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# Combination therapy with oncolytic viruses and immune checkpoint inhibitors

Matthew Chiu [1,2], Edward Armstrong [1,2], Vicki Jennings [1], Shane Foo [1], Eva Crespo-Rodriguez[1], Galabina Bozhanova [1], Emmanuel Patin [1], Martin McLaughli[1], David Mansfield[1], Gabby Baker[1], Lorna Grove[1], Malin Pedersen[1], Joan Kyula[1], Victoria Roulstone[1], Anna Wilkins[3], Fiona McDonald[2], Kevin Harrington[1,2], Alan Melcher[1,2].

[1]The Institute of Cancer Research, London, UK

[2]The Royal Marsden NHS Foundation Trust, London, UK

[3]The Francis Crick Institute, London, UK

## Abstract

**Introduction:** Immune checkpoint inhibitors (ICI) have dramatically improved the outcome for cancer patients across multiple tumour types. However the response rates to ICI monotherapy remain relatively low, in part due to some tumours cultivating an inherently "cold" immune microenvironment. Oncolytic viruses (OV) have the capability to promote a "hotter" immune microenvironment which can improve the efficacy of ICI.

**Areas covered:** In this article we conducted a literature search through Pubmed/Medline to identify relevant articles in both the pre-clinical and clinical settings for combining OVs with ICIs and discuss the impact of this approach on treatment as well as changes within the tumour microenvironment. We also explore the future directions of this novel combination strategy.

**Expert opinion:** The imminent results of the Phase 3 study combining pembrolizumab with or without T-Vec injection are eagerly awaited. OV/ICI combinations remain one of the most promising avenues to explore in the success of cancer immunotherapy.

**Keywords:** cancer, combination treatment, immune checkpoint inhibitors, immunotherapy, oncolytic viruses

**Article highlights:**

- Oncolytic viruses (OV) not only selectively infect, replicate inside and kill tumour cells directly, they also have the capability to promote a "hotter" immune microenvironment which can improve the efficacy of ICI.
- T-Vec is the first oncolytic virus to receive FDA and EMA approved in patients with metastatic melanoma.
- Preclinical and clinical studies across many tumour types have shown that OVs promote CD4+, CD8+ T cell tumour infiltration and increase tumoural expression of PD-L1.
- OVs can also be modified to include specific therapeutic transgenes that can enhance the effect of OV/ICI combinations.
- Multiple clinical trials of ICI/OV combinations are ongoing and some have already reported encouraging therapeutic effects.



**1. Introduction**

The concept of harnessing the patients' own immune system to treat cancer can be dated back to the early 19th century, when William B. Coley used an injected mixture of heat-killed *Streptococcus pyogenes* and *Bacillus prodigiosus* (now reclassified as *Serratia marcescens*) to induce an inflammatory response in his patients and, in some cases, achieved impressive responses (1). Since then our understanding and advancement in the field of cancer immunology have improved, with immune checkpoint inhibitors (ICIs) being clinically the most impactful step forward over the last decade. The discoveries leading to the development of ICIs for cancer treatment led to James P. Allison and Tasuku Honjo being awarded the 2018 Nobel Prize in Physiology or Medicine (2). ICIs block the negative regulators of T cell function, leading to T cell activation. Amongst these agents, programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitors have been the most successful to date, resulting in clear clinical benefit in multiple tumour sites (3-6). Currently, the US Food and Drug Administration (FDA) has approved six ICIs for clinical use: ipilimumab, nivolumab, pembrolizumab, atezolizumab, avelumab and durvalumab. However, despite their inarguable success, the percentage of patients with cancer estimated to respond to ICIs is still relatively low (7).

In an effort to enhance the responses to ICIs, combination strategies with conventional cancer therapeutics have been explored. An example of this is in the KEYNOTE189 study in which patients with metastatic non-squamous non-small cell lung cancer (NSCLC) were randomised to receive either pembrolizumab (an anti-PD-1 antibody) in combination with conventional first-line chemotherapy, or to conventional chemotherapy alone (8). The results demonstrated an improvement in 12-month overall survival (OS) of 69.2% vs. 49.4% in favour of the pembrolizumab combination group. ICIs have also been combined with radiotherapy for patients with stage III, unresectable NSCLC after conventional concurrent chemoradiotherapy (9). The addition of durvalumab (an anti-PD-L1 antibody) was shown to significantly increase 24-month OS rate (66.3% vs. 55.6%) compared to patients who received placebo. Both of these exemplar trials have led to changes in clinical practice.

Another promising class of immunotherapy is oncolytic viruses (OVs), which can be naturally occurring or genetically modified (Figure 1). They have a dual mechanism of action; OVs were originally developed selectively to infect, replicate and cause direct lysis of tumour cells, but they are

now also recognised to promote an anti-tumour immune response via the induction of immunogenic cell death (10). Virotherapy can lead to release of tumour-associated antigens (TAA) after cancer cell lysis as well as production of pro-inflammatory cytokines (11). The result is the ability of OVs to convert an immunosuppressive ("cold") tumour microenvironment (TME) to a "hotter" immunostimulatory one.

However, thus far, OVs have had limited single agent activity in clinical trials. In addition, OVs given systemically must be able to avoid degradation prior to reaching the tumour target. Intratumoural delivery may overcome this aspect of intravenous delivery however is logistically problematic to deliver in patients with widespread sites of metastatic disease. Furthermore, pre-existing anti-viral neutralising antibodies can exist in the human population to many oncolytic viruses, especially those which are vaccinated against, and this may limit effective delivery (12). Despite these challenges, there is particular interest in the potential benefit in combining OVs with ICIs. The effectiveness of anti-PD-1/PD-L1 therapy has been shown to be related to the immune TME, with tumours lacking lymphocyte infiltration and an IFN-γ gene signature being less responsive to ICIs (13). Therefore, the ability of OVs to convert the tumour to a more immune cell-rich environment should result in better therapeutic responses to ICIs (Figure 2). This paradigm has stimulated active, ongoing research combining OVs and ICI treatments in cancer therapy (Table 1). In this review, we will explore the pre-clinical and clinical studies looking at this promising novel strategy.

## 2. DNA viruses

### 2.1. Herpes Virus

#### 2.1.1. Pre-clinical work

Herpesviridae represent a family of approximately 100 viruses sharing common structural features. Each virus has a linear 120-230kb double-stranded DNA genome maintained in a toroidal conformation and surrounded by an icosadeltahedral nucleocapsid (14). They possess many qualities which make them good oncolytic viruses, such as broad tumour tropism, high lytic activity, large genomes that can be easily manipulated to insert multiple therapeutic transgenes; they are also potent activators of both innate and adaptive immunity.

Herpes simplex virus 1 (HSV-1) has been the most extensively studied as a backbone for oncolytic viral therapy and there have been multiple preclinical and early-phase clinical trials that have shown benefit in combination with PD-1/PD-L1 blockade. For example, Chen et al. (15) showed that combination therapy with HSV1716 and anti-PD-1 antibody led to "hot" immune changes within the tumour, including increased infiltration of effector CD4+ and CD8+ T cells, but no similar increase in FoxP3+ T-regulatory (Treg) cells. These changes extended survival in tumour-bearing mice compared to those untreated or receiving OV or ICI monotherapy in models of rhabdomyosarcoma. The therapeutic benefit was lost in athymic nude mice, suggesting that adaptive immunity was essential for the success of treatment. Du et al. (16) also noted improved overall survival in mice with metastatic melanoma brain lesions with combination treatment of oncolytic HSV (oHSV) and anti-PD-L1 blockade. In that study, oHSV was loaded onto mesenchymal stem cells (MSC-oHSV), which had an affinity to migrate towards tumour sites and, hence, delivered the OV directly to cancer cells after systemic injection in murine models. Combination therapy improvements were found to be associated with increased tumoural IFNy-producing CD8+ T cells.

Similar treatment advantages were observed by Saha et al. in a syngeneic murine glioblastoma (GBM) model (17). In these experiments, oHSV G47Δ was encoded with an IL-12 therapeutic transgene, which helps to promote proliferation of activated T cells and NK cells, stimulate Th1 differentiation and induce IFN-γ production. The so-called G47Δ-mIL-12 virus, in combination with anti-PD-1 or anti-PD-L1, again increased tumour infiltration of CD8+ T cells and the CD8+:Treg ratio, which led to a modest prolongation of survival in mice harbouring GBM compared to either monotherapy. However, when a CTLA-4 antibody was added to the doublet treatment of virus/anti-PD-1, an impressive 77% of treated mice with GBM were cured and protected from tumour rechallenge 6 months after initial treatment. This therapeutic benefit was associated with an increase in proliferating T cells, reduction of Tregs, influx of macrophages with polarisation to an anti-tumoural "M1"-type, and a significant reduction of PD-L1+ cells. Studies depleting/inhibiting CD4+, CD8+ and macrophages, showed that all are required for the triple combination therapeutic efficacy. Interestingly, it was also noted that depletion of a single cell type led to dramatic changes in the other immune cell types, highlighting the complex interconnections between different immune cell types in the immune TME in the context of therapy.

Macrophages were also found to play an important role in the synergistic response to oHSV and anti-PD-1 combination in another murine glioblastoma model (18). In that study, oHSV was generated to include a payload cassette to drive the expression of human UL16-binding protein 3 (ULBP3). This class 1 major histocompatibility complex-like (MHC-like) molecule has pro-inflammatory effects on myeloid cells (19). oHSV$^{ULBP3}$-treated tumours were more infiltrated with CD8+ T cells than their PBS control-treated counterparts, and this influx was further enhanced with co-treatment with anti-PD-1. In a bilateral glioblastoma model, invasion of tumour-associated macrophages (TAMs) was observed in the locally injected and distant untreated tumour with oHSV$^{ULBP3}$. The addition of anti-PD-1 augmented this anenestic increase of TAMs and critically switched the immunosuppressive repolarisation of these cells to generate a "hotter" immune microenvironment that correlated with better tumour control.

Current immune checkpoint antibodies require systemic administration and can be associated with adverse effects. A potential strategy to reduce toxicity is for local antibody delivery via expression of the ICI from an oncolytic viral vector. This approach was used by Passato et al. (20) who engineered an oHSV virus to express a single-fragment variable (scFv) antibody against PD-1 to produce the novel NG34scFvPD-1 virus. NG34scFvPD-1 was shown to produce and secrete scFvPD-1 protein, which was able to bind to PD-1 in different mouse GBM cells after viral infection. *In vivo*, the NG34scFvPD-1 virus significantly prolonged survival in GBM-bearing immunocompetent mice compared with those treated with parental NG24 virus/untreated controls, or tumour-bearing immunodeficient athymic mice, thereby reiterating the importance of an intact immune system for therapeutic response.

Our lab was recently involved in the development of a new cell fusion-enhanced oncolytic immunotherapy platform based on HSV-1 and their combination with checkpoint inhibition (21). In this study, new clinical HSV strains were isolated and developed to enhance their oncolytic potential. The immunogenicity of cell death was increased with the insertion of a gene encoding the envelope glycoprotein of gibbon ape leukemia virus (GALV) in a truncated, constitutively highly fusogenic form named GALV-GP-R$^-$. Treatment with this modified HSV-1 virus, expressing both granulocyte-macrophage colony-stimulating factor (GM-CSF) and GALV-GP-R$^-$, showed significant tumour regression both locally (injected tumour) and systemically (non-injected tumour) in bilateral 9L gliosarcoma tumour-bearing rats. An increase in CD8+ T cells and PD-L1 expression was also observed in both injected and contralateral non-injected tumours. Consistent with this, combination

treatment with PD-1 blockade led to significant enhancement of tumour regression, both locally and systemically in immune competent mice bearing established A20 lymphoma tumours. Furthermore, modifying HSV-1 to express not only GM-CSF and GALV-GP-R$^-$, but also to encode anti-CTLA-4 or other immune co-stimulatory pathway-activating ligands including CD40L, OX40L and 4-1BBL, further augmented anti-tumour effects when combined with PD-1 blockade. The therapeutic effects from combination treatment were durable as cured mice were again resistant to subsequent tumour rechallenge, consistent with the development of an effective memory immune response.

### 2.1.2. Clinical trials

#### Talimogene laherparepvec (T-VEC)

Talimogene laherparepvec (T-VEC) is a modified oHSV1 with deletions in the ICP34.5 and ICP47 genes resulting in enhanced tumour tropism and decreased neurovirulence (22). The addition of a GM-CSF transgene also improves the immune modulatory effects of the virus (23). T-VEC became the first FDA-approved oncolytic virus for clinical use based on results of the phase III OPTiM (Oncovex [GM-CSF] Pivotal Trial in Melanoma) trial (24). In this study, intralesional injection of T-VEC led to a statistically significant improvement in durable overall response rate when compared to GM-CSF alone (16.2% vs 2.1%, $p<0.001$), in patients with unresectable stage IIIB or IV melanoma. Importantly, an anenestic response was also noted, as 15% of measurable visceral (non-injected) lesions reduced in size by ≥50% following T-VEC treatment. The final analyses revealed a median OS benefit of 23.3 months vs. 18.9 months in the T-VEC and GM-CSF arms, respectively, while also exhibiting a tolerable safety profile with low rates of grade 3/4 adverse events (AEs) (25). T-VEC was also found to alter the tumour immune microenvironment by reducing the number of CD4+ Tregs, CD8+ T suppressor cells and myeloid derived suppressor cells (MDSC) (26).

With the success of T-VEC monotherapy and its ability to modulate the immune response to tumours, it was postulated that there would be a greater therapeutic benefit in combination with ICIs. This led to a Phase Ib/II trial of T-VEC in combination with ipilimumab (an anti-CTLA-4 antibody) for patients with previously untreated unresectable stage IIIB-IV melanoma. The first part of the study recruited nineteen patients in total and resulted in an objective response rate (ORR) of 50%; 44% of patients had a durable response lasting ≥6months and there were no documented dose-limiting toxicities (27). The subsequent phase II part of the trial randomised patients with advanced melanoma to either ipilimumab alone or in combination with T-VEC. The outcome demonstrated an ORR in 39% of patients receiving T-VEC and ipilimumab compared to 18% in patients receiving ipilimumab monotherapy. Importantly distant non-injected sites also showed anenestic responses with visceral lesions reducing in size in 52% of patients with combination treatment, compared to 23% of patients in the ipilimumab alone arm (28) (ClinicalTrials.gov: NCT01740297).

T-VEC has also been tested in combination with an anti-PD-1 antibody (pembrolizumab) for patients with advanced melanoma in the Masterkey-265 trial (ClinicalTrials.gov: NCT02263508). This is a phase Ib/III trial that has revealed promising results so far. The phase I component demonstrated an ORR of 62%, with a complete response rate (CRR) of 33% in patients receiving T-VEC + pembrolizumab combination therapy. The treatment was well tolerated with no dose-limiting toxicities occurring. Patients who responded to combination treatment had increased CD8+ T cells, elevated PD-L1 expression as well as IFN-γ gene expression on several cell subsets in tumours after induction T-VEC alone treatment (29). These data support the theory that oncolytic virotherapy can enhance the efficacy of ICI by altering the TME, making cold tumours hotter, and priming for more

effective checkpoint blockade. The subsequent phase III trial has now completed recruitment and outcome data are eagerly awaited.

**HF10**

HF10 is another oHSV1 that has natural deletions in UL43, UL49.5, UL55 and UL56 as well as overexpression of UL53 and UL54. These genomic alterations enhance viral tumour selectivity, viral replication and induce potent anti-tumour effects across different malignancies (30). Infection with HF10 has also been shown to increase tumour-infiltrating CD4+ and CD8+ T cells in breast, pancreatic and head and neck squamous cell cancers (30). HF10 was investigated as a combination treatment with ipilimumab in a phase I/II trial for patients with unresectable Stage IIIB-IV melanoma (ClinicalTrials.gov: NCT03153085). In this study, the authors found that patients who responded tended to have significant tumour infiltrations of CD8+ and CD4+ T cells, as well as higher expression of ICOS levels on CD4+ T cells, than non-responders, suggesting that these could be pharmacodynamic biomarkers for benefit (31). In another phase II trial of HF10 in combination with ipilumumab, again in patients with unresectable Stage IIIB-IV melanoma (ClinicalTrials.gov: NCT02272855), the treatment resulted in a best ORR of 41%, disease stability rate of 68% and no dose-limiting toxicities (32). There is a third phase II trial looking at HF10, this time in combination with nivolumab (an anti-PD-1 antibody) in patients with resectable Stage IIIB/C and IVa melanoma that is currently active (ClinicalTrials.gov: NCT03259425).

## 2.2. Vaccinia Virus

### 2.2.1. Pre-clinical work

Vaccinia viruses belong to the family of Poxviridae and consist of a linear double-stranded DNA genome (~190kb) within a brick-shaped envelope. They are suited as an oncolytic virus backbone due to their fast replication and dissemination, extensive safety knowledge as a smallpox vaccine and their ability to modulate immune responses (33, 34). They also have a large genome which enables therapeutic-enhancing DNA transgenes (up to 40kb) to be inserted (35).

Kleinpeter et al. exploited the large capacity of the Western Reserve strain of oncolytic vaccinia virus (oVV) to insert three forms of murine PD-1 (mPD-1) binders. The authors then assessed the expression of the resulting anti-PD-1 antibodies and their anti-tumour efficacy *in vitro* and *in vivo* in multiple tumour models. They showed the resulting viruses were indeed produced, assembled and able to block murine PD-1 ligand binding after virus infection, which subsequently led to improved survival in mice harbouring subcutaneous fibrosarcoma (36). Interestingly, the concentration of anti-PD-1 antibody expression was higher and persisted longer in mice receiving intratumoural injection of the vaccinia virus armed with the whole anti-PD-1 antibody (WR-mAb), compared to mice receiving an intratumoural injection of the antibody itself (36).

Consistently, groups have shown that oVV infection can increase the level of PD-L1 expression within the tumour after infection (37, 38). One such group armed vaccinia virus with a superagonist IL-15, which is a fusion protein of IL-15 and IL-15Ralpha (vvDD-IL15-Rα) (37). IL-15 is a cytokine that is capable of promoting survival, proliferation and activation of different immune cells such as natural killer (NK), NKT, and CD8+ T cells (39), as well as impacting on PD-1 and PD-L1 expression (40). Indeed, treatment with vvDD-IL15-Rα led to enhanced infiltration of NK and CD8+ T cells, as well as an increase in intratumoural PD-1 and PD-L1, although in that study it was not clear which cell types

in the TME accounted for this. The combination of virus with PD-1 blockade, as anticipated, led to more dramatic improvements in survival of mice bearing MC38 colon tumours than either monotherapy (37).

Liu et al. also showed increased PD-L1 expression on tumours and immune cells in murine colon and ovarian cancer models after infection with oVV, this time expressing CXCL11 (vvDD-CXCL11) (38). Subsequent combination treatment with anti-PD-L1 therapy led to reduced tumour burden and improved survival compared to single treatments in both murine models. This was attributed to increased tumour infiltration of CD8+ and CD4+ T cells, which were also more activated as indicated by enhanced IFN-γ, ICOS, granzyme B and perforin expression.

Our own group has shown that dendritic cells (DCs) may also play a role in the synergistic effect of vaccinia virus and anti-PD-1 blockade (41). In this study, GLV-1h68 (an attenuated vaccinia virus) (42) delivered via isolated limb perfusion (ILP), substantially improved responses to subsequent PD-1 blockade in a rodent model of high-grade extremity sarcoma. This combination treatment again increased the number and activation of tumour-infiltrating CD8+ cytotoxic and effector CD4+ T cells, without significant change in the number of Tregs, but the most marked difference was the substantial reduction in M2-like macrophages and accumulation of activated intratumoural DCs. The changes in T cells within the tumour immune microenvironment were maintained, even when tumours were treated with sub-therapeutic regimens that resulted in initial responses followed by local and distant relapses. However, the changes in DCs were only evident in tumours that were given curative treatment, suggesting that DCs play an essential part in activating the adaptive immune system and generating systemic anti-tumour immunity following local therapy (41). DCs are also known to express both PD-L1 and PD-L2 and blockade of the PD-1 axis may promote the ability of these cells to engage and stimulate effector T cell function (43). Others have also investigated combining oVV with anti-CTLA-4 (44, 45) resulting in positive therapeutic outcomes.

Studies by Fend et al. (44) and Rojas et al. (45) both found that the sequencing of virus and ICI administration was also important. Fend et al. noted both CTLA-4 and PD-1 blockade worked best shortly after oVV delivery in the murine MCA205 fibrosarcoma model. Rojas et al. similarly noted that the optimal combination strategy was to administer anti-CTLA-4 antibody after virus injection, as the therapeutic survival benefit was lost when both agents were given simultaneously in mice bearing renal adenocarcinoma.

### 2.2.2. Clinical trials

**Pexastimogene devacirepvec (Pexa-Vec)**

Pexastigmogene devacirepvec (Pexa-Vec; also known as JX-594) is a Wyeth strain oVV armed with both human GM-CSF and β-galactosidase transgenes designed to promote anti-tumour immunity (46). The immune consequences have been demonstrated in a clinical trial where patients with resectable malignancies received a single intravenous preoperative dose of Pexa-Vec, which demonstrated that virotherapy induced robust activation of NK and CD8+ T cells, as well as increasing PD-L1 expression within the tumour. Functional assays also revealed increased TAA recognition by T cells and increased pro-inflammatory cytokines within the serum such as IFNα, IFNβ, TRAIL and CXCL10 (47). Pexa-Vec has been investigated in combination with ICI in an ongoing phase 3 clinical trial in renal cell carcinoma (RCC). In this study Pexa-Vec treatment led to a 16-fold increase in tumoural CD8+ T cell infiltration as well as increasing expression of immune checkpoint molecules such as PD-1 (by 4 fold), CTLA-4 (by 2.3 fold) and LAG3 (by 3.1 fold). Combination of Pexa-

Vec with either PD-1 or CTLA-4 blockade led to further increase in intratumoural infiltration of activated CD8+ T cells and effective suppression of RCC growth (48). Currently, there are two recruiting clinical trials investigating Pexa-Vec in combination with ICI in refractory colorectal cancer (ClinicalTrials.gov: NCT03206073) and advanced/metastatic solid tumours (ClinicalTrials.gov: NCT02977156); disappointingly, a further trial in advanced hepatocellular carcinoma (NCT03071094) has recently failed.

### 2.3. Adenoviruses

#### 2.3.1. Pre-clinical work

Adenoviruses are non-enveloped, double-stranded DNA viruses with an icosahedral capsid that incorporates a 34-36kb genome (49). They are classified in serotypes according to the reactivity of the antibodies generated in their hosts, and in humans there are 57 serotypes classified into 7 subgroups (A-G). Group C from serotype 5 has been the most commonly used as a backbone for OVs (50). Oncolytic adenovirus (oADV) has been widely studied because, as with HSV and VV, they can accommodate large therapeutic transgenes and have a well-established patient safety profile (51). Their role in combination with ICI has also been a clear focus of active research.

One recent example is a study by Cevera-Carrascon et al. (52), which exploited the capacity of the adenovirus to accommodate transgenes for tumour necrosis factor alpha (TNFa) and interleukin-2 (IL-2). These cytokines are pro-inflammatory and stimulate T cell trafficking, activation and propagation (53). In murine B16.OVA melanoma models, despite the relatively poor infectivity of mouse cells, injection of this modified adenovirus in conjunction with anti-PD-1 blockade resulted in improvement in both tumour growth control and overall survival. This therapeutic treatment was associated with increased intratumoural cytotoxic CD8+ T cells compared with either single treatments. Furthermore, priming with intratumoural virus two days in advance of delivering an anti-PD-1 antibody, resulted in improved outcomes compared to combination treatment delivered simultaneously (52), again confirming that the sequencing of these immunotherapy agents is important.

Another group modified adenovirus by packaging the HSV thymidine kinase (HSV-tk) gene, an enzyme that metabolizes the prodrug ganciclovir into toxic nucleotide analogs, and investigated this agent as a gene-mediated cytotoxic immunotherapy (GMCI) in syngeneic murine glioblastoma models (GL261 and CT-2A) (54). Delivery of this virus followed by ganciclovir was found to induce immunogenic type I IFN responses, as well as upregulating PD-L1 expression within the tumour. The addition of anti-PD-1 antibody to GMCI, resulted in greater overall survival compared to either GMCI or anti-PD-1 therapy alone. Durable immune memory was also demonstrated in long-term (>100 days) survivors, as these mice were protected from subsequent tumour rechallenge.

Both Jiang et al. and Singh et al. published studies looking at oncolytic adenovirus armed with CD40 ligand, a T cell co-stimulatory receptor present on antigen-presenting cells (55, 56). In both studies, the modified virus was able to increase infiltration of cytotoxic CD8+ T cells and PD-L1 expression on tumour cells, when compared with their unmodified viruses. Both showed that combining these novel viruses with PD-L1 blockade led to improved overall survival in murine glioma (55) and melanoma models (56). Singh et al. also noted that CD40 armed adenovirus in combination with anti-PD-L1 therapy led to increase in expression of CTLA-4 on CD8+ T cells, and subsequently showed that by adding an anti-CTLA-4 antibody to the initial doublet treatment further improved survival.

This triple combination strategy also led to anenestic responses in non-virally injected contralateral flank tumours, demonstrating the induction of systemic anti-tumour immunity.

Another strategy to combine oADV and ICIs is to modify the virus to express PD-1 or PD-L1, as previously discussed for HSV. One example of this, is a study by Shin et al. who investigated an adenovirus that not only harboured a soluble form of PD-1 (sPD-1-Ig), but also HSV-tk, with the dual goal of enhancing tumour antigen release and priming, and blocking local immune resistance (57). They were able to show that tumour antigen released by HSV-tk-transduced tumours successfully primed tumour antigen-specific CD8 T cells via DCs, in addition to inducing direct tumour cell cytotoxicity. The addition of sPD-1-IG to the adenovirus further enhanced regression of murine tumours compared with adenovirus expressing only HSV-tk. This enhancement was found to be CD8+ T cell dependent, as depletion of CD8+ T cells abrogated the therapeutic effect.

A similar approach was used in a study by Tanoue et al. Here an oADV armed with a PD-L1 blocking mini-antibody (CAd-VEC PD-L1) was tested in combination with chimeric antigen receptor T cells (CAR T cells) (58). CAd-VEC PD-L1 infection exhibited oncolytic effects, along with production of PD-L1 blocking mini-body, locally at the tumour site in a human prostate cancer xenograft model. Combination with CAR T cell therapy prolonged median survival in these animals compared to either treatment alone, suggesting that PD-L1 mini-body expressed after CAd-VEC PD-L1 infection blocked the PD-1/PD-L1 interaction between the cancer and CAR T cells, while maintaining cancer cell oncolysis.

Finally, two further studies have modified adenoviruses to express tumour associated antigens (TAAs) (59, 60), and found that combination with anti-PD-L1 or anti-PD-1 treatment led to improved anti-tumour efficacy as a result of enhanced T cell activity.

### 2.3.2. Clinical trials

#### Tasadenoturev

Tasadenoturev (DNX-2401) is a tumour selective, replication-competent oADV that has been modified with a deletion in the E1A gene, which allows it to replicate only in cells defective in the retinoblastoma (RB) pathway. A dose-escalation phase I study showed that DNX-2401 was safe and capable of robust viral replication and tumour control in recurrent high-grade glioma patients. 72% of patients (18 out of 25) had tumour reduction and remarkably 20% (5 patients) survived for more than 3 years (61). Immunohistochemistry in a separate group of patients who had resected tumours after DNX-2401 revealed an increase in CD4+ and CD8+ cell density compared to pre-treatment specimens and there was no expression change of PD-1 or PD-L1. This led to a subsequent ongoing Phase II trial (CAPTIVE study/KEYNOTE 192; ClinicalTrials.gov: NCT02798406) looking at single intratumoural injection of DNX-2401 in combination with pembrolizumab in patients with recurrent glioblastoma. The interim results were published in November 2018 and showed that out of 23 patients treated, two had partial responses and there was a 100% 9-month survival for the first 7 patients with no treatment related deaths or discontinuations (62).

#### ONCOS-102

ONCOS-102 (AdV5/3- Δ24-GM-CSF) is an engineered oADV that expresses GM-CSF and its chimeric 5/3 capsid contains the fibre knob derived from Ad serotype 3, so infection occurs through the

desmogelin 2 receptor, that is often expressed on tumour cells (63). It possesses tumour specific qualities, as again there is a deletion in the E1A gene which makes viral replication only possible in cells with an abnormal Rb pathway. Preclinical studies have shown that ONCOS-102 in combination with pembrolizumab led to tumour reduction in humanised murine melanoma models, not only in virally injected tumours but also in non-injected sites (64, 65). A phase I clinical trial of ONCOS-102 in 12 patients with solid tumours showed anti-tumour effects associated with tumoural infiltration of CD8+ T cells, as well as an increase in tumour PD-L1 expression, in a subset of mesothelioma patients (66). This has led to a currently ongoing phase I pilot study of sequential ONCOS-102 and pembrolizumab in patients with advanced or unresectable melanoma (ClinicalTrials.gov: NCT03003676).

**ADV/HSV-tk**

As previously mentioned, adenovirus-mediated expression of herpes simplex virus thymidine kinase (ADV/HSV-tk) has been shown in many preclinical models (54, 57) to enhance tumour antigen priming of T cells, as well as increasing tumoural expression of PD-L1 and subsequent improved therapy in combination with ICI. This formed the basis of a phase II clinical trial looking at the safety and efficacy of in-situ ADV/HSV-tk plus valacyclovir in combination with stereotactic body radiation (SBRT) used as a window of opportunity treatment before pembrolizumab in patients with metastatic triple negative breast cancer (TNBC) and metastatic non-small cell lung cancer (NSCLC); this study (STOMP; ClinicalTrials.gov: NCT03004183) is currently ongoing.

**Enadenotucirev**

Enadenotucirev (previously described as ColoAd1) is a group B Ad11p/Ad3 chimeric oncolytic adenovirus which has shown potent tumour-selective cytotoxicity *in vitro* (67, 68) as well as antineoplastic properties in orthotopic human xenograft models *in vivo* (67, 69). Two phase I multicentre studies, Mechanism of Action (MOA) (70) and EVOLVE (71), have shown the feasibility of administering enadenotucirev intravenously with manageable safety profiles across a range of epithelial tumour types. In both trials, systemic delivery of this oncolytic virus was associated with CD8+ T cells recruitment into the tumour microenvironment which can potentially cause synergistic antitumour effects if combined with an ICI. This has led to an ongoing phase I study (SPICE; ClinicalTrials.gov: NCT02636036) to investigate the safety and tolerability of intravenously delivered enadenotucirev combined with nivolumab (a PD-1 inhibitor) for the treatment of epithelial carcinomas. A recent abstract publication of 31 patients with metastatic colorectal cancer within this trial, with median 4 prior lines of therapy, showed favourable overall survival outcome (median OS of 12.6 months) and 6 out of 8 matched biopsy samples showed evidence of increased CD8+ T cell infiltration and upregulation of markers of T cell activation (72).

### 2.4. Myxoma Virus

#### 2.4.1. Pre-clinical work

Myxoma virus is a rabbit-specific pathogenic DNA virus that has been shown to be an effective oncolytic virus in various human tumour types (73, 74). Bartee et al. generated a novel recombinant myxoma virus (vPD-1) which secretes soluble PD-1 into the tumour microenvironment from infected

cells (75). This modified virus was able to eradicate tumours in ~59% (12/22) of mice bearing B16/F10 melanoma and the effect was found to be CD8+ dependant. In a later study, the authors found that malignant T cells which did not express PD-L1, were highly susceptible to oncolytic virus therapy, suggesting that PD-L1 expression might play a role in the efficacy of viral therapy (76). Currently there are no clinical trials looking at myxoma virus in combination with ICIs.

## 3. RNA viruses

### 3.1. Reovirus

#### 3.1.1. Pre-clinical work

Respiratory Enteric Orphan virus (reovirus) is a double-stranded RNA virus from the Reoviridae family with demonstrated preferential replication and oncolysis in cancer cells. Reovirus is approximately 80 nm in diameter and is comprised of a protein shell with outer and inner components that altogether create an icosahedral capsid housing ten segments of double-stranded RNA. The oncolytic properties of reovirus appear to be partly dependent on activated Ras signalling. In humans it causes few, if any, clinical symptoms. However, when symptomatic, reovirus infection is characterized by mild enteric and respiratory symptoms (77).

In a murine subcutaneous B16 melanoma model, treatment with an anti-PD-1 antibody in combination with intratumoural reovirus resulted in increased survival compared to both control and monotherapy groups. Checkpoint inhibition was shown to improve the ability of NK cells to kill reovirus-infected tumour cells, reduce the activity of immunosuppressive Treg cells, and increase the adaptive CD8+ T cell-dependent antitumor response. Rajani et al. also demonstrated through depletion antibody experiments that NK and CD8+ T cells, but not CD4+ T cells, were responsible for the anti-tumour efficacy (78).

Beneficial immune changes were also observed by Mostafa et al. who demonstrated that reovirus upregulated tumour cell expression of PD-L1 *in vitro*. *In vivo*, reovirus monotherapy significantly reduced disease burden and enhanced survival in treated mice, which was further enhanced by PD-1 blockade. Reovirus therapy increased the number of intratumoural regulatory T cells, which was reversed by the addition of PD-1 blockade. Dual treatment of reovirus plus anti-PD-1 led to the generation of a systemic adaptive anti-tumour immune response evidenced by an increase in tumour-specific IFN-γ producing CD8+ T cells, and protection against tumour rechallenge (79).

The ability of reovirus to upregulate PD-L1 *in vitro* was also shown by Kelly et al. in a murine myeloma tumour cell model; in addition, intravenous reovirus could sensitise to anti PD-L1 antibody therapy (80).

Investigating the underlying immune mechanisms of reovirus, Ilett et al. showed that systemic reovirus can be internalised by dendritic cells, and viral transportation to tumours in mice can be increased with pre-treatment with GM-CSF. In a prime boost strategy they further combined systemic reovirus with subsequent systemic administration of a vesicular stomatitis oncolytic virus expressing melanoma antigens (VSV-ASMEL). When this approach was used in a triple combination with anti-PD-1 antibody it led to significantly enhanced survival with long-term cures (81).

The benefit of combination therapy was also identified by Samson et al. who demonstrated in mice that systemic reovirus could reach and be detected in tumours implanted into the brain. They also showed, in a window-of-opportunity clinical study, that intravenous infusion of oncolytic reovirus

could cross the blood brain barrier and infect tumour cells subsequently resected as part of standard clinical care, both in high-grade glioma and in brain metastases; this was associated with an increased CD8+ T cell tumour infiltration relative to patients not treated with virus. Reovirus upregulated the PD-1/PD-L1 axis in patient tumours and, on taking this data back to the laboratory, the authors found that reovirus/anti-PD-1 was an effective combination in a murine glioma model (82).

#### 3.1.2. Clinical trials

**Pelareorep**

Pelareorep, a proprietary isolate of the Dearing type 3 reovirus strain has been trialled in combination with single agent gemcitabine in chemotherapy-naïve patients with advanced pancreatic adenocarcinoma (ClinicalTrials.gov: NCT00998322). A proposed concern for such OV with chemotherapy combinations is the potential for the favourable virally induced changes in the tumour immune microenvironment to be reduced by leukocyte-depleting cytotoxic chemotherapy. Furthermore, chemotherapy may reduce viral neutralising antibodies and, whilst this may allow increased viral replication and anti-tumour oncolysis, .it also risks increasing the potential for virally mediated toxicity (83). Nevertheless, this phase II study enrolled 34 patients and results included one partial response, 23 stable disease, and 5 progressive disease (ClinicalTrials.gov: NCT00998322). The median overall survival was 10.2 months, with a 1- and 2-year survival rate of 45% and 24%, respectively. Treatment was well tolerated with manageable non-haematological toxicities and tumour analysis showed increased PD-L1 expression (83). As a result of this data, 11 further patients with metastatic pancreatic adenocarcinoma were recruited to receive the combination of chemotherapy, pembrolizumab and intravenous pelareorep (ClinicalTrials.gov: NCT02620423). The trial concluded that the combination showed a manageable safety profile. Of the five efficacy-evaluable patients, one had a partial response and two showed stable disease. On-treatment tumour biopsies showed an increased CD8+ T cell infiltrate and activated caspase-3 level within tumours (84). Blood samples were taken at the beginning of therapy and approximately three weeks later, to conduct an exploratory analysis of T cell changes in patients treated with the above triple combination. This demonstrated a high level of peripheral T cell repertoire clonal expansion, mostly through the expansion of new T cell clones. A correlation was identified between early and durable clonal expansion and survival (85).

### 3.2. Rotavirus

#### 3.2.1. Pre-clinical work

Rotavirus is another double-stranded RNA virus from the Reoviridae family and is the most common cause of severe diarrhoeal disease in infants and young children globally (86). The genome of 18kb is surrounded by a three-layered icosahedral non-enveloped protein capsid (87).

Shekarian et al. repurposed rotaviral vaccine strains and showed that these had selective oncolytic activity, including in vivo antitumor activity against a neuroblastoma and B cell lymphoma tumour model resistant to anti–CTLA-4 and anti–PD-L1 treatment. The group demonstrated a rapid and significant increase in tumour-infiltrating myeloid cells and, although there were no major variations in the CD4, CD8 and Treg proportions, a highly significant proportion of CD8+ T cells up-regulated markers including OX40 and CD137 on their surface, suggesting an activation of tumour-infiltrative

CD8+ T cells upon IT rotavirus therapy. CTLA-4 was also strongly up-regulated at the cell surface of both CD8+ and Treg cells and rotaviral therapy also induced type I IFN signalling and upregulation of PD-L1 gene expression. Testing the synergistic effects of the triple combination of IT rotavirus with systemic anti–PD-L1 and anti-CTLA-4 antibodies led to high cure rates with subsequent protection against tumour rechallenge. Furthermore, an anenestic benefit was observed in the non-injected tumours when using a bi-flank model. Although these vaccine strains are not in oncology clinical trials at present, such clinical translation should be feasible given these agents represent a clinically approved source of oncolytic virus (88).

### 3.3. Coxsackie Virus

#### 3.3.1. Pre-clinical work

The non-enveloped positive sense single-stranded group of Coxsackie RNA viruses have a 25 to 35 nm capsid of icosahedral symmetry and belong to the Picornaviridae family (89). They can cause a spectrum of human diseases ranging from the common cold to aseptic meningitis. Grouped into A and B subtypes, the wild-type A21 has been selected to be taken forward as a commercial oncolytic agent (90). The virus utilises Intercellular Adhesion Molecule 1 (ICAM-1) for cell entry (91, 92), and this viral receptor has been shown to be upregulated in certain human malignancies (93).

Coxsackievirus A21 has been investigated in several pre-clinical models including NSCLC, bladder cancer and melanoma. In a murine model of metastatic NSCLC, anti-PD-1 monotherapy showed an enhanced survival benefit compared to control. Conversely systemic viral therapy alone showed no benefit; however, the combination with anti PD-1 led to a survival advantage that was significantly extended beyond that of checkpoint inhibition alone. Although anti-CTLA monotherapy improved outcome, the addition of virotherapy did not further extend survival. These findings were replicated in a subcutaneous melanoma model treated with intratumoural virus (94).

#### 3.3.2. Clinical trials

**Coxsackie A21**

The data from a 16 patient extension arm to the phase II CALM trial (ClinicalTrials.gov: NCT01636882) which required pre-and post-treatment biopsies either side of at least one intratumoural injection of coxsackie A21 (CAVATAK®) in patients with advanced melanoma, showed notable changes within the tumour microenvironment. These included an increased tumoural infiltration of CD3+ and CD8+ cells and expression of PD-L1, in addition to the upregulation of a range of immune checkpoint inhibitory molecules.

These findings led to intratumoural CAVATAK® being investigated in phase Ib trials in combination with ipilimumab in the MITCI trial (ClinicalTrials.gov: NCT02307149), and in combination with pembrolizumab in the CAPRA study (ClinicalTrials.gov: NCT02565992), both in patients with advanced melanoma. Furthermore, CAVATAK® given intravenously with ipilimumab in metastatic uveal melanoma (ClinicalTrials.gov: NCT03408587) and with pembrolizumab in the phase Ib KEYNOTE 200 (STORM) trial (ClinicalTrials.gov: NCT02043665), initially in a range of solid tumour malignancies, and then in an expansion cohort for patients with advanced NSCLC and bladder cancers.

The latest presented data from the MITCI trial demonstrated an ORR of 50% in anti-PD-1 naïve and 33% in anti-PD-1 refractory patients, which compares favourably with data for ipilimumab alone in these settings, albeit patient numbers are small. Similarly, the CAPRA trial has shown an ORR of 59% from a total of 27 evaluated patients (80).

The KEYNOTE-200 (STORM) trial showed the combination was generally well tolerated with no limiting toxicities (only 8% and 0% grade 3 and 4 toxicities respectively), and clinical activity was seen in some patients. Preliminary paired biopsy results have demonstrated an increase in tumoural PD-L1 staining intensity at day 15 after treatment (95).

### 3.4. Polio Virus

#### 3.4.1. Pre-clinical work

Polio virus is another member of the Picornaviridae family with a 7.5 kb genome (96) and remains endemic in two countries (97). Poliovirus has an intriguing tropism for CD155, an immune checkpoint molecule virtually universally expressed in malignant T cells in solid neoplasia, as well as in myeloid and endothelial cells, thereby providing rationale to pursue attenuated poliovirus as a potential OV (98). Although approval has been given, caution will need to be taken due to the potential that PVSRIPO is a live attenuated (Sabin) type 1 poliovirus vaccine containing a foreign internal ribosomal entry site (IRES) of human rhinovirus type 2. This IRES substitution in PVSRIPO attenuates the wild type poliovirus' ability to cause meningoencephalomyelitis (98). In a pre-clinical model of metastatic triple negative breast cancer, Force et al. found that a single injection of PVSRIPO was equivalent to repeated anti-PD-1 or PD-L1 treatments in decreasing TNBC tumour burden. PVSRIPO combined with anti-PD-1 or anti-PD-L1 therapy provided improved tumour growth inhibition compared to monotherapies (99).

#### 3.4.2. Clinical trials

**Polio virus (PVSRIPO)**

PVSRIPO was recently evaluated in a trial which enrolled 61 patients with recurrent grade IV malignant glioma (ClinicalTrials.gov: NCT01491893). The OV was delivered intratumourally via a pressure gradient, through a catheter surgically inserted during a confirmatory biopsy procedure. Two deaths occurred during the trial. One patient had a seizure related to cerebral oedema that was probably related to autopsy-confirmed tumour progression and another from complications of an intracranial haemorrhage while receiving anticoagulation and bevacizumab (given to reduce peritumoural oedema to limit the use of glucocorticoid treatment). Whilst not the primary trial endpoint, median overall survival was 12.5 months, with an overall survival rate at both 24 and 36 months of 21% (100). Following these results, a phase I trial (ClinicalTrials.gov: NCT03564782) will investigate intralesional PVSRIPO in combination with nivolumab in patients with anti-PD-1 resistant melanoma (ClinicalTrials.gov: NCT04125719) and in combination with atezolizumab (ClinicalTrials.gov: NCT03973879) in patients with recurrent glioblastoma. Given the low but relevant risk of vaccine-derived polioviruses (VDPVs) in the context of the worldwide attempt to eradicate polio, caution will have to be given to the treatment related use of live-attenuated (albeit modified) poliovirus in order to avoid risking the release of oncolytic polioviruses (OVPVs) into the wider population.

### 3.5. Vesicular Stomatitis Virus (VSV)

#### 3.5.1. Pre-clinical work

Vesicular Stomatitis Virus (VSV) is an 11kb single stranded enveloped virus of the Rhabdoviridae family (101). In its wild type form it typically causes a self-limiting illness in cattle characterised by mucosal vesicles and ulcers (102). A recombinant form of VSV is being investigated in humans as a potential vaccine for the Ebola virus (103). Attenuated forms of VSV have demonstrated oncolytic properties because of their ability to selectively replicate in type 1 IFN deficient cancer cells, whereas normal cells are protected through their intact IFN response to viral infection (104).

Shen et al. utilised this function in testing systemic VSV-IFNβ-NIS. This VSV encodes human IFNβ (hIFNβ) or murine IFNβ (mIFNβ) in order to enhance tumour cell selectivity. In addition, encoding the sodium iodide symporter (NIS) transgene facilitates non-invasive imaging of virus spread, and gives the potential to enhance future therapeutic efficacy with concurrent radioiodine therapy. Combining this viral construct with anti-PD-L1 antibody therapy in a syngeneic murine model of acute myeloid leukaemia (AML) enhanced antitumor activity compared with treatment with virus or checkpoint antibody alone. This was associated with an increase in tumoural CD4+ and CD8+ T cell infiltration. Furthermore, depletion of NK or CD8+ immune cells resulted in a loss of efficacy of the virus and checkpoint combination, suggesting the necessity for a combined innate and adaptive immune response to enhance survival (105).

Furthermore, Cockle et al. showed initially that VSV encoding the TAAs HIF 2alpha, Sox-10 and c-myc had a therapeutic effect in an intracranial B16 melanoma murine model. This approach was also found to be effective in an unrelated GL261 primary murine glioma model. Survival from this VSV-TAA strategy was enhanced in combination with anti-PD-1 treatment. *Ex vivo* experiments suggested the anti-PD-1 addition uncovered a Th1 response against glioma cells and mimicked the depletion of regulatory T cells. The survival benefit was increased further with combination checkpoint blockade following the addition of an anti-CTLA antibody but (106).

#### 3.5.2. Clinical trials

**VSV- hIFNβ-NIS**

The VSV-hIFNβ-NIS construct has been taken forward into clinical trial in combination with avelumab (an anti-PD-L1 antibody) in patients with refractory metastatic solid tumours (ClinicalTrials.gov: NCT02923466) with a planned expansion phase in patients with metastatic colorectal cancer. In addition, it is being tested in combination with pembrolizumab in patients with refractory solid tumour malignancies with planned dose increased and expansion arms in refractory NSCLC and HNSCC (ClinicalTrials.gov: NCT03647163).

### 3.6. Maraba Virus

#### 3.6.1. Pre-clinical work

Maraba virus also belongs to the vesiculovirus genus of the Rhabdoviridae family. It has a life cycle within Brazilian phlebotomine sandflies, thereby avoiding the risk of livestock infection associated with VSV. A lack of pre-existing antibodies in the human population gives further credence to its

therapeutic potential. Preclinical interest has focused on a genetically modified version of the wildtype virus, known as MG1. The five sub-unit negative-sense RNA genome allows transgene inserts to bolster its immunogenic potential (107).

In a murine model of triple negative breast cancer, Bourgeois-Daigneault et al. demonstrated that neoadjuvant treatment with Maraba prior to surgical tumour resection could improve survival and result in a reduction in both size and number of the subsequent development of lung metastases. Improved survival in this study was also replicated using neoadjuvant HSV, VSV or adenovirus. Following Maraba infection, tumour cell PD-L1 levels increased, as did the percentage of intratumoural regulatory T cells. This prompted testing of neoadjuvant Maraba with postoperative combination anti-CTLA-4 and anti-PD-1 immune checkpoint inhibition. This led to significantly improved survival compared to both untreated mice and those undergoing either immune checkpoint or Maraba single arm therapy (108).

#### 3.6.2. Clinical trials

MG1 has been taken forward into human clinical trials with modifications to encode tumour antigens within the virus. The first, MG1-MAGEA3, is modified to express the melanoma associated antigen MAGE-A3. The normal function of this protein in healthy cells is unknown but it has been identified on tumours including NSCLC, melanoma and certain haematological malignancies. Preclinical work has shown that priming with a non-replicating adenovirus also expressing MAGE-A3 (Ad-MAGEA3) followed by boosting with MG1-MAGEA3 can induce MAGE-A3-specific CD4+ and CD8+ T cells, with the latter showing a marked expansion and persisting for several months in non-human primates (109). This approach is being evaluated in patients with advanced solid tumours expressing MAGE-A3 (ClinicalTrials.gov: NCT02285816) and with the addition of pembrolizumab in patients with previously treated advanced NSCLC (ClinicalTrials.gov: NCT02879760). Further trials are planned in patients with metastatic melanoma and cutaneous squamous cell skin cancer (ClinicalTrials.gov: NCT03773744).

The second modified Maraba virus encodes attenuated forms of the Human Papilloma Virus (HPV) E6 and E7 proteins (MG1-E6E7) (110). In their wild-type form, these proteins associate with the tumour suppressors p53 and pRB respectively, in infected cells driving the development of HPV related malignancies (111). In a similar prime-boost approach, the sequence of E6E7 encoded adenovirus (Ad-E6E7) followed by MG1-E6E7 is being investigated in combination with atezolizumab (an anti-PD-L1 antibody) in patients with advanced or recurrent HPV associated tumours (ClinicalTrials.gov: NCT03618953).

### 3.7. Measles Virus

#### 3.7.1. Pre-clinical work

Measles virus is a 15kb single stranded enveloped RNA virus belonging to the Paramyxovirdae family (112). It remains a leading cause of vaccine-preventable childhood mortality, with an estimated 7 million people affected by measles in 2016 leading to 89,780 deaths (113).

Attenuated measles virus strains deriving from the Edmonston vaccine lineage (MV-Edm) have been shown to have tumour selective oncolytic properties through their ability to infect tumour cells overexpressing CD46 while exhibiting little cytopathic effect in non-malignant T cells (114).

Hardcastle et al. showed in vitro that measles virus infection of glioma cell lines induced the release of damage associated molecular pattern (DAMP) molecule production and upregulated PD-L1 levels. They retargeted the virus by redirecting viral entry via epidermal growth factor receptor (EGFR) (MV-EGFR), and showed *in vivo* that combination treatment with anti-PD-1 therapy resulted in an increase in inflammatory cell influx on Magnetic Resonance Imaging (MRI) and an increase in activated CD8+ T cells on flow cytometry. The combination treatment demonstrated a significant survival benefit above either single therapies and this gain was lost when tested in immunodeficient mice (115).

Engeland et al. also engineered attenuated measles virus by encoding antibodies against CTLA-4 and PD-L1 (MV-aCTLA-4 and MV-aPD-L1). Using an immunocompetent murine model of malignant melanoma, treatment with MV-aPD-L1 mediated checkpoint modulation demonstrated an increase in CD8+ T cell tumour infiltration, increase in IFN gamma-expressing CD8+ T cell production and a decrease in regulatory T cells. Delayed tumour progression and improved median overall survival were observed for animals treated with measles virus encoding anti-CTLA-4 and anti-PD-L1 when compared to controls. Comparison of intratumoural MV-aPD-L1 with intratumoural MV and intraperitoneal aPD-L1 did not show any survival difference; however, intraperitoneal aCTLA-4 in combination with unmodified MV showed better survival outcomes compared to intratumoural MV-aCTLA-4, demonstrating the challenge of not compromising efficacy with the use of targeted virally delivered checkpoint inhibition (116).

#### 3.7.2. Clinical Trials

A phase I trial of MV-NIS in combination with atezolizumab in patients with metastatic NSCLC (ClinicalTrials.gov: NCT02919449) enrolled 4 patients but was terminated early due to low recruitment.

### 3.8. Newcastle Disease Virus

#### 3.8.1. Pre-clinical work

Newcastle Disease Virus (NDV) is a 15kb single stranded RNA virus from the Paramyxoviridae family (117) and can affect many domestic and wild bird species, notably poultry. In humans it can cause conjunctivitis and influenza-like symptoms. NDV selectively replicates in cells with deficiency in apoptotic and innate immune responses, leading to a more inflammatory tumour microenvironment - hence its potential as an immuno-oncolytic agent (118).

Zamarin et al. found that localized intratumoural therapy of B16 melanoma with NDV induced inflammatory responses, leading to lymphocytic infiltrates and a resulting antitumor effect in distant (non-virally injected) tumours without distant virus spread. However, when different tumour types, in this case B16 melanoma and MC38 colon carcinoma tumours, were injected in each flank, NDV injection on one side did not lead to regression of the opposing tumour, suggesting that the NDV-induced anti-tumour immune response is likely antigen-restricted to the injected tumour. Combining NDV with systemic CTLA-4 blockade led to the rejection of pre-established distant tumours and protection from tumour rechallenge, indicating the development of anti-tumoural memory. Tumour analysis showed the therapeutic effect was associated with infiltration of ICOS, Granzyme B, and Ki-67-expressing CD8+ and CD4+ effector but not regulatory T cells and, through

depletion antibody experiments, found to be dependent on CD8+ cells, natural killer cells, and type I interferon (119).

Building on this data, the same group engineered a recombinant NDV expressing ICOS ligand (NDV-ICOSL). In the same bilateral flank B16 melanoma model, intratumoural administration of NDV-ICOSL resulted in enhanced infiltration with activated T cells in both virus-injected and distant tumours. Combination with anti-CTLA-4 again proved effective, leading to the rejection of both tumours (120). This study also showed that, despite tumour infiltration of effector T lymphocytes in response to NDV, there was ongoing inhibition through PD-L1 acting as a mechanism of early and late adaptive immune resistance. This led to testing of an *in vivo* bilateral B16-F10 model, where tumours were treated with NDV injected into a single flank tumour, together with concomitant systemic PD-1 or PD-L1 blocking antibody. The combination approach resulted in complete regression of both NDV-injected and distant tumours in the majority of treated animals, an effect that was not seen with either treatment alone, leading to long-term survival. Similar effects were observed in a bilateral-flank CT26 colon carcinoma murine model (121).

#### 3.8.2. Clinical Trials

A recombinant strain of NDV (MEDI5395) has recently entered a first-in-human dose-escalation and expansion phase I trial, given either sequentially or concurrently with durvalumab (ClinicalTrials.gov: NCT03889275).

### 3.9. Sindbis Virus

#### 3.9.1. Pre-clinical work

Sindbis virus (SV) is an alphavirus belonging to the Togoviridae family and is transmitted by culex mosquitoes. It is 60-70 nm in diameter with a single-stranded 11 kb RNA genome within an icosahedral capsid (122) and has been genetically modified to be replication defective in order to enhance safety (123). In humans, SV is often asymptomatic but can result in fever, arthralgia, rash and malaise.

Scherwitzl et al. tested SV expressing the immunogenic TAA NYESO-1 (SV-NYESO1) in a murine model. They found that SV-NYESO1 treatment combined with anti-PD-1 resulted in an avid systemic and intratumoural immune response, involving reduced presence of granulocytic myeloid-derived suppressor cells in tumours and an increase in the activation of splenic and tumour-infiltrating T cells. Combined virus and checkpoint therapy also induced enhanced cytotoxic activity of T cells against NYESO-1-expressing tumours, and resulted in complete clearance of NYESO-1-expressing tumours *in vivo* and immunity against tumour rechallenge (124).

### 3.10. Semliki Forest

#### 3.10.1. Pre-clinical work

Semliki Forest Virus (SFV) is another alphavirus with a viral genome of approximately 11.5 kb (123). The synergistic effect of SFV and checkpoint inhibitors was demonstrated by Quetglas et al. who combined an intratumoural non-replicative SFV vector expressing IL-12 with checkpoint inhibition in bilaterally implanted MC38 colon and B16-OVA melanoma murine models. Flow cytometry data

demonstrated that upregulation of tumoural PD-L1 expression could be induced by SFV encoding IL-12, but not with SFV with a control transgene. The combination of SFV-IL-12 with checkpoint inhibitor induced tumour regression of both the treated and untreated flanks, and prolonged survival using both PD-1 and PD-L1 antibody blockade. However, checkpoint combination with a parental SFV showed only a modest benefit, thereby highlighting the added benefit from this virus encoding IL-12 (125).

Ballesteros-Briones et al. developed both a Semliki Forest virus (SFV) and adeno-associated virus (AAV) vector expressing an anti-PD-L1 antibody. Following intratumoural injection in a colorectal murine model, both viral vectors led to similar local PD-L1 expression at 24 hours. SFV-anti-PD-L1 led to complete regressions in over 40% of tumours, and was superior to AAV-anti-PD-L1, as well as to anti-PD-L1 antibody given systemically or locally. SFV-anti-PD-L1 also induced anenestic effects and was effective against a B16-ovalbumin (OVA) melanoma model. SFV-anti-PD-L1 promoted tumour-specific CD8+ T cell infiltration in both tumour models and upregulated the co-stimulatory markers 4-1BB and OX40 in tumoural CD8+ T cells in the MC38 colorectal model (126).

## 4. Emerging approaches

Following the approval of agents targeting the PD-1/PD-L1 and CTLA-4 axis, further research is investigating additional immunomodulatory strategies, including novel combinations with oncolytic viruses. These include combining or encoding oncolytic viruses with chemokines to attract effector T cells (127-130), and costimulatory molecules or ligands of the TNF superfamily (131-142). Furthermore, arming OVs with bispecific T cell engager (BiTE) molecules to enhance the adaptive immune response through engaging CD3 positive T cells with TAAs is being studied (143-147). In addition, OVs encoding specific antigens may have potential use alongside chimeric antigen receptor-modified T cell (CAR T cell) therapies (58, 148-151), particularly in view of recent CAR T cell clinical trial outcome data (152-154). These additional concepts are reviewed in detail elsewhere (155-160).

## 5. Conclusion

The last decade has seen numerous advances in the outcomes of patients with a range of malignancies due to the development of immunotherapeutic treatments, particularly immune checkpoint inhibitors. Despite these successes, many patients do not respond to these therapies and this has led to efforts to enhance ICI responsiveness and overcome ICI resistance.

Oncolytic viruses are potentially an effective adjunct to enhance anti-tumour responses in patients that fail to respond to immune checkpoint blockade. OVs demonstrate particular selectivity towards cancer cells and can favourably alter the local tumour microenvironment by increasing effector T cell infiltration and PD-L1 expression as shown in both pre-clinical and clinical models.

There is therefore an established rationale for combining oncolytic viruses with checkpoint inhibitors. Promising pre-clinical data has resulted in several clinical trials investigating such a strategy and their results are keenly awaited.

## 6. Expert Opinion

Immunotherapy for the treatment of cancer using ICI has revolutionised the outcome for patients with a range of tumours over the last decade. Despite proven benefit for a number of diseases including melanoma, renal, head and neck, and lung cancer, there remains a significant proportion of patients with these tumours who do not respond or who develop resistance. Furthermore, there are a number of tumours where response rates to ICI are very low or effectively absent, such as brain tumours and ovarian or pancreatic cancer. As our understanding of the biological basis of success (or failure) with ICI treatment has grown, focus has increased on converting inherently immunologically 'cold' tumours to 'hot', which respond better to ICI in general across tumour types. What is actually meant by the immune 'heat' of a tumour remains uncertain, but the concept essentially describes a situation where there is some immune response to the tumour ongoing (albeit ineffective), rather than the immune system remaining entirely unresponsive to the presence of the cancer. Measures of how hot a tumour is include the presence of a significant effector immune cell infiltrate, high tumour mutational burden and interferon gamma gene expression.

Amongst the strategies being tested in combination with ICI to turn cold tumours hot, and hence prime for more effective checkpoint blockade, OV are a group of novel agents with significant promise. If the presence of a virus within a tumour can alert the immune system to mount a response which was previously absent, improved benefit with ICI may follow. Combinations of ICI with chemotherapy and/or radiotherapy are also being tested, but there should be no better agent than a virus to wake up the immune system to attack. OV were initially developed as direct cytotoxic agents and, in the early days, the immune system was seen as a foe not friend for therapy, as it would initiate an anti-viral response which would shut down virus replication and tumour cell lysis, and hence restrict benefit. However, this paradigm has now been entirely reversed, with OV widely accepted as a type of immunotherapy themselves, acting as a danger signal within tumours to initiate immune attack. This response includes immune activation against both virus and tumour, but the balance between these two targets, and how and why they evolve in the way they do, remains poorly understood. Is the innate or adaptive response most important, and how do these interact? Which are the tumour antigens most important in any adaptive response in patients, neoantigens and/or others? It is arguably a surprise that any anti-tumour response at all arises in the context of OV, given the strength of anti-viral immune activation. We still understand far too little about the mechanistic biology of immunotherapy as a whole, including virotherapy, and progress in the basic science underlying single agent/combination success will be critical if we are to maximise benefit for patients over the next few years.

The lead current OV agent is a herpes virus (talimogene laherperepvec, T-Vec), which is clinically approved for the treatment of melanoma by intratumoural injection. Whilst a significant step, more clinical success is needed to maintain momentum in the field. The trial which led to the approval of T-Vec was fortunate in its timing, in that it preceded the success of BRAF inhibitors as well as anti-PD-1/anti-CTLA-4 checkpoint blockade in melanoma; the imminent results of the Phase 3 study of pembrolizumab with or without T-Vec injection, will be a significant milestone in the field.

Other urgent questions, particularly in the clinic, include whether any systemic delivery of OV (which is more practical and acceptable for widespread application in the clinic), rather than intratumoural injection, can be effective. Also, how do we select from the huge range of OV of different types,

with differing transgenes, for the expensive step of progression into clinical manufacture and testing? How do we optimise OV pre-clinically in terms of immunogenic, as well as cytotoxic, potential, particularly for OV which are difficult to test in immunocompetent mouse models, such as adenovirus, coxsackie and measles? Whilst many challenges remain in both the pre-clinical and clinical arenas, OV/ICI combination remains one of the most promising avenues to explore in terms of the next step-up in the success of cancer immunotherapy.

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Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

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**Table 1: Oncolytic Viruses in Combination with Immune Checkpoint Inhibitors - Pre-clinical studies**

| Virus | Phylogeny | Genomic Modifications | Immune Checkpoint Combination | Disease model | Reference |
|---|---|---|---|---|---|
| Herpes virus | Herpesviridae DNA | | anti-PD1 | Rhabdomyosarcoma | 15 |
| | | Mesenchycal stem cells | anti-PDL1 | Melanoma | 16 |
| | | IL-12 | anti-PD-1 | Glioma | 17 |
| | | ULBP3 | anti-PD-1 | Glioma | 18 |
| | | Soluble PD-1 | anti-PD-1 | Glioma | 20 |
| | | GM-CSF | anti-PD-1 | Lymphoma | 21 |
| Vaccinia virus | Poxviridae | aPD-1 Ab | anti-PD-1 | Fibrosarcoma | 36 |
| | | IL15 | anti-PD-1 | Colon | 37 |
| | | CXCL11 | anti-PD-L1 | Colon and ovarian | 38 |
| | | | anti-PD-1 | Sarcoma | 42 |
| | | | anti-PD-1 and anti-CTLA4 | Sarcoma | 44 |
| | | | anti-CTLA4 | Renal | 45 |
| Adenovirus | Adenoviridae | TNFa and IL2 | anti-PD-1 | Melanoma | 52 |
| | | HSV-tk | anti-PD-1 | Glioma | 54 |
| | | CD40 | anti-PD-L1 | Glioma | 55 |
| | | CD40 | anti-PD-L1 and anti-CTLA4 | Melanoma | 56 |
| | | Soluble PD-1 and HSV-tk | anti-PD-1 | Colon | 57 |
| | | aPD-L1 mAb | anti-PD-L1 | Prostate | 58 |
| | | TAA | anti-PD-1 and anti-41BB | Melanoma | 59 |
| | | TAA | anti-PD-1 | Prostate | 60 |
| Myxoma virus | Poxviridae | Soluble PD-1 | anti-PD-1 | Melanoma | 75 |
| Reovirus | Reoviridae dsRNA (reovirus) | | anti-PD-1 | Melanoma | 78 |
| | | | anti-PD-L1 | Myeloma | 80 |
| | | | anti-PD-1 | Glioma | 82 |
| | | | anti-PD-1 | Melanoma | 81 |
| | | | anti-PD-1 | Breast | 79 |
| | Reoviridae dsRNA (rotavirus) | | anti-PD-1 | Neuroblastoma | 88 |
| | | | anti-CTLA-4 | | 88 |

| | | | | | |
|---|---|---|---|---|---|
| | | | anti-PD-1 | B cell lymphoma | 88 |
| | | | anti-CTLA-4 | | 88 |

**Table 1 (continued)**

| Virus | Phylogeny | Genomic Modifications | Immune Checkpoint Combination | Disease model | Reference |
|---|---|---|---|---|---|
| Measles | Paramyxoviridae ss-RNA | MV-EGFR | anti-PD-1 | Glioma | 115 |
| | | MV-aCTLA-4 | anti-CTLA-4 | Melanoma | 116 |
| | | MV-aPD-L1 | anti-PD-L1 | Melanoma | 116 |
| VSV | Rhabdoviridae ss-RNA | VSV-IFNB-NIS | anti-PD-L1 | AML | 105 |
| | | TAA (HIF 2alpha, Sox-10 and c-myc) | anti-PD-1 | Melanoma and Glioma | 106 |
| | | | anti-CTLA-4 and anti-PD-1 | Melanoma and Glioma | 106 |
| | | | anti-CTLA-4 | Melanoma and Glioma | 106 |
| Maraba virus | Rhabdoviridae ss-RNA | MG1 | anti-CTLA-4 and anti-PD-1 | Breast | 108 |
| NDV | Paramyxoviridae ss-RNA | NDV-NS1 | anti-CTLA-4 | Melanoma | 119 |
| | | | | Colon | 119 |
| | | | | Prostate | 119 |
| | | NDV-NS1 | anti-PD-1 | Melanoma | 121 |
| | | | anti-PD-L1 | Melanoma | 121 |
| | | NDV-ICOSL | anti-CTLA-4 | Melanoma | 120 |
| Coxsackievirus | Picornaviridae ss+RNA | Coxsackie A21 | anti-PD-1 | NSCLC | 94 |
| | | | | Melanoma | 94 |
| | | Coxsackie A21 | anti-CTLA-4 | NSCLC | 94 |
| | | | | Melanoma | 94 |
| Poliovirus | Picornaviridae ss+RNA | PVSRIPO | anti-PD-1 | TNBC | 99 |
| | | | anti-PD-L1 | | 99 |
| SV | Togoviridae ss+RNA | SV-NYESO1 | | Colon expressing NYESO1 | 124 |
| SFV | Togoviridae ss+RNA | SFV-IL-12 | anti-PD-1 | Melanoma and Colon | 125 |
| | | | anti-PD-L1 | Melanoma and Colon | 125 |
| | | SFV-aPD-L1 | | | 126 |

**Table 2: Oncolytic Viruses in Combination with Immune Checkpoint Inhibitors - Clinical studies**

| Virus | Clinical Trial Number | Virus Treatment | Immune Checkpoint Combination | Disease model | Estimated/Actual enrollment | Phase | |
|---|---|---|---|---|---|---|---|
| Herpesvirus | NCT01740297 | T-VEC | Ipilimumab | Metastatic melanoma | 217 | Ib/II | Actual enrollment |
| | NCT02263508 | | Pembrolizumab | Metastatic melanoma | 713 | 1b/III | Actual enrollment |
| | NCT03069375 | | Pembrolizumab | Metastatic/locally advanced sarcoma | 60 | II | Actual enrollment |
| | NCT03153085 | HF10 | Ipilimumab | Metastatic melanoma | 28 | II | Actual enrollment |
| | NCT02272855 | | Ipilimumab | Metastatic melanoma | 46 | II | Actual enrollment |
| | NCT03259425 | | Nivolumab | Metastatic melanoma | 7 | II | Actual enrollment |
| Vaccinia virus | NCT03206073 | Pexa-Vec | Durvalumab/Tremelimumab | Colon | 35 | I/II | |
| | NCT03071094 | | Nivolumab | Hepatocellular carcinoma | 30 | I/Ia | |
| | NCT02977156 | | Ipilimumab | Solid tumours | 66 | I | |
| Adenovirus | NCT02798406 | Tasadenoturev | Pembrolizumab | Glioblastoma | 49 | II | Actual enrollment |
| | NCT03003676 | ONCOS-102 | Pembrolizumab | Metastatic melanoma | 24 | I | |
| | NCT02963831 | | Durvalumab | Advanced Peritoneal malignancies | 78 | I/II | |
| | NCT03004183 | ADV/HSV-tk | Pembrolizumab | Triple negative breast cancer/metastatic NSCLC | 57 | II | |
| | NCT02636036 | Enadenotucirev | Nivolumab | Epithelial tumours | 135 | I | |
| Reovirus | NCT02620423 | | Pembrolizumab | Advanced pancreatic adenocarcinoma | 11 | I | Actual enrollment |

**Table 2 (continued)**

| Virus | Clinical Trial Number | Virus Treatment | Immune Checkpoint Combination | Disease model | Estimated/Actual enrollment | Phase | |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| VSV | NCT02923466 | VSV-IFNβ-NIS | Avelumab | Refractory solid tumours, Colon | 93 | I | |
| | NCT03647163 | | Pembrolizumab | Solid tumours, NSCLC, HNSCC | 23 | I | |
| Maraba virus | NCT02879760 | MG1-MAGEA3 | Pembrolizumab | Advanced NSCLC | 75 | I/II | |
| | NCT03618953 | MG1-E6E7 | Atezolizumab | Recurrent or metastatic HPV associated malignancies | 75 | I/Ib | |
| | NCT03773744 | MG1-MAGEA3 | Pembrolizumab | Metastatic Melanoma, cutaneous squamous cell skin cancer | 40 | Ib | |
| Measles virus | NCT02919449 | MV-NIS | Atezolizumab | NSCLC | 4 | I | Actual enrollment |
| NDV | NCT03889275 | MEDI5395 | Durvalumab | Advanced solid tumours | 164 | I | |
| Coxsackievirus | NCT02307149 | Coxsackie A21 | Ipilimumab | Advanced melanoma | 59 | Ib | |
| | NCT03408587 | | Ipilimumab | Metastatic uveal melanoma | 11 | I | Actual enrollment |
| | NCT02565992 | | Pembrolizumab | Advanced melanoma | 50 | Ib | |
| | NCT02824965 | | Pembrolizumab | NSCLC | 11 | I | Actual enrollment |
| | NCT02043665 | | Pembrolizumab | Solid tumours with NSCLC and bladder expansion | 90 | I/II | |
| Poliovirus | NCT04125719 | PVSRIPO | Nivolumab | Advanced melanoma | 30 | I | |
| | NCT03973879 | | Atezolizumab | Glioblastoma | 31 | Ib/2 | |

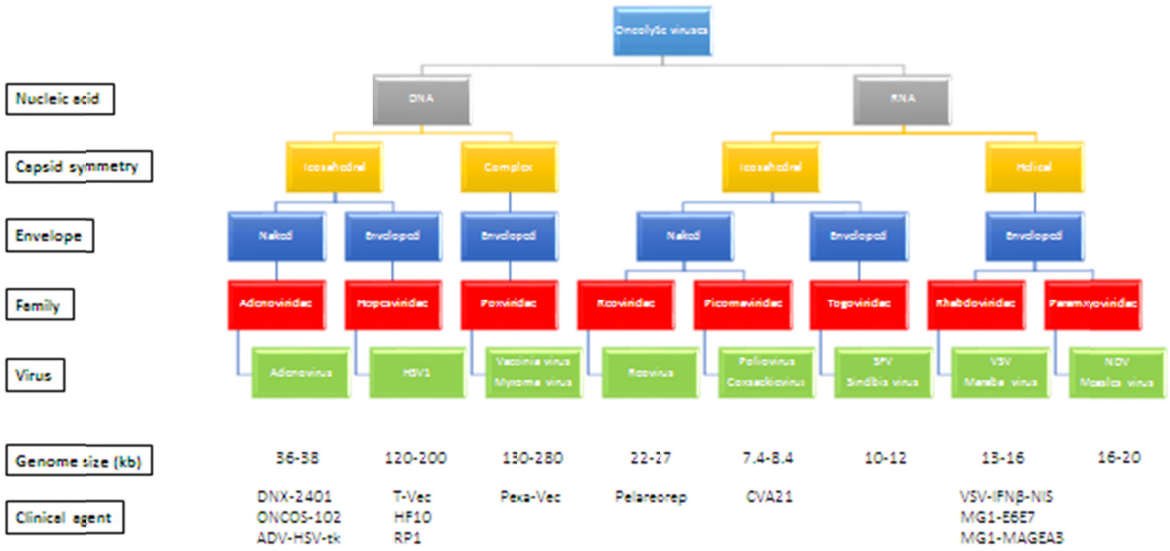


Figure 1. OV classification and viruses currently undergoing or have undergone clinical trials with combination of ICIs. HSV1: Herpes simplex virus type 1; SFV: Semliki forest virus; VSV: Vesicular stomatitis virus; NDV: Newcastle disease virus

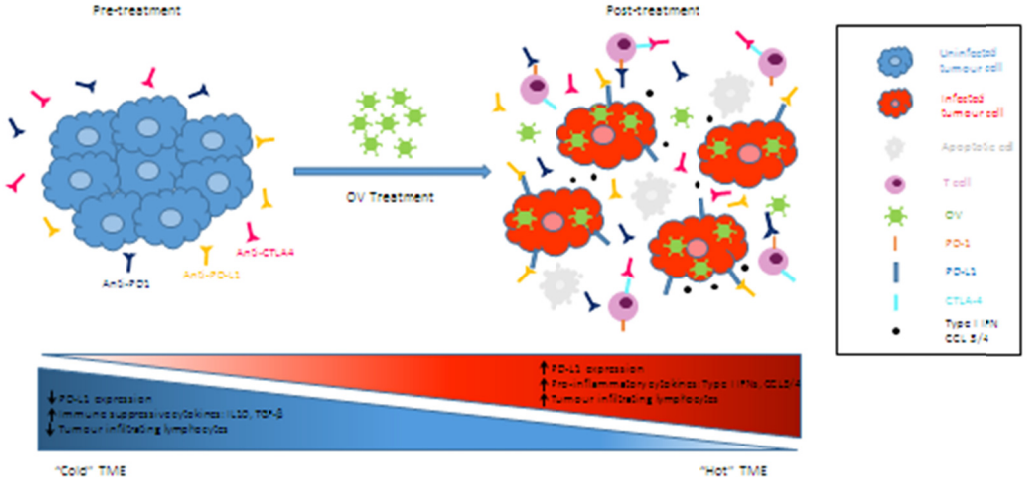


Figure 2. Oncolytic viruses (OV) have the ability to turn a "cold" tumour microenvironment (TME) to "hot". Prior to OV treatment, checkpoint inhibition is therapeutically inefficient as the tumour expresses low levels of PD-L1 (therefore no target for anti-PD-L1 antibodies) and is poorly infiltrated with immune cells (therefore no target for anti-PD1 and anti-CTLA4 antibodies). After OV infection, the tumour increases expression of PD-L1 and releases pro-inflammatory cytokines e.g. type I IFNs and CCL3/4, which attract immune cell infiltration, thereby increasing the efficacy of immune checkpoint blockade. OV: oncolytic virus; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; IFN: interferon; CCL3/4: Chemokine (C-C motif) ligand 3 or 4.