, by performing immunofluorescence using an anti-ICP4 primary antibody. However, very few GFP+ cells could be observed by fluorescence microscopy (around 1 or 2 cells in each well of a 24-multiwell plate, in which 10^5 primary monocytes infected with an MOI 5 or 10 were seeded). Correspondingly, low viral titres could be detected, and only up to 24 hours post infection. These findings may point at an interruption of the viral replication cycle at a very early stage in most infected cells. On the other hand, in the coculture conditions, numerous clusters of CellTracker™ +/GFP+ cells (presumably, infected monocytes expressing EGFP) could be visualized 24 hours and 48 hours post infection, often in close proximity to infected (GFP+) cancer cells. At later time points, the widespread EGFP-positivity of cancer cells made pictures difficult to interpret. We hypothesize that differentiation of monocytes triggered by cancer cells, a phenomenon which was already described, boosts the replication of the oncolytic virus during coculture, compared to monocyte monoculture.

This would be a very interesting finding, since it would mean that the oncolytic virus remains relatively “silent” in carrier cells until it reaches its target (the TME), where it undergoes enhanced replication. We will perform further experiments to confirm this hypothesis, taking into account different possible underlying mechanisms. If viral replication is enhanced by soluble factors released by MDA-MB-231 cells, a simple but effective experiment would be to culture infected primary monocytes in cell-free MDA-MB-231 cells supernatant and describe the output, in terms of EGFP-positivity and infectious viral particles.

If the mechanism is not due to soluble factors but to cell-associated factors or cell-to-cell contact, more subtle strategies would be required, for example immunofluorescence with a primary antibody against a late HSV-1 gene (expressed only when the viral replication cycle is completed) or other types of single-cell analysis. Performing the coculture assay, we were aware of the risk of a carryover of free virions along with the infected cells. However, while this possibility seems to be not very relevant considering the much higher viral titres obtained in cocultures compared to monocultures, we tried to exclude it by washing cells three times in PBS 1X before coculture, and titrating viral infectious particles in PBS from the third washing.

Infected THP-1 cells and primary monocytes could migrate towards the supernatants of MDA-MB-231 cells and of another breast cancer cell line (MCF-7), as measured by Boyden chamber migration in comparison with serum-free medium alone. Beyond such simple *in vitro* assays, which have obvious limitations, it is challenging to find complex systems that can mimic trafficking of human carrier cells through a vascular system. While the animal model recapitulates most features of “real” human tumors, the use of animals is expensive and relatively slow, thus making them suboptimal for initial screening studies. We therefore established a collaboration with Prof. Dr Stefan Kochanek and Dr. Lea Krutzke at the Department of Gene Therapy of the University of Ulm (Germany), to exploit the chorioallantoic membrane (CAM) model, in which human cancer cells grow as a small

mass on the CAM of embryonated chicken eggs. The established model used human squamous laryngeal carcinoma UM-SC-11B cells, which were suitable for our purposes due to the broad tropism of HSV-1, especially for cells of epithelial origin.

Embryonated chicken eggs at an early stage of developments are not legally considered living animals and do not have a functional immune system, however they have a developed circulatory system, allowing intravascular administration of therapeutics[12]. Therefore, we hypothesized that the CAM model was optimal to assess the capacity of monocytic cells infected with oHSV-1 to migrate selectively through a complex vasculature to reach tumor masses. Indeed, 5 days after intravascular injection of human THP-1 cells infected with a multiplicity of infection (MOI) 3 PFU/cell, we could detect clusters of carrier cells (staining positive for CD14) and clusters of infected cells (staining positive for ICP4) in tumors by immunohistochemistry (IHC). The pattern of IHC positivity suggests that a perivascular accumulation of carrier cells occurs. On the other hand, in other organs from the chicken embryos which were large enough to be isolated (liver and kidney) we found either no positive cells or sparse positive cells, probably circulating in the vasculature.

To further confirm this finding, we used a part of the formalin-fixed and TissueTek-embedded samples for DNA extraction and subsequent amplification of a small segment of the viral UL29 gene by real time PCR. Despite many limitations (small sample size with low DNA yield, non-homogeneous distribution of infected cells in the samples, and possible formalin contamination), the tumors from 4 eggs tested positive. Our results indicate that infected carrier cells migrate efficiently in a vascular system and have a tropism for tumors. Nevertheless, this system can be maintained only for a limited time span, does not allow an evaluation of the therapeutic effect and has no active immune system. Therefore, we regard it as a preliminary step, which establishes that monocytes are suitable carriers for further studies in an animal model.

Finally, a successful systemic distribution by carrier cells offers opportunities but also poses challenges. For example, circulating monocytes can migrate into the central nervous system (CNS) and this is particularly relevant for us for two reasons, i.e. 1)HSV-1 is a neurotropic virus and a cause of viral encephalitis, 2)on the other hand this delivery system can be used also to treat intracranial tumors.

The Δγ34.5 deletion has been consistently reported to be neuroattenuating, not only in preclinical animal studies, but also in clinical trials[126]. However, as explained elsewhere (Summary and Introduction), ΔUs12 partially rescues the replicative capacity of Δγ34.5. To the best of our knowledge, Δγ34.5/ΔUs12-oHSV1s were never used for intracranial injection in clinical trials. Therefore, we decided to further neuroattenuate the virus by inserting multiple target sequences of the neuron-specific mir124 miRNA downstream of UL29, which is essential for the replication of the viral genome and is also a novel target for miRNA-based attenuation of oncolytic HSV-1[92]. We compared the replication of Δγ34.5/ ΔUs12-FLuc HSV-1 (ΔΔFluc, parental virus) to Δγ34.5/ ΔUs12-UL29mir124 HSV-1 (ΔΔmir124) in an artificial system (293T cells overexpressing mir124) and a more natural system (brain organoids). In both systems, the replication of ΔΔmir124 was significantly inferior, as measured by plaque titration assay.

More experiments are ongoing to measure the diffusion of different viruses in the 3D structure of the organoids (by IHC) and downregulation of the UL29 mRNA in both 293T cells and brain organoids, by reverse transcriptase real time PCR.

In conclusion, the data obtained with human monocytes, either from a cell line or as primary cells, confirm that they can be further evaluated as carriers for an oncolytic HSV-1. It is also possible that biological changes in monocytes after close contact with cancer cells enhance viral replication, which will be the focus of future experiments.

In a first step towards the animal model, we also evaluated replication and transgene expression in a mouse monocyte/macrophage cell line with Balb/c genetic background (J774A.1). Interestingly, while viral replication could be detected 24 hours post infection, it rapidly declined and no newly produced virions were detectable 72 hours post infection. This pattern suggests that J774A.1 cells are not intrinsically refractory to HSV-1 replication, but rather that some inducible mechanism is involved. The most straightforward hypothesis is that the interferon signaling pathway is involved. A previous study reported that OV-infected macrophages migrated to tumors within a few hours in mice [124], therefore the production of virions up to 24 hours post infection should provide a sufficient window of opportunity.

However, in order to evaluate the mechanism which restricts oHSV-1 replication at later time points and to extend this window of opportunity, we plan to treat cells with an inhibitor of the interferon JAK-STAT pathway and measure its effect on viral production and cell survival. This experiment is also functional to the next step of the project, in which we will test oHSV-1 replication in primary mouse monocytes.

Many different solid tumors could be treated with this approach. In the selection of an appropriate animal model, we will focus on immunocompetent animal models with either metastatic or deep-seated orthotopic tumors. Possible candidates include glioblastoma (which exploits the capacity of monocytes to migrate into the CNS and our new neuroattenuation system), metastatic triple negative breast carcinoma (the spontaneously metastatic 4T1 Balb/c model), or other cancers with a very negative prognosis, such as pancreatic carcinoma or hepatocellular hepatocarcinoma.

References

1. Russell, S.J.; Peng, K.W.; Bell, J.C. Oncolytic Virotherapy. *Nature Biotechnology* 2012, *30*, 658–670.

2. Conry, R.M.; Westbrook, B.; McKee, S.; Norwood, T.G. Talimogene Laherparepvec: First in Class Oncolytic Virotherapy. *Human Vaccines and Immunotherapeutics* 2018, *14*, 839–846.

3. Reale, A.; Vitiello, A.; Conciatori, V.; Parolin, C.; Calistri, A.; Palù, G. Perspectives on Immunotherapy via Oncolytic Viruses. *Infectious Agents and Cancer* 2019, *14*.

4. Haines, B.B.; Denslow, A.; Grzesik, P.; Lee, J.S.; Farkaly, T.; Hewett, J.; Wambua, D.; Kong, L.; Behera, P.; Jacques, J.; et al. ONCR-177, an Oncolytic HSV-1 Designed to Potently Activate Systemic Antitumor Immunity. *Cancer Immunology Research* **2021**, *9*, 291–308, doi:10.1158/2326-6066.CIR-20-0609.

5. Zendedel, E.; Atkin, S.L.; Sahebkar, A. Use of Stem Cells as Carriers of Oncolytic Viruses for Cancer Treatment. *Journal of cellular physiology* **2019**, *234*, 14906–14913, doi:10.1002/jcp.28320.

6. Hadryś, A.; Sochanik, A.; McFadden, G.; Jazowiecka-Rakus, J. Mesenchymal Stem Cells as Carriers for Systemic Delivery of Oncolytic Viruses. *European Journal of Pharmacology* 2020, *874*.

7. Leoni, V.; Gatta, V.; Palladini, A.; Nicoletti, G.; Ranieri, D.; Dall'Ora, M.; Grosso, V.; Rossi, M.; Alviano, F.; Bonsi, L.; et al. Systemic Delivery of HER2-Retargeted Oncolytic-HSV by Mesenchymal Stromal Cells Protects from Lung and Brain Metastases. *Oncotarget* **2015**, *6*, 34774–34787, doi:10.18632/oncotarget.5793.

8. Melzer, M.K.; Zeitlinger, L.; Mall, S.; Steiger, K.; Schmid, R.M.; Ebert, O.; Krackhardt, A.; Altomonte, J. Enhanced Safety and Efficacy of Oncolytic VSV Therapy by Combination with T Cell Receptor Transgenic T Cells as Carriers. *Molecular therapy oncolytics* **2019**, *12*, 26–40, doi:10.1016/j.omto.2018.12.001.

9. Chen, Z.; Feng, X.; Herting, C.J.; Garcia, V.A.; Nie, K.; Pong, W.W.; Rasmussen, R.; Dwivedi, B.; Seby, S.; Wolf, S.A.; et al. Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma. *Cancer Research* **2017**, *77*, 2266–2278, doi:10.1158/0008-5472.CAN-16-2310.

10. Laviron, M.; Boissonnas, A. Ontogeny of Tumor-Associated Macrophages. *Frontiers in immunology* 2019, *10*, 1799.

11. Teh, Y.C.; Ding, J.L.; Ng, L.G.; Chong, S.Z. Capturing the Fantastic Voyage of Monocytes through Time and Space. *Frontiers in Immunology* 2019, *10*.

12. Krutzke, L.; Allmendinger, E.; Hirt, K.; Kochanek, S. Chorioallantoic Membrane Tumor Model for Evaluating Oncolytic Viruses. *Human Gene Therapy* **2020**, *31*, 1100–1113, doi:10.1089/hum.2020.045.

13. Sung, H.; Ferlay, J.; Siegel, R.L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians* **2021**, doi:10.3322/caac.21660.

14. S, V.; M, L.; J, F.; F, B. Cervical Cancer in Africa, Latin America and the Caribbean and Asia: Regional Inequalities and Changing Trends. *International journal of cancer* **2017**, *141*, 1997–2001, doi:10.1002/IJC.30901.

15. L, C.; A, M.; S, G. HIV-Associated Malignancies in Sub-Saharan Africa: Progress, Challenges, and Opportunities. *Current opinion in HIV and AIDS* **2017**, *12*, 89–95, doi:10.1097/COH.0000000000000329.

16. MS, K.; M, S. Impact of Human Papillomavirus Vaccination in Reducing Cancer. *Primary care* **2020**, *47*, 529–537, doi:10.1016/J.POP.2020.05.007.

17. SP, B.; M, T.; PJ, L.; K, P.; BAHA, A.; S, V.; JE, S.; MA, G.; CE, B.; JC, H. Tissue-Based Biomarkers for the Risk Stratification of Men With Clinically Localized Prostate Cancer. *Frontiers in oncology* **2021**, *11*, doi:10.3389/FONC.2021.676716.

18. C, C.; H, F.; P, A.; LG, A.; Z, E.; E, H.; A, N.; B, N.; P, K.; B, L. Survival Patterns of Invasive Lobular and Invasive Ductal Breast Cancer in a Large Population-Based Cohort with Two Decades of Follow Up. *Breast (Edinburgh, Scotland)* **2021**, *59*, 294–300, doi:10.1016/J.BREAST.2021.07.011.

19. Wu, Q.; Siddharth, S.; Sharma, D. Triple Negative Breast Cancer: A Mountain yet to Be Scaled despite the Triumphs. *Cancers* **2021**, *13*, doi:10.3390/CANCERS13153697.

20. A, B.; A, G.; JU, M. Current State of Breast Cancer Diagnosis, Treatment, and Theranostics. *Pharmaceutics* **2021**, *13*, doi:10.3390/PHARMACEUTICS13050723.

21. Roche - Roche Provides Update on Tecentriq US Indication for PD-L1-Positive, Metastatic Triple-Negative Breast Cancer Available online: https://www.roche.com/media/releases/med-cor-2021-08-27.htm (accessed on 26 September 2021).

22. D, A.; B, T.; B, A.; F, H.; C, S.; C, W.; A, S.; D, B.; M, B.; R, A. Pancreatic Cancer: Yesterday, Today and Tomorrow. *Future oncology (London, England)* **2016**, *12*, 1929–1946, doi:10.2217/FON-2016-0010.

23. Weller, M.; Bent, M. van den; Tonn, J.C.; Stupp, R.; Preusser, M.; Cohen-Jonathan-Moyal, E.; Henriksson, R.; Rhun, E. le; Balana, C.; Chinot, O.; et al. European Association for Neuro-Oncology (EANO) Guideline on the Diagnosis and Treatment of Adult Astrocytic and Oligodendroglial Gliomas. *The Lancet Oncology* **2017**, *18*, e315–e329, doi:10.1016/S1470-2045(17)30194-8.

24. Poon, M.T.C.; Sudlow, C.L.M.; Figueroa, J.D.; Brennan, P.M. Longer-Term (≥ 2 Years) Survival in Patients with Glioblastoma in Population-Based Studies Pre- and Post-2005: A Systematic Review and Meta-Analysis. *Scientific Reports 2020 10:1* **2020**, *10*, 1–10, doi:10.1038/s41598-020-68011-4.

25. A, B.; R, A.; D, A. The Actual 5-Year Survivors of Pancreatic Ductal Adenocarcinoma Based on Real-World Data. *Scientific reports* **2020**, *10*, doi:10.1038/S41598-020-73525-Y.

26. Weiss, R.A. A Perspective on the Early Days of RAS Research. *Cancer and Metastasis Reviews* **2020**, *39*, 1023–1028, doi:10.1007/S10555-020-09919-1.

27. R, R. Small Molecule Inhibitors Targeting the EGFR/ErbB Family of Protein-Tyrosine Kinases in Human Cancers. *Pharmacological research* **2019**, *139*, 395–411, doi:10.1016/J.PHRS.2018.11.014.

28. Longo, D.L. Imatinib Changed Everything. *New England Journal of Medicine* **2017**, *376*, 982–983, doi:10.1056/NEJME1700833.

29. Ribatti, D.; Solimando, A.G.; Pezzella, F. The Anti-Vegf(R) Drug Discovery Legacy: Improving Attrition Rates by Breaking the Vicious Cycle of Angiogenesis in Cancer. *Cancers* **2021**, *13*, doi:10.3390/CANCERS13143433.

30. Y, Z.; X, W. Targeting the Wnt/β-Catenin Signaling Pathway in Cancer. *Journal of hematology & oncology* **2020**, *13*, doi:10.1186/S13045-020-00990-3.

31. Porta, C.; Paglino, C.; Mosca, A. Targeting PI3K/Akt/MTOR Signaling in Cancer. *Frontiers in Oncology* **2014**, *4 APR*, doi:10.3389/FONC.2014.00064.

32. JJ, C.; SM, B.; JR, Z. Hippo Pathway Inhibition by Blocking the YAP/TAZ-TEAD Interface: A Patent Review. *Expert opinion on therapeutic patents* **2018**, *28*, 867–873, doi:10.1080/13543776.2018.1549226.

33. AR, M.; SC, R.; F, M.; S, M. RAS-Targeted Therapies: Is the Undruggable Drugged? *Nature reviews. Drug discovery* **2020**, *19*, 533–552, doi:10.1038/S41573-020-0068-6.

34. K, S.; T, M. Drug Tolerance to EGFR Tyrosine Kinase Inhibitors in Lung Cancers with EGFR Mutations. *Cells* **2021**, *10*, doi:10.3390/CELLS10071590.

35. EA, K.; S, K.; J, T.; SR, J.; F, S. Epidermal Growth Factor Receptor Expression and Resistance Patterns to Targeted Therapy in Non-Small Cell Lung Cancer: A Review. *Cells* **2021**, *10*, doi:10.3390/CELLS10051206.

36. Marin, J.J.G.; Sanchon-Sanchez, P.; Cives-Losada, C.; Carmen, S. del; González-Santiago, J.M.; Monte, M.J.; Macias, R.I.R. Novel Pharmacological Options in the Treatment of Cholangiocarcinoma: Mechanisms of Resistance. *Cancers* **2021**, *13*, doi:10.3390/CANCERS13102358.

37. S, B.; MC, L.; L, O. Neratinib Resistance and Cross-Resistance to Other HER2-Targeted Drugs Due to Increased Activity of Metabolism Enzyme Cytochrome P4503A4. *British journal of cancer* **2017**, *116*, 620–625, doi:10.1038/BJC.2016.445.

38. Lin, Z.; Zhang, Q.; Luo, W. Angiogenesis Inhibitors as Therapeutic Agents in Cancer: Challenges and Future Directions. *European Journal of Pharmacology* **2016**, *793*, 76–81, doi:10.1016/J.EJPHAR.2016.10.039.

39. Sennino, B.; McDonald, D.M. Controlling Escape from Angiogenesis Inhibitors. *Nature Reviews Cancer* **2012**, *12*, 699–709, doi:10.1038/NRC3366.

40. Kumagai, S.; Koyama, S.; Nishikawa, H. Antitumour Immunity Regulated by Aberrant ERBB Family Signalling. *Nature Reviews Cancer* **2021**, *21*, 181–197, doi:10.1038/S41568-020-00322-0.

41. Ni, X.; Tao, J.; Barbi, J.; Chen, Q.; Park, B. v.; Li, Z.; Zhang, N.; Lebid, A.; Ramaswamy, A.; Wei, P.; et al. YAP Is Essential for Treg-Mediated Suppression of Antitumor Immunity. *Cancer Discovery* **2018**, *8*, 1026–1043, doi:10.1158/2159-8290.CD-17-1124.

42. Ahn, M.J.; Sun, J.M.; Lee, S.H.; Ahn, J.S.; Park, K. EGFR TKI Combination with Immunotherapy in Non-Small Cell Lung Cancer. *Expert Opinion on Drug Safety* **2017**, *16*, 465–469, doi:10.1080/14740338.2017.1300656.

43. Dobosz, P.; Dzieciątkowski, T. The Intriguing History of Cancer Immunotherapy. *Frontiers in Immunology* **2019**, *10*, 2965, doi:10.3389/FIMMU.2019.02965.

44. Haen, S.P.; Löffler, M.W.; Rammensee, H.G.; Brossart, P. Towards New Horizons: Characterization, Classification and Implications of the Tumour Antigenic Repertoire. *Nature Reviews Clinical Oncology* **2020**, *17*, 595–610, doi:10.1038/S41571-020-0387-X.

45. S, M.; HL, K. High-Dose Interleukin-2 (IL-2) for the Treatment of Melanoma: Safety Considerations and Future Directions. *Expert opinion on drug safety* **2017**, *16*, 1347–1357, doi:10.1080/14740338.2017.1382472.

46. BQ, T.; Q, W.; R, L.; C, P.; G, L.; F, S.; JW, W.; BJ, P.; IH, F.; JLG, C. Evolution of Cancer Vaccines-Challenges, Achievements, and Future Directions. *Vaccines* **2021**, *9*, doi:10.3390/VACCINES9050535.

47. Taylor-Papadimitriou, J.; Burchell, J.M.; Graham, R.; Beatson, R. Latest Developments in MUC1 Immunotherapy. *Biochemical Society transactions* **2018**, *46*, 659–668, doi:10.1042/BST20170400.

48. N, C.; J, S.; C, Y. HER2-Based Immunotherapy for Breast Cancer. *Cancer biotherapy & radiopharmaceuticals* **2018**, *33*, 169–175, doi:10.1089/CBR.2017.2327.

49. Mizukoshi, E.; Kaneko, S. Telomerase-Targeted Cancer Immunotherapy. *International Journal of Molecular Sciences* **2019**, *20*, doi:10.3390/IJMS20081823.

50. Gp100 Peptide Vaccine in Melanoma. *New England Journal of Medicine* **2011**, *365*, 771–771, doi:10.1056/NEJMC1107536.

51. Tagliamonte, M.; Petrizzo, A.; Tornesello, M.L.; Buonaguro, F.M.; Buonaguro, L. Antigen-Specific Vaccines for Cancer Treatment. *Human Vaccines and Immunotherapeutics* **2014**, *10*, 3332–3346, doi:10.4161/21645515.2014.973317.

52. Weon, J.L.; Potts, P.R. The MAGE Protein Family and Cancer. *Current Opinion in Cell Biology* **2015**, *37*, 1–8, doi:10.1016/J.CEB.2015.08.002.

53. Thomas, R.; Al-Khadairi, G.; Roelands, J.; Hendrickx, W.; Dermime, S.; Bedognetti, D.; Decock, J. NY-ESO-1 Based Immunotherapy of Cancer: Current Perspectives. *Frontiers in Immunology* **2018**, *9*, doi:10.3389/FIMMU.2018.00947.

54. T, K.; J, O. The Potential of Donor T-Cell Repertoires in Neoantigen-Targeted Cancer Immunotherapy. *Frontiers in immunology* **2017**, *8*, doi:10.3389/FIMMU.2017.01718.

55. Handy, C.E.; Antonarakis, E.S. Sipuleucel-T for the Treatment of Prostate Cancer: Novel Insights and Future Directions. *Future Oncology* **2018**, *14*, 907–917, doi:10.2217/FON-2017-0531.

56. Eagar, T.N.; Karandikar, N.J.; Bluestone, J.A.; Miller, S.D. The Role of CTLA-4 in Induction and Maintenance of Peripheral T Cell Tolerance., doi:10.1002/1521-4141.

57. C, G.; G, L.; Y, S. A Snapshot of the PD-1/PD-L1 Pathway. *Journal of Cancer* **2021**, *12*, 2735–2746, doi:10.7150/JCA.57334.

58. Chauvin, J.-M.; Zarour, H.M. TIGIT in Cancer Immunotherapy. *Journal for ImmunoTherapy of Cancer* **2020**, *8*, e000957, doi:10.1136/JITC-2020-000957.

59. Acharya, N.; Sabatos-Peyton, C.; Anderson, A.C. Tim-3 Finds Its Place in the Cancer Immunotherapy Landscape. *Journal for Immunotherapy of Cancer* **2020**, *8*, doi:10.1136/JITC-2020-000911.

60. PW, H.; JW, C. Immune Checkpoint Inhibitors Win the 2018 Nobel Prize. *Biomedical journal* **2019**, *42*, 299–306, doi:10.1016/J.BJ.2019.09.002.

61. Joyce, J.A.; Fearon, D.T. T Cell Exclusion, Immune Privilege, and the Tumor Microenvironment. *Science* 2015, *348*, 74–80.

62. Pitt, J.M.; Vétizou, M.; Daillère, R.; Roberti, M.P.; Yamazaki, T.; Routy, B.; Lepage, P.; Boneca, I.G.; Chamaillard, M.; Kroemer, G.; et al. Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors. *Immunity* 2016, *44*, 1255–1269.

63. Arduino, P.G.; Porter, S.R. Herpes Simplex Virus Type 1 Infection: Overview on Relevant Clinico-Pathological Features. *Journal of Oral Pathology and Medicine* **2008**, *37*, 107–121, doi:10.1111/J.1600-0714.2007.00586.X.

64. RF, N.; GJ, van D.; PG, M.; HA, N.; WI, van der M. Importance of Herpes Simplex Virus Type-1 (HSV-1) in Primary Genital Herpes. *Acta dermato-venereologica* **2006**, *86*, 129–134, doi:10.2340/00015555-0029.

65. Gorfinkel, I.S.; Aoki, F.; McNeil, S.; Dionne, M.; Shafran, S.D.; Zickler, P.; Halperin, S.; Langley, J.; Bellamy, A.; Schulte, J.; et al. Seroprevalence of HSV-1 and HSV-2 Antibodies in Canadian Women Screened for Enrolment in a Herpes Simplex Virus Vaccine Trial. *International Journal of STD and AIDS* **2013**, *24*, 345–349, doi:10.1177/0956462412472822.

66. Issakwisa, H.M.; Mbwile, G.R.; Mbwanji, G.F.; Nassoro, D.D.; Ntinginya, N.E.; Nsojo, A.A. Seroprevalence of Herpes Simplex Virus Type 1 among People Living with HIV in Mbeya, Tanzania. *BMC Infectious Diseases* **2020**, *20*, doi:10.1186/S12879-020-05301-2.

67. Munawwar, A.; Gupta, S.; Sharma, S.K.; Singh, S. Seroprevalence of HSV-1 and 2 in HIV-Infected Males with and without GUD: Study from a Tertiary Care Setting of India. *Journal of Laboratory Physicians* **2018**, *10*, 326–331, doi:10.4103/JLP.JLP_7_18.

68. Singh, N.; Tscharke, D.C. Herpes Simplex Virus Latency Is Noisier the Closer We Look. *Journal of Virology* **2020**, *94*, doi:10.1128/JVI.01701-19.

69. JP, S.; A, M. Herpes Simplex Virus Encephalitis Update. *Current opinion in infectious diseases* **2019**, *32*, 239–243, doi:10.1097/QCO.0000000000000554.

70. Bradshaw, M.J.; Venkatesan, A. Herpes Simplex Virus-1 Encephalitis in Adults: Pathophysiology, Diagnosis, and Management. *Neurotherapeutics* **2016**, *13*, 493–508, doi:10.1007/S13311-016-0433-7.

71. Roberto, L.; Pierlanfranco, D.; Annalisa, B.; Cristina, M.; Fulvia, M.; Chiara, R.; Ludovica, S.; Giuseppe, G.; Marta, M.; Marco, C. Herpes Simplex Virus (HSV) Pneumonia in the Non-Ventilated Immunocompromised Host: Burden and Predictors. *Journal of Infection* **2019**, *78*, 127–133, doi:10.1016/J.JINF.2018.09.010.

72. Calix, R.X.; Loeliger, K.B.; Burn, M.S.; Campbell, K.H. Acute Herpes Simplex Virus Hepatitis in Pregnancy. *Obstetrics and gynecology* **2020**, *135*, 396–400, doi:10.1097/AOG.0000000000003613.

73. Ahmad, I.; Wilson, D.W. Hsv-1 Cytoplasmic Envelopment and Egress. *International Journal of Molecular Sciences* **2020**, *21*, 1–34, doi:10.3390/IJMS21175969.

74. X, X.; Y, C.; Q, L. HSV-1 Tegument Protein and the Development of Its Genome Editing Technology. *Virology journal* **2016**, *13*, doi:10.1186/S12985-016-0563-X.

75. Heming, J.D.; Conway, J.F.; Homa, F.L. Herpesvirus Capsid Assembly and DNA Packaging. *Advances in Anatomy Embryology and Cell Biology* **2017**, *223*, 119–142, doi:10.1007/978-3-319-53168-7_6.

76. O, K.; A, A. The Fate of Incoming HSV-1 Genomes Entering the Nucleus. *Current issues in molecular biology* **2021**, *41*, 221–266, doi:10.21775/CIMB.041.221.

77. Arii, J.; Kawaguchi, Y. The Role of HSV Glycoproteins in Mediating Cell Entry. *Advances in Experimental Medicine and Biology* **2018**, *1045*, 3–21, doi:10.1007/978-981-10-7230-7_1.

78. Praena, B.; Bello-Morales, R.; López-Guerrero, J.A. Hsv-1 Endocytic Entry into a Human Oligodendrocytic Cell Line Is Mediated by Clathrin and Dynamin but Not Caveolin. *Viruses* **2020**, *12*, doi:10.3390/V12070734.

79. Campadelli-Fiume, G.; Menotti, L.; Avitabile, E.; Gianni, T. Viral and Cellular Contributions to Herpes Simplex Virus Entry into the Cell. *Current Opinion in Virology* **2012**, *2*, 28–36, doi:10.1016/J.COVIRO.2011.12.001.

80. Rajčáni, J.; Andrea, V.; Ingeborg, R. Peculiarities of Herpes Simplex Virus (HSV) Transcription: An Overview. *Virus Genes* **2004**, *28*, 293–310, doi:10.1023/B:VIRU.0000025777.62826.92.

81. Dremel, S.E.; DeLuca, N.A. Genome Replication Affects Transcription Factor Binding Mediating the Cascade of Herpes Simplex Virus Transcription. *Proceedings of the National Academy of Sciences of the United States of America* **2019**, *116*, 3734–3739, doi:10.1073/PNAS.1818463116.

82. Bigalke, J.M.; Heuser, T.; Nicastro, D.; Heldwein, E.E. Membrane Deformation and Scission by the HSV-1 Nuclear Egress Complex. *Nature Communications* **2014**, *5*, doi:10.1038/NCOMMS5131.

83. Banerjee, A.; Kulkarni, S.; Mukherjee, A. Herpes Simplex Virus: The Hostile Guest That Takes Over Your Home. *Frontiers in Microbiology* **2020**, *11*, doi:10.3389/FMICB.2020.00733.

84. Wilcox, D.R.; Longnecker, R. The Herpes Simplex Virus Neurovirulence Factor Γ34.5: Revealing Virus–Host Interactions. *PLoS Pathogens* **2016**, *12*, doi:10.1371/JOURNAL.PPAT.1005449.

85. Brown, S.M.; MacLean, A.R.; McKie, E.A.; Harland, J. The Herpes Simplex Virus Virulence Factor ICP34.5 and the Cellular Protein MyD116 Complex with Proliferating Cell Nuclear Antigen through the 63-Amino-Acid Domain Conserved in ICP34.5, MyD116, and GADD34. *Journal of Virology* **1997**, *71*, 9442–9449, doi:10.1128/JVI.71.12.9442-9449.1997.

86. Orvedahl, A.; Alexander, D.; Tallóczy, Z.; Sun, Q.; Wei, Y.; Zhang, W.; Burns, D.; Leib, D.A.; Levine, B. HSV-1 ICP34.5 Confers Neurovirulence by Targeting the Beclin 1 Autophagy Protein. *Cell Host and Microbe* **2007**, *1*, 23–35, doi:10.1016/J.CHOM.2006.12.001.

87. Manivanh, R.; Mehrbach, J.; Knipe, D.M.; Leib, D.A. Role of Herpes Simplex Virus 1 Γ34.5 in the Regulation of IRF3 Signaling. *Journal of Virology* **2017**, *91*, doi:10.1128/JVI.01156-17.

88. Burgos, J.S.; Serrano-Saiz, E.; Sastre, I.; Valdivieso, F. ICP47 Mediates Viral Neuroinvasiveness by Induction of TAP Protein Following Intravenous Inoculation of Herpes Simplex Virus Type 1 in Mice. *Journal of NeuroVirology* **2006**, *12*, 420–427, doi:10.1080/13550280601009546.

89. Liu, B.L.; Robinson, M.; Han, Z.Q.; Branston, R.H.; English, C.; Reay, P.; McGrath, Y.; Thomas, S.K.; Thornton, M.; Bullock, P.; et al. ICP34.5 Deleted Herpes Simplex Virus with Enhanced Oncolytic, Immune Stimulating, and Anti-Tumour Properties. *Gene Therapy* 2003, *10*, 292–303.

90. KF, B.; Z, Y.; DH, D.; DM, K. Identification of a Divalent Metal Cation Binding Site in Herpes Simplex Virus 1 (HSV-1) ICP8 Required for HSV Replication. *Journal of virology* **2012**, *86*, 6825–6834, doi:10.1128/JVI.00374-12.

91. Weerasooriya, S.; DiScipio, K.A.; Darwish, A.S.; Bai, P.; Weller, S.K. Herpes Simplex Virus 1 ICP8 Mutant Lacking Annealing Activity Is Deficient for Viral DNA Replication. *Proceedings of the National Academy of Sciences of the United States of America* **2019**, *116*, 1033–1042, doi:10.1073/PNAS.1817642116.

92. Darwish, A.S.; Grady, L.M.; Bai, P.; Weller, S.K. ICP8 Filament Formation Is Essential for Replication Compartment Formation during Herpes Simplex Virus Infection. *Journal of Virology* **2016**, *90*, 2561–2570, doi:10.1128/JVI.02854-15.

93. Song, B.; Liu, X.; Wang, Q.; Zhang, R.; Yang, T.; Han, Z.; Xu, Y. Adenovirus-Mediated ShRNA Interference against HSV-1 Replication in Vitro. *Journal of NeuroVirology* **2016**, *22*, 799–807, doi:10.1007/S13365-016-0453-4.

94. E, K.; SJ, R. History of Oncolytic Viruses: Genesis to Genetic Engineering. *Molecular therapy : the journal of the American Society of Gene Therapy* **2007**, *15*, 651–659, doi:10.1038/SJ.MT.6300108.

95. Kaufman, H.L.; Kohlhapp, F.J.; Zloza, A. Oncolytic Viruses: A New Class of Immunotherapy Drugs. *Nature Reviews Drug Discovery* 2015, *14*, 642–662.

96. Nguyen, T.; Avci, N.G.; Shin, D.H.; Martinez-Velez, N.; Jiang, H. Tune Up In Situ Autovaccination against Solid Tumors with Oncolytic Viruses. *Cancers* **2018**, *10*, doi:10.3390/CANCERS10060171.

97. Puzanov, I.; Milhem, M.M.; Minor, D.; Hamid, O.; Li, A.; Chen, L.; Chastain, M.; Gorski, K.S.; Anderson, A.; Chou, J.; et al. Talimogene Laherparepvec in Combination With Ipilimumab in Previously Untreated, Unresectable Stage IIIB-IV Melanoma. *Journal of Clinical Oncology* **2016**, *34*, 2619, doi:10.1200/JCO.2016.67.1529.

98. AM, N.; MR, F.; SM, G.; Y, H.; S, T.; D, A.; S, M.; KK, C.; S, P.; S, A.; et al. Randomized Phase 2 Trial of the Oncolytic Virus Pelareorep (Reolysin) in Upfront Treatment of Metastatic Pancreatic Adenocarcinoma. *Molecular therapy : the journal of the American Society of Gene Therapy* **2016**, *24*, 1150–1158, doi:10.1038/MT.2016.66.

99. YR, S.; T, Z.; K, E. Oncolytic Viruses: Emerging Options for the Treatment of Breast Cancer. *Medical oncology (Northwood, London, England)* **2017**, *34*, doi:10.1007/S12032-017-0899-0.

100. AH, J.; F, E.-M.; RG, V.; PJ, S.; AA, M.; S, G. Progress in Clinical Oncolytic Virus-Based Therapy for Hepatocellular Carcinoma. *The Journal of general virology* **2015**, *96*, 1533–1550, doi:10.1099/VIR.0.000098.

101. Bretscher, C.; Marchini, A. H-1 Parvovirus as a Cancer-Killing Agent: Past, Present, and Future. *Viruses* **2019**, *11*, doi:10.3390/V11060562.

102. Leber, M.F.; Neault, S.; Jirovec, E.; Barkley, R.; Said, A.; Bell, J.C.; Ungerechts, G. Engineering and Combining Oncolytic Measles Virus for Cancer Therapy. *Cytokine and Growth Factor Reviews* **2020**, *56*, 39–48, doi:10.1016/J.CYTOGFR.2020.07.005.

103. Kurisetty, V.V.S.; Heiber, J.; Myers, R.; Pereira, G.S.; Goodwin, J.W.; Federspiel, M.J.; Russell, S.J.; Peng, K.W.; Barber, G.; Merchan, J.R. Preclinical Safety and Activity of Recombinant VSV-IFN-β in an Immunocompetent Model of Squamous Cell Carcinoma of the Head and Neck. *Head and Neck* **2014**, *36*, 1619–1627, doi:10.1002/HED.23502.

104. JF, H.; GN, B. Vesicular Stomatitis Virus Expressing Tumor Suppressor P53 Is a Highly Attenuated, Potent Oncolytic Agent. *Journal of virology* **2011**, *85*, 10440–10450, doi:10.1128/JVI.05408-11.

105. L, R.; J, S.; AC, J.-R.; Y, W.; A, S.; Y, B.-S.; JY, Y.; GM, S.; R, K.; J, Z.; et al. PTEN Expression by an Oncolytic Herpesvirus Directs T-Cell Mediated Tumor Clearance. *Nature communications* **2018**, *9*, doi:10.1038/S41467-018-07344-1.

106. Béguin, J.; Foloppe, J.; Maurey, C.; Laloy, E.; Hortelano, J.; Nourtier, V.; Pichon, C.; Cochin, S.; Cordier, P.; Huet, H.; et al. Preclinical Evaluation of the Oncolytic Vaccinia Virus TG6002 by

Translational Research on Canine Breast Cancer. *Molecular Therapy - Oncolytics* **2020**, *19*, 57–66, doi:10.1016/J.OMTO.2020.08.020.

107. Ghouse, S.M.; Nguyen, H.M.; Bommareddy, P.K.; Guz-Montgomery, K.; Saha, D. Oncolytic Herpes Simplex Virus Encoding IL12 Controls Triple-Negative Breast Cancer Growth and Metastasis. *Frontiers in Oncology* **2020**, *10*, doi:10.3389/FONC.2020.00384.

108. Heiniö, C.; Havunen, R.; Santos, J.; de Lint, K.; Cervera-Carrascon, V.; Kanerva, A.; Hemminki, A. TNFa and IL2 Encoding Oncolytic Adenovirus Activates Pathogen and Danger-Associated Immunological Signaling. *Cells* **2020**, *9*, doi:10.3390/CELLS9040798.

109. Fukuhara, H.; Ino, Y.; Todo, T. Oncolytic Virus Therapy: A New Era of Cancer Treatment at Dawn. *Cancer Science* 2016, *107*, 1373–1379.

110. Ferguson, M.S.; Lemoine, N.R.; Wang, Y. Systemic Delivery of Oncolytic Viruses: Hopes and Hurdles. *Advances in Virology* **2012**, *2012*, doi:10.1155/2012/805629.

111. Y, K.; JD, D.; D, K.; BC, F.; JM, G.; T, F.; T, K.; M, L.; KK, T. Comparison of Intravenous versus Intraperitoneal Administration of Oncolytic Herpes Simplex Virus 1 for Peritoneal Carcinomatosis in Mice. *Cancer gene therapy* **2009**, *16*, 291–297, doi:10.1038/CGT.2008.83.

112. Tsai, V.; Johnson, D.E.; Rahman, A.; Wen, S.F.; LaFace, D.; Philopena, J.; Nery, J.; Zepeda, M.; Maneval, D.C.; Demers, G.W.; et al. Impact of Human Neutralizing Antibodies on Antitumor Efficacy of an Oncolytic Adenovirus in a Murine Model. *Clinical Cancer Research* **2004**, *10*, 7199–7206, doi:10.1158/1078-0432.CCR-04-0765.

113. Alemany, R.; Suzuki, K.; Curiel, D.T. Blood Clearance Rates of Adenovirus Type 5 in Mice. *Journal of General Virology* **2000**, *81*, 2605–2609, doi:10.1099/0022-1317-81-11-2605.

114. Luo, Y.; Lin, C.; Ren, W.; Ju, F.; Xu, Z.; Liu, H.; Yu, Z.; Chen, J.; Zhang, J.; Liu, P.; et al. Intravenous Injections of a Rationally Selected Oncolytic Herpes Virus as a Potent Virotherapy for Hepatocellular Carcinoma. *Molecular Therapy - Oncolytics* **2019**, *15*, 153–165, doi:10.1016/J.OMTO.2019.09.004.

115. Bradley, H.; Markowitz, L.E.; Gibson, T.; McQuillan, G.M. Seroprevalence of Herpes Simplex Virus Types 1 and 2—United States, 1999–2010. *The Journal of Infectious Diseases* **2014**, *209*, 325–333, doi:10.1093/INFDIS/JIT458.

116. Russell, S.J.; Barber, G.N. Oncolytic Viruses as Antigen-Agnostic Cancer Vaccines. *Cancer Cell* **2018**, *33*, 599–605, doi:10.1016/J.CCELL.2018.03.011.

117. Andtbacka, R.H.I.; Ross, M.; Puzanov, I.; Milhem, M.; Collichio, F.; Delman, K.A.; Amatruda, T.; Zager, J.S.; Cranmer, L.; Hsueh, E.; et al. Patterns of Clinical Response with Talimogene Laherparepvec (T-VEC) in Patients with Melanoma Treated in the OPTiM Phase III Clinical Trial. *Annals of Surgical Oncology* **2016**, *23*, 4169–4177, doi:10.1245/s10434-016-5286-0.

118. Chastkofsky, M.I.; Pituch, K.C.; Katagi, H.; Zannikou, M.; Ilut, L.; Xiao, T.; Han, Y.; Sonabend, A.M.; Curiel, D.T.; Bonner, E.R.; et al. Mesenchymal Stem Cells Successfully Deliver Oncolytic Virotherapy to Diffuse Intrinsic Pontine Glioma. *Clinical Cancer Research* **2020**, doi:10.1158/1078-0432.ccr-20-1499.

119. Smith, C.A.; Bates, P.; Rivera-Gonzalez, R.; Gu, B.; DeLuca, N.A. ICP4, the Major Transcriptional Regulatory Protein of Herpes Simplex Virus Type 1, Forms a Tripartite Complex with TATA-Binding Protein and TFIIB. *Journal of Virology* **1993**, *67*, 4676–4687, doi:10.1128/JVI.67.8.4676-4687.1993.

120. Tischer, B.K.; von Einem, J.; Kaufer, B.; Osterrieder, N. Two-Step Red-Mediated Recombination for Versatile High-Efficiency Markerless DNA Manipulation in Escherichia Coli. *BioTechniques* **2006**, *40*, 191–197, doi:10.2144/000112096.

121. Krenn, V.; Bosone, C.; Burkard, T.R.; Spanier, J.; Kalinke, U.; Calistri, A.; Salata, C.; Rilo Christoff, R.; Pestana Garcez, P.; Mirazimi, A.; et al. Organoid Modeling of Zika and Herpes Simplex Virus 1 Infections Reveals Virus-Specific Responses Leading to Microcephaly. *Cell Stem Cell* **2021**, *28*, 1362-1379.e7, doi:10.1016/J.STEM.2021.03.004.

122. S, Z.; P, K.-D.; D, K.; A, S.; V, F.; K, B.; S, O.; N, M.; S, C.-K.; P, V. de P.; et al. Combined Testing for Herpes Simplex Virus and Mycobacterium Tuberculosis DNA in Cerebrospinal Fluid of Patients with Aseptic Meningitis in Burkina Faso, West Africa. *Journal of clinical laboratory analysis* **2019**, *33*, doi:10.1002/JCLA.22719.

123. Bunuales, M.; Garcia-Aragoncillo, E.; Casado, R.; Quetglas, J.I.; Hervas-Stubbs, S.; Bortolanza, S.; Benavides-Vallve, C.; Ortiz-De-Solorzano, C.; Prieto, J.; Hernandez-Alcoceba, R. Evaluation of Monocytes as Carriers for Armed Oncolytic Adenoviruses in Murine and Syrian Hamster Models of Cancer. *Human Gene Therapy* **2012**, *23*, 1258–1268, doi:10.1089/hum.2012.043.

124. Peng, K.W.; Dogan, A.; Vrana, J.; Liu, C.; Ong, H.T.; Kumar, S.; Dispenzieri, A.; Dietz, A.B.; Russell, S.J. Tumor-Associated Macrophages Infiltrate Plasmacytomas and Can Serve as Cell Carriers for Oncolytic Measles Virotherapy of Disseminated Myeloma. *American Journal of Hematology* **2009**, *84*, 401–407, doi:10.1002/ajh.21444.

125. Grosche, L.; Kummer, M.; Steinkasserer, A. What Goes around, Comes around - HSV-1 Replication in Monocyte-Derived Dendritic Cells. *Frontiers in Microbiology* **2017**, *8*, doi:10.3389/FMICB.2017.02149.

126. R, R.; G, C.; V, P.; J, N.; D, H.; D, B.; R, P.; A, M.; J, H.; E, M.; et al. Toxicity Evaluation of Replication-Competent Herpes Simplex Virus (ICP 34.5 Null Mutant 1716) in Patients with Recurrent Malignant Glioma. *Gene therapy* **2000**, *7*, 859–866, doi:10.1038/SJ.GT.3301184.

Final remark

Se le cellule *carrier* sono dei "cavalli di Troia" che permettono ai virus oncolitici di entrare nel microambiente tumorale, ci aiuterà certamente leggere come il poeta descriveva la costruzione dell'originale:

[...]Fracti bello fatisque repulsi
ductores Danaum, tot iam labentibus annis,
instar montis equum divina Palladis arte
aedificant sectaque intexunt abiete costas:
votum pro reditu simulant, ea fama vagatur.
Huc delecta virum sortiti corpora furtim
includunt caeco lateri penitusque cavernas
ingentis uterumque armato milite complent.

Virgilio, *Eneide,* II

Reale et al. Infectious Agents and Cancer (2019) 14:5
https://doi.org/10.1186/s13027-018-0218-1

Infectious Agents and Cancer

REVIEW Open Access

Perspectives on immunotherapy via oncolytic viruses

Alberto Reale, Adriana Vitiello, Valeria Conciatori, Cristina Parolin, Arianna Calistri and Giorgio Palù*

Abstract

Background: With few exceptions, current chemotherapy and radiotherapy protocols only obtain a slightly prolonged survival with severe adverse effects in patients with advanced solid tumors. In particular, most solid malignancies not amenable to radical surgery still carry a dismal prognosis, which unfortunately is also the case for relapsing disease after surgery. Even though targeted therapies obtained good results, clinical experience showed that tumors eventually develop resistance. On the other hand, earlier attempts of cancer immunotherapy failed to show consistent efficacy. More recently, a deeper knowledge of immunosuppression in the tumor microenvironment (TME) allowed the development of effective drugs: in particular, monoclonal antibodies targeting the so-called immune checkpoint molecules yielded striking and lasting effects in some tumors. Unfortunately, these monoclonal antibodies are not effective in a majority of patients and are ineffective in several solid malignancies. Furthermore, due to their mechanism of action, checkpoint inhibitors often elicit autoimmune-like disease.

Main body: The use of viruses as oncolytic agents (OVs) was considered in the past, while only recently OVs revealed a connection with immunotherapy. However, their antitumoral potential has remained largely unexplored, due to safety concerns and some limitations in the techniques to manipulate viruses. OV research was recently revived by a better knowledge of viral/cancer biology and advances in the methodologies to delete virulence/immune-escape related genes from even complex viral genomes or "to arm" OVs with appropriate transgenes. Recently, the first oncolytic virus, the HSV-1 based Talimogene Laherparepvec (T-VEC), was approved for the treatment of non-resectable melanoma in USA and Europe.

Conclusion: OVs have the potential to become powerful agents of cancer immune and gene therapy. Indeed, in addition to their selective killing activity, they can act as versatile gene expression platforms for the delivery of therapeutic genes. This is particularly true for viruses with a large DNA genome, that can be manipulated to address the multiple immunosuppressive features of the TME. This review will focus on the open issues, on the most promising lines of research in the OV field and, more in general, on how OVs could be improved to achieve real clinical breakthroughs in cancers that are usually difficult to treat by immunotherapy.

Keywords: Oncolytic virus, Oncolytic virotherapy, Cancer immunotherapy, Cancer gene therapy, Oncolytic HSV-1, Tumor microenvironment

Background

The pharmacological therapy of cancer represents one of the greatest challenges for contemporary medicine. State-of-the-art chemotherapy and radiotherapy protocols can be curative in some hematologic malignancies, such as Hodgkin lymphoma and acute lymphoid leukemia (ALL), and can be successfully combined with other therapeutic solutions like autologous stem cell transplantation [1, 2]. Targeted therapies have also emerged that changed the natural course of diseases like chronic myeloid leukemia or promyelocytic myeloid leukemia [3, 4]. Even for ALL resistant to current therapies, the use of chimeric antigen receptor (CAR)-T cellular therapy provided a major breakthrough [5].

The situation is much bleaker for non-hematologic neoplasms. With very few exceptions, in this case, the hope of a cure rests mainly on the possibility of a radical surgical excision at the moment of diagnosis. If this is not possible, due to extensive local invasion or metastatic dissemination,

* Correspondence: giorgio.palu@unipd.it
Department of Molecular Medicine, University of Padua, Via A. Gabelli, 63, 35121 Padua, Italy

prognosis remains dismal [6, 7]. Great expectations were associated with targeted therapies, such as small molecule tyrosine kinase inhibitors (TKIs) or monoclonal antibodies directed against receptors overexpressed by cancer cells. Even though these approaches obtained good results in selected patients, in terms of prolonged survival, with a good toxicity profile, it soon became evident that tumors usually develop resistance [8, 9].

Another possible therapeutic strategy is immunotherapy. Although it has been known for quite a long time that the immune system can recognize and kill cancer cells, previous attempts of immunotherapy based on the administration of recombinant cytokines, anti-cancer vaccines or in vitro expanded tumor infiltrating lymphocytes (TILs) did not provide enough efficacy [10, 11]. Still, there were some remarkable exceptions, as a small subset of metastatic melanoma and of clear cell renal carcinoma patients showed long-term remissions after treatment with high doses of recombinant interleukin 2 (rIL-2) [12]. In recent years, new light was shed on mechanisms involved in cancer immunology, and, especially, on the immunosuppressive features of the tumor microenvironment (TME), which mediate escape from tumoricidal immune responses. In particular, cancer has the ability to exploit mechanisms involved in the maintenance of immune peripheral tolerance, either i) directly, by expressing immune checkpoint molecule ligands which dampen the activity of cytotoxic T cells, such as Programmed Death Ligand-1 (PDL-1), or ii) indirectly, by recruiting immune cells with immunosuppressive features, such as CD4+ CD25+ Foxp3+ T regulatory cells (Tregs), immature myeloid-derived suppressor cells (MDSCs), or M2 macrophages [13, 14]. These cells usually express checkpoint molecule ligands and secrete soluble cytokines (e.g. IL-10) or enzymes (arginase and IDO) that hinder cytotoxic T responses. These and other actors, like cancer associated fibroblasts and downregulation of MHC class I molecules by cancer cells, are probably playing a role in TME immunosuppression. Based on these considerations, new cancer immunotherapies were developed, based on checkpoint inhibition by means of monoclonal antibodies directed against Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), Programmed Death-1 (PD-1), or its ligands PDL-1 and PDL-2 [15]. Anti-CTLA-4 humanized antibodies, as ipilimumab, were the first to show therapeutic efficacy against melanoma [16]. On the other hand, anti-PD1 and anti-PDL1 mAbs seem to have a broader spectrum of action (including NSCLC and possibly small subsets of pancreatic and breast cancer), while triggering less autoimmune toxicity [17]. However, also in cancer types considered susceptible to checkpoint inhibitors, more than 50% of patients fail to respond to treatment. In this context, the combination of different checkpoint inhibitors (anti-CTLA-4 and anti-PD1 Abs) yielded better results in melanoma patients, but with increased toxicity [18].

Oncolytic viruses (OVs) are defined as viruses able to selectively replicate in and kill cancer cells [19]. The history of OVs is quite long, since already at the beginning of the twentieth century physicians observed that cancer patients experienced partial disease remissions after natural infections [20]. It was, therefore, hypothesized that cancer cells were somehow more vulnerable to viral infections, and that attenuated viral strains could be used in cancer therapy. However, many factors, including safety concerns, the development of cytotoxic chemotherapy, and the lack of tools to manipulate viruses, hindered research in this field. In recent years OV studies were revived by better knowledge of viral gene function and advancements in molecular biology, which allow precise modifications of viral genomes to maximize both efficacy and safety. Over the last years a new paradigm emerged according to which OVs might also function as a form of immunotherapy [21]. Indeed, it has been shown that the proinflammatory stimuli provided by viruses can overcome the TME immunosuppression and, thereby, elicit a systemic antitumoral immune response. Such a response was observed also when OVs were injected locally (intratumoral injection), rather than systemically [22]. It was demonstrated that the first OV approved for cancer treatment in North America and Europe, the HSV-1 based talimogene laherparepvec (T-VEC), has an immunological mechanism of action, which also causes the regression of uninjected and uninfected metastases [23].

Nevertheless, OVs are still not powerful enough, especially for scarcely immunogenic or immunosuppressive solid tumors, which unfortunately are quite frequent in the population, like pancreatic adenocarcinoma, triple negative breast cancer, hepatocellular carcinoma [24–26]. This lack of efficacy is somehow unexpected, as OVs should make the TME significantly more immunogenic due to inflammation and the presence of viral antigens. Such a consideration fuels the feeling that major improvements in the OV therapy field are at hand.

This review will focus on open issues regarding OVs, and especially their interaction with the TME and the host immune system. The answer to these questions will probably be crucial to fully exploit the therapeutic potential of OVs.

Main text

As explained above, OVs are emerging as a new, promising form of immunotherapy. In recent years a remarkable array of different OVs has been tested in preclinical cancer models or in phase I/II clinical trials [27]. This plethora of viruses includes, among the others, attenuated strains of human pathogens, such as adenoviruses (AdVs) [28],

herpes simplex type 1 (HSV-1) [29], vaccinia virus (VACV) [30], measles [31], mumps virus [32] and influenza A virus [33], or viruses that are naturally poorly pathogenic for humans, including the orthoreovirus strain T3D [34], Newcastle Disease Virus (NDV) [35], vesicular stomatitis virus (VSV) [36], Maraba Virus [37], the rodent H-1 parvovirus [38] and the picornavirus Mengovirus [39], a long list far from being complete [Table 1]. Perhaps the most striking common feature of these heterogeneous OVs is their outstanding safety profile. Indeed, severe adverse effects were very rare and it was unusual that therapy had to be discontinued due to toxicity [40]. Unfortunately, safety was not always matched by efficacy, and so far only the HSV-1 based talimogene laherparepvec was effective enough to be authorized for routine clinical use. Also, efficacy was markedly higher in immunogenic tumors such as melanoma [41].

Therefore, despite the fact that OVs hold great therapeutic potential, it is clear that they need to be further improved. A better understanding of their in vivo mechanisms of action and pharmacokinetics, as well as a clearer picture of the complex interplay between viruses and host are some of the crucial aspects to be further elucidated to design safer and more effective OVs. Under this respect, different questions remain to be addressed:

1. How "attenuated" should an attenuated OV be? The question may sound trivial, the answer being "attenuated enough to replicate only in cancer cells". However, the application of this oversimplified principle can have dire consequences, as it does not consider the real complexity of tumors. Many OVs were designed to be able to replicate only in actively dividing cells, for example by deletion of specific genes (like HSV or VACV thymidine kinase and ribonucleotide reductase), according to the idea that cancer cells are actively replicating while healthy cells are not [42]. Unfortunately, many cancer cells within a tumor mass are not undergoing replication. To make things worse, many non-tumoral cells are present in the TME (including macrophages, endothelial cells, lymphocytes, fibroblasts, MDSCs). It has been shown that these cells do not support the replication of OVs designed according to the aforementioned principle and can, therefore, protect malignant cells from viral diffusion [43]. Furthermore, due to the well known cancer heterogeneity, it is hazardous to assume that all cancer cells in all patients will display the same specific molecular characteristic, like mutations within certain oncosuppressor genes and/or overexpression of single pathways [44] and to assume that OV specificity towards cancer cells could rely only on these features [45, 46]. Indeed, evidence demonstrate that the increased susceptibility of cancer cells towards viral replication is the result of variable combinations of alterations mainly in antiviral response and cell cycle regulation pathways. Thus, a more sensible approach for generating OVs, when starting from well-known human pathogens, might be to attenuate them in such a way that they cannot cause the dangerous forms of disease they are associated with (Table 1). In the case of HSV-1, in immunocompetent adults nearly all severe morbidity and mortality is caused by dissemination and replication in neurons, resulting in encephalitis. Therefore, genome modifications that attenuate HSV-1 virulence in neurons might be sufficient to generate a safe OV, despite the fact that the virus retains, at least partially, its ability to replicate in "healthy" fibroblasts or epithelial cells. This feature might even be useful, as it enables the virus to be more effective in the TME, as appears to be in the case of T-VEC.
2. What defines a solid in vitro model to screen for selectivity of OVs towards cancer cells? This question persists despite the fact that, according to what we have suggested above, the focus could be shifted from "unable to replicate in nonmalignant cells" to "unable to replicate in specific target cells relevant for human disease" (neurons in the case of HSV). The problem is associated with the definition itself of "nonmalignant" applied to tissue culture cells. Cell lines, even when incapable of forming tumors once inoculated in immunosuppressed mice, are often immortalized and have very different features from their in vivo counterparts, which can lead to OVs replication in these "healthy" cells. Furthermore, cell lines (including cancer cell lines) are often unpredictable in their susceptibility to viral replication: even viruses with broad cell tropism will occasionally produce very low titers in some cell lines [unpublished observations]. This raises the issue of finding a real "healthy" cell line in which the OV under evaluation is not replicating because the cell line does not have malignant characteristics, rather than because that cell line is characterized by refractoriness to that virus. Primary cells, although technically more demanding, could partially overcome some of these difficulties. On the other hand, organoids derived from malignant and healthy tissue, that are becoming a widely employed in vitro model for several types of studies, would have the further advantage of letting cells grow in a 3D environment, more closely mimicking the in vivo situation [47]. Thus, organoids might represent

Table 1 A necessarily incomplete overview of currently investigated or clinically available oncolytic viruses

	Adenoviruses (AdVs)	Herpes simplex virus- 1 (HSV-1)	H-1 Parvovirus	Vaccinia virus (VACV)	Measles virus (MeV)	Maraba virus (MARV)	Orthoreovirus (T3D)
Family	*Adenoviridae*	*Herpesviridae*	*Parvoviridae*	*Poxviridae*	*Paramyxoviridae*	*Rhabdoviridae*	*Reoviridae*
Nucleic acid	dsDNA	dsDNA	ssDNA	dsDNA	ssRNA, negative sense	ssRNA, negative sense	dsRNA, segmented
Genome length	~ 30–35 kb	~ 150 kb	~ 5 kb	~ 190 kb	~ 15–16 kb	~ 11 kb	~ 23 kb
Wild-type virus associated diseases	Depending on serotypes, common cause of mild community-acquired respiratory, ocular, gastrointestinal infections.	Immunocompetent host: primary gingivostomatitis or genital lesions, reactivation from latency (cold sores). Occasionally encephalitis. Immunocompromised host: disseminated disease, multiorgan involvement.	Rodent virus. No disease in humans during clinical trials.	Strains derived from animal poxviruses, used as smallpox vaccine. Usually mild local reaction at the site of inoculation. In immunocompromised hosts severe, progressive disease (vaccinia necrosum).	Measles. Severe complications include giant cell pneumonia, subacute sclerosing panencephalitis (SSPE).	Virus isolated from a brazilian sandfly. Limited evidence of natural infection in humans.	Infection usually asymptomatic.
Available therapy	Live vaccine employed by the US army. No established therapy	Highly effective nucleoside analogues (acyclovir, famciclovir, penciclovir, etc.).	None	Disease rare due to smallpox vaccine programs interruption. Cidofovir possibly active.	Measles-mumps-rubella (MMR) vaccine. Ribavirin possibly useful in severe infections.	None	None
Examples of eploited oncolytic attenuation strategies	E1B55K deletion restricts replication to p53-deficient cells; E3 deletion; E1ACR2 deletion Viral retargeting to receptors expressed only on cancer cells.	Γ34.5 deletion abolishes neurovirulence. UL23 (thymidilate kinase) and ICP6 (ribonucleotide reductase) deletion limit replication to actively dividing cells. Viral retargeting to receptors expressed only on cancer cells.	Not needed – virus does not replicate in healthy human cells and needs actively dividing cells.	Thymidine kinase gene deletions restrict replication to dividing cells.	Use of attenuated vaccine strains (e.g Edmonston strain) as oncolytic agents.	Double mutant strain with mutations in G protein (Q242R) and M protein (L123 W) reportedly oncotropic.	Not needed – virus does not cause significant disease in humans.
Clinical advancement stage	Many phase I or phase I/II clinical trials ongoing for several malignancies including lung, ovarian, pancreatic cancer, glioblastoma and melanoma	Talimogene laherparepvec (®Imlygic, Amgen) approved in the US and EU for metastatic melanoma in the wake of a phase 3 clinical trial (NCT00769704)	Phase I/II clinical trial in primary or recurrent glioblastoma multiforme (NCT01301430)	Phase 3 randomized clinical trial for hepatocellular carcinoma, Pexa-Vec (NCT02562755)	Phase I and II clinical trials with different kinds of tumors, including ovarian cancer, multiple myeloma, and pleural mesothelioma.	Three currently recruiting, open label phase I/II clinical trials for MAGE-A3 expressing solid tumors, non small cell lung cancer and HPV associated tumors (NCT02285816; NCT02879760; NCT03618953)	Several phase I and II clinical trials. One phase 3 clinical trial in association with chemotherapy for head and neck cancer (NCT01166542)

Features described include the genome type and length (which gives an idea of viral capacity as gene therapy vectors), wild-type virus pathogenicity, availability of effective therapies for a "worst case scenario", the main strategies devised to make viruses selective for cancer cells, and eventually the clinical trial stage reached by viral vectors. ssDNA= single stranded deoxyribonucleic acid; dsDNA= double stranded DNA; ssRNA= single stranded ribonucleic acid; dsRNA= double stranded RNA

the best in vitro model to test OV specificity towards cancer cells.

3. What is the most appropriate animal model to test safety and efficacy of OVs? Most studies so far relied on SCID or nude mice, which are a readily available model in which murine or human cancer cell lines can thrive because of immunosuppression [48]. Still, this feature (especially the absence of a competent T cell response) profoundly alters the mechanism of action of OVs. In these models, direct oncolysis by the virus could actually be the main effector mechanism that prolongs survival of the animals, which is probably not what happens in human patients. On the other hand, the use of immunocompetent mice could partially overcome this difficulty. However, the differences between the murine and human immune systems are still a hurdle, especially when the ability of the OV of influencing the immune response against cancer cells is under evaluation. Humanized mice, i.e.completely immunodeficient mice which receive a human hematopoietic stem cells (HSC) transplant plus human fetal thymus and liver tissue to guarantee T cell maturation, could provide an answer to this difficult problem [49].
4. What is the exact role of the host immune system/OV interaction in determining the success of virotherapy for tumors? Some investigators argue that the immune system has a deleterious effect, since it could wipe away the OV, especially if deriving from a human pathogen widely present in the population, before it can kill a sufficient number of cancer cells. As a consequence, efforts were spent on concealing the virus from the immune system, or on using viruses for which a preexisting immunity in the general population is unlikely [50]. However, this concept is clearly rooted in the older paradigm of OVs directly killing cancer cells rather than in the recent idea of OVs as tools for immunotherapy. Indeed, recent data strongly suggest that the release of danger signals and inflammation due to OV replication, along with immune system activation against infected cells, account for an important part of the antitumoral potential of the OV itself [51]. Furthermore, in the case of T-VEC, no differences in therapeutic effects were observed between HSV-1 seronegative and seropositive patients. Recently, a study using a mouse model of melanoma treated with the paramyxovirus NDV showed enhanced antitumoral activity in mice with preexisting immunity to NDV [52]. Finally, after intratumoral treatment with T-VEC, it was observed that uninjected lesions, including some visceral metastases, underwent regression. In a recent clinical trial, regression was heralded by activated CD8+ lymphocytes infiltration and was enhanced by checkpoint inhibitors [53]. Such a pattern is consistent with an immune response elicited by viral injection in multiple accessible lesions but effective also against uninjected lesions in which the virus was not detectable.
5. What is the best route of administration for an OV? It is often stated that an ideal OV should be systemically injectable, for some good reasons: essentially, the possibility to infect both primary tumor and metastases, and the fact that this route is relatively non-invasive and injections can be frequently repeated [54]. However, although some OVs (VACV, T3D orthoreovirus, H-1 parvovirus), were administered intravenously to human patients without severe side effects, the most used route is the local (intratumoral) injection [55]. This is the case also for the only approved OV, T-VEC. Intratumoral delivery is usually chosen because of safety concerns after intravenous injection, or, especially in the case of HSV-1, to minimize the chance that preexisting circulating antibodies might neutralize the virus before it reaches its target, as discussed above [56]. Nevertheless, as mentioned above, in the case of T-VEC, despite the intratumoral injection, uninjected lesions and visceral metastases displayed a regression, likely due to the immune response elicited by the virus [41].
6. Viruses have an important feature, which makes them particularly appealing as cancer therapeutics: they are not just cancer cell killers or a proinflammatory stimulus, but they can also serve as platforms for the delivery/expression of transgenes. This feature allows the development of OVs “armed” with therapeutic genes, some of which are already under evaluation in clinical trials. One example is again T-VEC that, in addition to specific mutations within viral genes, carries two copies of the human granulocyte-monocyte colony stimulating factor (hGM-CSF) encoding sequence, under the transcriptional control of the human CMV immediate early promoter [57]. However, most of these engineered viruses only express a single immunostimulatory cytokine or ligand [58, 59]. As a result, these OVs do not exploit the wealth of information that was recently accumulated on cancer immunology and the TME [60], and may even be outdated. For instance, recent comprehensive reviews cast a dubious light on the usefulness of hGM-CSF in cancer immunotherapy [61]. The issue with cancer immunotherapy is not simply boosting a “sleeping” immune response, but the fact that cancer cells actively use

immunosuppressive mechanisms and recruit tolerogenic cells. OVs could be used to locally deliver high and constant concentrations of single-chain antibodies or other protein ligands that disrupt those immunosuppressive features. Under this respect, many different strategies can be devised, including the expression of enzymes that degrade the abundant extracellular matrix present in some tumors (desmoplastic reaction) or of dominant negative forms of immunosuppressive cytokines (for example TGF-β) [62].

7. More specifically, could there be space for engineered OVs expressing checkpoint inhibitors, as single chain antibodies, whole antibodies, or proteins that may have the same function? Potent immune checkpoint inhibitors (CKIs) which are delivered systemically are already available, and most investigators are focusing on synergism between existing checkpoint inhibitors and OVs [63]. However, CKIs expressed as therapeutic genes by OVs would probably have the advantage of a prolonged and localized delivery in the TME, which might, in principle, avoid the autoimmune side effects usually associated with systemic CKIs.
8. Is there the possibility of a more extensive cell-specific reprogramming of viruses? Ideally, once an OV reaches the TME, it should produce different effects in different types of cells. Of course, it should replicate in cancer cells and cause their death while sparing surrounding normal tissue and/or non causing severe diseases. However there could be further nuances. For example, an OV could be designed to specifically trigger a Th1 phenotype in infected macrophages or to replicate also in cancer associated fibroblasts or endothelial cells that might become a more persistent "factory" of therapeutic gene products. Transgenes under the transcriptional control of cell-specific promoter might serve to this end. While "promoter retargeting" has been explored to enhance viral replication in cancer cells, such an approach to the diversity of the TME has not been investigated yet, at least to our knowledge.

Conclusions

Cancer immunotherapy is establishing new paradigms in the treatment of advanced stage solid malignancies. Together with immune checkpoint inhibitors, OVs are increasingly recognized as a promising therapeutic tool in this field. The use of OVs on patients has become a clinical reality in the case of talimogene laherparepvec, also known as T-VEC, for metastatic melanoma, and recent

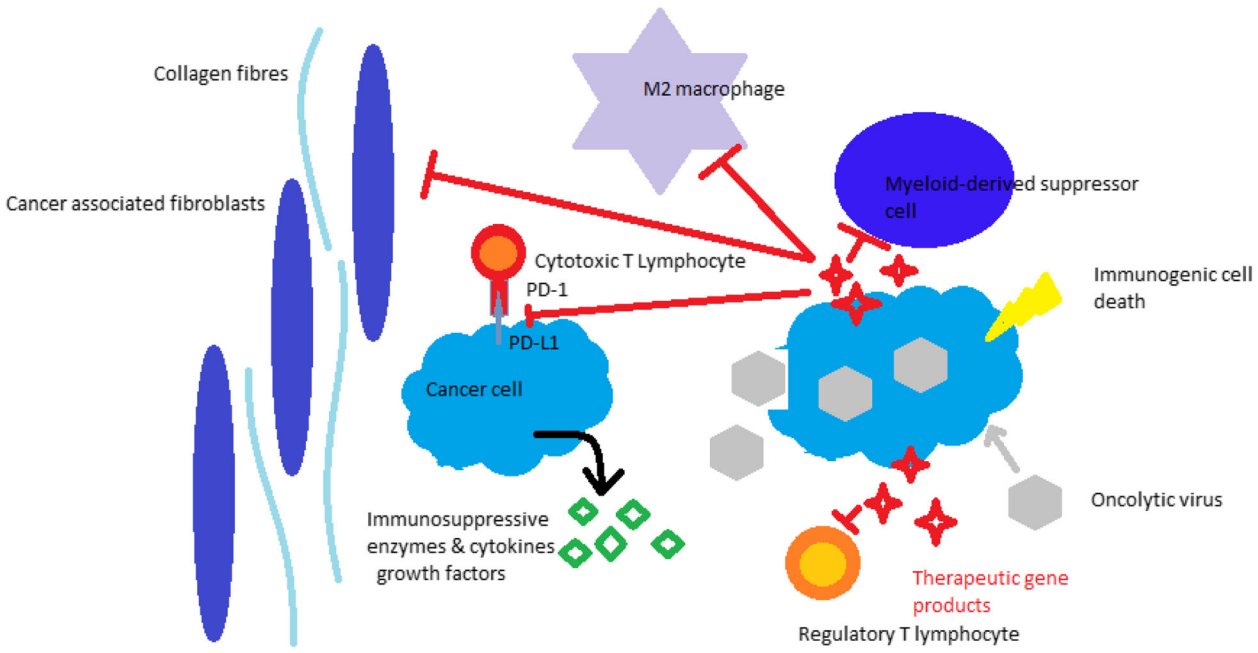


Fig. 1 Schematic representation of cancer cells (cyan) in their immunosuppressive microenvironment, which they shape by secreting cytokines and growth factors. Immune checkpoint molecules and immunosuppressive enzymes released in the milieu inhibit cytotoxic T lymphocytes. Infection of cancer cells by an oncolytic virus (OV, gray) disrupts the immunosuppressive features of the microenvironment by triggering immunogenic cell death and releasing proinflammatory substances. OVs can also be armed with therapeutic genes targeting non malignant cells that support tumor growth and immune escape, such as cancer associated fibroblasts, M2 macrophages, myeloid derived suppressor cells (MDSCs), and regulatory T lymphocytes

clinical trials strongly suggest that the combination of talimogene with CKIs could be particularly effective in this setting [23, 53]. Despite this successes, OV treatment of cancers other than melanoma, which is usually considered a very immunogenic tumor, has given limited clinical results [64, 65]. Several recent studies have been trying to characterize the antitumoral immune response after OV therapy both in mouse models and in patients enrolled in clinical trials [53, 66]. However, current basic and translational research on OVs is mainly focused on safety (which, however, has never been a real issue over decades of clinical trials), on various combinations of OVs with chemo and radiotherapy or CKIs, and on the quest for "exotic" non-human viruses, whose ability to infect and lyse a significant number of human cancer cells in vivo remains questionable [67].

The feeling that OVs are not being exploited to their potential is increased by the lack of new ideas on the use of OVs as platforms to express factors aimed at increasing their killing ability and the immunomodulatory effect. Under this respect, there have not been many novelties in the last years, at least to our knowledge. Indeed, in the field of OVs, viral engineering has been mainly employed for the attenuation of the human pathogen under evaluation, for its transcriptional [68] or receptorial [69] retargeting, for the expression of suicide genes or single immunostimulatory cytokines (as in the case of talimogene). Thus, there is wide space for the design of innovative OVs to better achieve, for instance, TME modulation. The ideal candidate would be a large dsDNA virus that can allow the insertion of multiple transgenes within its genome, without losing its ability to replicate in and kill cancer cells, and for which "robust" gene editing techniques are available.

Finally, the big challenge that OVs are facing is the therapy of immunologically "cold" tumors which are usually failing to respond to checkpoint inhibitors due to the absence of a lymphocyte infiltrate. The presence of a virus (especially of a replication competent virus) can profoundly alter the TME by enhancing the immune cell infiltrate and generating proinflammatory cues. Is this enough to make cold tumors sensitive to immune checkpoint inhibition? It must be considered that these tumors often display an immunosuppressive immune cell infiltrate or a fibrotic microenvironment, which could protect malignant cells from the immunogenic stimuli provided by the virus. Armed OVs might be once again the solution to this problem. Indeed, therapeutic gene products, released at high concentrations in situ by infected cells, could synergize with OVs by killing immunosuppressive cells or inhibiting their activity. Furthermore, OVs could be engineered to express enzymes that degrade the fibrotic extracellular matrix, thus helping to tackle very "difficult" tumor microenvironments [Fig. 1].

Abbreviations

CKI: Checkpoint Inhibitors; CTLA-4: Cytotoxic T Lymphocyte Antigen 4; hGM-CSF: human Granulocyte Monocyte Colony Stimulating Factor; HSV: Herpes Simplex Virus; OV: Oncolytic Virus; PD-1: Programmed Death 1; PD-L1: Programmed Death Ligand 1; TME: Tumor Microenvironment

Availability of data and materials

Not applicable.

Authors' contributions

All Authors contributed in writing the review. All authors read and provided critical revision of the manuscript and approved the final version.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The Authors declare that they have no competing interests.

Publisher's Note

Received: 18 October 2018 Accepted: 20 December 2018
Published online: 11 February 2019

References

1. Ansell SM. Hodgkin lymphoma: diagnosis and treatment. In: Mayo Clinic proceedings (2015). (Vol. 90, no. 11, pp. 1574-1583). Elsevier.
2. Hefazi M, Litzow MR. Recent advances in the biology and treatment of T cell acute lymphoblastic leukemia. Curr Hematol Malig Rep. 2018:1–10.
3. Soverini S, Mancini M, Bavaro L, Cavo M, Martinelli G. Chronic myeloid leukemia: the paradigm of targeting oncogenic tyrosine kinase signaling and counteracting resistance for successful cancer therapy. Mol Cancer. 17(1).
4. Kayser S, Schlenk RF, Platzbecker U. Management of patients with acute promyelocytic leukemia. Leukemia. 2018;1.
5. Phelan KW, Advani AS. Novel Therapies in Acute Lymphoblastic Leukemia. Current hematologic malignancy reports.2018; 1–11.
6. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2017;68.
7. de Groot PM, Wu CC, Carter BW, Munden RF. The epidemiology of lung cancer. Transl Lung Cancer Res. 2018;7(3).
8. Gainor JF, Shaw AT. Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer. J Clin Oncol. 2013;31.
9. Jarboe J, Gupta A, Saif W. Therapeutic human monoclonal antibodies against Cancer. 2014.In: Steinitz M. (eds) human monoclonal antibodies. Methods in molecular biology, vol 1060. Humana press, Totowa, NJ.
10. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. Immunol Rev. 2014;257(1).
11. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. Immunity. 2013(1):39.
12. Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. The Journal of Immunology. 2014;192(12).
13. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nature immunology. 2013;14(10).
14. Liu Y, Cao X. Immunosuppressive cells in tumor immune escape and metastasis. J Mol Med. 2016;94(5).
15. Seidel JA, Otsuka A, Kabashima K. Anti-PD-1 and anti-CTLA-4 therapies in Cancer: mechanisms of action, efficacy, and limitations. Front Oncol. 2018;8.
16. Hodi FS, O'day SJ, McDermott DF, Weber RW, Sosman JA, Haanen J., et al. Improved survival with ipilimumab in patients with metastatic melanoma. New England J Med. 2010; 363(8).
17. Palmieri DJ, Carlino MS. Immune Checkpoint Inhibitor Toxicity. Curr Oncol Rep. 2018;20:9.
18. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Previously Untreated Melanoma. N Engl J Hum Serv. 2015;373(1).

19. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nature Biotechnology. 2012;30(7).
20. Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. Mol Ther. 2007;15(4).
21. Kaufman HL, Kohlhapp FJ, Zloza A. Oncolytic viruses: a new class of immunotherapy drugs. Nature reviews Drug discovery. 2015;14(9).
22. Nguyen T, Avci NG, Shin DH, Martinez-Velez N, Jiang H. Tune Up In Situ Autovaccination against Solid Tumors with Oncolytic Viruses. Cancers. 2018; 10(6).
23. Puzanov I, Milhem MM, Minor D, Hamid O, Li A, Chen L, et al . Talimogene laherparepvec in combination with ipilimumab in previously untreated, unresectable stage IIIB-IV melanoma. J Clin Oncology. 2016;34(22).
24. Noonan AM, Farren MR, Geyer SM, Huang Y, Tahiri S, Ahn D, et al. Randomized phase 2 trial of the oncolytic virus pelareorep (reolysin) in upfront treatment of metastatic pancreatic adenocarcinoma. Molecular Therapy. 2016;(6):24.
25. Suryawanshi YR, Zhang T, Essani K. Oncolytic viruses: emerging options for the treatment of breast cancer. Med Oncology. 2017;34(3).
26. Jebar AH, Errington-Mais F, Vile RG, Selby PJ, Melcher AA, Griffin S. Progress in clinical oncolytic virus-based therapy for hepatocellular carcinoma. J Gen Virol. 2015;96(7).
27. Pol J, Buqué A, Aranda F, Bloy N, Cremer I, Eggermont A, et al. Trial Watch—Oncolytic viruses and cancer therapy. Oncoimmunology. 2016;5(2).
28. Shaw AR, Suzuki M. Recent advances in oncolytic adenovirus therapies for cancer. Current opinion in virology. 2016;21.
29. Sokolowski NA, Rizos H, Diefenbach RJ. Oncolytic virotherapy using herpes simplex virus: how far have we come? Oncolytic virotherapy. 2015;4.
30. Haddad D. Genetically engineered vaccinia viruses as agents for cancer treatment, imaging, and transgene delivery. Front Oncol. 2017;7.
31. Aref S, Bailey K, Fielding A. Measles to the rescue: A review of oncolytic measles virus. Viruses. 2016;8(10).
32. Ammayappan A, Russell SJ, Federspiel MJ. Recombinant mumps virus as a cancer therapeutic agent. Molecular Therapy-Oncolytics. 2016;3.
33. Pizzuto MS, Silic-Benussi M, Ciminale V, Elderfield RA, Capua I, Barclay WS. An engineered avian-origin influenza A virus for pancreatic ductal adenocarcinoma virotherapy. Journal of General Virology. 2016;97(9).
34. Bourhill T, Mori Y, Rancourt DE, Shmulevitz M, Johnston RN. Going (Reo) viral: factors promoting successful revival oncolytics infection. *Viruses.* 2018;*10*(8).
35. Tayeb S, Zakay-Rones Z, Panet A. Therapeutic potential of oncolytic Newcastle disease virus: a critical review. Oncolytic virotherapy. 2015;4.
36. Bishnoi S, Tiwari R, Gupta S, Byrareddy SN, Nayak D. Oncotargeting by Vesicular Stomatitis Virus (VSV): Advances in Cancer Therapy. Viruses. 2018 ; 10(2).
37. Pol JG, Zhang L, Bridle BW, Stephenson KB, Rességuier J, Hanson S, et al. Maraba virus as a potent oncolytic vaccine vector. Molecular Therapy. 2014; 22(2).
38. Angelova AL, Witzens-Harig M, Galabov AS, Rommelaere J. The Oncolytic Virotherapy era in cancer management: prospects of applying H-1 parvovirus to treat blood and solid cancers. Frontiers in oncology.2017; 7.
39. Ruiz AJ, Hadac EM, Nace RA, Russell SJ. MicroRNA-detargeted mengovirus for oncolytic virotherapy. J Virol. 2016;JVI-02810.
40. Matsuda T, Karube H, Aruga A. A Comparative Safety Profile Assessment of Oncolytic Virus Therapy Based on Clinical Trials. Therapeutic innovation & regulatory science.2017; 52(4).
41. Andtbacka RH, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, et al Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. Journal of clinical Oncology. 2015; 33(25).
42. Irwin CR, Hitt MM, Evans DH. Targeting nucleotide biosynthesis: a strategy for improving the oncolytic potential of DNA viruses. Front Oncol. 2017;7.
43. Delwar ZM, Kuo Y, Wen YH, Rennie, PS, Jia W. Oncolytic virotherapy blockade by microglia and macrophages requires STAT1/3. Cancer research. 2017;78(3).
44. Rye IH, Trinh A, Saetersdal A, Nebdal D, Lingjaerde OC, Almendro V, et al. Intra-tumor heterogeneity defines treatment-resistant HER2+ breast tumors. Mol Oncol. 2018.
45. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013; 501(7467).
46. Sarinella F, Calistri A, Sette P, Palu G, Parolin C. Oncolysis of pancreatic tumour cells by a γ34. 5-deleted HSV-1 does not rely upon Ras-activation, but on the PI 3-kinase pathway. Gene Ther. 2006;13(14).
47. Drost J, Clevers H. Organoids in cancer research. Nat Rev Cancer. 2018;1.
48. Lai Y, Wei X, Lin S, Qin L, Cheng L, Li P. Current status and perspectives of patient-derived xenograft models in cancer research. J Hematol Oncol. 2017;(1):10.
49. Carrillo MA, Zhen A, Kitchen SG. The use of the humanized mouse model in gene therapy and immunotherapy for HIV and cancer. Front Immunol. 2018;9.
50. Power AT, Wang J, Falls TJ, Paterson JM, Parato KA, Lichty BD, et al. Carrier cell-based delivery of an oncolytic virus circumvents antiviral immunity. Mol Ther. 2007;15(1).
51. Guo ZS, Liu Z, Bartlett DL. Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity. Front Oncol. 2014;4:74.
52. Ricca JM, Oseledchyk A, Walther T, Liu C, Mangarin L, Merghoub T, et al. Pre-existing immunity to oncolytic virus potentiates its immunotherapeutic efficacy. Mol Ther. 2018;26(4).
53. Ribas A, Dummer R, Puzanov I, VanderWalde A, Andtbacka RH, Michielin O, et al. *Oncolytic virotherapy promotes intratumoral T cell infiltration and improves anti-PD-1 immunotherapy. Cell. 2017;170(6).*
54. Fisher K. Striking out at disseminated metastases: the systemic delivery of oncolytic viruses. Current opinion in molecular therapeutics. 2006; 8(4).
55. Wang Y, Marelli G, Howells A, Lemoine N. Oncolytic viral therapy and the immune system: a double-edged sword against cancer. Front Immunol. 2018;9.
56. Fukuhara H, Ino Y, Todo T. Oncolytic virus therapy: a new era of cancer treatment at dawn. Cancer Sci. 2016;107:10.
57. Liu BL, Robinson M, Han ZQ, Branston RH, English C, Reay P, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther. 2003;10(4).
58. Leoni V, Vannini A, Gatta V, Rambaldi J, Sanapo M, Barboni C, et al. A fully-virulent retargeted oncolytic HSV armed with IL-12 elicits local immunity and vaccine therapy towards distant tumors. PLoS pathogens.2018; 14(8).
59. Kowalsky SJ, Liu Z, Feist M, Berkey SE, Ma C, Ravindranathan R, et al. Superagonist IL-15-Armed Oncolytic Virus Elicits Potent Antitumor Immunity and Therapy That Are Enhanced with PD-1 Blockade. Mol Ther. 2018;(10):26.
60. Binnewies M, Roberts EW, Kersten K, Chan V, Fearon DF, Merad M, et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat Med. 2018;1.
61. Hoeller C, Michielin O, Ascierto PA, Szabo Z, Blank CU. Systematic review of the use of granulocyte–macrophage colony-stimulating factor in patients with advanced melanoma. Cancer Immun. 65(9).
62. Kloss CC, Lee J, Zhang A, Chen F, Melenhorst JJ, Lacey SF, et al. Dominant-Negative TGF-β Receptor Enhances PSMA-Targeted Human CAR T Cell Proliferation And Augments Prostate Cancer Eradication. Mol Ther. 2018;26(7).
63. Chen CY, Hutzen B, Wedekind MF, Cripe TP. Oncolytic virus and PD-1/PD-L1 blockade combination therapy. Oncolytic virotherapy. 2018;7.
64. Downs-Canner S, Guo ZS, Ravindranathan R, Breitbach CJ, O'malley ME, Jones HL, et al. Phase 1 study of intravenous oncolytic poxvirus (vvDD) in patients with advanced solid cancers. Mol Ther. 2016;24(8).
65. Streby KA, Geller JI, Currier MA, Warren PS, Racadio JM, Towbin AJ, et al. Intratumoral injection of HSV1716, an oncolytic herpes virus, is safe and shows evidence of immune response and viral replication in young cancer patients. Clin Cancer Res. 2017;23(14).
66. Zhang J, Tai LH, Ilkow CS, Alkayyal AA, Ananth AA, De Souza CT, et al. Maraba MG1 virus enhances natural killer cell function via conventional dendritic cells to reduce postoperative metastatic disease. Mol Ther. 2014;22(7).
67. Burke MJ. Oncolytic Seneca Valley Virus: past perspectives and future directions. Oncolytic Virotherapy. 2016;5.
68. Kambara H, Okano H, Chiocca EA, Saeki Y. An oncolytic HSV-1 mutant expressing ICP34.5 under control of a nestin promoter increases survival of animals even when symptomatic from a brain tumor. Cancer Res. 2005; 65(7).
69. Campadelli-Fiume G, Petrovic B, Leoni V, Gianni T, Avitabile E, Casiraghi C, Gatta V. Retargeting strategies for oncolytic herpes simplex viruses. Viruses. 2016;8(3).

EBioMedicine 47 (2019) 4–5

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

Commentary

A clinical trial investigating biodistribution and shedding of an oncolytic virus

Alberto Reale, Arianna Calistri, Giorgio Palù *

Department of Molecular Medicine, University of Padua, Italy

Oncolytic viruses (OVs) are an emerging class of antitumoral therapeutics, that combine selective cancer cell killing and an immunotherapeutic effect, by facilitating the recognition of tumor antigens by the immune system [1]. To the present day, only one replication-competent OV, based on herpes simplex virus type 1 (HSV-1), talimogene laherparepvec or T-VEC (IMLYGIC®, Amgen) has been approved for clinical use in the US and the EU by intralesional injection against metastatic melanoma [2]. T-VEC is a genetically modified OV, carrying deletions in the ICP 34.5 (γ34.5) gene (attenuating virulence, in particular in neurons), in the ICP47 (Us12) gene (enhancing, among other effects, antigen presentation), and expressing a therapeutic gene (the human granulocyte-monocyte colony stimulating factor, GM-CSF) [3]. Recent "real world" clinical data confirm the efficacy of T-VEC against melanoma beyond clinical trials [4]. Although T-VEC proved to be quite safe [5], the main adverse effects being flu-like symptoms and fatigue, concerns still remain about the pharmacokinetics of the recombinant virus and the possibility of its transmission to healthcare workers and close contacts of the treated patient. In an article in *EBioMedicine,* Andtbacka and colleagues report the results of a phase 2 clinical trial investigating biodistribution, shedding and transmissibility of T-VEC in 60 patients with melanoma [6]. Following administration, injected lesions were covered with occlusive dressing according to the therapeutic protocol. Presence of viral DNA was assessed by a T-VEC specific quantitative real time PCR in blood, urine, swabs from injected lesions, exterior of dressings, oral and anogenital mucosa. Positive swabs were further tested for the presence of infectious virus. Close contacts of patients who developed suspect herpetic lesions were also tested. Interestingly, T-VEC DNA was detectable in the blood of most patients and in 31.7% urine samples during the first cycles of therapy, irrespective of previous HSV-1 serological status, while only a minority of patients was positive in oral (8.3%) and anogenital swabs (8%). During the following cycles of treatments and safety follow-up controls T-VEC specific PCR became rapidly negative. Of note and not surprisingly, the surface of the injected lesions resulted positive for viral DNA in 100% of patients at least once during cycles 1 to 4, and in 14% of patients during safety follow-up visits. Exterior of occlusive dressings was also positive in 80% of patients during cycles 1 to 4. Finally, only a small percentage of swabs obtained from the surface of injected lesions (7 out of 740 samples) was positive for infectious virus.

Three patients had cutaneous herpetic lesions with detectable T-VEC DNA from uninjected sites. Three close contacts had possible herpetic lesions. One of them declined testing, while the other two resulted PCR negative. One healthcare provider had a suspect lip lesion, which was also negative for T-VEC. The Authors also report overall safety and efficacy data. Most patients had adverse effects consisting mainly of chills (65%) and fatigue (56.7%). Serious related adverse effects were reported in 8 patients, and treatment was permanently discontinued in 3 patients.

The reported overall response rate (ORR) was 35%. Remarkably, 9 patients (15%) had a complete response, while 12 patients (20%) had a partial response, consistent with data of previous clinical trials [7].

Overall the reported results confirm the safety profile of T-VEC and, most importantly, the very low possibility of transmission to contacts and healthcare workers, when recommended precautions and protocols are applied. An issue that could require further inquiry is the presence of T-VEC DNA in the blood of most patients at the beginning of treatment. Even though it seems unlikely that a transient presence of DNA mirrors a real "viremia" with possible effects on metastases, this possibility should be evaluated, together with other systemic effects of the virus (for example, on the inflammatory response and modulation of the immune system). A remaining open question involves the *in vivo* kinetics of the expression of the T-VEC therapeutic gene (GM-CSF), which could be interesting both for the evaluation of its effect and for comparison with therapeutic genes expressed by other investigational HSV-1 based OVs.

T-VEC indeed represents a valuable tool in the treatment of melanoma, and it has been shown to synergize with immune checkpoint inhibitors (ICIs) in clinical trials [8], in particular in those tumors with a low baseline lymphocyte infiltrate that are poorly responsive to ICI monotherapy. On the other hand, the full therapeutic potential of OVs is probably still to be unleashed. Thus, the research goes on with different aims, such as devising OVs (i) that can be delivered systemically [9], (ii) that require an inferior number of injections, or (iii) that can extend their immunotherapeutic potential beyond melanoma, to other

DOI of original article: https://doi.org/10.1016/j.ebiom.2019.07.066.
* Corresponding author.
E-mail address: giorgio.palu@unipd.it (G. Palù).

https://doi.org/10.1016/j.ebiom.2019.08.011

malignancies with a dismal prognosis and a poor response to immunotherapy [10]. In this setting, OVs are very attractive as a possible *trait d'union* between direct cancer cell lysis, immunotherapy and gene therapy.

Disclosure

The authors have nothing to disclose.

References

[1] Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nat Biotechnol 2012;30(7).
[2] Conry RM, Westbrook B, McKee S, Norwood TG. Talimogene laherparepvec: first in class oncolytic virotherapy. Hum Vaccin Immunother 2018;14(4):839–46.
[3] Liu BL, Robinson M, Han ZQ, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther 2003;10(4).
[4] Franke V, Berger DM, Klop WM, et al. High response rates for T-VEC in early metastatic melanoma (stage IIIB/C-IVM1a). Int J Cancer 2019;145:974–8.
[5] Gangi A, Zager JS, et al. Expert Opin Drug Saf 2017;16(2):265–9.
[6] Andtbacka R, Amatruda T, Nemunaitis J, et al. Biodistribution, shedding, and transmissibility of the oncolytic virus talimogene laherparepvec in patients with melanoma. EBioMedicine 2019 https://www.ebiomedicine.com/article/S2352-3964(19)30508-0/fulltext.
[7] Andtbacka RHI, Kaufman HL, Collichio F, et al. Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. J Clin Oncol 2015;33(25).
[8] Ribas A, Dummer R, Puzanov I, et al. Oncolytic virotherapy promotes intratumoral T cell infiltration and improves anti-PD-1 immunotherapy. Cell 2017;170(6).
[9] Fisher K. Striking out at disseminated metastases: the systemic delivery of oncolytic viruses. Curr Opin Mol Ther 2006;8:4.
[10] Pearl TM, Markert JM, Cassady KA, Ghonime MG. Oncolytic virus-based cytokine expression to improve immune activity in brain and solid tumors. Mol Ther Oncolytics 2019;13:14–21.

cells

MDPI

Review

Why Cells and Viruses Cannot Survive without an ESCRT

Arianna Calistri *, Alberto Reale, Giorgio Palù and Cristina Parolin

Department of Molecular Medicine, University of Padua, 35121 Padua, Italy; alberto.reale@studenti.unipd.it (A.R.); giorgio.palu@unipd.it (G.P.); cristina.parolin@unipd.it (C.P.)
* Correspondence: arianna.calistri@unipd.it

Abstract: Intracellular organelles enwrapped in membranes along with a complex network of vesicles trafficking in, out and inside the cellular environment are one of the main features of eukaryotic cells. Given their central role in cell life, compartmentalization and mechanisms allowing their maintenance despite continuous crosstalk among different organelles have been deeply investigated over the past years. Here, we review the multiple functions exerted by the endosomal sorting complex required for transport (ESCRT) machinery in driving membrane remodeling and fission, as well as in repairing physiological and pathological membrane damages. In this way, ESCRT machinery enables different fundamental cellular processes, such as cell cytokinesis, biogenesis of organelles and vesicles, maintenance of nuclear–cytoplasmic compartmentalization, endolysosomal activity. Furthermore, we discuss some examples of how viruses, as obligate intracellular parasites, have evolved to hijack the ESCRT machinery or part of it to execute/optimize their replication cycle/infection. A special emphasis is given to the herpes simplex virus type 1 (HSV-1) interaction with the ESCRT proteins, considering the peculiarities of this interplay and the need for HSV-1 to cross both the nuclear-cytoplasmic and the cytoplasmic-extracellular environment compartmentalization to egress from infected cells.

Keywords: ESCRT; viruses; cellular membranes; extracellular vesicles; HSV-1

Citation: Calistri, A.; Reale, A.; Palù, G.; Parolin, C. Why Cells and Viruses Cannot Survive without an ESCRT. *Cells* **2021**, *10*, 483. https://doi.org/10.3390/cells10030483

Academic Editor: Alexander E. Kalyuzhny

Received: 4 February 2021
Accepted: 21 February 2021
Published: 24 February 2021

1. Introduction

Membrane-surrounded organelles characterize eukaryotic cells and guarantee the compartmentalization of distinctive processes and functions. Intracellular membranes not only maintain the integrity of these compartments but, thanks to finely tuned vesicle trafficking, also play a pivotal role in the crosstalk between organelles themselves. Dynamic, constant and controlled remodeling processes enable the exchange of signals, information and materials between membranes that is crucial for the functioning of biological systems [1]. Two main types of membrane involutions can be formed. The first type of vesicles excludes the cytosolic environment and occurs during "classical" budding events, such as endocytosis. The second type of vesicles, instead, originates from a "reverse-topology" membrane scission which includes the cytosol and is mediated by the endosomal sorting complex required for transport (ESCRT) machinery [2]. This network of proteins is involved in different essential cellular processes, such as cytokinesis, autophagy, multivesicular body (MVB) and extracellular vesicle (EV) biogenesis, plasma, nuclear and endolysosomal membrane repair [2,3]. This list is far from being exhaustive, but represents the pathways involving ESCRTs that are better characterized. Viruses, being obligate intracellular parasites, have evolved to hijack highly conserved cellular pathways throughout their replication cycle [4]. Thus, and not surprisingly, viruses exploit the host intracellular membrane trafficking machinery to execute crucial steps of infection, such as (i) entering the target cell; (ii) transporting their genomic materials to the site of replication; (iii) if enveloped, acquiring their external lipid coating; (iv) exiting from infected cells. At the same time, several enveloped and non-enveloped viruses induce profound membrane remodeling/proliferation in infected cells to create specialized compartments where viral

genome replication and/or new virion assembly occurs [5–7]. Interestingly, some insect and plant viruses are able to modify mitochondrial and peroxisomal membranes for their replication [7]. Finally, EVs play a broad-spectrum role in the pathogenesis of viral infection. Indeed, viruses not only adopt exosomes to accomplish specific steps of their life cycle, but also exploit these EVs to transfer both viral and cellular factors, such as proteins and non-coding RNAs, outside the infected cells to promote infection and to escape from the immune system [8].

Here, we review the similarities and differences of various ESCRT-dependent cellular processes, including EV biogenesis, emphasizing mechanisms of ESCRT recruitment by viruses. Furthermore, we focus on how the herpes simplex virus type 1 (HSV-1), a complex DNA-enveloped virus, interacts with ESCRT proteins to cross the nuclear envelope and egress from infected cells.

2. The ESCRT Machinery: An Overview

The ESCRT machinery and its associated factors include a network of different proteins (roughly 20 in yeast and 30 in mammals) that are sequentially recruited to the inner surface of the membrane necks of vesicles, mostly budding away from the cytosol (the so-called "reverse topology" budding event). ESCRT proteins were originally identified in budding yeasts in studies aimed at the identification of factors involved in the biogenesis of the MVBs [9,10]. MVBs contain intraluminal vesicles (ILVs) that arise from the budding of the limiting endosomal membrane into the lumen of the organelle. When MVBs fuse to the lysosomes, the content of those ILVs is degraded [11]. This mechanism of degradation has been well described in the case of both misfolded cell surface proteins and of G-coupled proteins and tyrosine kinase receptors, channels and transporters, which need to be downregulated after responding to specific stimuli [12]. The nascent ILVs are connected to the limiting membrane by a narrow membrane neck, which must be cut to release them into the lumen. In MVB biogenesis, the ESCRT machinery drives both budding and scission of ILVs. In different pathways, proteins other than the ESCRTs are responsible for the formation of the vesicle/membrane neck, while ESCRTs and associated factors drive membrane scission. Although ESCRT machinery is the master player of reverse topology budding events, some exceptions to this rule are reported in the literature. For instance, in the peroxisome biogenesis of budding yeasts, one of the ESCRTs, namely ESCRT-III, which is described in the next paragraph, is involved in a classical topology membrane scission event. Indeed, it allows the scission of pre-peroxisomal vesicles from the endoplasmic reticulum (ER) membrane into the cytosol [13].

The core of the ESCRT machinery consists of three complexes (ESCRT-I, ESCRT-II, ESCRT-III), as well as of the associated protein Alix (BRO1 in yeast) and the AAA-type ATP-ase, the vacuolar protein sorting (VPS) 4 [2,3,10]. ESCRT-I is a stalk-shaped heterotetrameric complex of proteins encompassing tumor suppressor gene 101, TSG101 (Vps23 in yeast), VPS28, VPS37 (from A to D), and UBAP1, MVB12A, or MVB12B [14–16]. ESCRT-II is a Y-shaped complex constituted by VPS22 (also known as EAP30), VPS36 (also known as EAP45), and two copies of VPS25 (also known as EAP20) [17]. ESCRT-III is formed by the so-called charged multivesicular body (CHMP) proteins 1 to 7, i.e., CHMP1A/B (Did2 in yeast); CHMP2A/B (Vps2 in yeast); CHMP3, (Vps24 in yeast); CHMP4A/B/C (Snf7 in yeast); CHMP5 (Vps60 in yeast); CHMP6 (Vps20 in yeast) and CHMP7 (Chm7 in yeast). Finally, increased sodium tolerance-1 (IST1) is also part of this complex [18]. While ESCRT-III is directly involved in remodeling and severing membranes, ESCRT-I and ESCRT-II often function together as assembly factors for ESCRT-III. Indeed, those two complexes cooperate to bring ESCRT-III to the site of the membranes where budding events are going to take place [19]. On the other hand, Alix [20,21] can work as an alternative way for the recruitment and activation of ESCRT-III. Indeed, Alix, via its Bro1 domain [22], can directly bind CHMP4. In some cases, the His domain-containing protein tyrosine phosphatase HD-PTP, an Alix-like protein also bearing a Bro1 domain, can replace Alix itself in recruiting CHMP4 [23,24]. An additional Bro1 containing protein,

Bro1 domain/CAAX motif containing-protein (BROX), has been suggested to function in an Alix-like manner [25]. ESCRT-III assembly at the side of vesicle budding and membrane pinching off are dynamic processes. The AAA+ ATPase VPS4 [26] has a crucial role in recycling ESCRT-III subunits by extracting ESCRT-III monomers from the assembly. VPS4 functions as a hexamer and is characterized by an N-terminal microtubule interaction and transport (MIT) domain [27], along with a catalytic domain [28]. The MIT domain can bind to the MIT-interaction motifs (MIMs) that are present within ESCRT-III components. In particular, VPS4 has been shown to strongly bind to the MIM containing ESCRT-III proteins CHMP1, CHMP2, CHMP6, and IST1 [29,30]. ESCRT-III and VPS4 are universally involved in ESCRT-dependent membrane dynamic processes [31] that are reviewed in the following sections. Thus, it is not surprising that both ESCRT-III and VPS4 have homologs in archaea [32–36]. At the basis of most of the reverse topology fission events, indeed, are sequential steps involving ESCRT-III (Figure 1).

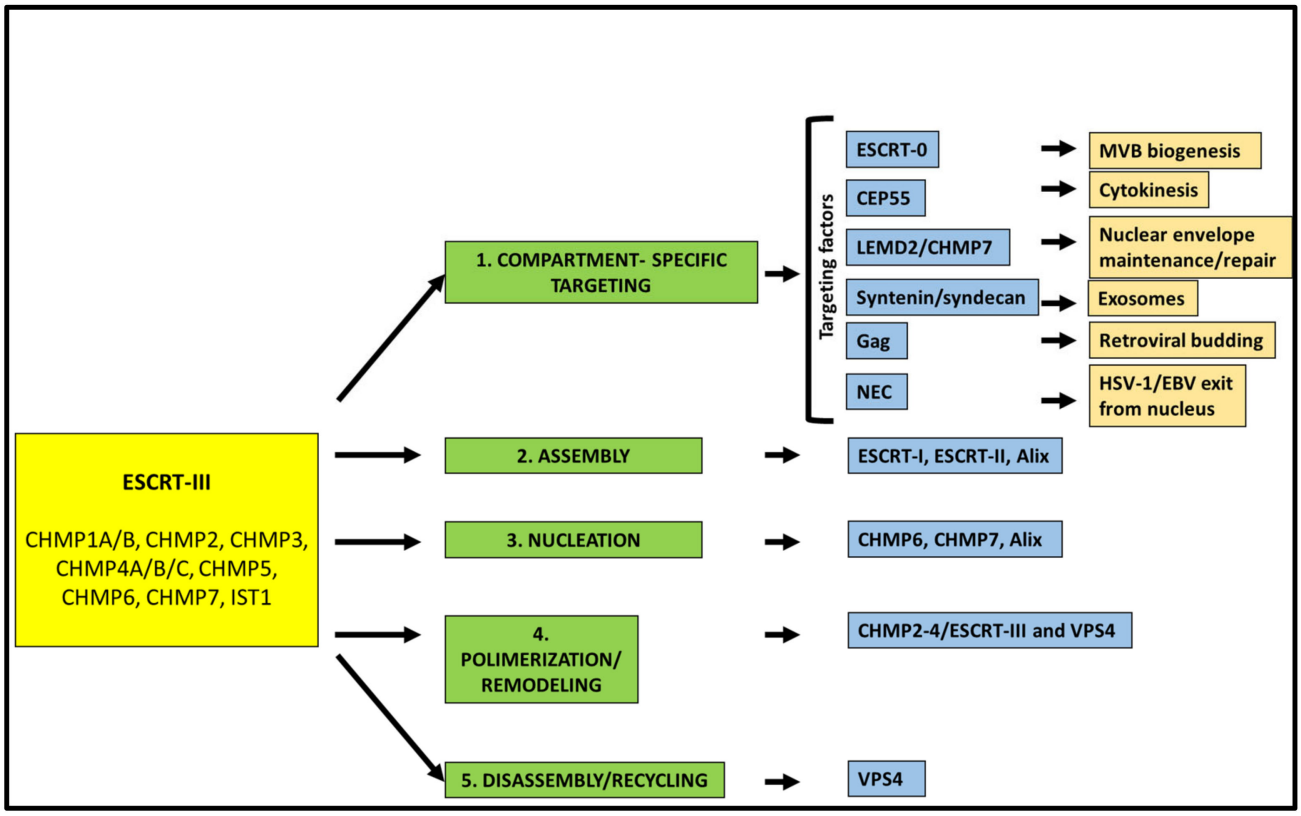


Figure 1. Schematic representation of the sequential steps leading to ESCRT-III polymerization and functioning. The main factors involved in each step are reported, along with the biological processes in which ESCRT-III plays a role based on its compartment-specific recruitment (light orange rectangles).

First, ESCRT-III is targeted to the correct membrane/cell compartment. In most cases, this occurs thanks to proteins that act as compartment-specific targeting factors by forming molecular bridges for the recruitment of the ESCRT machinery (Figure 1). Several of these factors have been characterized over the years, including the cellular and viral proteins ESCRT-0, the centrosomal protein 55 (CEP55), syndecan/syntenin, Gag, LEMD2 (LEM Domain 2 protein)–CHMP7, BFRF1 and UL31/34. Each of these proteins contributes to the targeting of the ESCRT machinery towards a certain cellular compartment and facilitates a specific function of this complex. For instance, and as better detailed below, syndecan/syntenin are involved in the biogenesis of exosomes, CEP55 in the ESCRT-III mediated cell abscission during cytokinesis, BFRF1 and UL31/34 enable the egress of two herpesviruses, Epstein–Barr virus (EBV) and the herpes simplex virus type 1 (HSV-1), respectively, from the inner nuclear membrane (INM) of infected cells. Usually,

ESCRT-III does not directly interact with these proteins, but it needs the involvement of assembly factors as the above-mentioned ESCRT-I/ESCRT-II, Alix and HD-PTP. In particular, Alix and HD-PTP, by binding both ESCRT-III and ESCRT-I [37,38], function as ESCRT-II alternatives. After assembly, nucleation and polymerization of ESCRT-III take place. In these steps, ESCRT-III components, and in particular CHMP4, need to be activated, a crucial process that can be mediated by both ESCRT-I/ESCRT-II or ESCRT-0/Alix [39,40]. ESCRT-III function relies on a finely tuned balance between the upstream factors that allow filament nucleation and the downstream proteins, including VPS4, that control filament polymerization rate [10]. If this balance is altered, the consequences for the cell can be detrimental. CHMP4-containing ESCRT filaments, as well as filaments composed of other ESCRT-III proteins (i.e., CHMP-2 and CHMP-3), are highly dynamic, a feature essential to determine membrane remodeling and scission. Furthermore, CHMP4 interacts with CHMP3 leading to the sliding of polymers that contribute to the ability of ESCRT-III filaments to change their architecture to adapt to the different phases of the membrane fission [41]. Interestingly, there is evidence that also VPS4 plays a role during the ESCRT-III remodeling process, in addition to functioning as a recycling factor for the proteins composing this complex [42].

3. ESCRT Machinery Functions in Fundamental Cellular Pathways

Membrane remodeling followed by reverse topology budding events and/or fission enables different fundamental cellular processes, including biogenesis of MVBs; cytokinesis; plasma and intracellular membrane maintenance/repair/reformation; micro- and macroautophagy. The involvement of the ESCRT machinery in these pathways, with a focus on results obtained in mammalian cells, is discussed in the next paragraphs and summarized in Figure 2.

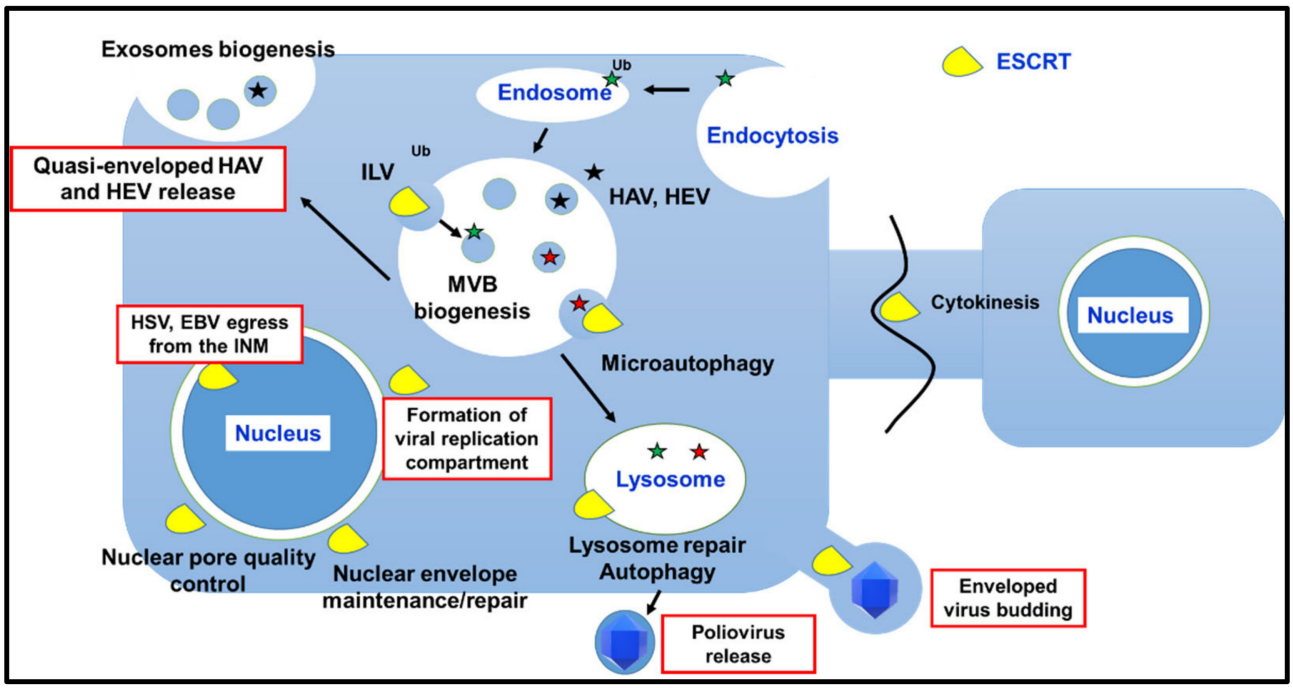


Figure 2. Schematic representation of the better-characterized cellular and viral processes (highlighted by red rectangles) that involve ESCRT-machinery or specific ESCRT factors. HAV stands for hepatitis A virus; HEV for hepatitis E virus; HSV for herpes simplex virus; EBV for Epstein–Barr virus; INM for inner nuclear membrane. The involvement of ESCRT machinery in the viral replication cycles is discussed in the next paragraphs.

3.1. ESCRTs Involvement in MVB Biogenesis

Historically, one of the first studied functions of ESCRTs was their central role in the biogenesis of MVBs. Typically, MVBs are involved in protein trafficking throughout the endosomal-lysosomal pathway [43]. Transmembrane proteins endocytosed from the plasma membrane face two main fates: (i) they can be recycled back to the plasma membrane or the Golgi apparatus (GA) or can be retained to the limiting membrane of the MVB itself; (ii) they can be targeted to the ILVs and thus degraded, once mature MVBs fuse with the lysosomes. The ESCRT machinery is involved in both protein sorting as well as in ILV biogenesis and budding. MVBs display other important biological functions mostly related to the release of EVs, which can significantly influence intercellular signaling as well as the extracellular microenvironment [44–46]. Indeed, ILVs can give rise to exosomes upon a fusion of MVBs with the plasma membrane [43,47]. In conclusion, intracellular trafficking of MVBs, which is mainly regulated by Rab proteins, follows at least three different pathways: degradation (fusion with lysosomes), back-fusion (recycling of proteins) and secretion (release of exosomes). Central to all is the biogenesis of ILVs, which begins in early endosomes [48,49]. Proteins intended for degradation are usually marked by ubiquitin. Ubiquitin is a highly conserved protein of 76 amino acids that can be covalently linked to target proteins through a multistep process known as ubiquitylation [50]. Ubiquitin plays a central role in the MVB biogenesis pathway. Indeed, mono-ubiquitylation is necessary and sufficient to trigger the ESCRT-dependent endosomal sorting of membrane proteins and their degradation through the MVB/lysosomal pathway [51,52]. Moreover, ubiquitin plays a role in the recruitment and function of ESCRT components during the ILVs formation. Endosomal proteins, typically marked by lysine-63-linked ubiquitin, enter into the nascent ILVs by interacting with a ubiquitin-binding complex, known as ESCRT-0, constituted by HRS and STAM (Vps27 and Hse1 in budding yeast, respectively) [2,53]. ESCRT-0 is recruited to the early endosome limiting membrane by the interaction of an HRS FYVE domain with phosphatidylinositol 3-phosphate [54,55], a lipid that enriches this type of membrane. Once assembled as a heterotetrameric complex (two HRS and two STAM subunits) [56], ESCRT-0 can efficiently bind ubiquitylated cargoes since it displays several ubiquitin-binding domains (UBDs). Clathrin, along with ESCRT-0, concentrates ubiquitylated proteins in specific patches where sorted cargoes are handed over to ESCRT-I, once again thanks to ubiquitin recognition. Indeed, TSG101 contains a ubiquitin E2 variant (UEV) domain that binds ubiquitin. Furthermore, the mutually exclusive ESCRT-I components MVB12 or UBAP1 can bind ubiquitin through the ubiquitin associated (UBA) or the solenoid of overlapping UBA (SOUBA) domains, respectively. ESCRT-I then binds ESCRT-II, which contains a GRAM-like ubiquitin-binding in Eap45 (GLUE) domain. Finally, ESCRT-III and VPS4 are recruited to the sites of the endosomal membranes where cargo proteins have been concentrated, leading to membrane deformation, ILVs formation and budding [42,57–59]. As protein ubiquitylation is crucial for ESCRT-mediated cargo sorting into ILVs, and ESCRT components themselves (i.e., HRS, TSG101 and Alix) can be ubiquitylated, it is not surprising that both ubiquitin ligases and de-ubiquitylating enzymes are associated with the ESCRT machinery, with a key regulative role [60]. Of note, ESCRT-III has no known UBDs, and cargo deubiquitylation, at least in budding yeast, takes place prior to ILVs sorting, a process that contributes to maintaining the correct cytosolic levels of free ubiquitin (Figure 2). Finally, in mammalian cells, ILVs biogenesis can occur independently from ESCRT-0 and ESCRT-I. In this alternative pathway, Alix binds and sorts cargo proteins irrespectively of their ubiquitylation state [61–63]. Interestingly, the proteoglycan syndecan can recruit the ESCRT machinery via Alix along with syntenin-1, which functions as a bridge between the first two factors [64]. The syndecan-syntenin-Alix complex is, then, involved in the engagement of ESCRT-III during the biogenesis of exosomes. Exosomes are the smallest subclass of EVs with a diameter ranging from 30 to 120 nm. In addition to the Alix-mediated mechanisms, also classical ESCRT-I/ESCRT-II-dependent engagement of ESCRT-III has been described in exosome formation, as well as ESCRT machinery-independent processes [8]. Exosomes play a key role in cell-to-cell

signaling and are emerging as important tools for diagnosis/treatment of diseases, cancer included, and as prognostics markers. Indeed, their cargo, which encompasses not only proteins but also lipids and nucleic acids, such as micro-ribonucleic acids (miRNAs) and long non-coding RNAs (lncRNAs), as well as functional messenger RNAs (mRNAs), is protected and can be delivered to target cells or quantified upon vesicle harvesting [8].

3.2. Role of ESCRT Machinery in Autophagy

Taking into account its role in the endolysosomal pathway, the involvement of the ESCRT machinery in autophagy is not unexpected. Indeed, autophagy is a process central for cell survival, as, among the best characterized of its functions, it mediates degradation of large intracellular materials (as organelles) by lysosomes. The autophagosome is a double-membrane structure that can originate from a variety of cellular membranes, including the ER, mitochondria and plasma membrane [65]. Autophagy is divided into macroautophagy and microautophagy. In the first case, cytoplasmic content is surrounded by a double membrane phagosome that can then fuse with lysosomes to degrade intraluminal substances. Macroautophagy plays a crucial role not only for cell catabolism, but also as a defense against intracellular pathogens and for removing damaged organelles. Microautophagy, instead, is based on the budding of ILVs from the endosome/lysosome membranes for degradation of specific cytosolic cargoes. In this sense, although the process closely resembles the MVB biogenesis, microautophagy is a distinct pathway with respect to MVB formation, as it does not involve endocytosed proteins but cytosolic molecules. ESCRT machinery plays a role in both types of autophagy [66–68]. This is not surprising in the case of microautophagy due to the aforementioned similarities. However, ESCRT-III and VPS4 are also recruited to the forming autophagosome, where they participate in the sealing of the double membrane coating. Furthermore, when ESCRT functions are blocked, cells accumulate autophagosomes, thus suggesting that ESCRTs are also part of the machinery allowing the fusion of autophagosomes with lysosomes [69,70].

3.3. Role of the ESCRT Machinery in Cytokinesis

Over the years, different studies carried on both in vitro and in model organisms (i.e., the fruit fly Drosophila melanogaster and the nematode Caenorhabditis elegans) have revealed the involvement of specific ESCRT factors also in the final phases of cell cytokinesis, the process is known as abscission [71–75]. Specifically, TSG101 and Alix play a pivotal role in this context, as they are directly engaged to the midbody ring by interactions with the centrosomal protein of 55 kDa (CEP55) [71,72,74,76,77]. Furthermore, TSG101 binds to septin 9, one of the septin ring proteins [78]. TSG101 and Alix work in parallel, allowing ESCRT-III nucleation and assembly at the midbody ring [79,80]. ESCRT-III is bound to the membrane via the microtubule interacting and trafficking domain containing 1 (MITD1) protein [81,82] and polymerizes in filaments that stretch out like arms from each side of the midbody ring. VPS4 functions by continuously remodeling these arms [42,58,83]. In the late phases of abscission, part of ESCRT-III and VPS4 localizes at the level of a secondary and thinner intracellular bridge between daughter cells that is generated by actin cytoskeleton remodeling along with microtubule reorganization and fusion of endocytic vesicles to the plasma membrane. ESCRT-III recruits the AAA-ATPase spastin that determines microtubule severing, followed by cell scission [75,84–86]. Interestingly, ESCRT-III and VPS4 homologs have also been characterized in archaea [35,87], where they work in concert with the cell division machinery (Cdv) to regulate abscission. Thus, it is likely that ESCRT-III/VPS4 involvement in the late phases of cytokinesis represents the ancestral role of the ESCRT machinery. Finally, the ESCRT-III function is modulated by abscission checkpoint regulators through different mechanisms [73,88,89]. In particular, CHMP4C plays a crucial role in this context. Indeed, it has been recently shown that a CHMP4C polymorphism (CHMP4CA232T), which disrupts the abscission checkpoint, leads to genome instability and is associated with different types of cancer [90]. On the other hand, and not surprisingly, failures in general of the process of abscission are con-

nected with tumorigenesis [91]. This finding suggests that ESCRT-III components may be connected to oncogenesis via the induction of non-checkpointed aneuploidy. On the other hand, and not surprisingly, failures in the process of abscission, by leading to cells with an unstable tetraploid content, are, in general, connected with tumorigenesis [91]. Under this respect, different roles in cancer development are emerging for CEP55 [92]. Furthermore, it has been reported that when full chromosomes or part of them do not properly segregate, they can recruit their own nuclear membrane and form micronuclei (MN). Importantly, due to protein distribution defects within the inner membrane, during interphase, a large fraction of MN collapses. In this context, ruptures of the membrane are not repaired and trigger MN disruption followed by massive DNA damage and genomic instability [93]. Recently, Vietri and coworkers have elegantly linked this process, known as catastrophic nuclear envelope collapse, to the MN inability of limiting CHMP7-LEMD2 accumulation to the site of lesions. The role of this complex in recruiting ESCRT-III/VP4 to the site of nuclear envelope ruptures is discussed in more detail in Section 3.4.2. As a consequence, ESCRT-III over-accumulates, causing dramatic membrane distortion, DNA stress, and, in the end, chromosome fragmentation [94]. This work, on one hand, further supports the notion that ESCRT-III nucleation and polymerization need to be finely regulated in physiological processes to keep cells healthy and alive. On the other hand, it clearly indicates a role for ESCRT-III in genome instability and in tumorigenesis. Interestingly, different ESCRT components have been linked to cancer development by mechanisms other than defective cytokinesis. Among these mechanisms, which have been extensively reviewed elsewhere [95–97], is the control of downregulation of tumor-related receptors by their correct sorting into MVBs, as well as some of the functions displayed by ESCRT proteins in the nucleus that are discussed later in this review.

3.4. Involvement of the ESCRT Machinery in Damage Repair of Cellular Membranes

3.4.1. Plasma Membrane Repair

Plasma membrane damages can occur in response to different pathophysiological insults, pathogen-mediated ones included. These lesions compromise cell life, thus need to be rapidly repaired. Recruitment of lysosomes to the plasma membrane triggered by Ca^{2+} influxes is one of the main repair mechanisms for lesions ranging from 200 to 500 nm in size [98,99]. ESCRT machinery, by contrast, appears to be involved in repairing smaller lesions (<100 nm) of the cell surface. In this case, Ca^{2+} influx determines a rapid localization of CHMP4B to the sites of damages. Additional ESCRT-III components along with VPS4 are then recruited to the sites of the lesions, although CHMP4B seems to play a major role in injury resealing [99–101]. Interestingly, Alix and TSG101 are the main ESCRT-III assembly factors in this context, while ESCRT-0 and ESCRT-II, as well as additional known assembly cofactors (CHMP6), seem to be dispensable [101]. In particular, Alix interacts with the plasma membrane where it binds TSG101 in a Ca2+ dependent manner [102]. Thus, ESCRT-III plays a crucial protective role in contrasting membrane lesion-mediated types of cell death [103,104]. As an example, it has been shown that ESCRT-III can rescue cells from early-stages of necroptosis, at least temporarily, by acting on the membrane microdomains that are permeabilized by mixed lineage kinase domain-like pseudokinase (MLKL), one of the necroptotic proteins functioning at the plasma membrane [104].

3.4.2. Nuclear Envelope Maintenance and Repair

ESCRT-III is a key player also in the safeguard of nuclear membrane integrity. The nuclear envelope is responsible for the compartmentalization of the cell genome and, thus, of all the nuclear activities. Therefore, its rupture is associated with diseases [105]. At the structural level, the nuclear envelope is a double membrane encompassing the INM and the outer nuclear membrane (ONM), as well as nuclear pore complexes (NPCs) that are involved in controlling the trafficking of macromolecules in and out of the nucleus. Nuclear envelope ruptures can be either a physiological or a pathological event. Indeed, during a normal cell cycle, the nuclear membrane breaks down to allow the interaction

between the mitotic spindle and chromosomes. Next, the nuclear envelope reassembles to form the nuclei of the daughter cells [106]. ESCRT-III and VPS4 are involved in the late steps of the reassembly process by executing membrane fission required for sealing the nuclear envelope [107,108]. As seen in the cytokinesis abscission, ESCRT-III recruits the AAA-ATPase spastin to accomplish microtubule severing, a step that is crucial for nuclear compartmentalization [107–109]. Interestingly, in this case, recruitment of ESCRT-III does not occur through the canonical ESCRT targeting/bridging factors. By contrast, it is mediated by CHMP7, an accessory ESCRT-III protein [107] that works in concert with the nuclear envelope protein LEMD2 and with additional regulator factors [109,110]. This fundamental function of ESCRT-III appears to be evolutionarily conserved, thus further supporting the notion that ESCRT-III is the central complex of ESCRT machinery [111]. In addition to the involvement in nuclear membrane reconstitution upon mitosis, ESCRT-III core subunits and VPS4 have been described to play a role in sealing the usually transient nuclear envelope damages that occur during the interphase and under pathological conditions [105,112–114]. Interestingly it has been recently reported that also the barrier to autoantigen factor (BAF) functions in nuclear envelope repair, most likely upstream of ESCRT-III [115]. Thus, BAF may be involved in an alternative pathway to maintain/reconstitute nucleocytoplasmic compartmentalization in cells lacking ESCRT-III. Finally, ESCRT-III appears to act also in the NPC quality surveillance [116].

3.4.3. Endolysosomal Membrane Repair

Injuries in endolysosomal membranes can be caused by different chemical and biological stresses, as well as by pathogens. Recent evidence indicates that damages in these membranes lead to the Ca^{2+} and ESCRT-I-mediated recruitment of ESCRT-III. By contrast, ESCRT-0 is not required [117,118]. Interestingly, both TSG101 and Alix are engaged by damaged lysosomal membranes and seem to cooperate in early events after injuries [117–119]. Data suggest that ESCRT-III represents the first line of intervention in lysosome repair. Indeed, it does not require large ruptures to be activated in its sealing function [117]. The role played by the ESCRT machinery in the repair of lysosome damages has been better analyzed in the context of injuries caused by pathogens, e.g., intracellular bacteria that exploit phagosomes for their survival. For instance, it has been reported that ESCRT-III is localized to the phagolysosomes that contain replicating Coxiella burnetii, most likely to repair membrane lesions induced by bacterial factors/proliferation [120]. Importantly, replication of the bacterium is impaired by TSG101 depletion [118], indicating that ESCRT machinery is crucial to maintain the integrity of the bacterium-colonized phagolysosomes and, thus, to sustain bacterial replication. ESCRT-III is also engaged by Mycobacterium tuberculosis-damaged phagolysosomes. In this way, bacterial virulence factors are prevented from being released in the cytosol [121]. Overall, these findings point to the ESCRT machinery as a potential target for antimicrobial drug design.

In conclusion, ESCRT machinery is involved in many fundamental cellular processes, and when it is not correctly working or it is not finely regulated, it is linked to diseases, cancer included [97]. The importance of ESCRT machinery is further highlighted by the result of knockout studies carried on in mice. Indeed, the loss of TSG101, VPS25, and CHMP5, components of ESCRT-I, ESCRT-II, and ESCRT-III, respectively, is embryonically lethal in mice [122–124]. By contrast, deletions of core ESCRT genes in the yeast Saccharomyces cerevisiae are not lethal and enabled the study of ESCRTs in MVB biogenesis. In addition to yeast, Drosophila [125,126] and Arabidopsis thaliana [127] have also been employed as models to analyze the effects of the removal of specific ESCRTs on different cellular pathways. Finally, works based on strategies (mainly small interfering RNAs and more recently CRISPR-Cas9 editing) aimed at silencing the expression of ESCRT factors/cofactors in mammalian cells have been crucial to elucidate the key players in each process and their interplay. Some of these studies, focused on the main cellular processes addressed in this review, are summarized in Table 1.

Table 1. Table reports the key players for the major processes in mammalian cells involving ESCRTs. The results of silencing studies supporting the role played by these key players are also displayed.

Cellular Process	Key Player/s	Effect of Silencing	References
MVB Biogenesis	ESCRT-0 (HRS)	HRS depletion: enlarged MVBs with few ILVs TSG101 depletion: MVB formation strongly reduced	[53,128–130]
	ESCRT-I (TSG101)	ESCRT-III/Vps24 depletion: smaller MVBs in clusters	
	ESCRT-III/VPS4	HRS TSG101, Vps22 and Vps24 co-depletion: MVBs and ILVs still formed	
Autophagy	ESCRT-I (VPS37A)	VPS37A depletion: accumulation of phagophores due to defects in autophagosome completion	[66,67]
	ESCRT-III (CHMP2A)/VPS4	CHMP2A depletion: accumulation of immature autophagosomal structures; impairment of autophagic flux; inhibition of phagophore sealing during mitophagy	
		CHMP2A, CHMP3, CHMP7 depletion: increase in immature autophagosomal membranes under starvation	
		CHMP2A, CHMP4B and VPS4 depletion: inhibition of mitophagy	
Cytokinesis	ESCRT-I (TSG101)/ESCRT-II	Alix depletion: an increase in multinuclear cells; furrow regression; a failure in CHMP4C recruitment to the midbody; CHMP4B still recruited	[73,74,80,88,131]
	Alix	TSG101 and Alix co-depletion: failure in CHMP4B recruitment to the midbody; multinucleation non aggravated	
	ESCRT-III (CHMP-6,CHMP4B,CHMP4C)/VPS4	Alix, VPS22, and CHMP6 co-depletion: CHMP4B is not recruited to the intercellular bridge	
		CHMP4C depletion: altered cytokinetic arrest in the presence of chromosomal problems; furrow regression and binucleation	
Cell Membrane Repair	ESCRT-I (TSG101)	Alix, CHMP2B VPS4 depletion: failure of the repairing process followed by cell death (CHMP4B and VPS4 silencing)	[99,101]
	Alix	CHMP2A depletion: impairment of the repairing process	
	ESCRT-III (CHMP4B)/VPS4	CHMP3 depletion: no significant effect	
Nuclear Membrane Repair	ESCRT-III (CHMP4B, CHMP7)/VPS4	Alix, HD-PTP, HRS, TSG101 depletion: no effects on CHMP4B recruitment to the site of ruptures	[107,108]
		CHMP7depletion: failure of CHMP4B recruitment to the nuclear envelope	
Lysosomal Membrane Repair	ESCRT-I (TSG101)	HRS depletion: no effect on CHMP4B recruitment to lysosomes	[118,119]
	Alix	TSG101 depletion: consistent delay in CHMP4B recruitment CHMP2A depletion: increased accumulation of CHMP4B on damaged lysosomes	
	ESCRT-III (CHMP2A, CHMP4B)/VPS4	Alix depletion: no detectable effect on CHM4B recruitment	
		TSG101 and Alix co-depletion: almost complete abrogation of CHMP4B recruitment; failure of recovering of damaged lysosomes	

4. ESCRT Machinery and Viral Replication Cycle

Being obligate intracellular parasites, viruses have evolved to hijack fundamental and highly conserved cellular pathways/machineries to execute each step of their replication. This concept is true without any exception, and it is a feature shared among viruses infecting both prokaryotic and eukaryotic cells. Taking into account the crucial functions played by ESCRT proteins, and especially by ESCRT-III and VPS4, in cell life, it does not come as a surprise that viruses can interfere with ESCRT-mediated processes or directly exploit the ESCRT machinery to maximize their chances to establish a successful infection in the host. The main steps of viral replication involving the entire ESCRT machinery or some selected components are reported in Figure 2.

First, viruses have evolved to either antagonize or take advantage of ESCRT-driven processes as autophagy and EV secretion. The interplay between autophagy and viruses has been excellently reviewed elsewhere [132]. We mention the involvement of autophagy in viral transmission later. As far as EVs are concerned, it is interesting to note that they can act both as promoters or inhibitors of viral spreading and infection [8,133]. This dual effect is well exemplified by EVs released from human immunodeficiency virus type 1 (HIV-1)-infected cells. On one hand, EVs can be loaded with HIV proteins that, once transferred to neighboring cells, increase their susceptibility to viral infection. Among these proteins, the accessory Nef can be recruited into EVs [134] and, upon delivery to latently infected cells, it can activate viral replication [135]. Furthermore, Nef-containing EVs have been described to exert several effects on $CD4^+$ T cells. For instance, it has been reported to induce senescence and death or to suppress their cytotoxic activity [136,137]. At the same time, Nef-positive EVs affect humoral immune response by inhibiting the production of IgA and IgG by B-lymphocytes [138]. Interestingly, it has been reported that EVs released from HIV-1 infected cells are capable of transferring viral co-receptors to recipient cells, thus increasing their susceptibility to viral infection [139,140]. Finally, EVs have also been found to carry the HIV transactivation response element (TAR) [141]. TAR is a stem-loop-shaped sequence present at the 5′ end of HIV transcripts that interacts with Tat, the main viral transactivator protein, and upregulates viral RNA production. The TAR molecule delivered by EVs could be processed into miRNAs that target a Bcl-2 interacting protein, resulting in a block of apoptosis and thus supporting virus production [141]. On the other hand, it has been shown that EVs produced by infected cells can contain APOBEC3G, one of the main host anti-HIV proteins, while the viral protein Vif, which antagonizes APOBEC3G, is not recruited into EVs [142]. APOBEC3G-loaded EVs would then render recipient cells more resistant to infection. Another example of EVs carrying anti-HIV factors is the vesicles containing the antiviral soluble host molecule cGAMP that acts through interferon and innate immune responses [143]. In conclusion, the interplay between EVs and HIV appears to be extremely complex and not fully disclosed yet [144]. Additional examples of viruses adopting exosomes to facilitate their spreading are the hepatitis B virus [145], HTLV-1 [146] as well as rhinovirus. In the latter case, virus infected cells secrete EVs that induce the upregulation of viral receptors in monocytes, thus increasing the spectrum of cells that can be infected by the virus [147]. Interestingly, it has been recently reported that also cells infected with viruses belonging to the Coronaviridae family, coronaviruses, may produce exosomes with different functions in the replication cycle, pathogenesis and cell response to infection [148]. A coronavirus, the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), is the virus causing the current pandemic. In this context, it has been shown that exosomes can transfer the angiotensin-converting enzyme 2 (ACE2), the main receptor that allows SARS-CoV-2 entry, to other cells rendering them susceptible to viral infection [149]. However, the same EVs might also work as a decoy for circulating SARS-CoV-2 [150], thus suggesting a potential role as therapeutic agents. The idea of using exosomes to interfere with viral infections is not novel [151], as well as the possibility to develop vaccination strategies based on EVs loaded with viral antigens/nucleic acids to stimulate antiviral immune responses, as it naturally occurs from infected cells. Finally, it must be mentioned that EVs can also transfer different miRNAs and even long non-coding RNAs that can

affect viral spreading/pathogenesis as well as the host immune system [152–154]. The role of EVs in viral transmission is further discussed in Section 4.4 below.

On the other hand, viruses are able to exploit the ESCRT machinery to execute their life cycle. Viral replication encompasses different steps that can be summarized as follows: entry into target cells, uncoating of the viral genome, replication and transcription of the viral genome, assembly of new viral progeny, egress from infected cells (Figure 3).

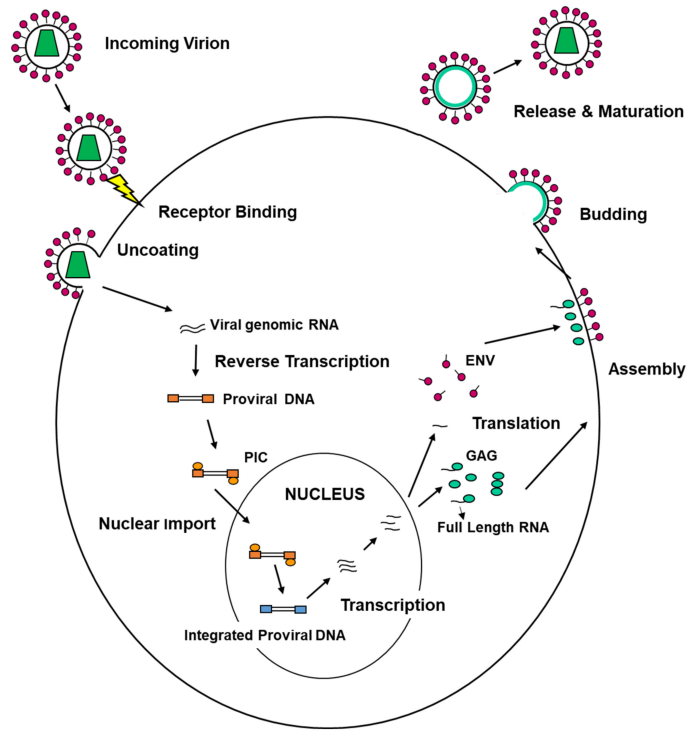


Figure 3. Schematic representation of the main steps in a typical viral life cycle. The figure reports the replication of HIV-1. Upon entry into target cells, mediated by fusion of the viral envelope with the cell surface, the viral genome, which is single-stranded positive RNA (two molecules per viral particle), is partially uncoated from the virion structural proteins, although it is shielded by viral and cellular factors, not entirely known yet (pre-integration complex, PIC). PIC travels to the nucleus and, at the same time, the viral RNA is retrotranscribed by one of the viral enzymes into double-stranded DNA (provirus). Once entered the nucleus, the proviral DNA is integrated into the cellular genome and becomes part of it. Indeed, it is the cellular RNA polymerase II that transcribes the integrated provirus in mRNAs that are translated into the cytosol to give rise to the structural proteins Gag-Pol and Env. The first one drives—in concert with cellular proteins—the assembly and egress of the progeny virions. The latter enriches the viral envelope and is essential to bind the cellular receptors during the next replication round. Regulatory and accessory proteins are also translated. Furthermore, full-length genomic RNAs are also produced to be packaged into the novel budding particles. Once released in the extracellular environment, the new virions terminate their maturation, and they become infectious. Different viruses, due to the specific biological features (in a particular type of genome and presence or absence of an envelope), might differently execute the steps of their life cycle. However, the main steps remain the same: entry, uncoating, genome replication and transcription, protein translation, assembly of progeny virions, egress from infected cells. These stages may present certain differences in the different viral families, also due to the different biological features that characterize each family itself (with the type of genomic nucleic acids and presence or absence of a lipid envelope enwrapping the particle as the main ones in this context). What remains a common trait is the necessity for the virus to interact with the cellular apparatus throughout its replication. This is also the main reason why viruses can alter cell functions, thus causing a disease, and are all pathogenic.

Seminal studies aimed at dissecting the mechanisms allowing the egress of HIV-1 from infected cells discovered the role played by the ESCRT machinery in the viral life cycle. Some of these studies have also contributed, over the years, to our understanding of the functioning of the machinery in fundamental cellular processes, despite its involvement in viral replication. It is now clear that different viruses can exploit ESCRT proteins or part of them to accomplish virtually all phases of their replication cycle. Interestingly, the interaction between ESCRTs and viruses is of ancient origin, as it has been shown that in Archaea, ESCRT-III components and VPS4 homologs support the replication of the Sulfolobus-turreted icosahedral virus [2]. We summarize below some of the functions of the ESCRT machinery in the viral life cycle with a special accent on the late steps of HSV-1 replication.

Finally, supporting a pivotal role of the ESCRT machinery in the viral life cycle, the host immune system evolved to target this pathway to control infections. In this context, the interferon-stimulated gene 15 (ISG15) represents a key player as it targets the ESCRT proteins in budding complexes to block the release of viruses [155,156]. Furthermore, specific polymorphisms within the 5′ sequence of TSG101 encoding gene (between the −183 and +181 nucleotides with respect to the translation start codon) have been associated with the acquired immunodeficiency syndrome (AIDS) progression, likely due to an effect on plasma HIV-1 load [157]. Overall, these findings strongly suggest that ESCRT proteins and TSG101, in particular, may be interesting targets for the development of antiviral drugs. In this context, pump inhibitors of the prazole family, which interact with TSG101, have been already tested and proved to efficiently inhibit a number of enveloped viruses [158,159], as we further discuss later.

4.1. ESCRT Machinery and Viral Entry

To enter into animal cells, viruses must cross the plasma membrane. This non-trivial process can be executed by two main routes: (1) fusion of the viral envelope (if present) with the cellular membrane, a strategy that does not cause membrane injuries [160]; (2) receptor-mediated endocytosis of viral particles that occurs for both enveloped and naked viruses. Members belonging to Picornaviridae, a family encompassing small naked RNA viruses, in most cases enter host cells by creating a pore within the endosomal membrane to translocate their genome in the cytosol where it is replicated [161]. Viruses that exploit the endolysosomal pathway need to escape from it to gain access to the cytosol before undergoing degradation or recycling back to the plasma membrane [162]. In the case of enveloped viruses, this step is carried out by fusion of their envelope with the endosomal membrane, a process that, as mentioned above, should not determine ruptures of the phospholipid bilayer. On the other hand, naked viruses usually cause lesions while crossing the endosomal membranes. This process would result in the exposure of the organelle content in the cytosol with toxic effects and triggering of inflammation. Although most of the mechanisms facilitating this step and the network of cell/viral proteins playing a role are still to be fully elucidated, evidence indicates that ESCRT machinery may play a role in this context, especially when small injuries are involved. It must be mentioned that, at least to our knowledge, this aspect has not been investigated yet. However, it is interesting to note that several viruses display, within virion structural proteins, conserved and short proline-rich motifs, known as late domains (L-domains) that are better described later and that interact with ESCRT and ESCRT related factors [163]. These domains, when exposed in entering viral particles, could act to recruit the ESCRT machinery, for instance, to control membrane damage. Interestingly one of these L-domains, the PPxY motif, is present in the adenoviral membrane lytic protein VI [164]. Adenoviruses are naked DNA viruses that have been extensively studied also with respect to the mechanism of entry into target cells. Upon receptor-mediated endocytosis, adenoviral particles undergo capsid perturbation with the release of the protein VI. Protein VI displays membrane lytic activity [165], and it is crucial for viral infectivity [166]. Membrane damages create pores large enough for enabling viral particle access to the cytosol [167,168]. Protein VI is also known to counteract

cellular autophagy, which is activated to remove damaged endosomes [169]. Currently, it is not clear whether this process relies on the ESCRT machinery, although protein VI can interact through a PPxY motif with the Nedd4 ubiquitin ligase, one of the E3 enzymes playing a role in certain ESCRT-mediated processes, as better detailed below. It would be of importance to investigate whether protein VI can also engage the ESCRT machinery prior to exerting its control on autophagy by preventing the cell from repairing small endosomal lesions. Indeed, this could represent a novel and additional mechanism of cell-intrinsic response to a viral infection that may even go beyond the adenoviral entry.

As additional hints on the role that ESCRT components play in viral entry, it has been demonstrated that Kaposi's sarcoma herpesvirus (KHSV) hijacks the ESCRT machinery for efficient entry in endothelial cells [170]. HRS and TSG101 are key factors in this context. Interestingly, the involvement of TSG101 is common among viruses besides KHSV that enter target cells by micropinocytosis. Indeed, this ESCRT-I component has been shown to contribute to the entry of the Crimean-Congo hemorrhagic fever virus (CCHFV), human papillomaviruses, and echovirus-1 [171–173]. Furthermore, in the case of KHSV, TSG101 is also important for the correct trafficking of viral particles through the endosomal pathway [170]. In the case of CCHFV, a tick-borne bunyavirus that can cause a severe clinical manifestation in humans, not only silencing of TSG101 but also depletion of Vps24, VPS4 and Alix inhibits infection. Moreover, virions are trapped in the MVBs when cells are treated with bafilomycin A. This finding suggests that these organelles are the site of virus-endosome membrane fusion. In addition, the highly pathogenic Old World arenavirus Lassa virus (LASV) and the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) have been described to exploit HRS, Tsg101, ESCRT-III factors, as well as VPS4 to enter into cells [174]. Finally, a study adopting a genome-wide RNA-interference screening has suggested a role for ESCRT components necessary for ILV formation in rotavirus cell entry [175]

4.2. ESCRT Machinery Involvement in the Formation of Viral Replication/Assembly Compartments

Once they get access to the cell, several RNA and DNA viruses remodel cellular membranes and exploit host proteins to build specialized compartments where viral genome replication takes place. In these virus-induced "factories", viral and cellular factors involved in the process are concentrated and preserved from innate immune response mechanisms. Replication compartments originate from different cell membranes, including the plasma membrane, ER, GA, peroxisomes, mitochondria and endosomes [7]. Their biogenesis resembles the ILVs formation process. However, in this case, a membrane fission event does not take place. In addition to genome replication, these compartments can also facilitate the assembly of viral progeny. Working as a shield towards antiviral defenses, the replication compartments are especially useful for most RNA viruses. Indeed, during replication of their genome, high copies of double-stranded RNAs are produced that are the main trigger of the interferon response. Interestingly, ESCRT components have been shown to play a role in the formation of replication compartments of different plus-stranded RNA plant viruses [176–179]. Flaviviridae, a family of single-stranded positive-sense RNA viruses that include some human pathogens as dengue virus (DENV) and Japanese encephalitis virus (JEV), induce remodeling of the ER membranes to form compartments for genome replication and assembly of viral particles. An elegant study performed by adopting a mass spectrometry approach has shown that several ESCRT factors are re-localized to these compartments. In agreement with the functional relevance of this finding, RNAi screening production of both DENV and JEV particles was significantly affected by lack of TSG101 or of specific ESCRT-III components. However, it was unaffected by depletion of VPS4, thus suggesting that a unique and specific set of ESCRT factors contributes to the biogenesis of the flavivirus-induced replication compartment [180]. It must be mentioned that, while in the case of plant viruses, the role of the ESCRT-machinery is clearly linked to the formation of the replication compartments, in the case of other viruses, flaviviruses included, it is very difficult to distinguish between a role in this process

or purely in particle assembly. This is particularly true for certain large DNA viruses, as the ones belonging to the Herpesviridae family, the herpesviruses, whose genome replication and particle assembly occur in the nucleus and in the cytosol, respectively. For instance, both VPS4 and CHMP1 have been reported to localize in the proximity of the human cytomegalovirus (HCMV)-induced cytoplasmic assembly compartments [181]. However, it is very difficult to understand whether these proteins participate in the biogenesis of these specialized sites of viral maturation, to the process of viral morphogenesis or to both.

4.3. ESCRT Machinery and Viral Egress from Infected Cells

Enveloped viruses are enwrapped by host membranes and usually egress from infected cells without necessarily causing cell death. Examples of enveloped viruses are HIV, herpesviruses, influenza virus, flaviviruses and coronaviruses (SARS-CoV-2 included). Naked viruses are not surrounded by host membranes and typically are released upon cell lysis [65,182]. Examples of un-enveloped viruses are adenovirus, poliovirus, norovirus, HAV. Enveloped viruses leave the cells through a complex process known as budding that requires two main membrane modifications, (i) a deformation around the assembling virions and (ii) fission, resulting in the detachment of the viral particles from the cellular surface. Budding takes place not only at the plasma membrane but also into intracellular organelles that then fuse with the plasma membrane, releasing viral particles in the external environment [183]. Assembly of virions and budding is usually strictly linked processes. As mentioned above, studies aimed at the dissection of the molecular mechanisms enabling HIV-1 budding have been instrumental for understanding how enveloped viruses are released from infected cells and for unrevealing the viral/cellular protein network involved in the process. In 1991, Göttlinger and coworkers identified in the C-terminal p6 domain of HIV-1 protein Gag, which encodes for the main structural components of the virion, i.e., matrix, nucleocapsid and capsid, a highly conserved PT/SAP motif playing a crucial role in the detachment of budded virions from the cell surface [184]. Starting from this seminal finding, additional research has led to the identification in the Gag protein of all known retroviruses of short proline-rich sequences, named late assembly or L-domains [185], functionally equivalent to the HIV-1 PT/SAP motif. To date, in addition to the PT/SAP motif, typical of most lentiviruses, two further L-domains have been well characterized: the PPXY-type L-domain present in the Gag proteins of oncoretroviruses, first, described in [185], and the YPXnL-type motif, identified in the Gag protein of the equine infectious anemia virus (EIAV) [186]. Besides retroviruses, L-domains and L-domain-like sequences have been identified in the structural proteins of most RNA-enveloped viruses, such as rhabdoviruses, filoviruses, arenaviruses, and paramyxoviruses [186], and in some DNA-enveloped viruses [187–191]. Interesting exceptions have been reported. For instance, neither the hepatitis C virus nor the influenza virus appears to have canonical L-domains or L-domain-like sequences [192,193], while the Bluetongue virus (BTV) presents both a PSAP and a PPXY motif, but at the level of the nonstructural protein NS3 [194]. Early studies indicated that L-domains act independently from their position in the viral protein and can be exchanged between unrelated viruses without losing their ability to mediate budding [195–197]. Furthermore, different types of L-domains often occur in combination; for instance, the HIV-1 p6 contains, in addition to its primary PTAP-type L-domain, an auxiliary L-domain of the YPXnL type [20,197,198], and the Ebola virus has a PTAPPEY domain which combines the PTAP and the PPXY types of L-domains [199]. Overall, these features suggested that the L-domains may represent docking sites for a set of cellular proteins belonging to a specific pathway exploited by retroviruses to execute budding efficiently. The first indication on this pathway came from previous observations that cellular proteins, such as the epithelial Na^+ channel, contained sequences overlapping with the L-domains and that their endocytosis was dependent on these motifs [199,200] and on ubiquitylation [201]. At the same time, studies were supporting the hypothesis on the role of ubiquitin in the viral budding process [50]. In particular, we were able to show that ubiquitin residues involved in retroviral budding were the ones known to play

a role in endocytosis [202]. Importantly, in 2001, the Carter and Sundquist laboratories discovered that the PT/SAP motif of HIV-1 p6 binds to TSG101 and that this interaction is crucial for viral budding [203,204]. Later, we show that Alix also plays a role in this context, and it is recruited to the site of budding through a YPXnL domain mapping in the Gag-p6 [20]. Finally, the PPXY type of L-domains was found to bind Nedd4 like ubiquitin ligases that facilitate ESCRT-machinery engagement [50,205]. Over the last two decades, several studies aimed at dissecting the complex viral/cellular protein network that enables most of the RNA-enveloped viruses to hijack the ESCRT-machinery. An updated review on this topic focused on retroviruses was recently published by Rose and coworkers [206], and several excellent and more general ones are available [12,18]. Thus, we do not further describe these aspects here. However, taking inspiration mostly from studies focused on retroviruses, we would like to point out some clear common traits among mechanisms adopted by enveloped viruses to usurp ESCRT-machinery during egress from infected cells: (1) ESCRT-III and VPS4 represent the master players in this process; (2) early data suggested that, while Alix and ESCRT-I were sufficient to recruit ESCRT-III to the side of HIV-1 budding, ESCRT-II was dispensable. By contrast, ESCRT-II is crucial for the exit of the avian sarcoma leukemia virus (ASLV). ASLV Gag displays a PPxY type L-domain [207], while, as already mentioned, HIV-1 Gag contains both a PT/SAP and a YPXnL L-domain. Overall, these findings support the notion that different L domains connect retroviral structural proteins with a different array of ESCRT proteins to execute budding; (3) on the other hand, it has been shown that mutations affecting Alix engagement do not entirely inhibit HIV-1 budding, suggesting a compensatory role for ESCRT-II [208,209] or for additional BRO1 containing proteins in this process. Importantly, most retroviruses have evolved redundant mechanisms to hijack ESCRT-III/VPS4 to the site of budding, as exemplified by HIV [210], and as we reported, for instance, in the case of the closely related feline immunodeficiency virus (FIV) [211]. Strikingly, budding of HIV-1 and FIV devoid of functional L-domains can be efficiently rescued by overexpression of Nedd4-like ubiquitin ligases, although both lentiviruses lack a PPxY motif within their Gag [212–215]. Finally, retroviruses have been shown to engage ESCRT components also through Gag ubiquitylation [50,204,216], as well as through the Gag Nucleocapsid region [53,211,217–219]; (4) RNA-enveloped viruses that seem to egress independently from the ESCRT-machinery, e.g., the influenza virus [220], encode proteins that might functionally replace ESCRT-III [193]; (5) large DNA-enveloped viruses can adopt ESCRT factors to egress from infected cells, but with some clear peculiarities that are discussed later in the case of HSV-1.

4.4. EVs and Autophagy in Viral Transmission

As the healthy counterpart, virus infected cells can secrete EVs that can exert different functions as described above. These EVs can shed viral proteins, and nucleic acids and either trigger immune responses or affect recipient cells, favoring viral spreading [8]. Interestingly, EVs and enveloped viral particles share common biogenesis machinery, and in some cases, it is difficult to isolate EVs containing viral molecules from forms of defective viral particles as they display similar density, size, and composition [8]. As mentioned above, viruses are distinguished in enveloped and naked viruses. However, in 2013, this concept was challenged by the finding of both types of particles in the supernatant of liver cells infected with the hepatitis A virus (HAV), as well as in vivo [221,222]. A follow-up study demonstrated that HAV enveloped particles could be found inside MVBs and ESCRT depletion inhibited their release from infected cells, thus suggesting exosomes as an egress pathway. Furthermore, these findings also indicate that the envelopment of HAV relies on the same cellular machinery exploited by most of the known enveloped viruses [65]. Importantly, HAV-containing exosomes, named "quasi-enveloped" HAV (eHAV), are able to protect viral particles from antibody-mediated neutralization. In addition to HAV, also hepatitis E virus (HEV), another naked virus, could be found in MVB. The release of eHEV particles is likely to occur via the exosomal pathway [223,224].

Interestingly, exosomes seem to play a role also in the egress of HIV. Indeed, Gould and coworkers demonstrated that EVs secreted by dendritic cells infected with HIV were able to transfer the infectious virus to CD4+ T lymphocytes [225]. Similar results were reported for hepatitis C virus (HCV). Exosomes loaded with HCV were indeed isolated from infected liver cell lines [226]. In addition, hepatic exosomes allowed transmission of infectious HCV particles in vitro and were resistant to antibody neutralization, at least partially. In agreement, HCV release was found to follow both an exosome-driven and an exosome-free route, as in the case of HAV. Importantly, exosome-associated HCV particles were infectious and resistant to neutralizing antibodies [227]. Furthermore, it was also demonstrated that EVs could deliver HCV replication-competent subgenomic RNAs allowing infection of recipient cells, even in the absence of infectious particles and independently from known viral receptors [228]. This latter aspect is particularly relevant as it would shield the virus from neutralization, an additional immune evasion strategy that may be exploited by other viruses. In this respect, also EVs secreted from Zika virus (ZIKV) or foot and mouth disease virus (FMDV) infected cells contain viral RNAs and proteins and can support viral transmission as well as function as an escape strategy from neutralization [229,230]. Furthermore, infectious particles of severe fever with thrombocytopenia syndrome (SFTS) virus, a tick-borne bunyavirus associated with hemorrhagic fever, were found in exosomes and were shown to infect cells, bypassing the need of classical viral receptors [231]. Finally, DENV-infected insect cells produce EVs loaded with virus-like particles that are able to infect other cells [232].

EVs can contribute to the spreading of infection also by increasing the spectrum of cells susceptible to viral entry and/or viral infectivity. One of the mechanisms allowing this EV-mediated activity is the delivery of viral receptors from the producing cell to the recipient one, as described above. Moreover, in the case of HIV-1, it has been shown that resting CD4+ T lymphocytes, which are usually refractory to infection, become permissive to viral replication by the combined function of the TNF-converting enzyme ADAM17 and Nef, delivered by exosomes [136]. Additionally, Nef-containing EVs can alter lipid rafts on the cell surface, facilitating HIV entry by fusion and thus increasing infectivity [233]. It must be noticed that certain viruses that acquire their envelope by budding into cellular organelles, e.g., the HSV-1, can hijack the exosomes to facilitate their transmission, at least from certain cell types [234,235]. We discuss HSV-1 egress from infected cells later in this review.

Surprisingly, studies carried on with cells infected by certain members of the Picornaviridae family, e.g., poliovirus, revealed that also these viruses could egress from host cells in vesicles before cell lysis [236]. However, these vesicles did not derive from MVBs, but rather from autophagosomes [236,237]. As already mentioned, autophagosomes by fusing with lysosomes display a pivotal role in different aspects of cell biology. However, it is known that these structures can also fuse with the plasma membrane to release molecules and organelles in the extracellular environment, a process known as "secretory autophagy" [237]. Thus, the above-mentioned viruses hijack this specialized type of autophagy to egress from infected cells. Indeed, virus-containing autophagosomes, which in the case of the poliovirus originate from the ER, do not fuse with lysosomes but with the plasma membrane and release vesicles enwrapped in a single membrane and loaded with viral particles [236]. By contrast, to exosome-mediated uptake of viruses, autophagosome-derived vesicles drive viral entry into recipient cells via the respective physiological receptors. This finding suggests that vesicles are disrupted upon binding with the new host cell, releasing particles that need to bind to the surface receptors [236]. Furthermore, lipid recognition seems to play a role as well. Indeed, proteins able to bind the phosphatidylserine, a lipid enriching the autophagosome-derived vesicles, inhibited viral infection [236].

Overall, these findings indicate that vesicles can contribute to cell egress and transmission of both naked and enveloped viruses. The emerging picture is complex, and the impacts on the host immune response and viral pathogenicity urge further studies.

5. The Travel of Herpes Simplex Virus Type 1 from the Nucleus to the Extracellular Environment: Is There a Role for the ESCRT Machinery?

The Herpesviridae are large, enveloped DNA viruses that not only replicate their genome within the nucleus, but also start the process of assembling progeny particles in the same cellular compartment. Newly assembled nucleocapsids need to cross the nuclear membranes to translocate into the cytoplasm. In the cytosol, nucleocapsids are enwrapped in proteins that constitute what is known as tegument, an amorphous coat that is typical of herpes viral particles. Next, tegument surrounded nucleocapsids bud into vesicles derived from host organelles to acquire their envelope. Finally, these vesicles fuse with the plasma membrane to release the mature viral progeny in the extracellular environment. Therefore, the biogenesis of herpesviral particles is a multistep process involving at least two budding and fusion events and complex traffic of nascent virions from inside the nucleus to outside the cell. Notably, ESCRT components play a role along this complex process, although with peculiar and distinct features with respect to the better-clarified strategies evolved by retroviruses and several other families of enveloped RNA viruses to hijack the ESCRT machinery during budding. One of the most striking peculiarities in the assembly and maturation of herpesviral particles is the first budding from the nuclear envelope. If ESCRTs are involved in this process, they need to be available within the nuclear environment. This is indeed the case. When TSG101 was described as a key component of the cellular machinery involved in HIV-1 budding, this finding came as a surprise as this factor was mainly known for its nuclear functions, as also suggested by its name (tumor suppressor gene 101). Indeed, TSG101 was initially characterized for its ability to inhibit not only the transcriptional activity of the nuclear hormone receptor superfamily [238,239], but also for its ability to suppress the activity of certain viral promoters and transcriptional activators [239]. As an example, TSG01 can interact with Rta, a transactivator of EBV lytic genes, and, as a consequence, it can be recruited to the viral promoters [240]. TSG101 improves binding of Rta to these promoters and its transactivating activity. Furthermore, TSG101 is known to play a role in the p53 pathway [241]. Indeed, TSG101 inhibits ubiquitylation and degradation of the mouse double minute 2 homolog (MDM2) protein. MDM2 is an E3 ubiquitin ligase, which targets p53 for degradation by recognizing its N-terminal transactivation domain (TAD). Furthermore, MDM2 can also inhibit p53 transcriptional activation. The effect of TSG101 on MDM2 stability results in p53 downregulation accompanied by a stimulation of the cell cycle [242]. As in the case of TSG101, also ESCRT-II components were first described as nuclear proteins. Indeed, the yeast orthologues of ESCRT-II subunits are named ELL-associated proteins (EAPs). ELL binds to the RNA polymerase II, functioning as an elongation factor. Interestingly, also in rats, ELL interacts with three factors belonging to ESCRT-II (EAP20/Vps25, EAP30/Vps22, and EAP45/hVps36), giving rise to a heterotetrameric complex that enhances transcription elongation, at least in vitro [243,244]. Overall, ESCRT-II appears to interfere with the expression of specific genes by regulating the rate of promoter initiation of transcription. Finally, mammalian ESCRT-III subunits (CHMPs) were initially studied for their functions in the nucleus as suggested by their initial name, i.e. chromatin-modifying proteins, that was then changed into charged multivesicular body proteins upon discovery of their cytoplasmic functions. These two names are currently used interchangeably. In the context of CHMP nuclear activities, several studies focused on CHMP1. This protein is characterized by a predicted bipartite nuclear localization signal, and it is found both in the cytosol and in the nuclear matrix. Interestingly, nuclear CHMP1 appears to be post-translationally modified with respect to the cytosolic counterpart. This modification may contribute to the regulation of CHMP1 compartmentalization or to the reason for a different activity of the protein in the two cellular spaces. In the nucleus, CHMP1 interacts with polycomb-like proteins that, along with the other components of the polycomb group (PcG), enable chromatin condensation and gene silencing during development. Furthermore, it has been suggested that CHMP1 might be involved in chromosome condensation during mitosis [245]. Interestingly, in a screening aimed at the identification of human CHMP interactors, 19 out of 45 novel-fished-out factors were nucleus-related

proteins [246]. Finally, several CHMPs take part in nuclear sumoylation pathways. These distinct functions of the nucleoplasmic ESCRT components with respect to the activity displayed by the cytoplasmic counterpart are a striking example of the importance/role of cell compartmentalization.

Interestingly, however, herpesviruses, at least HSV-1 and EBV that have been studied under this respect exploit nucleoplasmic ESCRTs to facilitate nuclear inner membrane remodeling and fission in the first envelopment step, as better explained below. Thus, in this context, nuclear ESCRTs function as their cytoplasmic counterpart. HSV-1 belongs to the Alphaherpesvirinae subfamily of the Herpesviridae, and it is a neurotropic virus ubiquitous in the human population. HSV-1 usually causes localized mucocutaneous lesions in the facial region. Primary infection often occurs after direct interpersonal contact and precedes the retrograde transport of the virus to the sensory nerve ganglia, where it establishes a lifelong latent infection with recurrent infections [247]. In fragile patients, HSV-1 can systemically disseminate and cause fatal infections. In particular, HSV-1 can cause acute encephalitis and severe neonatal infections. The process by which HSV-1 virions exit the nuclear and then the cytoplasmic membranes of infected cells is very complex and not fully elucidated yet. Viral DNA replication and gene transcription take place in the nucleus where, during productive (lytic) infection, nucleocapsids are also assembled. Althoug a sufficient dilatation of nuclear pores to allow egress of viral capsids was described [248], the most widely accepted and supported model postulates that HSV-1 capsids by budding from the INM acquire a lipid envelope, which then fuses with the ONM [249]. A central role is played by the herpesviral nuclear egress complex (NEC) that is composed of viral proteins encoded by the UL31 and UL34 genes. NEC forms a hexagonal lattice on the inner surface of the nuclear membrane, and it is sufficient to induce perinuclear vesicles in uninfected cells expressing alphaherpesviral NEC proteins alone. Furthermore, HSV-1 NEC can mediate the budding of membrane vesicles in the absence of endogenous cellular proteins and ATP [249]. The UL34 protein is likely the real effector, but in the absence of the UL31 protein, it fails to localize to the nucleus, as it lacks a nuclear import signal. It is noteworthy that HSV-1s bearing deletions or dominant-negative mutations within the UL34 gene display highly reduced viral infectious titers with a corresponding accumulation of intranuclear viral capsids; however, some infectious virions can still be recovered. Different mechanisms may be activated in this case, including nuclear pore enlargement (see above) or nuclear envelope breakdown, but the relevance of such alternative pathways under physiological conditions is unclear. NEC-mediated budding is also likely to have a quality control function, selecting mature, DNA-filled C-capsids rather than type A and B capsids [250]. Conversely, budding is probably negatively regulated to reduce the number of capsid-less vesicles, for example, by phosphorylation of UL31 by the US3 viral kinase. Regulation of budding is even more complex and could involve tegument proteins that localize to the nucleus, such as UL36, UL47, UL16, UL11 and UL21, along with nonstructural proteins like UL24 [251] and the neurovirulence factor ICP34.5 [252]. De-envelopment of perinuclear enveloped virions (PEVs) is not as well characterized as envelopment: NEC alone is not able to perform it efficiently, and viral glycoproteins gB and gH play a role in the process; however, both proteins are dispensable for it [249].

It has been recently shown that HSV-1 employs nucleoplasmic ESCRT proteins to bud across the INM. In particular, UL34 can interact with Alix in infected cells, and UL31/UL34 leads to the localization of CHMP4B to the nuclear envelope. At the same time, virions accumulate in the perinuclear space of cells depleted of CHMP4A/B/C or Alix, likely because scission at the INM is inhibited [253]. Overall, these data indicate that HSV-1 NEC via Alix can recruit CHMP4 to the site of budding at the INM. However, a dominant-negative version of VPS4 did not abrogate transit of nascent nucleocapsids to the cytosol [253–256], suggesting no major nuclear egress defects. On the other hand, it has been demonstrated that NEC alone is sufficient to drive membrane scission, at least in vitro [257]. Furthermore, Alix does not appear to be essential for HSV-1 replication [258].

Thus, CHMP4/VPS4 would be necessary to optimize the rate of HSV-1 budding into the perinuclear space, but not strictly required. On the other hand, HSV-1 infection has been shown to trigger autophagy at the nuclear membranes [259,260], leading to degradation of lamins that constitute a barrier for HSV-1 nuclear egress [260,261]. Under this respect, ESCRT-III/VPS4 could facilitate HSV-1 budding from the INM by functioning in the biogenesis of the autophagosome and thus in laminin destruction [66,262]. Finally, ESCRT-III may be recruited to help the nuclear envelope stressed by HSV-1 budding nucleocapsid to maintain its integrity, although, as described above, CHMP-7 and not Alix is the factor recruiting CHMP4B at the INM during this process [263]. Of note, as discussed below, TSG101 has been recently implicated in HSV-1 ability to cross the nuclear envelope. Interestingly also the NEC of EBV, constituted by BFLF2 and BFRF1 proteins, has been described to interact with ESCRT components during EBV capsid nuclear envelopment [264]. In this case, not only Alix, CHMP4B and VPS4 play a role, but also the ubiquitin ligase Itch/AIP4 that belongs to the Nedd4 family of E3 enzymes. As in the case of retroviral Gag, BFRF1, which interacts with Itch, is ubiquitylated at several lysine residues [265]. Mutations of these residues affect the release of enveloped particles from infected cells, likely due to an impairment of the EBV particle trafficking from the nucleus to the cytosol [265]. Although some viral tegument proteins localize to the nucleus, as mentioned above, most of them are acquired in the cytosol: subsequently, virions undergo secondary envelopment by budding into cytoplasmic membranes. The exact nature of this membrane is still under debate. HSV-1 capsids have been observed enveloping at structures morphologically resembling the trans-Golgi network (TGN) [266]. This finding would be consistent with the lipid composition of the final HSV-1 envelope [267]. An alternative model identifies endocytic tubules deriving from the plasma membrane and containing viral envelope proteins at the site of cytoplasmic envelopment [268]. Following secondary envelopment, HSV-1 hijacks secretory pathways to exit the cell by exocytosis, though at late-stages of infection, some virions are released following cell death and lysis. Among tegument proteins, UL36 and UL37 are crucial for envelopment [269] as they mediate both interactions with cytoskeleton proteins to transport virions towards the Golgi apparatus and with envelope glycoproteins. Other tegument proteins are essential (UL48) or auxiliary (e.g., UL51 and UL7) for secondary envelopment [262]. The precise identity of the compartment in which secondary envelopment takes place is still unknown, though it surely involves post-Golgi membranes, which may include TGN or endosomes, or possibly membranes of heterogeneous origin [262]. Viral glycoproteins are trafficked to the same post-Golgi membranes via different mechanisms. In particular, while some viral surface proteins have clear localization signals, others do not [188]. Knockdown of Rab6A, involved in intracellular trafficking, inhibits viral secondary envelopment [268]. Enveloped virions then are translocated to the plasma membrane by a mechanism, which is associated with proteins directly involved in the secretory pathway, such as Rab GTPases, GAP-43, kinesin-1 and SNAP-2, as well as upstream regulators like protein kinase [247]. Since the discovery that the dominant-negative version of VPS4 (VPS-EQ) affects HSV-1 cytoplasmic envelopment and replication [254], several studies have connected ESCRT machinery also to this step of viral egress from infected cells. For instance, it has been shown that dominant forms of different CHMPs can also inhibit HSV-1 replication [258]. In particular, electron microscopy studies suggested that in cells overexpressing VPS-EQ, partially enveloped nucleocapsids of HSV-1 accumulate in the cytosol as the final step of membrane fission into the lumen of a cytoplasmic organelle did not occur [256]. This phenotype is reminiscent of the one found for HIV-1 in cells expressing the same dominant-negative protein or in the case of mutation of the PT/SAP L-domain [20,184]. Importantly, a number of HSV-1 proteins contain binding motifs for Alix as well as for TSG101 [258]. However, neither the dominant-negative version of these proteins nor their depletion affects HSV-1 yield [258]. Interestingly, it has been reported that proton pump inhibitors of the prazole family, by interfering with TSG101, affect not only the budding of HIV-1 but also of different enveloped viruses, EBV included [158,159], and, more recently, in

a pre-print manuscript by Leis and coworkers, of HSV-1 and HSV-2 [270]. It must be noted that the drugs appear to inhibit HSV-1 transit through the nuclear membrane and not the final envelopment in the cytosol. In any case, these studies, on one hand, further connect TSG101 to the egress of different viruses, including HSV-1, from infected cells. On the other hand, they support the possibility of adopting ESCRT proteins as a target for antiviral therapy. Despite the presence of a PPxY domain in HSV-1 UL56 protein, there is no evidence that interaction with Nedd4 is involved in ubiquitin-mediated recruitment of the ESCRT machinery to execute viral envelopment. In agreement, the viral RING finger E3 ubiquitin ligase ICP0 does not seem to play a role in ESCRT engagement.

In conclusion, although TSG101 may play a role as suggested by the prazole-based data, the mechanism adopted by HSV-1 to recruit ESCRT-III to the side of the final envelopment is still under evaluation. Interestingly, different studies have focused on the viral tegument and envelope proteins as potential ESCRT-III recruiting factors/cofactors. Under this respect, we were able to show that glycoprotein B (gB), one of the most highly conserved glycoproteins across the Herpesviridae family, was sorted into MVB membranes [188]. In cells expressing VPS4-EQ, the site of intracellular gB accumulation and maturation was altered, indicating that gB traffic was dependent on a functional MVB pathway. Interestingly, gB was ubiquitylated in both infected and transfected cells. A partial deletion of the gB cytoplasmic tail resulted in a dramatic reduction of ubiquitination, as well as of progeny virus assembly and release to the extracellular compartment. Our data support the view that the sorting of gB to MVB membranes may represent a critical step in HSV envelopment and egress [188]. On the other hand, the major and highly conserved tegument protein UL36 appears to be a very promising candidate due to its essential role in the final envelopment of HSV-1 particles. Supporting this hypothesis, UL36 encompasses an amino-terminal functional deubiquitylase (DUB) domain [271,272] that would fit with the role of ubiquitin in ESCRT-mediated viral budding described above. We were able to show that HSV-1 UL36 regulates the ubiquitination of TSG101 [189]. However, as UL36 DUB activity is not essential for HSV-1 replication [258,273], we could not conclude that this finding is relevant in the context of viral envelopment. Under this respect, an elegant study carried on by adopting light and scanning electron microscopy strongly suggested a role for UL36 in the recruitment of capsids to the site of ESCRT-III/Vps4 localization [256], although this effect may be indirect. Finally, a recent study has demonstrated that the tegument protein UL51 has an impressive structural similarity to the ESCRT-III subunit CHMP4B, and can give rise to long ESCRT-III-like polymers in vitro [274]. Thus, UL51 could facilitate CHMP polymerization or may even functionally replace CHMPs originating ESCRT-III-like filaments. Conceivably, any envelope and tegument proteins involved in cytoplasmic envelopment could facilitate the recruitment of ESCRT components as well as the docking of nucleocapsid-containing vesicles at the assembly sites. Studies may also be complicated by the redundant functions of different viral factors.

Overall, although ESCRT components are clearly involved in HSV-1 final envelopment and egress from infected cells, there are still several open questions to address before fully understanding all the peculiar aspects of these complex late steps of viral replication.

6. Conclusions and Outlook

The ESCRT machinery is involved in many fundamental cellular pathways. In eukaryotic cells, ESCRTs contribute to both maintenance of cell compartmentalization, as well as to the biogenesis of vesicles and thus to the crosstalk among organelles and between the cell and the extracellular environment. Over the past years, efforts have been made to gain further insights into the molecular mechanisms enabling most of the ESCRT-mediated pathways. However, there are still unmet questions that need to be addressed, including a better understanding of the precise role of the individual interactions and steps allowing the recruitment of the same set of proteins to different locations to facilitate or play key roles in a variety of processes. Viruses interact with the ESCRT components in different steps of their replication cycle and/or influence ESCRT-mediated actions to their advantage.

Thus, the study of viral/ESCRT protein interplay might contribute to unraveling aspects of the ESCRT machinery functions as well as of its regulation that are still under debate. In this context, HSV-1 and herpesviruses, in general, might be of great interest as they exploit ESCRT components in peculiar ways that differ from the ones that have been well characterized for HIV-1 and most RNA-enveloped viruses. Particularly interesting is the fact that HSV-1 uses both nuclear and cytoplasmic ESCRT factors to cross the nuclear and plasma membrane barriers. Furthermore, nascent virions engage cytoskeleton elements as well as secretory pathways to travel throughout the cell and exit in the extracellular environment. Thus, studying the missing links of this travel may contribute to our knowledge not only on the ESCRT machinery functions, but also on the molecular exchanges between intracellular organelles and compartments.

Author Contributions: Conceptualization, A.C., A.R., G.P. and C.P.; writing—original draft preparation, A.C., A.R., G.P. and C.P.; writing—review and editing, A.C., A.R., G.P. and C.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rajwar, A.; Morya, V.; Kharbanda, S.; Bhatia, D. DNA Nanodevices to Probe and Program Membrane Organization, Dynamics, and Applications. *J. Membr. Biol.* **2020**, *253*, 577–587. [CrossRef] [PubMed]
2. Vietri, M.; Radulovic, M.; Stenmark, H. The many functions of ESCRTs. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 25–42. [CrossRef]
3. Campsteijn, C.; Vietri, M.; Stenmark, H. Novel ESCRT functions in cell biology: Spiraling out of control? *Curr. Opin. Cell Biol.* **2016**, *41*, 1–8. [CrossRef]
4. Robinson, M.; Schor, S.; Barouch-Bentov, R.; Einav, S. Viral journeys on the intracellular highways. *Cell Mol. Life Sci.* **2018**, *75*, 3693–3714. [CrossRef] [PubMed]
5. Lv, Y.; Zhou, S.; Gao, S.; Deng, H. Remodeling of host membranes during herpesvirus assembly and egress. *Protein Cell* **2019**, *10*, 315–326. [CrossRef] [PubMed]
6. Zhang, J.; Lan, Y.; Sanyal, S. Membrane heist: Coronavirus host membrane remodeling during replication. *Biochimie* **2020**, *179*, 229–236. [CrossRef] [PubMed]
7. Miller, S.; Krijnse-Locker, J. Modification of intracellular membrane structures for virus replication. *Nat. Rev. Microbiol.* **2008**, *6*, 363–374. [CrossRef]
8. Martins, S.T.; Alves, L.R. Extracellular Vesicles in Viral Infections: Two Sides of the Same Coin? *Front. Cell Infect. Microbiol.* **2020**, *10*, 593170. [CrossRef]
9. Katzmann, D.J.; Babst, M.; Emr, S.D. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. *Cell* **2001**, *106*, 145–155. [CrossRef]
10. Schoneberg, J.; Lee, I.H.; Iwasa, J.H.; Hurley, J.H. Reverse-topology membrane scission by the ESCRT proteins. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 5–17. [CrossRef]
11. Hanson, P.I.; Cashikar, A. Multivesicular body morphogenesis. *Annu. Rev. Cell Dev. Biol.* **2012**, *28*, 337–362. [CrossRef] [PubMed]
12. Ahmed, I.; Akram, Z.; Iqbal, H.M.N.; Munn, A.L. The regulation of Endosomal Sorting Complex Required for Transport and accessory proteins in multivesicular body sorting and enveloped viral budding—An overview. *Int. J. Biol. Macromol.* **2019**, *127*, 1–11. [CrossRef] [PubMed]
13. Mast, F.D.; Herricks, T.; Strehler, K.M.; Miller, L.R.; Saleem, R.A.; Rachubinski, R.A.; Aitchison, J.D. ESCRT-III is required for scissioning new peroxisomes from the endoplasmic reticulum. *J. Cell Biol.* **2018**, *217*, 2087–2102. [CrossRef] [PubMed]
14. Kostelansky, M.S.; Sun, J.; Lee, S.; Kim, J.; Ghirlando, R.; Hierro, A.; Emr, S.D.; Hurley, J.H. Structural and functional organization of the ESCRT-I trafficking complex. *Cell* **2006**, *125*, 113–126. [CrossRef] [PubMed]
15. Kostelansky, M.S.; Schluter, C.; Tam, Y.Y.; Lee, S.; Ghirlando, R.; Beach, B.; Conibear, E.; Hurley, J.H. Molecular architecture and functional model of the complete yeast ESCRT-I heterotetramer. *Cell* **2007**, *129*, 485–498. [CrossRef] [PubMed]
16. Weissenhorn, W.; Gottlinger, H. ESCRT-I part II: Forming the real ESCRT-I complex. *Cell Host Microbe* **2007**, *2*, 1–2. [CrossRef] [PubMed]
17. Hierro, A.; Sun, J.; Rusnak, A.S.; Kim, J.; Prag, G.; Emr, S.D.; Hurley, J.H. Structure of the ESCRT-II endosomal trafficking complex. *Nature* **2004**, *431*, 221–225. [CrossRef] [PubMed]
18. Horvath, P.; Muller-Reichert, T. A Structural View on ESCRT-Mediated Abscission. *Front. Cell Dev. Biol.* **2020**, *8*, 586880. [CrossRef] [PubMed]
19. Gill, D.J.; Teo, H.; Sun, J.; Perisic, O.; Veprintsev, D.B.; Emr, S.D.; Williams, R.L. Structural insight into the ESCRT-I/-II link and its role in MVB trafficking. *EMBO J.* **2007**, *26*, 600–612. [CrossRef]

20. Strack, B.; Calistri, A.; Craig, S.; Popova, E.; Gottlinger, H.G. AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* **2003**, *114*, 689–699. [CrossRef]
21. von Schwedler, U.K.; Stuchell, M.; Muller, B.; Ward, D.M.; Chung, H.Y.; Morita, E.; Wang, H.E.; Davis, T.; He, G.P.; Cimbora, D.M.; et al. The protein network of HIV budding. *Cell* **2003**, *114*, 701–713. [CrossRef]
22. Kim, J.; Sitaraman, S.; Hierro, A.; Beach, B.M.; Odorizzi, G.; Hurley, J.H. Structural basis for endosomal targeting by the Bro1 domain. *Dev. Cell* **2005**, *8*, 937–947. [CrossRef] [PubMed]
23. Ali, N.; Zhang, L.; Taylor, S.; Mironov, A.; Urbe, S.; Woodman, P. Recruitment of UBPY and ESCRT exchange drive HD-PTP-dependent sorting of EGFR to the MVB. *Curr. Biol.* **2013**, *23*, 453–461. [CrossRef] [PubMed]
24. Parkinson, M.D.; Piper, S.C.; Bright, N.A.; Evans, J.L.; Boname, J.M.; Bowers, K.; Lehner, P.J.; Luzio, J.P. A non-canonical ESCRT pathway, including histidine domain phosphotyrosine phosphatase (HD-PTP), is used for down-regulation of virally ubiquitinated MHC class I. *Biochem. J.* **2015**, *471*, 79–88. [CrossRef]
25. Zhai, Q.; Landesman, M.B.; Robinson, H.; Sundquist, W.I.; Hill, C.P. Structure of the Bro1 domain protein BROX and functional analyses of the ALIX Bro1 domain in HIV-1 budding. *PLoS ONE* **2011**, *6*, e27466. [CrossRef] [PubMed]
26. Monroe, N.; Hill, C.P. Meiotic Clade AAA ATPases: Protein Polymer Disassembly Machines. *J. Mol. Biol.* **2016**, *428*, 1897–1911. [CrossRef]
27. Hurley, J.H.; Yang, D. MIT domainia. *Dev. Cell* **2008**, *14*, 6–8. [CrossRef]
28. Monroe, N.; Han, H.; Gonciarz, M.D.; Eckert, D.M.; Karren, M.A.; Whitby, F.G.; Sundquist, W.I.; Hill, C.P. The oligomeric state of the active Vps4 AAA ATPase. *J. Mol. Biol.* **2014**, *426*, 510–525. [CrossRef] [PubMed]
29. Obita, T.; Saksena, S.; Ghazi-Tabatabai, S.; Gill, D.J.; Perisic, O.; Emr, S.D.; Williams, R.L. Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. *Nature* **2007**, *449*, 735–739. [CrossRef]
30. Stuchell-Brereton, M.D.; Skalicky, J.J.; Kieffer, C.; Karren, M.A.; Ghaffarian, S.; Sundquist, W.I. ESCRT-III recognition by VPS4 ATPases. *Nature* **2007**, *449*, 740–744. [CrossRef] [PubMed]
31. Christ, L.; Raiborg, C.; Wenzel, E.M.; Campsteijn, C.; Stenmark, H. Cellular Functions and Molecular Mechanisms of the ESCRT Membrane-Scission Machinery. *Trends Biochem. Sci.* **2017**, *42*, 42–56. [CrossRef]
32. Samson, R.Y.; Dobro, M.J.; Jensen, G.J.; Bell, S.D. The Structure, Function and Roles of the Archaeal ESCRT Apparatus. *Subcell Biochem.* **2017**, *84*, 357–377. [CrossRef]
33. Ghazi-Tabatabai, S.; Obita, T.; Pobbati, A.V.; Perisic, O.; Samson, R.Y.; Bell, S.D.; Williams, R.L. Evolution and assembly of ESCRTs. *Biochem Soc. Trans.* **2009**, *37*, 151–155. [CrossRef]
34. Samson, R.Y.; Bell, S.D. Ancient ESCRTs and the evolution of binary fission. *Trends Microbiol.* **2009**, *17*, 507–513. [CrossRef]
35. Samson, R.Y.; Obita, T.; Hodgson, B.; Shaw, M.K.; Chong, P.L.; Williams, R.L.; Bell, S.D. Molecular and structural basis of ESCRT-III recruitment to membranes during archaeal cell division. *Mol. Cell* **2011**, *41*, 186–196. [CrossRef]
36. Samson, R.Y.; Obita, T.; Freund, S.M.; Williams, R.L.; Bell, S.D. A role for the ESCRT system in cell division in archaea. *Science* **2008**, *322*, 1710–1713. [CrossRef]
37. Bissig, C.; Gruenberg, J. ALIX and the multivesicular endosome: ALIX in Wonderland. *Trends Cell Biol.* **2014**, *24*, 19–25. [CrossRef] [PubMed]
38. Tabernero, L.; Woodman, P. Dissecting the role of His domain protein tyrosine phosphatase/PTPN23 and ESCRTs in sorting activated epidermal growth factor receptor to the multivesicular body. *Biochem. Soc. Trans.* **2018**, *46*, 1037–1046. [CrossRef] [PubMed]
39. Tang, S.; Henne, W.M.; Borbat, P.P.; Buchkovich, N.J.; Freed, J.H.; Mao, Y.; Fromme, J.C.; Emr, S.D. Structural basis for activation, assembly and membrane binding of ESCRT-III Snf7 filaments. *Elife* **2015**, *4*. [CrossRef]
40. Tang, S.; Buchkovich, N.J.; Henne, W.M.; Banjade, S.; Kim, Y.J.; Emr, S.D. ESCRT-III activation by parallel action of ESCRT-I/II and ESCRT-0/Bro1 during MVB biogenesis. *Elife* **2016**, *5*. [CrossRef]
41. Banjade, S.; Tang, S.; Shah, Y.H.; Emr, S.D. Electrostatic lateral interactions drive ESCRT-III heteropolymer assembly. *Elife* **2019**, *8*. [CrossRef]
42. Schoneberg, J.; Pavlin, M.R.; Yan, S.; Righini, M.; Lee, I.H.; Carlson, L.A.; Bahrami, A.H.; Goldman, D.H.; Ren, X.; Hummer, G.; et al. ATP-dependent force generation and membrane scission by ESCRT-III and Vps4. *Science* **2018**, *362*, 1423–1428. [CrossRef]
43. Peng, X.; Yang, L.; Ma, Y.; Li, Y.; Li, H. Focus on the morphogenesis, fate and the role in tumor progression of multivesicular bodies. *Cell Commun. Signal.* **2020**, *18*, 122. [CrossRef]
44. Eitan, E.; Suire, C.; Zhang, S.; Mattson, M.P. Impact of lysosome status on extracellular vesicle content and release. *Ageing Res. Rev.* **2016**, *32*, 65–74. [CrossRef] [PubMed]
45. Latifkar, A.; Ling, L.; Hingorani, A.; Johansen, E.; Clement, A.; Zhang, X.; Hartman, J.; Fischbach, C.; Lin, H.; Cerione, R.A.; et al. Loss of Sirtuin 1 Alters the Secretome of Breast Cancer Cells by Impairing Lysosomal Integrity. *Dev. Cell* **2019**, *49*, 393–408.e397. [CrossRef] [PubMed]
46. Yoon, S.; Kovalenko, A.; Bogdanov, K.; Wallach, D. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. *Immunity* **2017**, *47*, 51–65.e57. [CrossRef]
47. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. *Cell* **2019**, *177*, 428–445.e418. [CrossRef]
48. Raiborg, C.; Rusten, T.E.; Stenmark, H. Protein sorting into multivesicular endosomes. *Curr. Opin. Cell Biol.* **2003**, *15*, 446–455. [CrossRef]

49. Raiborg, C.; Stenmark, H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **2009**, *458*, 445–452. [CrossRef]
50. Calistri, A.; Munegato, D.; Carli, I.; Parolin, C.; Palu, G. The ubiquitin-conjugating system: Multiple roles in viral replication and infection. *Cells* **2014**, *3*, 386–417. [CrossRef]
51. Mukhopadhyay, D.; Riezman, H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science* **2007**, *315*, 201–205. [CrossRef]
52. Piper, R.C.; Luzio, J.P. Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes. *Curr. Opin. Cell Biol.* **2007**, *19*, 459–465. [CrossRef] [PubMed]
53. Bache, K.G.; Brech, A.; Mehlum, A.; Stenmark, H. HRS regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell Biol.* **2003**, *162*, 435–442. [CrossRef]
54. Raiborg, C.; Bache, K.G.; Gillooly, D.J.; Madshus, I.H.; Stang, E.; Stenmark, H. HRS sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat. Cell Biol.* **2002**, *4*, 394–398. [CrossRef]
55. Gillooly, D.J.; Morrow, I.C.; Lindsay, M.; Gould, R.; Bryant, N.J.; Gaullier, J.M.; Parton, R.G.; Stenmark, H. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* **2000**, *19*, 4577–4588. [CrossRef]
56. Mayers, J.R.; Fyfe, I.; Schuh, A.L.; Chapman, E.R.; Edwardson, J.M.; Audhya, A. ESCRT-0 assembles as a heterotetrameric complex on membranes and binds multiple ubiquitinylated cargoes simultaneously. *J. Biol. Chem.* **2011**, *286*, 9636–9645. [CrossRef] [PubMed]
57. Henne, W.M.; Buchkovich, N.J.; Zhao, Y.; Emr, S.D. The endosomal sorting complex ESCRT-II mediates the assembly and architecture of ESCRT-III helices. *Cell* **2012**, *151*, 356–371. [CrossRef] [PubMed]
58. Mierzwa, B.E.; Chiaruttini, N.; Redondo-Morata, L.; von Filseck, J.M.; Konig, J.; Larios, J.; Poser, I.; Muller-Reichert, T.; Scheuring, S.; Roux, A.; et al. Dynamic subunit turnover in ESCRT-III assemblies is regulated by Vps4 to mediate membrane remodelling during cytokinesis. *Nat. Cell Biol.* **2017**, *19*, 787–798. [CrossRef]
59. Adell, M.A.; Vogel, G.F.; Pakdel, M.; Muller, M.; Lindner, H.; Hess, M.W.; Teis, D. Coordinated binding of Vps4 to ESCRT-III drives membrane neck constriction during MVB vesicle formation. *J. Cell Biol.* **2014**, *205*, 33–49. [CrossRef] [PubMed]
60. Clague, M.J.; Liu, H.; Urbe, S. Governance of endocytic trafficking and signaling by reversible ubiquitylation. *Dev. Cell* **2012**, *23*, 457–467. [CrossRef]
61. Dores, M.R.; Paing, M.M.; Lin, H.; Montagne, W.A.; Marchese, A.; Trejo, J. AP-3 regulates PAR1 ubiquitin-independent MVB/lysosomal sorting via an ALIX-mediated pathway. *Mol. Biol. Cell* **2012**, *23*, 3612–3623. [CrossRef]
62. Dores, M.R.; Lin, H.; Neil, J.G.; Mendez, F.; Trejo, J. The alpha-arrestin ARRDC3 mediates ALIX ubiquitination and G protein-coupled receptor lysosomal sorting. *Mol. Biol. Cell* **2015**, *26*, 4660–4673. [CrossRef] [PubMed]
63. Dores, M.R.; Grimsey, N.J.; Mendez, F.; Trejo, J. ALIX Regulates the Ubiquitin-Independent Lysosomal Sorting of the P2Y1 Purinergic Receptor via a YPX3L Motif. *PLoS ONE* **2016**, *11*, e0157587. [CrossRef]
64. Baietti, M.F.; Zhang, Z.; Mortier, E.; Melchior, A.; Degeest, G.; Geeraerts, A.; Ivarsson, Y.; Depoortere, F.; Coomans, C.; Vermeiren, E.; et al. Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* **2012**, *14*, 677–685. [CrossRef]
65. Altan-Bonnet, N. Extracellular vesicles are the Trojan horses of viral infection. *Curr. Opin. Microbiol.* **2016**, *32*, 77–81. [CrossRef]
66. Takahashi, Y.; He, H.; Tang, Z.; Hattori, T.; Liu, Y.; Young, M.M.; Serfass, J.M.; Chen, L.; Gebru, M.; Chen, C.; et al. An autophagy assay reveals the ESCRT-III component CHMP2A as a regulator of phagophore closure. *Nat. Commun.* **2018**, *9*, 2855. [CrossRef]
67. Zhen, Y.; Spangenberg, H.; Munson, M.J.; Brech, A.; Schink, K.O.; Tan, K.W.; Sorensen, V.; Wenzel, E.M.; Radulovic, M.; Engedal, N.; et al. ESCRT-mediated phagophore sealing during mitophagy. *Autophagy* **2020**, *16*, 826–841. [CrossRef]
68. Sahu, R.; Kaushik, S.; Clement, C.C.; Cannizzo, E.S.; Scharf, B.; Follenzi, A.; Potolicchio, I.; Nieves, E.; Cuervo, A.M.; Santambrogio, L. Microautophagy of cytosolic proteins by late endosomes. *Dev. Cell* **2011**, *20*, 131–139. [CrossRef] [PubMed]
69. Filimonenko, M.; Stuffers, S.; Raiborg, C.; Yamamoto, A.; Malerod, L.; Fisher, E.M.; Isaacs, A.; Brech, A.; Stenmark, H.; Simonsen, A. Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J. Cell Biol.* **2007**, *179*, 485–500. [CrossRef]
70. Rusten, T.E.; Simonsen, A. ESCRT functions in autophagy and associated disease. *Cell Cycle* **2008**, *7*, 1166–1172. [CrossRef]
71. Carlton, J.G.; Martin-Serrano, J. Parallels between cytokinesis and retroviral budding: A role for the ESCRT machinery. *Science* **2007**, *316*, 1908–1912. [CrossRef] [PubMed]
72. Carlton, J.G.; Agromayor, M.; Martin-Serrano, J. Differential requirements for Alix and ESCRT-III in cytokinesis and HIV-1 release. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10541–10546. [CrossRef] [PubMed]
73. Carlton, J.G.; Caballe, A.; Agromayor, M.; Kloc, M.; Martin-Serrano, J. ESCRT-III governs the Aurora B-mediated abscission checkpoint through CHMP4C. *Science* **2012**, *336*, 220–225. [CrossRef] [PubMed]
74. Morita, E.; Sandrin, V.; Chung, H.Y.; Morham, S.G.; Gygi, S.P.; Rodesch, C.K.; Sundquist, W.I. Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. *EMBO J.* **2007**, *26*, 4215–4227. [CrossRef]
75. Guizetti, J.; Schermelleh, L.; Mantler, J.; Maar, S.; Poser, I.; Leonhardt, H.; Muller-Reichert, T.; Gerlich, D.W. Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments. *Science* **2011**, *331*, 1616–1620. [CrossRef]
76. Lee, H.H.; Elia, N.; Ghirlando, R.; Lippincott-Schwartz, J.; Hurley, J.H. Midbody targeting of the ESCRT machinery by a noncanonical coiled coil in CEP55. *Science* **2008**, *322*, 576–580. [CrossRef]
77. Mierzwa, B.; Gerlich, D.W. Cytokinetic abscission: Molecular mechanisms and temporal control. *Dev. Cell* **2014**, *31*, 525–538. [CrossRef]

78. Karasmanis, E.P.; Hwang, D.; Nakos, K.; Bowen, J.R.; Angelis, D.; Spiliotis, E.T. A Septin Double Ring Controls the Spatiotemporal Organization of the ESCRT Machinery in Cytokinetic Abscission. *Curr. Biol.* **2019**, *29*, 2174–2182.e2177. [CrossRef] [PubMed]
79. Goliand, I.; Nachmias, D.; Gershony, O.; Elia, N. Inhibition of ESCRT-II-CHMP6 interactions impedes cytokinetic abscission and leads to cell death. *Mol. Biol. Cell* **2014**, *25*, 3740–3748. [CrossRef]
80. Christ, L.; Wenzel, E.M.; Liestol, K.; Raiborg, C.; Campsteijn, C.; Stenmark, H. ALIX and ESCRT-I/II function as parallel ESCRT-III recruiters in cytokinetic abscission. *J. Cell Biol.* **2016**, *212*, 499–513. [CrossRef] [PubMed]
81. Hadders, M.A.; Agromayor, M.; Obita, T.; Perisic, O.; Caballe, A.; Kloc, M.; Lamers, M.H.; Williams, R.L.; Martin-Serrano, J. ESCRT-III binding protein MITD1 is involved in cytokinesis and has an unanticipated PLD fold that binds membranes. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 17424–17429. [CrossRef] [PubMed]
82. Lee, S.; Chang, J.; Renvoise, B.; Tipirneni, A.; Yang, S.; Blackstone, C. MITD1 is recruited to midbodies by ESCRT-III and participates in cytokinesis. *Mol. Biol. Cell.* **2012**, *23*, 4347–4361. [CrossRef] [PubMed]
83. Goliand, I.; Adar-Levor, S.; Segal, I.; Nachmias, D.; Dadosh, T.; Kozlov, M.M.; Elia, N. Resolving ESCRT-III Spirals at the Intercellular Bridge of Dividing Cells Using 3D STORM. *Cell Rep.* **2018**, *24*, 1756–1764. [CrossRef] [PubMed]
84. Elia, N.; Sougrat, R.; Spurlin, T.A.; Hurley, J.H.; Lippincott-Schwartz, J. Dynamics of endosomal sorting complex required for transport (ESCRT) machinery during cytokinesis and its role in abscission. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4846–4851. [CrossRef]
85. Reid, E.; Connell, J.; Edwards, T.L.; Duley, S.; Brown, S.E.; Sanderson, C.M. The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. *Hum. Mol. Genet.* **2005**, *14*, 19–38. [CrossRef]
86. Yang, D.; Rismanchi, N.; Renvoise, B.; Lippincott-Schwartz, J.; Blackstone, C.; Hurley, J.H. Structural basis for midbody targeting of spastin by the ESCRT-III protein CHMP1B. *Nat. Struct. Mol. Biol.* **2008**, *15*, 1278–1286. [CrossRef] [PubMed]
87. Lindas, A.C.; Karlsson, E.A.; Lindgren, M.T.; Ettema, T.J.; Bernander, R. A unique cell division machinery in the Archaea. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 18942–18946. [CrossRef]
88. Thoresen, S.B.; Campsteijn, C.; Vietri, M.; Schink, K.O.; Liestol, K.; Andersen, J.S.; Raiborg, C.; Stenmark, H. ANCHR mediates Aurora-B-dependent abscission checkpoint control through retention of VPS4. *Nat. Cell Biol.* **2014**, *16*, 550–560. [CrossRef] [PubMed]
89. Caballe, A.; Wenzel, D.M.; Agromayor, M.; Alam, S.L.; Skalicky, J.J.; Kloc, M.; Carlton, J.G.; Labrador, L.; Sundquist, W.I.; Martin-Serrano, J. ULK3 regulates cytokinetic abscission by phosphorylating ESCRT-III proteins. *Elife* **2015**, *4*, e06547. [CrossRef]
90. Sadler, J.B.A.; Wenzel, D.M.; Williams, L.K.; Guindo-Martinez, M.; Alam, S.L.; Mercader, J.M.; Torrents, D.; Ullman, K.S.; Sundquist, W.I.; Martin-Serrano, J. A cancer-associated polymorphism in ESCRT-III disrupts the abscission checkpoint and promotes genome instability. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E8900–E8908. [CrossRef]
91. Lens, S.M.A.; Medema, R.H. Cytokinesis defects and cancer. *Nat. Rev. Cancer* **2019**, *19*, 32–45. [CrossRef] [PubMed]
92. Tandon, D.; Banerjee, M. Centrosomal protein 55: A new paradigm in tumorigenesis. *Eur. J. Cell Biol.* **2020**, *99*, 151086. [CrossRef] [PubMed]
93. Hatch, E.M.; Fischer, A.H.; Deerinck, T.J.; Hetzer, M.W. Catastrophic nuclear envelope collapse in cancer cell micronuclei. *Cell* **2013**, *154*, 47–60. [CrossRef] [PubMed]
94. Vietri, M.; Schultz, S.W.; Bellanger, A.; Jones, C.M.; Petersen, L.I.; Raiborg, C.; Skarpen, E.; Pedurupillay, C.R.J.; Kjos, I.; Kip, E.; et al. Unrestrained ESCRT-III drives micronuclear catastrophe and chromosome fragmentation. *Nat. Cell Biol.* **2020**, *22*, 856–867. [CrossRef]
95. Tanaka, N.; Kyuuma, M.; Sugamura, K. Endosomal sorting complex required for transport proteins in cancer pathogenesis, vesicular transport, and non-endosomal functions. *Cancer Sci.* **2008**, *99*, 1293–1303. [CrossRef]
96. Mattissek, C.; Teis, D. The role of the endosomal sorting complexes required for transport (ESCRT) in tumorigenesis. *Mol. Membr. Biol.* **2014**, *31*, 111–119. [CrossRef] [PubMed]
97. Alfred, V.; Vaccari, T. When membranes need an ESCRT: Endosomal sorting and membrane remodelling in health and disease. *Swiss Med. Wkly.* **2016**, *146*, w14347. [CrossRef]
98. Andrews, N.W.; Almeida, P.E.; Corrotte, M. Damage control: Cellular mechanisms of plasma membrane repair. *Trends Cell Biol.* **2014**, *24*, 734–742. [CrossRef]
99. Jimenez, A.J.; Maiuri, P.; Lafaurie-Janvore, J.; Divoux, S.; Piel, M.; Perez, F. ESCRT machinery is required for plasma membrane repair. *Science* **2014**, *343*, 1247136. [CrossRef] [PubMed]
100. Grassel, L.; Fast, L.A.; Scheffer, K.D.; Boukhallouk, F.; Spoden, G.A.; Tenzer, S.; Boller, K.; Bago, R.; Rajesh, S.; Overduin, M.; et al. The CD63-Syntenin-1 Complex Controls Post-Endocytic Trafficking of Oncogenic Human Papillomaviruses. *Sci. Rep.* **2016**, *6*, 32337. [CrossRef]
101. Scheffer, L.L.; Sreetama, S.C.; Sharma, N.; Medikayala, S.; Brown, K.J.; Defour, A.; Jaiswal, J.K. Mechanism of Ca(2)(+)-triggered ESCRT assembly and regulation of cell membrane repair. *Nat. Commun.* **2014**, *5*, 5646. [CrossRef]
102. Sun, S.; Zhou, X.; Corvera, J.; Gallick, G.E.; Lin, S.H.; Kuang, J. ALG-2 activates the MVB sorting function of ALIX through relieving its intramolecular interaction. *Cell Discov.* **2015**, *1*, 15018. [CrossRef] [PubMed]
103. Ruhl, S.; Shkarina, K.; Demarco, B.; Heilig, R.; Santos, J.C.; Broz, P. ESCRT-dependent membrane repair negatively regulates pyroptosis downstream of GSDMD activation. *Science* **2018**, *362*, 956–960. [CrossRef] [PubMed]
104. Gong, Y.N.; Guy, C.; Olauson, H.; Becker, J.U.; Yang, M.; Fitzgerald, P.; Linkermann, A.; Green, D.R. ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell* **2017**, *169*, 286–300.e216. [CrossRef]

105. Robijns, J.; Houthaeve, G.; Braeckmans, K.; De Vos, W.H. Loss of Nuclear Envelope Integrity in Aging and Disease. *Int Rev. Cell Mol. Biol.* **2018**, *336*, 205–222. [CrossRef]
106. Guttinger, S.; Laurell, E.; Kutay, U. Orchestrating nuclear envelope disassembly and reassembly during mitosis. *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 178–191. [CrossRef]
107. Vietri, M.; Schink, K.O.; Campsteijn, C.; Wegner, C.S.; Schultz, S.W.; Christ, L.; Thoresen, S.B.; Brech, A.; Raiborg, C.; Stenmark, H. Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing. *Nature* **2015**, *522*, 231–235. [CrossRef] [PubMed]
108. Olmos, Y.; Hodgson, L.; Mantell, J.; Verkade, P.; Carlton, J.G. ESCRT-III controls nuclear envelope reformation. *Nature* **2015**, *522*, 236–239. [CrossRef] [PubMed]
109. Ventimiglia, L.N.; Cuesta-Geijo, M.A.; Martinelli, N.; Caballe, A.; Macheboeuf, P.; Miguet, N.; Parnham, I.M.; Olmos, Y.; Carlton, J.G.; Weissenhorn, W.; et al. CC2D1B Coordinates ESCRT-III Activity during the Mitotic Reformation of the Nuclear Envelope. *Dev. Cell* **2018**, *47*, 547–563.e546. [CrossRef]
110. Martinelli, N.; Hartlieb, B.; Usami, Y.; Sabin, C.; Dordor, A.; Miguet, N.; Avilov, S.V.; Ribeiro, E.A., Jr.; Gottlinger, H.; Weissenhorn, W. CC2D1A is a regulator of ESCRT-III CHMP4B. *J. Mol. Biol.* **2012**, *419*, 75–88. [CrossRef]
111. Pieper, G.H.; Sprenger, S.; Teis, D.; Oliferenko, S. ESCRT-III/Vps4 Controls Heterochromatin-Nuclear Envelope Attachments. *Dev. Cell* **2020**, *53*, 27–41. [CrossRef] [PubMed]
112. Maciejowski, J.; Hatch, E.M. Nuclear Membrane Rupture and Its Consequences. *Annu. Rev. Cell Dev. Biol.* **2020**, *36*, 85–114. [CrossRef] [PubMed]
113. Denais, C.M.; Gilbert, R.M.; Isermann, P.; McGregor, A.L.; te Lindert, M.; Weigelin, B.; Davidson, P.M.; Friedl, P.; Wolf, K.; Lammerding, J. Nuclear envelope rupture and repair during cancer cell migration. *Science* **2016**, *352*, 353–358. [CrossRef] [PubMed]
114. Raab, M.; Gentili, M.; de Belly, H.; Thiam, H.R.; Vargas, P.; Jimenez, A.J.; Lautenschlaeger, F.; Voituriez, R.; Lennon-Dumenil, A.M.; Manel, N.; et al. ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. *Science* **2016**, *352*, 359–362. [CrossRef] [PubMed]
115. Halfmann, C.T.; Sears, R.M.; Katiyar, A.; Busselman, B.W.; Aman, L.K.; Zhang, Q.; O'Bryan, C.S.; Angelini, T.E.; Lele, T.P.; Roux, K.J. Repair of nuclear ruptures requires barrier-to-autointegration factor. *J. Cell Biol.* **2019**, *218*, 2136–2149. [CrossRef] [PubMed]
116. Webster, B.M.; Colombi, P.; Jager, J.; Lusk, C.P. Surveillance of nuclear pore complex assembly by ESCRT-III/Vps4. *Cell* **2014**, *159*, 388–401. [CrossRef] [PubMed]
117. Skowyra, M.L.; Schlesinger, P.H.; Naismith, T.V.; Hanson, P.I. Triggered recruitment of ESCRT machinery promotes endolysosomal repair. *Science* **2018**, *360*. [CrossRef]
118. Radulovic, M.; Schink, K.O.; Wenzel, E.M.; Nahse, V.; Bongiovanni, A.; Lafont, F.; Stenmark, H. ESCRT-mediated lysosome repair precedes lysophagy and promotes cell survival. *EMBO J.* **2018**, *37*. [CrossRef] [PubMed]
119. Radulovic, M.; Stenmark, H. ESCRTs in membrane sealing. *Biochem. Soc. Trans.* **2018**, *46*, 773–778. [CrossRef]
120. Mansilla Pareja, M.E.; Bongiovanni, A.; Lafont, F.; Colombo, M.I. Alterations of the Coxiella burnetii Replicative Vacuole Membrane Integrity and Interplay with the Autophagy Pathway. *Front. Cell Infect. Microbiol.* **2017**, *7*, 112. [CrossRef]
121. Mittal, E.; Skowyra, M.L.; Uwase, G.; Tinaztepe, E.; Mehra, A.; Koster, S.; Hanson, P.I.; Philips, J.A. Mycobacterium tuberculosis Type VII Secretion System Effectors Differentially Impact the ESCRT Endomembrane Damage Response. *mBio* **2018**, *9*. [CrossRef] [PubMed]
122. Wagner, K.U.; Krempler, A.; Qi, Y.; Park, K.; Henry, M.D.; Triplett, A.A.; Riedlinger, G.; Rucker, I.E.; Hennighausen, L. Tsg101 is essential for cell growth, proliferation, and cell survival of embryonic and adult tissues. *Mol. Cell Biol.* **2003**, *23*, 150–162. [CrossRef] [PubMed]
123. Handschuh, K.; Feenstra, J.; Koss, M.; Ferretti, E.; Risolino, M.; Zewdu, R.; Sahai, M.A.; Benazet, J.D.; Peng, X.P.; Depew, M.J.; et al. ESCRT-II/Vps25 constrains digit number by endosome-mediated selective modulation of FGF-SHH signaling. *Cell Rep.* **2014**, *9*, 674–687. [CrossRef] [PubMed]
124. Shim, J.H.; Xiao, C.; Hayden, M.S.; Lee, K.Y.; Trombetta, E.S.; Pypaert, M.; Nara, A.; Yoshimori, T.; Wilm, B.; Erdjument-Bromage, H.; et al. CHMP5 is essential for late endosome function and down-regulation of receptor signaling during mouse embryogenesis. *J. Cell Biol.* **2006**, *172*, 1045–1056. [CrossRef]
125. Thompson, B.J.; Mathieu, J.; Sung, H.H.; Loeser, E.; Rorth, P.; Cohen, S.M. Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. *Dev. Cell* **2005**, *9*, 711–720. [CrossRef] [PubMed]
126. Vaccari, T.; Rusten, T.E.; Menut, L.; Nezis, I.P.; Brech, A.; Stenmark, H.; Bilder, D. Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in Drosophila by efficient isolation of ESCRT mutants. *J. Cell Sci.* **2009**, *122*, 2413–2423. [CrossRef]
127. Spitzer, C.; Schellmann, S.; Sabovljevic, A.; Shahriari, M.; Keshavaiah, C.; Bechtold, N.; Herzog, M.; Muller, S.; Hanisch, F.G.; Hulskamp, M. The Arabidopsis elch mutant reveals functions of an ESCRT component in cytokinesis. *Development* **2006**, *133*, 4679–4689. [CrossRef]
128. Razi, M.; Futter, C.E. Distinct roles for Tsg101 and HRS in multivesicular body formation and inward vesiculation. *Mol. Biol. Cell* **2006**, *17*, 3469–3483. [CrossRef] [PubMed]

129. Bache, K.G.; Stuffers, S.; Malerod, L.; Slagsvold, T.; Raiborg, C.; Lechardeur, D.; Walchli, S.; Lukacs, G.L.; Brech, A.; Stenmark, H. The ESCRT-III subunit hVps24 is required for degradation but not silencing of the epidermal growth factor receptor. *Mol. Biol. Cell* **2006**, *17*, 2513–2523. [CrossRef] [PubMed]
130. Stuffers, S.; Sem Wegner, C.; Stenmark, H.; Brech, A. Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic* **2009**, *10*, 925–937. [CrossRef] [PubMed]
131. Capalbo, L.; Montembault, E.; Takeda, T.; Bassi, Z.I.; Glover, D.M.; D'Avino, P.P. The chromosomal passenger complex controls the function of endosomal sorting complex required for transport-III Snf7 proteins during cytokinesis. *Open Biol.* **2012**, *2*, 120070. [CrossRef]
132. Xiao, Y.; Cai, W. Autophagy and Viral Infection. *Adv. Exp. Med. Biol.* **2020**, *1207*, 425–432. [CrossRef]
133. Purvinsh, L.; Gorshkov, A.; Brodskaia, A.; Vasin, A. Extracellular Vesicles in Viral Pathogenesis: A Case of Dr. Jekyll and Mr. Hyde. *Life* **2021**, *11*, 45. [CrossRef]
134. Raymond, A.D.; Diaz, P.; Chevelon, S.; Agudelo, M.; Yndart-Arias, A.; Ding, H.; Kaushik, A.; Jayant, R.D.; Nikkhah-Moshaie, R.; Roy, U.; et al. Microglia-derived HIV Nef+ exosome impairment of the blood-brain barrier is treatable by nanomedicine-based delivery of Nef peptides. *J. Neurovirol.* **2016**, *22*, 129–139. [CrossRef] [PubMed]
135. Arenaccio, C.; Anticoli, S.; Manfredi, F.; Chiozzini, C.; Olivetta, E.; Federico, M. Latent HIV-1 is activated by exosomes from cells infected with either replication-competent or defective HIV-1. *Retrovirology* **2015**, *12*, 87. [CrossRef]
136. Arenaccio, C.; Chiozzini, C.; Columba-Cabezas, S.; Manfredi, F.; Affabris, E.; Baur, A.; Federico, M. Exosomes from human immunodeficiency virus type 1 (HIV-1)-infected cells license quiescent CD4+ T lymphocytes to replicate HIV-1 through a Nef- and ADAM17-dependent mechanism. *J. Virol.* **2014**, *88*, 11529–11539. [CrossRef]
137. Lenassi, M.; Cagney, G.; Liao, M.; Vaupotic, T.; Bartholomeeusen, K.; Cheng, Y.; Krogan, N.J.; Plemenitas, A.; Peterlin, B.M. HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. *Traffic* **2010**, *11*, 110–122. [CrossRef]
138. Xu, W.; Santini, P.A.; Sullivan, J.S.; He, B.; Shan, M.; Ball, S.C.; Dyer, W.B.; Ketas, T.J.; Chadburn, A.; Cohen-Gould, L.; et al. HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits. *Nat. Immunol.* **2009**, *10*, 1008–1017. [CrossRef] [PubMed]
139. Mack, M.; Kleinschmidt, A.; Bruhl, H.; Klier, C.; Nelson, P.J.; Cihak, J.; Plachy, J.; Stangassinger, M.; Erfle, V.; Schlondorff, D. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: A mechanism for cellular human immunodeficiency virus 1 infection. *Nat. Med.* **2000**, *6*, 769–775. [CrossRef] [PubMed]
140. Rozmyslowicz, T.; Majka, M.; Kijowski, J.; Murphy, S.L.; Conover, D.O.; Poncz, M.; Ratajczak, J.; Gaulton, G.N.; Ratajczak, M.Z. Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *AIDS* **2003**, *17*, 33–42. [CrossRef]
141. Narayanan, A.; Iordanskiy, S.; Das, R.; Van Duyne, R.; Santos, S.; Jaworski, E.; Guendel, I.; Sampey, G.; Dalby, E.; Iglesias-Ussel, M.; et al. Exosomes derived from HIV-1-infected cells contain trans-activation response element RNA. *J. Biol. Chem.* **2013**, *288*, 20014–20033. [CrossRef] [PubMed]
142. Khatua, A.K.; Taylor, H.E.; Hildreth, J.E.; Popik, W. Exosomes packaging APOBEC3G confer human immunodeficiency virus resistance to recipient cells. *J. Virol.* **2009**, *83*, 512–521. [CrossRef] [PubMed]
143. Bridgeman, A.; Maelfait, J.; Davenne, T.; Partridge, T.; Peng, Y.; Mayer, A.; Dong, T.; Kaever, V.; Borrow, P.; Rehwinkel, J. Viruses transfer the antiviral second messenger cGAMP between cells. *Science* **2015**, *349*, 1228–1232. [CrossRef] [PubMed]
144. Dias, M.V.S.; Costa, C.S.; da Silva, L.L.P. The Ambiguous Roles of Extracellular Vesicles in HIV Replication and Pathogenesis. *Front. Microbiol.* **2018**, *9*, 2411. [CrossRef]
145. Li, S.; Li, S.; Wu, S.; Chen, L. Exosomes Modulate the Viral Replication and Host Immune Responses in HBV Infection. *Biomed. Res. Int.* **2019**, *2019*, 2103943. [CrossRef]
146. Jaworski, E.; Narayanan, A.; Van Duyne, R.; Shabbeer-Meyering, S.; Iordanskiy, S.; Saifuddin, M.; Das, R.; Afonso, P.V.; Sampey, G.C.; Chung, M.; et al. Human T-lymphotropic virus type 1-infected cells secrete exosomes that contain Tax protein. *J. Biol. Chem.* **2014**, *289*, 22284–22305. [CrossRef] [PubMed]
147. Miura, T.A. Respiratory epithelial cells as master communicators during viral infections. *Curr. Clin. Microbiol. Rep.* **2019**, *6*, 10–17. [CrossRef]
148. Hassanpour, M.; Rezaie, J.; Nouri, M.; Panahi, Y. The role of extracellular vesicles in COVID-19 virus infection. *Infect. Genet. Evol* **2020**, *85*, 104422. [CrossRef]
149. Wang, J.; Li, J.; Cheng, C.; Liu, S. Angiotensin-converting enzyme 2 augments the effects of endothelial progenitor cells-exosomes on vascular smooth muscle cell phenotype transition. *Cell Tissue Res.* **2020**, *382*, 509–518. [CrossRef] [PubMed]
150. Cocozza, F.; Nevo, N.; Piovesana, E.; Lahaye, X.; Buchrieser, J.; Schwartz, O.; Manel, N.; Tkach, M.; Thery, C.; Martin-Jaular, L. Extracellular vesicles containing ACE2 efficiently prevent infection by SARS-CoV-2 Spike protein-containing virus. *J. Extracell Vesicles* **2020**, *10*, e12050. [CrossRef]
151. Ipinmoroti, A.O.; Matthews, Q.L. Extracellular Vesicles: Roles in Human Viral Infections, Immune-Diagnostic, and Therapeutic Applications. *Pathogens* **2020**, *9*, 1056. [CrossRef] [PubMed]
152. Wang, S.C.; Li, C.Y.; Chang, W.T.; Cheng, W.C.; Yen, C.H.; Tu, W.Y.; Lin, Z.Y.; Lin, C.C.; Yeh, M.L.; Huang, C.F.; et al. Exosome-derived differentiation antagonizing non-protein coding RNA with risk of hepatitis C virus-related hepatocellular carcinoma recurrence. *Liver Int.* **2020**. [CrossRef]

153. Zhang, J.; Li, X.; Hu, J.; Cao, P.; Yan, Q.; Zhang, S.; Dang, W.; Lu, J. Long noncoding RNAs involvement in Epstein-Barr virus infection and tumorigenesis. *Virol. J.* **2020**, *17*, 51. [CrossRef]
154. Yang, X.; Li, H.; Sun, H.; Fan, H.; Hu, Y.; Liu, M.; Li, X.; Tang, H. Hepatitis B Virus-Encoded MicroRNA Controls Viral Replication. *J. Virol.* **2017**, *91*. [CrossRef] [PubMed]
155. Pincetic, A.; Kuang, Z.; Seo, E.J.; Leis, J. The interferon-induced gene ISG15 blocks retrovirus release from cells late in the budding process. *J. Virol.* **2010**, *84*, 4725–4736. [CrossRef]
156. Kuang, Z.; Seo, E.J.; Leis, J. Mechanism of inhibition of retrovirus release from cells by interferon-induced gene ISG15. *J. Virol.* **2011**, *85*, 7153–7161. [CrossRef]
157. Bashirova, A.A.; Bleiber, G.; Qi, Y.; Hutcheson, H.; Yamashita, T.; Johnson, R.C.; Cheng, J.; Alter, G.; Goedert, J.J.; Buchbinder, S.; et al. Consistent effects of TSG101 genetic variability on multiple outcomes of exposure to human immunodeficiency virus type 1. *J. Virol.* **2006**, *80*, 6757–6763. [CrossRef] [PubMed]
158. Strickland, M.; Ehrlich, L.S.; Watanabe, S.; Khan, M.; Strub, M.P.; Luan, C.H.; Powell, M.D.; Leis, J.; Tjandra, N.; Carter, C.A. Tsg101 chaperone function revealed by HIV-1 assembly inhibitors. *Nat. Commun.* **2017**, *8*, 1391. [CrossRef]
159. Watanabe, S.M.; Ehrlich, L.S.; Strickland, M.; Li, X.; Soloveva, V.; Goff, A.J.; Stauft, C.B.; Bhaduri-McIntosh, S.; Tjandra, N.; Carter, C. Selective Targeting of Virus Replication by Proton Pump Inhibitors. *Sci. Rep.* **2020**, *10*, 4003. [CrossRef]
160. Ketter, E.; Randall, G. Virus Impact on Lipids and Membranes. *Annu. Rev. Virol.* **2019**, *6*, 319–340. [CrossRef] [PubMed]
161. Tuthill, T.J.; Bubeck, D.; Rowlands, D.J.; Hogle, J.M. Characterization of early steps in the poliovirus infection process: Receptor-decorated liposomes induce conversion of the virus to membrane-anchored entry-intermediate particles. *J. Virol.* **2006**, *80*, 172–180. [CrossRef]
162. Lozach, P.Y.; Huotari, J.; Helenius, A. Late-penetrating viruses. *Curr. Opin. Virol* **2011**, *1*, 35–43. [CrossRef] [PubMed]
163. Votteler, J.; Sundquist, W.I. Virus budding and the ESCRT pathway. *Cell Host Microbe* **2013**, *14*, 232–241. [CrossRef]
164. Wodrich, H.; Henaff, D.; Jammart, B.; Segura-Morales, C.; Seelmeir, S.; Coux, O.; Ruzsics, Z.; Wiethoff, C.M.; Kremer, E.J. A capsid-encoded PPxY-motif facilitates adenovirus entry. *PLoS Pathog.* **2010**, *6*, e1000808. [CrossRef] [PubMed]
165. Wiethoff, C.M.; Wodrich, H.; Gerace, L.; Nemerow, G.R. Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J. Virol.* **2005**, *79*, 1992–2000. [CrossRef] [PubMed]
166. Moyer, C.L.; Wiethoff, C.M.; Maier, O.; Smith, J.G.; Nemerow, G.R. Functional genetic and biophysical analyses of membrane disruption by human adenovirus. *J. Virol.* **2011**, *85*, 2631–2641. [CrossRef]
167. Prchla, E.; Plank, C.; Wagner, E.; Blaas, D.; Fuchs, R. Virus-mediated release of endosomal content in vitro: Different behavior of adenovirus and rhinovirus serotype 2. *J. Cell Biol.* **1995**, *131*, 111–123. [CrossRef] [PubMed]
168. Farr, G.A.; Zhang, L.G.; Tattersall, P. Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 17148–17153. [CrossRef]
169. Montespan, C.; Marvin, S.A.; Austin, S.; Burrage, A.M.; Roger, B.; Rayne, F.; Faure, M.; Campell, E.M.; Schneider, C.; Reimer, R.; et al. Multi-layered control of Galectin-8 mediated autophagy during adenovirus cell entry through a conserved PPxY motif in the viral capsid. *PLoS Pathog.* **2017**, *13*, e1006217. [CrossRef]
170. Kumar, B.; Roy, A.; Veettil, M.V.; Chandran, B. Insight into the Roles of E3 Ubiquitin Ligase c-Cbl, ESCRT Machinery, and Host Cell Signaling in Kaposi's Sarcoma-Associated Herpesvirus Entry and Trafficking. *J. Virol.* **2018**, *92*. [CrossRef]
171. Shtanko, O.; Nikitina, R.A.; Altuntas, C.Z.; Chepurnov, A.A.; Davey, R.A. Crimean-Congo hemorrhagic fever virus entry into host cells occurs through the multivesicular body and requires ESCRT regulators. *PLoS Pathog.* **2014**, *10*, e1004390. [CrossRef]
172. Broniarczyk, J.; Bergant, M.; Gozdzicka-Jozefiak, A.; Banks, L. Human papillomavirus infection requires the TSG101 component of the ESCRT machinery. *Virology* **2014**, *460–461*, 83–90. [CrossRef]
173. Karjalainen, M.; Rintanen, N.; Lehkonen, M.; Kallio, K.; Maki, A.; Hellstrom, K.; Siljamaki, V.; Upla, P.; Marjomaki, V. Echovirus 1 infection depends on biogenesis of novel multivesicular bodies. *Cell Microbiol.* **2011**, *13*, 1975–1995. [CrossRef] [PubMed]
174. Pasqual, G.; Rojek, J.M.; Masin, M.; Chatton, J.Y.; Kunz, S. Old world arenaviruses enter the host cell via the multivesicular body and depend on the endosomal sorting complex required for transport. *PLoS Pathog.* **2011**, *7*, e1002232. [CrossRef]
175. Silva-Ayala, D.; Lopez, T.; Gutierrez, M.; Perrimon, N.; Lopez, S.; Arias, C.F. Genome-wide RNAi screen reveals a role for the ESCRT complex in rotavirus cell entry. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 10270–10275. [CrossRef]
176. Barajas, D.; Jiang, Y.; Nagy, P.D. A unique role for the host ESCRT proteins in replication of Tomato bushy stunt virus. *PLoS Pathog.* **2009**, *5*, e1000705. [CrossRef]
177. Kovalev, N.; de Castro Martin, I.F.; Pogany, J.; Barajas, D.; Pathak, K.; Risco, C.; Nagy, P.D. Role of Viral RNA and Co-opted Cellular ESCRT-I and ESCRT-III Factors in Formation of Tombusvirus Spherules Harboring the Tombusvirus Replicase. *J. Virol.* **2016**, *90*, 3611–3626. [CrossRef] [PubMed]
178. Richardson, L.G.; Clendening, E.A.; Sheen, H.; Gidda, S.K.; White, K.A.; Mullen, R.T. A unique N-terminal sequence in the Carnation Italian ringspot virus p36 replicase-associated protein interacts with the host cell ESCRT-I component Vps23. *J. Virol.* **2014**, *88*, 6329–6344. [CrossRef] [PubMed]
179. Diaz, A.; Zhang, J.; Ollwerther, A.; Wang, X.; Ahlquist, P. Host ESCRT proteins are required for bromovirus RNA replication compartment assembly and function. *PLoS Pathog.* **2015**, *11*, e1004742. [CrossRef] [PubMed]
180. Tabata, K.; Arimoto, M.; Arakawa, M.; Nara, A.; Saito, K.; Omori, H.; Arai, A.; Ishikawa, T.; Konishi, E.; Suzuki, R.; et al. Unique Requirement for ESCRT Factors in Flavivirus Particle Formation on the Endoplasmic Reticulum. *Cell Rep.* **2016**, *16*, 2339–2347. [CrossRef]

181. Tandon, R.; AuCoin, D.P.; Mocarski, E.S. Human cytomegalovirus exploits ESCRT machinery in the process of virion maturation. *J. Virol.* **2009**, *83*, 10797–10807. [CrossRef]
182. Lindenbach, B.D. Virion assembly and release. *Curr. Top. Microbiol. Immunol.* **2013**, *369*, 199–218. [CrossRef]
183. Rossman, J.S.; Lamb, R.A. Viral membrane scission. *Ann. Rev. Cell Dev. Biol.* **2013**, *29*, 551–569. [CrossRef] [PubMed]
184. Gottlinger, H.G.; Dorfman, T.; Sodroski, J.G.; Haseltine, W.A. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 3195–3199. [CrossRef] [PubMed]
185. Wills, J.W.; Cameron, C.E.; Wilson, C.B.; Xiang, Y.; Bennett, R.P.; Leis, J. An assembly domain of the Rous sarcoma virus Gag protein required late in budding. *J. Virol.* **1994**, *68*, 6605–6618. [CrossRef]
186. Calistri, A.; Salata, C.; Parolin, C.; Palu, G. Role of multivesicular bodies and their components in the egress of enveloped RNA viruses. *Rev. Med. Virol.* **2009**, *19*, 31–45. [CrossRef] [PubMed]
187. Crump, C. Virus Assembly and Egress of HSV. *Adv. Exp. Med. Biol.* **2018**, *1045*, 23–44. [CrossRef] [PubMed]
188. Calistri, A.; Sette, P.; Salata, C.; Cancellotti, E.; Forghieri, C.; Comin, A.; Gottlinger, H.; Campadelli-Fiume, G.; Palu, G.; Parolin, C. Intracellular trafficking and maturation of herpes simplex virus type 1 gB and virus egress require functional biogenesis of multivesicular bodies. *J. Virol.* **2007**, *81*, 11468–11478. [CrossRef]
189. Calistri, A.; Munegato, D.; Toffoletto, M.; Celestino, M.; Franchin, E.; Comin, A.; Sartori, E.; Salata, C.; Parolin, C.; Palu, G. Functional Interaction Between the ESCRT-I Component TSG101 and the HSV-1 Tegument Ubiquitin Specific Protease. *J. Cell Physiol.* **2015**, *230*, 1794–1806. [CrossRef]
190. Garcia, M.L.; Reynolds, T.D.; Mothes, W.; Robek, M.D. Functional characterization of the putative hepatitis B virus core protein late domain using retrovirus chimeras. *PLoS ONE* **2013**, *8*, e72845. [CrossRef]
191. Hurley, J.H.; Hanson, P.I. Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 556–566. [CrossRef] [PubMed]
192. Ariumi, Y.; Kuroki, M.; Maki, M.; Ikeda, M.; Dansako, H.; Wakita, T.; Kato, N. The ESCRT system is required for hepatitis C virus production. *PLoS ONE* **2011**, *6*, e14517. [CrossRef]
193. Rossman, J.S.; Jing, X.; Leser, G.P.; Lamb, R.A. Influenza virus M2 protein mediates ESCRT-independent membrane scission. *Cell* **2010**, *142*, 902–913. [CrossRef]
194. Wirblich, C.; Bhattacharya, B.; Roy, P. Nonstructural protein 3 of bluetongue virus assists virus release by recruiting ESCRT-I protein Tsg101. *J. Virol.* **2006**, *80*, 460–473. [CrossRef] [PubMed]
195. Parent, L.J.; Bennett, R.P.; Craven, R.C.; Nelle, T.D.; Krishna, N.K.; Bowzard, J.B.; Wilson, C.B.; Puffer, B.A.; Montelaro, R.C.; Wills, J.W. Positionally independent and exchangeable late budding functions of the Rous sarcoma virus and human immunodeficiency virus Gag proteins. *J. Virol.* **1995**, *69*, 5455–5460. [CrossRef]
196. Yuan, B.; Campbell, S.; Bacharach, E.; Rein, A.; Goff, S.P. Infectivity of Moloney murine leukemia virus defective in late assembly events is restored by late assembly domains of other retroviruses. *J. Virol.* **2000**, *74*, 7250–7260. [CrossRef] [PubMed]
197. Martin-Serrano, J.; Yarovoy, A.; Perez-Caballero, D.; Bieniasz, P.D. Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12414–12419. [CrossRef]
198. Martin-Serrano, J.; Bieniasz, P.D. A bipartite late-budding domain in human immunodeficiency virus type 1. *J. Virol.* **2003**, *77*, 12373–12377. [CrossRef]
199. Strack, B.; Calistri, A.; Accola, M.A.; Palu, G.; Gottlinger, H.G. A role for ubiquitin ligase recruitment in retrovirus release. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13063–13068. [CrossRef]
200. Staub, O.; Dho, S.; Henry, P.; Correa, J.; Ishikawa, T.; McGlade, J.; Rotin, D. WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. *EMBO J.* **1996**, *15*, 2371–2380. [CrossRef]
201. Staub, O.; Gautschi, I.; Ishikawa, T.; Breitschopf, K.; Ciechanover, A.; Schild, L.; Rotin, D. Regulation of stability and function of the epithelial Na+ channel (ENaC) by ubiquitination. *EMBO J.* **1997**, *16*, 6325–6336. [CrossRef] [PubMed]
202. Strack, B.; Calistri, A.; Gottlinger, H.G. Late assembly domain function can exhibit context dependence and involves ubiquitin residues implicated in endocytosis. *J. Virol.* **2002**, *76*, 5472–5479. [CrossRef] [PubMed]
203. Garrus, J.E.; von Schwedler, U.K.; Pornillos, O.W.; Morham, S.G.; Zavitz, K.H.; Wang, H.E.; Wettstein, D.A.; Stray, K.M.; Cote, M.; Rich, R.L.; et al. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* **2001**, *107*, 55–65. [CrossRef]
204. VerPlank, L.; Bouamr, F.; LaGrassa, T.J.; Agresta, B.; Kikonyogo, A.; Leis, J.; Carter, C.A. Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 7724–7729. [CrossRef] [PubMed]
205. Kikonyogo, A.; Bouamr, F.; Vana, M.L.; Xiang, Y.; Aiyar, A.; Carter, C.; Leis, J. Proteins related to the Nedd4 family of ubiquitin protein ligases interact with the L domain of Rous sarcoma virus and are required for gag budding from cells. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11199–11204. [CrossRef] [PubMed]
206. Rose, K.M.; Hirsch, V.M.; Bouamr, F. Budding of a Retrovirus: Some Assemblies Required. *Viruses* **2020**, *12*, 1188. [CrossRef]
207. Pincetic, A.; Medina, G.; Carter, C.; Leis, J. Avian sarcoma virus and human immunodeficiency virus, type 1 use different subsets of ESCRT proteins to facilitate the budding process. *J. Biol. Chem.* **2008**, *283*, 29822–29830. [CrossRef] [PubMed]
208. Langelier, C.; von Schwedler, U.K.; Fisher, R.D.; De Domenico, I.; White, P.L.; Hill, C.P.; Kaplan, J.; Ward, D.; Sundquist, W.I. Human ESCRT-II complex and its role in human immunodeficiency virus type 1 release. *J. Virol.* **2006**, *80*, 9465–9480. [CrossRef] [PubMed]

209. Meng, B.; Ip, N.C.Y.; Abbink, T.E.M.; Kenyon, J.C.; Lever, A.M.L. ESCRT-II functions by linking to ESCRT-I in human immunodeficiency virus-1 budding. *Cell Microbiol.* **2020**, *22*, e13161. [CrossRef]
210. Hurley, J.H.; Cada, A.K. Inside job: How the ESCRTs release HIV-1 from infected cells. *Biochem. Soc. Trans.* **2018**, *46*, 1029–1036. [CrossRef]
211. Del Vecchio, C.; Celestino, M.; Celegato, M.; Palu, G.; Parolin, C.; Bouamr, F.; Calistri, A. Alix-Mediated Rescue of Feline Immunodeficiency Virus Budding Differs from That Observed with Human Immunodeficiency Virus. *J. Virol.* **2020**, *94*. [CrossRef] [PubMed]
212. Weiss, E.R.; Popova, E.; Yamanaka, H.; Kim, H.C.; Huibregtse, J.M.; Gottlinger, H. Rescue of HIV-1 release by targeting widely divergent NEDD4-type ubiquitin ligases and isolated catalytic HECT domains to Gag. *PLoS Pathog.* **2010**, *6*, e1001107. [CrossRef]
213. Usami, Y.; Popov, S.; Popova, E.; Gottlinger, H.G. Efficient and specific rescue of human immunodeficiency virus type 1 budding defects by a Nedd4-like ubiquitin ligase. *J. Virol.* **2008**, *82*, 4898–4907. [CrossRef] [PubMed]
214. Chung, H.Y.; Morita, E.; von Schwedler, U.; Muller, B.; Krausslich, H.G.; Sundquist, W.I. NEDD4L overexpression rescues the release and infectivity of human immunodeficiency virus type 1 constructs lacking PTAP and YPXL late domains. *J. Virol.* **2008**, *82*, 4884–4897. [CrossRef]
215. Calistri, A.; Del Vecchio, C.; Salata, C.; Celestino, M.; Celegato, M.; Gottlinger, H.; Palu, G.; Parolin, C. Role of the feline immunodeficiency virus L-domain in the presence or absence of Gag processing: Involvement of ubiquitin and Nedd4-2s ligase in viral egress. *J. Cell Physiol.* **2009**, *218*, 175–182. [CrossRef]
216. Sette, P.; Nagashima, K.; Piper, R.C.; Bouamr, F. Ubiquitin conjugation to Gag is essential for ESCRT-mediated HIV-1 budding. *Retrovirology* **2013**, *10*, 79. [CrossRef] [PubMed]
217. Popov, S.; Popova, E.; Inoue, M.; Gottlinger, H.G. Divergent Bro1 domains share the capacity to bind human immunodeficiency virus type 1 nucleocapsid and to enhance virus-like particle production. *J. Virol.* **2009**, *83*, 7185–7193. [CrossRef] [PubMed]
218. Dussupt, V.; Javid, M.P.; Abou-Jaoude, G.; Jadwin, J.A.; de La Cruz, J.; Nagashima, K.; Bouamr, F. The nucleocapsid region of HIV-1 Gag cooperates with the PTAP and LYPXnL late domains to recruit the cellular machinery necessary for viral budding. *PLoS Pathog.* **2009**, *5*, e1000339. [CrossRef]
219. Bello, N.F.; Dussupt, V.; Sette, P.; Rudd, V.; Nagashima, K.; Bibollet-Ruche, F.; Chen, C.; Montelaro, R.C.; Hahn, B.H.; Bouamr, F. Budding of retroviruses utilizing divergent L domains requires nucleocapsid. *J. Virol.* **2012**, *86*, 4182–4193. [CrossRef] [PubMed]
220. Gottlinger, H.G. Influenza exits the cell without an ESCRT. *Cell* **2010**, *142*, 839–841. [CrossRef]
221. Feng, Z.; Hirai-Yuki, A.; McKnight, K.L.; Lemon, S.M. Naked Viruses That Aren't Always Naked: Quasi-Enveloped Agents of Acute Hepatitis. *Ann. Rev. Virol.* **2014**, *1*, 539–560. [CrossRef] [PubMed]
222. Feng, Z.; Hensley, L.; McKnight, K.L.; Hu, F.; Madden, V.; Ping, L.; Jeong, S.H.; Walker, C.; Lanford, R.E.; Lemon, S.M. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. *Nature* **2013**, *496*, 367–371. [CrossRef] [PubMed]
223. Nagashima, S.; Takahashi, M.; Jirintai, S.; Tanggis; Kobayashi, T.; Nishizawa, T.; Okamoto, H. The membrane on the surface of hepatitis E virus particles is derived from the intracellular membrane and contains trans-Golgi network protein 2. *Arch. Virol* **2014**, *159*, 979–991. [CrossRef] [PubMed]
224. Nagashima, S.; Takahashi, M.; Kobayashi, T.; Tanggis; Nishizawa, T.; Nishiyama, T.; Primadharsini, P.P.; Okamoto, H. Characterization of the Quasi-Enveloped Hepatitis E Virus Particles Released by the Cellular Exosomal Pathway. *J. Virol.* **2017**, *91*. [CrossRef] [PubMed]
225. Izquierdo-Useros, N.; Naranjo-Gomez, M.; Erkizia, I.; Puertas, M.C.; Borras, F.E.; Blanco, J.; Martinez-Picado, J. HIV and mature dendritic cells: Trojan exosomes riding the Trojan horse? *PLoS Pathog.* **2010**, *6*, e1000740. [CrossRef] [PubMed]
226. Ramakrishnaiah, V.; Thumann, C.; Fofana, I.; Habersetzer, F.; Pan, Q.; de Ruiter, P.E.; Willemsen, R.; Demmers, J.A.; Stalin Raj, V.; Jenster, G.; et al. Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 13109–13113. [CrossRef]
227. Giannessi, F.; Aiello, A.; Franchi, F.; Percario, Z.A.; Affabris, E. The Role of Extracellular Vesicles as Allies of HIV, HCV and SARS Viruses. *Viruses* **2020**, *12*, 571. [CrossRef]
228. Longatti, A. The Dual Role of Exosomes in Hepatitis A and C Virus Transmission and Viral Immune Activation. *Viruses* **2015**, *7*, 6707–6715. [CrossRef]
229. Zhou, W.; Woodson, M.; Sherman, M.B.; Neelakanta, G.; Sultana, H. Exosomes mediate Zika virus transmission through SMPD3 neutral Sphingomyelinase in cortical neurons. *Emerg Microbes Infect.* **2019**, *8*, 307–326. [CrossRef]
230. Zhang, K.; Xu, S.; Shi, X.; Xu, G.; Shen, C.; Liu, X.; Zheng, H. Exosomes-mediated transmission of foot-and-mouth disease virus in vivo and in vitro. *Vet. Microbiol.* **2019**, *233*, 164–173. [CrossRef]
231. Silvas, J.A.; Popov, V.L.; Paulucci-Holthauzen, A.; Aguilar, P.V. Extracellular Vesicles Mediate Receptor-Independent Transmission of Novel Tick-Borne Bunyavirus. *J. Virol.* **2016**, *90*, 873–886. [CrossRef]
232. Reyes-Ruiz, J.M.; Osuna-Ramos, J.F.; De Jesus-Gonzalez, L.A.; Hurtado-Monzon, A.M.; Farfan-Morales, C.N.; Cervantes-Salazar, M.; Bolanos, J.; Cigarroa-Mayorga, O.E.; Martin-Martinez, E.S.; Medina, F.; et al. Isolation and characterization of exosomes released from mosquito cells infected with dengue virus. *Virus Res.* **2019**, *266*, 1–14. [CrossRef]
233. Dubrovsky, L.; Ward, A.; Choi, S.H.; Pushkarsky, T.; Brichacek, B.; Vanpouille, C.; Adzhubei, A.A.; Mukhamedova, N.; Sviridov, D.; Margolis, L.; et al. Inhibition of HIV Replication by Apolipoprotein A-I Binding Protein Targeting the Lipid Rafts. *mBio* **2020**, *11*. [CrossRef] [PubMed]

234. Bello-Morales, R.; Lopez-Guerrero, J.A. Extracellular Vesicles in Herpes Viral Spread and Immune Evasion. *Front. Microbiol.* **2018**, *9*, 2572. [CrossRef] [PubMed]
235. Bello-Morales, R.; Praena, B.; de la Nuez, C.; Rejas, M.T.; Guerra, M.; Galan-Ganga, M.; Izquierdo, M.; Calvo, V.; Krummenacher, C.; Lopez-Guerrero, J.A. Role of Microvesicles in the Spread of Herpes Simplex Virus 1 in Oligodendrocytic Cells. *J. Virol.* **2018**, *92*. [CrossRef] [PubMed]
236. Mutsafi, Y.; Altan-Bonnet, N. Enterovirus Transmission by Secretory Autophagy. *Viruses* **2018**, *10*, 139. [CrossRef]
237. Teo, Q.W.; van Leur, S.W.; Sanyal, S. Escaping the Lion's Den: Redirecting autophagy for unconventional release and spread of viruses. *FEBS J.* **2020**. [CrossRef] [PubMed]
238. Hittelman, A.B.; Burakov, D.; Iniguez-Lluhi, J.A.; Freedman, L.P.; Garabedian, M.J. Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. *EMBO J.* **1999**, *18*, 5380–5388. [CrossRef] [PubMed]
239. Watanabe, M.; Yanagi, Y.; Masuhiro, Y.; Yano, T.; Yoshikawa, H.; Yanagisawa, J.; Kato, S. A putative tumor suppressor, TSG101, acts as a transcriptional suppressor through its coiled-coil domain. *Biochem. Biophys. Res. Commun.* **1998**, *245*, 900–905. [CrossRef] [PubMed]
240. Chua, H.H.; Lee, H.H.; Chang, S.S.; Lu, C.C.; Yeh, T.H.; Hsu, T.Y.; Cheng, T.H.; Cheng, J.T.; Chen, M.R.; Tsai, C.H. Role of the TSG101 gene in Epstein-Barr virus late gene transcription. *J. Virol.* **2007**, *81*, 2459–2471. [CrossRef]
241. Ruland, J.; Sirard, C.; Elia, A.; MacPherson, D.; Wakeham, A.; Li, L.; de la Pompa, J.L.; Cohen, S.N.; Mak, T.W. p53 accumulation, defective cell proliferation, and early embryonic lethality in mice lacking tsg101. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1859–1864. [CrossRef]
242. Li, L.; Liao, J.; Ruland, J.; Mak, T.W.; Cohen, S.N. A TSG101/MDM2 regulatory loop modulates MDM2 degradation and MDM2/p53 feedback control. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 1619–1624. [CrossRef] [PubMed]
243. Schmidt, A.E.; Miller, T.; Schmidt, S.L.; Shiekhattar, R.; Shilatifard, A. Cloning and characterization of the EAP30 subunit of the ELL complex that confers derepression of transcription by RNA polymerase II. *J. Biol. Chem.* **1999**, *274*, 21981–21985. [CrossRef] [PubMed]
244. Kamura, T.; Burian, D.; Khalili, H.; Schmidt, S.L.; Sato, S.; Liu, W.J.; Conrad, M.N.; Conaway, R.C.; Conaway, J.W.; Shilatifard, A. Cloning and characterization of ELL-associated proteins EAP45 and EAP20. a role for yeast EAP-like proteins in regulation of gene expression by glucose. *J. Biol. Chem.* **2001**, *276*, 16528–16533. [CrossRef]
245. Stauffer, D.R.; Howard, T.L.; Nyun, T.; Hollenberg, S.M. CHMP1 is a novel nuclear matrix protein affecting chromatin structure and cell-cycle progression. *J. Cell Sci.* **2001**, *114*, 2383–2393. [PubMed]
246. Tsang, H.T.; Connell, J.W.; Brown, S.E.; Thompson, A.; Reid, E.; Sanderson, C.M. A systematic analysis of human CHMP protein interactions: Additional MIT domain-containing proteins bind to multiple components of the human ESCRT III complex. *Genomics* **2006**, *88*, 333–346. [CrossRef] [PubMed]
247. Miranda-Saksena, M.; Denes, C.E.; Diefenbach, R.J.; Cunningham, A.L. Infection and Transport of Herpes Simplex Virus Type 1 in Neurons: Role of the Cytoskeleton. *Viruses* **2018**, *10*, 92. [CrossRef] [PubMed]
248. Leuzinger, H.; Ziegler, U.; Schraner, E.M.; Fraefel, C.; Glauser, D.L.; Heid, I.; Ackermann, M.; Mueller, M.; Wild, P. Herpes simplex virus 1 envelopment follows two diverse pathways. *J. Virol.* **2005**, *79*, 13047–13059. [CrossRef]
249. Draganova, E.B.; Zhang, J.; Zhou, Z.H.; Heldwein, E.E. Structural basis for capsid recruitment and coat formation during HSV-1 nuclear egress. *Elife* **2020**, *9*, e56627. [CrossRef]
250. Klupp, B.G.; Granzow, H.; Mettenleiter, T.C. Nuclear envelope breakdown can substitute for primary envelopment-mediated nuclear egress of herpesviruses. *J. Virol.* **2011**, *85*, 8285–8292. [CrossRef] [PubMed]
251. Lymberopoulos, M.H.; Bourget, A.; Ben Abdeljelil, N.; Pearson, A. Involvement of the UL24 protein in herpes simplex virus 1-induced dispersal of B23 and in nuclear egress. *Virology* **2011**, *412*, 341–348. [CrossRef] [PubMed]
252. Wu, S.; Pan, S.; Zhang, L.; Baines, J.; Roller, R.; Ames, J.; Yang, M.; Wang, J.; Chen, D.; Liu, Y.; et al. Herpes Simplex Virus 1 Induces Phosphorylation and Reorganization of Lamin A/C through the gamma134.5 Protein That Facilitates Nuclear Egress. *J. Virol.* **2016**, *90*, 10414–10422. [CrossRef] [PubMed]
253. Arii, J.; Watanabe, M.; Maeda, F.; Tokai-Nishizumi, N.; Chihara, T.; Miura, M.; Maruzuru, Y.; Koyanagi, N.; Kato, A.; Kawaguchi, Y. ESCRT-III mediates budding across the inner nuclear membrane and regulates its integrity. *Nat. Commun.* **2018**, *9*, 3379. [CrossRef]
254. Crump, C.M.; Yates, C.; Minson, T. Herpes simplex virus type 1 cytoplasmic envelopment requires functional Vps4. *J. Virol.* **2007**, *81*, 7380–7387. [CrossRef]
255. Kharkwal, H.; Smith, C.G.; Wilson, D.W. Blocking ESCRT-mediated envelopment inhibits microtubule-dependent trafficking of alphaherpesviruses in vitro. *J. Virol.* **2014**, *88*, 14467–14478. [CrossRef]
256. Kharkwal, H.; Smith, C.G.; Wilson, D.W. Herpes Simplex Virus Capsid Localization to ESCRT-VPS4 Complexes in the Presence and Absence of the Large Tegument Protein UL36p. *J. Virol.* **2016**, *90*, 7257–7267. [CrossRef]
257. Reynolds, A.E.; Ryckman, B.J.; Baines, J.D.; Zhou, Y.; Liang, L.; Roller, R.J. U(L)31 and U(L)34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids. *J. Virol.* **2001**, *75*, 8803–8817. [CrossRef] [PubMed]
258. Pawliczek, T.; Crump, C.M. Herpes simplex virus type 1 production requires a functional ESCRT-III complex but is independent of TSG101 and ALIX expression. *J. Virol.* **2009**, *83*, 11254–11264. [CrossRef] [PubMed]

259. English, L.; Chemali, M.; Duron, J.; Rondeau, C.; Laplante, A.; Gingras, D.; Alexander, D.; Leib, D.; Norbury, C.; Lippe, R.; et al. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. *Nat. Immunol.* **2009**, *10*, 480–487. [CrossRef] [PubMed]
260. Radtke, K.; English, L.; Rondeau, C.; Leib, D.; Lippe, R.; Desjardins, M. Inhibition of the host translation shutoff response by herpes simplex virus 1 triggers nuclear envelope-derived autophagy. *J. Virol.* **2013**, *87*, 3990–3997. [CrossRef]
261. Turan, A.; Grosche, L.; Krawczyk, A.; Muhl-Zurbes, P.; Drassner, C.; Duthorn, A.; Kummer, M.; Hasenberg, M.; Voortmann, S.; Jastrow, H.; et al. Autophagic degradation of lamins facilitates the nuclear egress of herpes simplex virus type 1. *J. Cell Biol.* **2019**, *218*, 508–523. [CrossRef]
262. Owen, D.J.; Crump, C.M.; Graham, S.C. Tegument Assembly and Secondary Envelopment of Alphaherpesviruses. *Viruses* **2015**, *7*, 5084–5114. [CrossRef] [PubMed]
263. Ventimiglia, L.N.; Martin-Serrano, J. ESCRT machinery: Damage control at the nuclear membrane. *Cell Res.* **2016**, *26*, 641–642. [CrossRef] [PubMed]
264. Lee, C.P.; Liu, P.T.; Kung, H.N.; Su, M.T.; Chua, H.H.; Chang, Y.H.; Chang, C.W.; Tsai, C.H.; Liu, F.T.; Chen, M.R. The ESCRT machinery is recruited by the viral BFRF1 protein to the nucleus-associated membrane for the maturation of Epstein-Barr Virus. *PLoS Pathog.* **2012**, *8*, e1002904. [CrossRef] [PubMed]
265. Lee, C.P.; Liu, G.T.; Kung, H.N.; Liu, P.T.; Liao, Y.T.; Chow, L.P.; Chang, L.S.; Chang, Y.H.; Chang, C.W.; Shu, W.C.; et al. The Ubiquitin Ligase Itch and Ubiquitination Regulate BFRF1-Mediated Nuclear Envelope Modification for Epstein-Barr Virus Maturation. *J. Virol.* **2016**, *90*, 8994–9007. [CrossRef]
266. Granzow, H.; Klupp, B.G.; Fuchs, W.; Veits, J.; Osterrieder, N.; Mettenleiter, T.C. Egress of alphaherpesviruses: Comparative ultrastructural study. *J. Virol.* **2001**, *75*, 3675–3684. [CrossRef]
267. van Genderen, I.L.; Brandimarti, R.; Torrisi, M.R.; Campadelli, G.; van Meer, G. The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. *Virology* **1994**, *200*, 831–836. [CrossRef]
268. Hollinshead, M.; Johns, H.L.; Sayers, C.L.; Gonzalez-Lopez, C.; Smith, G.L.; Elliott, G. Endocytic tubules regulated by Rab GTPases 5 and 11 are used for envelopment of herpes simplex virus. *EMBO J.* **2012**, *31*, 4204–4220. [CrossRef] [PubMed]
269. Schipke, J.; Pohlmann, A.; Diestel, R.; Binz, A.; Rudolph, K.; Nagel, C.H.; Bauerfeind, R.; Sodeik, B. The C terminus of the large tegument protein pUL36 contains multiple capsid binding sites that function differently during assembly and cell entry of herpes simplex virus. *J. Virol.* **2012**, *86*, 3682–3700. [CrossRef]
270. Leis, J.; Luan, C.H.; Audia, J.E.; Dunne, S.F.; Heath, C.M. Ilaprazole and other novel prazole-based compounds that bind Tsg101 inhibit viral budding of HSV-1/2 and HIV from cells. *BioRxiv* **2020**. [CrossRef]
271. Kattenhorn, L.M.; Korbel, G.A.; Kessler, B.M.; Spooner, E.; Ploegh, H.L. A deubiquitinating enzyme encoded by HSV-1 belongs to a family of cysteine proteases that is conserved across the family Herpesviridae. *Mol. Cell* **2005**, *19*, 547–557. [CrossRef] [PubMed]
272. Schlieker, C.; Korbel, G.A.; Kattenhorn, L.M.; Ploegh, H.L. A deubiquitinating activity is conserved in the large tegument protein of the herpesviridae. *J. Virol.* **2005**, *79*, 15582–15585. [CrossRef]
273. Bolstad, M.; Abaitua, F.; Crump, C.M.; O'Hare, P. Autocatalytic activity of the ubiquitin-specific protease domain of herpes simplex virus 1 VP1-2. *J. Virol.* **2011**, *85*, 8738–8751. [CrossRef] [PubMed]
274. Butt, B.G.; Owen, D.J.; Jeffries, C.M.; Ivanova, L.; Hill, C.H.; Houghton, J.W.; Ahmed, M.F.; Antrobus, R.; Svergun, D.I.; Welch, J.J.; et al. Insights into herpesvirus assembly from the structure of the pUL7:pUL51 complex. *Elife* **2020**, *9*, e53789. [CrossRef] [PubMed]