Oncolytic virus immunotherapies in ovarian cancer: moving beyond adenoviruses

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Abstract
Ovarian cancer is the 5th most common cancer in UK women with a high relapse rate. The overall survival for ovarian cancer has remained low for decades prompting a real need for new therapies. Recurrent ovarian cancer remains confined in the peritoneal cavity in >80% of the patients, providing an opportunity for locoregional administration of novel therapeutics, including gene and viral therapy approaches. Immunotherapy is an expanding field, and includes oncolytic viruses as well as monoclonal antibodies, immune checkpoint inhibitors, and therapeutic vaccines.

Oncolytic viruses cause direct cancer cell cytolysis and immunogenic cell death and subsequent release of tumor antigens that will prime for a potent tumor-specific immunity. This effect may be further enhanced when the viruses are engineered to express, or coadministered with, immunostimulatory molecules. Currently, the most commonly used and well-characterized vectors utilized for virotherapy purposes are adenoviruses. They have been shown to work synergistically with traditional chemotherapy and radiotherapy and have met with success in clinical trials. However, pre-existing immunity and poor in vivo models limit our ability to fully investigate the potential of oncolytic adenovirus as effective immunotherapies which in turn fosters the need to develop alternative viral vectors. In this review we cover recent advances in adenovirus-based oncolytic therapies targeting ovarian cancer and recent advances in mapping immune responses to oncolytic virus therapies in ovarian cancer.

Keywords: immunotherapy, oncolytic virus, ovarian cancer

Introduction
Ovarian cancer
Ovarian cancer (OC) is the 5th most common cancer in UK women1 and the most lethal gynaecological malignancy owing to non-specific symptoms, lack of screening tests, and advanced stage diagnosis.2 Epithelial ovarian cancer (EOC) represents approximately 90% of all OC1 and consist of the following subtypes: clear cell (10%), endometrioid (10%), mucinous invasive (3%), low grade serous (<5%), and high grade serous (70%). These all share a common anatomical location yet have differing clinicopathologic features and sites of origin.3,4 Clear cell ovarian cancer is the second most common subtype of OC. Endometriosis, a disease characterized by the ectopic growth of endometrial glands and stroma, is a risk factor for this histotype.5 The prognosis for clear cell carcinoma is worse than high grade serous ovarian cancer (HGSOC) due to poorer sensitivity to platinum-based chemotherapy6 with patient response being 15%.7 Similar to clear cell OC, endometrioid OC is also associated with endometriosis.8 Invasive mucinous cancer is often a result of metastasis to the ovary from the gastrointestinal tract, including the colon, appendix, or stomach.9 Non-epithelial malignancies of the ovary like germ cell, sex cord stromal tumors, and granulosa cell tumor (sertoli leydig cell tumor) account for ~10% of all ovarian cancers.9

High-grade serous ovarian cancer is the most common histologic subtype of ovarian cancer. Early detection of the disease is one of the biggest challenges due to lack of early symptoms of the disease and late diagnosis with metastasis disseminated in the peritoneal cavity (peritoneal carcinomatosis).9,10 Transvaginal ultrasound and assessment of the blood cancer antigen 125 (CA125) levels are the current methods for early detection of HGSOC, but their effectiveness has been very limited.11,12 Accounting for approximately 70% of OC deaths,13,14 the 5-year survival rate has remained unchanged for over 50 years at a dismal 30%.4,14 There is compelling evidence to suggest that HGSOC may arise from secretory epithelial cells of the distal fallopian tube.13,15 A gene expression study published in 2008 reported that the expression profiles of HGSOC more closely resemble fallopian tube epithelium than ovarian surface epithelium (OSE).16 This is debated by others who have reported cortical inclusion cysts developing from the OSE as likely progenitors.17 Mullerian inclusions in the pelvic cavity and precursor cells of the ovary have also been proposed as the site of origin.18,19 Management of HGSOC involves total abdominal hysterectomy and bilateral salpingo-oophorectomy, followed by platinum-based chemotherapy, typically 3-weekly carboplatin in combination with paclitaxel for 6 cycles.2,18 Despite high (~80%) initial response to treatment,2,18 >70% of patients relapse. Early peritoneal and pleural spread is a feature of HGSOC with a majority of patients presenting with Federation of Gynecology and Obstetrics stage IIC or IV disease, and, in these cases where
the patients are not suitable for debulking surgery, 3 cycles of platinum-based neo-adjuvant chemotherapy followed by interval debulking surgery and adjuvant chemotherapy is the accepted approach. Recurrent ovarian cancer remains confined in the peritoneal cavity in >80% of the patients, providing an opportunity for locoregional administration of novel therapeutics, including gene and viral therapy approaches. Due to the current limitations of the established treatments for HGSOC there is an urgent need to develop new therapies to improve overall survival. Recently, the development of oncolytic viruses has shown great promise in treating a broad range of cancers including ovarian cancers.

**Introduction to oncolytic viruses**

The term oncolytic viruses (OV) is commonly employed to identify a viral strain which selectively infect and lyse tumor cells, spreading to adjacent tumor cells resulting in a self-sustaining cycle of anticancer activity. Cell lysis following viral infection, will depend on the virus used and the dose and results in cell death via various mechanisms including apoptosis, autophagy, pyroptosis, and necrosis. As a result, the cell death of targeted cancers will depend on the virus used and the dose and results in cell death via various mechanisms including apoptosis, autophagy, pyroptosis, and necrosis. As a result, the cell death of targeted cancers may be less significant in normal cells if the OV in question affected non-pathogenic versions are required if they are to be used as OV. An example of this is the Edmonston strain, used in vaccination, although clearly there is an issue with its use given the prevalence of immunity through vaccination.

OVs can enter both normal and cancerous cells, although this may be less significant in normal cells if the OV in question exploits adherent expression of cell surface receptors. However once inside a cell, the ability of an OV to replicate will be reduced in a normal cell due to its anti-viral response. Normal cells are able to mount an anti-viral response, through a variety of anti-viral response mediated by protein kinase R, frequently defective in tumor cells. This virus was shown, in stage IIIb to IV melanoma, to improve overall response rate, and overall survival.

| Virus family | Characteristic | Ref |
|-------------|----------------|-----|
| Measles     | Requires CD46 expression                      | 28  |
| Vesicular stomatitis | Targets cells with activated Ras pathway     | 28  |
| Reovirus    | Requires defective interferon and PKR signaling pathway | 29  |

**Table 1: Examples of oncolytic DNA and RNA viruses in cancer research**

### DNA viruses

| Designation | Virus family | Characteristic | Ref |
|-------------|--------------|----------------|-----|
| ONCOS-102   | Adenovirus   | GM-CSF expressing adenovirus | 23  |
| ONNX-015    | Adenovirus   | Deletion of E1B     | 24  |
| d920-947    | Adenovirus   | Deletion of E1A CR2 | 25  |
| Ad5-Δ24-RGD | Adenovirus   | Serves to integrate ωg3 and ωg5 | 26  |
| rV4         | Vaccinia     | lacZ and lac inserted into the TK gene | 27  |
| JX-594      | Vaccinia     | TK deletion, expresses human GM-CSF | 27  |
| vD0         | Vaccinia     | TK and VEGF deletions | 27  |
| HSV-1716    | Herpes simplex | Deletion of the gene ICP34.5 | 28  |
| Synco-2D    | Herpes simplex | Addition of membrane fusion capability | 28  |

### RNA viruses

| Virus family | Characteristic | Ref |
|-------------|----------------|-----|
| GM-CSF = granulocyte-macrophage colony-stimulating factor; RNA = ribonucleic acid. |
signaling pathways, which act to detect and remove viral pathogens or induce apoptosis of the cell.21

Recent advances in oncolytic adenovirus-based therapies in ovarian cancer

Currently the most commonly used and well-characterized vectors utilized for virotherapy purposes are adenoviruses. Human adenoviruses have been extensively studied in patients with malignancies and have demonstrated no severe side effects. Due to their intrinsic biological plasticity, adenoviruses are amenable to manipulation. Serotype 5 (Ad5) in particular has proven to be an attractive vector, allowing for a variety of modifications and efficient infectivity without being associated with a serious disease.26 For optimal oncolytic activity, adenovirus are required to specifically and efficiently infect and replicate within cancer cells. The replication of human adenoviruses is initiated by E1A which is responsible for dissociating the retinoblastoma (Rb)/E2F complex. This dissociation produces free transcription factor E2F activation thereby allowing the transcription of the adenoviral E1B, E2, E3, and E4 genes.37

There are 2 broad strategies to engineer conditionally replicative adenoviruses (CRAd). Firstly, the deletions of part of the E1A and E1B genome sequence impair replication in normal cell. Typically, Ad5-based CRAd contain a 24 base pair mutation in the E1A gene disrupting the Rb binding domain resulting in an E1A protein unable to release E2F and support viral replication. In tumor cells, however, genetic defects often result in the deregulation of Rb pathway thereby complementing the deleted viral genome functions and allowing conditional replication. The second strategy relies on placing the adenoviral genes under the control of a tumor-specific or tissue-specific promoter in order to achieve selective replication in cancer cells.

Enhancing CRAd infectivity and replication

One of the main limitations of CRAd, as well as other vectors used in virotherapy, is poor or insufficient transduction into target cells due in part to low specificity, high pre-existing immunity to vectors, and poor dissemination of the vector. In the case of Ad5-based CRAd, infection is mediated by the high immunity to vectors, and poor dissemination of the vector. In endometrium and cervix cancers as well as in breast and lung contrast CAR expression was reported to be upregulated in

An alternative approach has been to modify the fiber knobs of adenovirus vectors and create chimeric vectors. Human adenoviruses are classified into 6 distinct subgroups (A–F) with Ad5 belonging to subgroup C. One example of a chimeric adenovirus vector is the Ad5/3 fiber chimera. The fiber knob domain of serotype 3 (from subgroup B) binds primarily to CD46 and to CD80 and CD86 rather than to CAR.25,32 Kanerva et al.154 have shown that this chimeric vector displays improved cytopathic in ovarian cancer cells compared with the wildtype Ad5. Additionally, the further modified Ad5/3-Delta24 chimera demonstrated enhanced cytopathic activity in murine orthotopic models of ovarian cancer.53 More recently, another Ad5-chimeric vector has been engineered by pseudotyping Ad5 with the fiber knob domain from Ad48 (from subgroup D) called Ad5/kn48. Uusi-Kerttula et al.156 showed that this vector utilized CAR but not CD46 for cell entry. The vector was then further modified by the insertion of 20 to 11 peptide to redirect the vector to αvβ6 integrin. By targeting the prognostic cancer cell marker αvβ6 instead of CAR, the vector transduction was improved by ~60 fold in epithelial ovarian cancer cells. Using the less seroprevalent Ad48 also allowed for the circumvention of pre-existing anti-Ad5 immunity. Similarly, Hulin-Curtis and colleagues modified the Ad5 vector by pseudotyping it with the Ad35 fiber thereby achieving higher transduction via CD46 into primary epithelial ovarian cancer cells despite the presence of neutralizing antibodies and factors.57

Alongside the creation of chimeric vectors, specific modifications of adenoviral constructs as proved effective in enhancing CRAd. Incorporating an RGD motif into the fiber knob domain has been shown to improve the infection of both primary ovarian cancer cells and ovarian cancer cell lines by adenovirus vectors despite low CAR expression.58 More recently Bauerschmitz et al.59 demonstrated that the insertion of the RGD motif redirected viral interactions to integrins highly expressed by ovarian cancer cells, allowing CAR-independent infection enhanced oncolytic activity in orthotopic murine models of ovarian cancer. Gamble et al.60 went further and engineered an Ad5 vector containing an RGD motif in both the fiber motif and the capsid protein XI. This new vector demonstrated greater oncolytic activity in vitro in ovarian cancer cell lines. Ad5-delta24-RGD vectors have shown promise in early, phase I, clinical trials.61

A different approach to improve CRAd efficacy has been to modify vectors using tissue specific promoters (TSP) in order to enhance viral replication. Rein et al.62 modified an Ad5/3 chimeric vector to place E1A under the control of the TSP secretory leukoprotease inhibitor (SLPI). This Ad5/3/SLPI vector displayed efficient replication and oncolytic activity in primary ovarian cancer cells, orthotopic murine models, and multiple cell lines. Additionally, this novel vector showed reduced liver toxicity increasing its potential for clinical use. Other CRAd exploiting TSP include a chimeric Ad5/3 and Ad5-RGD vector which utilize the chemokine receptor CXCR4, as well as a CRAd-S.F5/3 modified to include the surviving promoter. All of these vectors have demonstrated increased selectivity for ovarian cancer cells and improved oncolysis in ovarian cancer cell lines and murine models along with decreased hepatic toxicity.63,64

Improving adenovirus efficacy by arming vectors

In order to improve the anti-tumor efficacy of CRAd, many vectors are being modified to encapsulate therapeutic transgenes. “Arming” viral vectors in this way is designed to enhance direct
In order to limit tumor proliferation, angiogenesis has also been targeted. An adenovirus construct was developed expressing a short hairpin RNA against VEGF (Ad-deltaB7-shVEGF) which elicited greater anti-tumor efficacy in human glioma xenografts. Similarly, an adenovirus vector (AdEHE2F) encoding soluble Flt-1 andDll4 to neutralize VEGF and Notch signaling, respectively displayed enhanced anti-tumor effects and dramatic reductions in total vasculature in breast cancer.

**CRAd developed to target chemo-resistant cells or reverse resistance**

A major limitation of current treatments for ovarian cancer is the development of chemotherapy resistance. Consequently, the development of novel CRAds aimed at ovarian cancers center on preferentially targeting resistant cells or reversing resistance and resensitizing cells to chemotherapy agents (Table 2). The mechanisms through which ovarian cancers acquire resistance to chemotherapy agents are still under debate.

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**Table 2**

Major reported strategies to enhance effectivity and the ability to overcome chemoresistance and modulate immune response of adenovirus and vaccinia based oncolytic viruses

| Aim                                      | Virus Family | Strategies | Targets                      | Advantages                                      | Disadvantages                                      | Ref |
|------------------------------------------|--------------|------------|------------------------------|-------------------------------------------------|---------------------------------------------------|-----|
| Enhancing infectivity/efficiency         | Adenovirus   | Chimeric vectors | Ad5/6 fiber chimera, Ad5/45 lev48 | Cell entry occurs via more prolific receptors   | Genome size limits insertions                      | 52,53 |
|                                          | Adenovirus   | Fiber knot modifications | Ad5-delta24-RGD | Cell entry occurs via more prolific receptors   |                                                   | 58–60 |
|                                          | Adenovirus   | Tissue specific promoters (TSP) | SLPI, CXCR4, Survivin | Increased selectivity and replication            |                                                   | 62–64 |
|                                          | Adenovirus   | Drug combinations | Trichostatin (TSA) | Increased CAR expression                        | High variability of CAR across tissue and disease types | 50  |
|                                          | Adenovirus   | Gene inserts (arming vector) | Relaxin, hyaluronidase | Improved viral dissemination                     | Genome size limits insertions                      | 66,67 |
| Overcoming chemo-resistant               | Adenovirus   | Tissue specific promoters (TSP) | Ad5/3-MDR1 | Increased specificity for chemo-resistant cells |                                                   | 71  |
|                                          | Adenovirus   | Gene inserts (Arming vector) | Mdr60D, STAT3 anti-sense DNA | Enhanced cisplatin cytotoxicity                  |                                                   | 72–74 |
|                                          | Adenovirus   | Gene inserts + TSP | Cre/Lexp under CD133 | Increased cytotoxicity when combined with cisplatin |                                                   | 75  |
| Evading innate and anti-viral immunity   | Adenovirus   | Viral coat modifications and polymer coating | PEGylation | Reduced neutralising antibody binding            | Risk of reducing infectivity                       | 21  |
|                                          | Adenovirus, semiliki forest virus vaccinia | Chimeric and alternative vectors | Ad5/6 chimera, semiliki forest virus, vaccinia | Lower prevalence of pre-existing neutralizing antibodies | Rare serotypes and vectors are typically less extensively characterised | 76–78 |
|                                          | Adenovirus   | Drug combinations | Cyclophosphamide anti- CTLA4 anti-TNFα, β3 integrin inhibition | Reduced anti-viral immune response (eg, reduced Treg, inflammation) leading to enhanced oncolysis | Lack of immunocompetent animal models limits advancements | 21,78–84 |
| Stimulating anti-tumor immunity          | Adenovirus, semiliki forest virus vaccinia | Gene inserts (Arming vector) | GMCSF, ovalbumin | Increased infiltration of tumor-specific CD8+ T cells | T cell response mediates both virus clearance and anti-tumor cytotoxicity | 85,86,78 |
|                                          | Vaccinia     | Gene inserts (Arming vector) | CXCR4 antagonist | Decreased G-MDCs and Tregs                      |                                                   | 87,88 |
|                                          | Vaccinia     | Checkpoint inhibitor combinations | α-POL1 + vaccinia | Increased CD8+ T cells and decreased Treg infiltration | T cell response mediates both virus clearance and anti-tumor cytotoxicity | 89  |

DNA = deoxyribonucleic acid.
Multidrug resistance gene 1 (MDR1) overexpression is believed to be a major factor limiting anticancer drugs. Rein et al.1,2 targeted chemotherapy resistant ovarian cancer cells by combining the TSP MDR1 (multidrug resistance gene 1) and an Ad5/3 chimeric vector. The resulting Ad5/3MDR1 constructs were shown to be a promising therapy to specifically target chemotherapy resistant cancer cells by displaying efficient oncolysis in chemo-resistant ovarian cancer cell lines, primary tumors, and orthotopic murine models.

CD133+ ovarian cancer cells have been identified as cancer stem cells which contribute to recurrence and chemoresistance.3,90 Long et al.75 modified an adenovirus vector to place the Cre/Loxp system under the control of a CD133 promoter to develop a new “suicide” gene therapy for ovarian cancer stem cells. They demonstrated that this new therapy initiated pro-apoptotic signaling pathways enhancing the cytotoxicity of cisplatin and promoted marked tumor suppression in xenografts. Targeting elements of the apoptotic pathway has proved to be an effective strategy to resensitise cancers to chemotherapy. Luan et al.91 targeted the p53 upregulated modulator of apoptosis PUMA in ovarian cancer cell lines and increased their chemo sensitivity. Similarly, Wang et al.92,93 increased the efficacy of cisplatin by arming an adenovirus with a manganese superoxide dismutase (MnSOD). They demonstrated that an overexpression of MnSOD resulted in a remarkable induction of apoptosis and armoring an adenovirus with a manganese superoxide dismutase (MnSOD). They demonstrated that an overexpression of MnSOD resulted in a remarkable induction of apoptosis and

Intratumoral injection can only be given in those tumors which are either clinically palpable or amenable to imaging guided injection. Intravenous administration can be limited by sequestration of the virus by the liver and spleen.29,94 Although OV have yet to be approved for the treatment of ovarian cancer they have met with positive outcomes in clinical trials. Past and current clinical trials using oncolytic viruses in ovarian cancer are summarized in the table below (Table 4).

| Advantages: Intratumoral injection | Disadvantages: Intratumoral injection | Advantages: Intravenous injection | Disadvantages: Intravenous injection | Advantages: Intraperitoneal injection | Disadvantages: Intraperitoneal injection |
|---------------------------------|-------------------------------------|---------------------------------|-------------------------------------|-------------------------------------|-------------------------------------|
| High infection rate of the tumor | Limited to clinically palpable or tumors amenable to image guided injection | Systemic delivery | High dose required | Good dissemination to secondary tumors within the peritoneum | Poor dissemination to metastatic sites |
| Low dose required               | Poor dissemination to metastatic sites | Ease of administration | High sequestration of the virus by the liver and spleen | Low sequestration and neutralization of the virus | High dissemination to metastatic sites | High neutralization by antibodies and serum factors |
| Very low sequestration and neutralization of the virus | High dissemination to metastatic sites |                                |                                      |                                      |                                      |

**Clinical trials of oncolytic virus in ovarian cancer**

There are numerous OV currently under investigation as potential therapeutic agents, however, to date only 1 OV has been government approved. This virus, approved in China in 2005 for nasopharyngeal cancer, is a modified adenovirus. Another herpes simplex OV, T-VEC, is expected to be approved by the Food and Drug Administration (FDA) for treatment of melanoma. They have been shown to work synergistically with traditional chemotherapy and radiotherapy.34,95 OV are typically administered via intratumoral or intravenous injection (Table 3).

**Oncolytic virus and the immune system**

The effectiveness of OV depends on various factors including the TME, alterations in tumor cell signaling pathways, and expression of immunosuppressive factors as well as the innate and adaptive immune response induced by the virus.21,22 Of the innate and adaptive immune system, it is thought that the adaptive immune system plays the most important role in the anti-tumor immunity induced by OV. While tumors develop many ways to evade and dampen immune responses, OV driven cell death is immunogenic as new tumor-specific antigens are released following cell lysis. These new circulating antigens are then free to stimulate systemic anti-tumor immune response which can lead to tumor regression at sites not infected by the OV.

While viruses can help to promote an immune response against the tumor cells, on the other hand, neutralising antiviral responses may block virus replication and further infection of other tumor cells.21 Therefore, the anti-tumor immune response is a critical part of the action of OV, but the immune activation can also lead to clearance of the virus. Strategies to prevent antibody binding include PEGylation of the viral coat and polymer coating. Additionally, low dose cyclophosphamide has been used to successfully reduce numbers of regulatory T cells (Tregs), while not impairing the cytotoxic T cell response.21,95 Tregs are also important in the prevention of tumor associated immunity. In ovarian cancer, Tregs in tumor infiltrating lymphocytes (TILs) are associated with a poor prognosis. Whereas the presence of other TILs is associated with delayed recurrence of epithelial ovarian cancer. The presence of tumor infiltrating CD3+ cells in ovarian cancer has also been found to increase patient survival and improve response to chemotherapy.23,26,97

Immune checkpoint inhibitors are able to activate lymphocytes within tumors. Immune checkpoints exist to modulate the immune response and are important in maintaining self-tolerance, however, tumors can use these pathways to aid immune resistance. The first class of immune checkpoint inhibitors approved by the FDA were cytotoxic T-lymphocyte associated antigen 4 (CTLA4) antibodies. CTLA4 is expressed by
standing of the immune response following OV treatment is largely limited in OV research is the lack of immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models. The majority of research is carried out in immunocompetent animal models.

• **Adenovirus Ad5-Δ24-RGD**
  - Phase I: Deletion in the E1A gene. Virus replication limited to cells deficient in the RB pathway. RGD motif binds to integrins αvβ3 and αvβ5.
  - Response: Completed. Demonstrated good safety profile, with potential antitumor activity.
  - Serious adverse events: None reported

- **Adenovirus Ad5-Δ24-GMCSF**
  - Compassionate use: Deletion in the E1A gene and expresses GMCSF.
  - Response: Well tolerated, induction of tumor-specific and virus-specific immunity. Efficacy seen in 63% patients.
  - Serious adverse events: None reported

- **Vaccinia virus**
  - Phase I: GL-ONC1
  - Response: Recruiting
  - Serious adverse events: None reported

- **Reovirus reolsyn**
  - Phase I: No modification
  - Response: Active, not recruiting. Pacitaxel with or without viral therapy
  - Serious adverse events: Study results not reported

**Table 4**

| Virus                  | Trial                      | Modification of virus                                                                 | Response                                      | Serious adverse events               | Ref          |
|-----------------------|----------------------------|---------------------------------------------------------------------------------------|-----------------------------------------------|--------------------------------------|--------------|
| Measles virus         | Phase I                    | Expresses carcinoembryonic antigen (CEA) to allow monitoring of viral gene expression | Well tolerated, dose-dependent activity was observed | Bowel obstruction, peritonitis NCT00408590 | 26           |
| Measles Virus MV-NIS  | Phase I/II                 | Infected mesenchymal stem cells                                                     | Recruiting                                    | Study results not reported NCT02068794 | 26           |
| Measles virus         | Phase II                   | Encodes thyroid sodium iodide symporter (MV-NIS)                                     | Recruiting                                    | Study results not reported NCT2364713 |              |
| Adenovirus Onyx-015   | Phase I                    | E1B gene deletion, selective replication in p-53 deficient cells.                   | No anti-tumor activity demonstrated. Abdominal discomfort seen, virus administered intraperitoneally. | Common toxicity criteria grade 3 abdominal pain and diarrhea | 26           |
| Adenovirus Ad5-Δ24-RGD| Phase I                    | Deletion in the E1A gene. Virus replication limited to cells deficient in the RB pathway. RGD motif binds to integrins αvβ3 and αvβ5. | Completed. Demonstrated good safety profile, with potential antitumor activity. | Study results not reported NCT00562003 | 26           |
| Adenovirus Ad5-Δ24-GMCSF| Compassionate use            | Deletion in the E1A gene and expresses GMCSF                                         | Well tolerated, induction of tumor-specific and virus-specific immunity. Efficacy seen in 63% patients. | None reported 92                  |              |
| Adenovirus Ad5/3- Δ24-GMCSF | Compassionate use       | p16-Rb pathway selective oncolytic adenovirus coding for GMCSF                      | Well tolerated, no severe adverse events. Clinical benefit seen in 8/12 patients. | None reported 93                  |              |
| Vaccinia virus         | Phase I                    | GL-ONC1                                                                               | Recruiting                                    | Study results not reported NCT02759588 | 94           |
| Vaccinia Virus JX-594  | Phase I                    | Modified to replicate in cells with activation of the epidermal growth factor receptor (EGFR)/ Ras pathway. | Completed. Selectively infects and replicates in cancer cells with no effect on normal tissues. | None reported NCT00625456 | 94           |
| Reovirus reolsyn       | Phase II                   | No modification                                                                       | Active, not recruiting. Pacitaxel with or without viral therapy | Study results not reported NCT01199263 |              |
| Reovirus reolsyn       | Phase I                    | No modification                                                                       | Completed                                     | Study results not reported NCT00602277 |              |

GM-CSF = granulocyte-macrophage colony-stimulating factor.

T cells and is involved in regulating the amplitude of T cell activation. Programmed cell death protein 1 (PD1) and its ligands (PDL1 and PDL2) are involved in limiting T cell activity during an inflammatory response. Immune checkpoint inhibitors could lead to a reduction of Treg numbers, and patients most likely to benefit from treatment with immune checkpoint inhibitors are those with pre-existing TILs.

Therefore, the combination of OV and immune checkpoint inhibitors is an exciting area of research. Several studies have shown that the combination of Newcastle Disease Virus (NDV) with CTLA-4 blockade in tumor bearing mice leads to regression of tumors, including those at sites distal to initial OV injection, and greater long-term survival. However, a significant limitation in OV research is the lack of immunocompetent animal models. The majority of research is carried out in immunosuppressed mice growing human tumors. This means our understanding of the immune response following OV treatment is incomplete, and it is vital to improve our understanding of this effect to further improve OV efficacy.

**Harnessing the immune component and immune limitation of adenovirus.** A major advantage of oncolytic viral therapies is their unique ability to stimulate host immune responses against cancers through the release of cancer specific antigens following immunogenic cell death. These effects can be further enhanced by arming CRAd with immune-modulatory compounds and transgenes. The attempts to influence the immune response in relation to CRAd fall broadly into 2 categories, firstly in an effort to minimize and bypass the host immune response to the viral vector itself in order to enhance the efficacy of the therapy and secondly to elicit an immune response targeted towards the cancer cells to maximize cell killing and removal.

Several strategies have shown success in altering immune response to enhance the efficacy of CRAd in ovarian cancer. By combining CRAd therapies with a pharmacological inhibition of tumor necrosis factor alpha (TNF-α) has been shown to increase adenovirus activity in ovarian cancer cell lines through the suppression of apoptosis inhibitors (cIAP1 and cIAP2). The inflammatory response initiated by CRAd was further target by Browne et al by inhibiting β3-integrins in ovarian cancer xenograft models thereby showing that CRAd therapies in β3 knockout mice released significantly less pro-inflammatory cytokines and presented with lower inflammatory hepatic toxicity. In a different approach, Ashshi et al were able to improve virus-induced cytotoxic effects by overexpression IL24, a member of the anti-inflammatory IL10 family, by arming a CRAd with IL-24. CRAd-IL24 infection resulted in significantly higher yields of infectious particles which translated into enhanced efficacy.

One of the most successful CRAd engineered to stimulate an immune response in ovarian cancer has been ONCOS-102, an Ad5/3 chimeric adenovirus expressing granulocyte-macrophage colony stimulating factor GM-CSF. ONCOS-102 has undergone clinical trials in which the treatment resulted in a progressive infiltration and activation of tumor-specific CD8+ T cells into the tumors of selected patients while the majority of patients showed...
Significantly (i.p.) injected with MOSEC ovarian tumor cells and then SFV and VV. Immunocompetent C57BL/6 mice were intraperitoneally infected several times with the same virus. To further increase the immunological antitumor effects of the viruses, oncolytic vaccinia virus (VV) led to mice prolonged survival compared with ovarian tumor-bearing mice with Semliki forest virus (SFV) and considering that intercalating the treatment of oncolytic viruses demonstrated that intercalating the treatment of oncolytic viruses can be a double-edged sword, on one hand, they can promote an immune response against the tumor cells but, on the other hand, neutralizing antiviral responses may block virus replication and ongoing infection of tumor cells. A study aiming to both enhance and limit CRAd effectivity.

Other oncolytic viruses used to boost the immune system against ovarian cancer. Alternatives to adenovirus-based oncolytic viruses such as measles, herpes simplex virus, reovirus, vaccinia virus, and sindbis viruses have also been employed for virotherapy as they have shown to target and lyse the tumor cells directly. Unlike adenoviruses, these alternative OV lend themselves more readily to studies elucidating the role of the immune system in virotherapies. Advanced metastatic tumors are often immunosuppressive and therefore challenging to treat with conventional immunotherapy, which is the case with ovarian cancer of epithelial origin. The therapeutic benefit of oncolytic viruses causing direct cytolysis and cancer-specific immunity has been demonstrated in several preclinical studies and preclinical studies have further demonstrated that the combination of oncolytic viruses and antigen-specific immunotherapy leads to enhanced anti-tumor effects against ovarian tumors.

As previously mentioned, the immune response to oncolytic viruses can be a double-edged sword, on one hand, they can promote an immune response against the tumor cells but, on the other hand, neutralizing antiviral responses may block virus replication and ongoing infection of tumor cells. A study aiming to circumvent the limitation of neutralizing antibodies against the Ad5 serotype in particular is high in humans with a prevalence of 50% to 90% in the developing world. Secondly, oncolytic viruses are unable to replicate in mouse tissue, as a result in vivo models are limited and determining the involvement of immune components in CRAd therapies is challenging.

A short-term increase in systemic pro-inflammatory cytokines and a marked increase in tumor-infiltrating lymphocytes. A recent study has demonstrated that the efficacy of CRAd is mediated by T-cell responses which regulate both the clearance of viruses and enhancing anti-tumor cytotoxicity. These findings indicate that influencing T-cell responses in a delicate balance able to both enhance and limit CRAd effectivity.

Using a virally-delivered CXCR4 antagonist to block the CXCL12/CXCR4 axis in combination with doxorubicin has been shown to increase survival in ovarian tumor-bearing mice by reversing the immunosuppressive phenotype of the TME while promoting antitumor immunity. In this study, the treatment of both orthotopic murine ID8-R and human CAOV2-R drug-resistant ovarian tumors in syngeneic C57BL/6 with an armed VV expressing the CXCR4 antagonist 12 hours before doxorubicin revealed reduction of metastatic spread and tumor growth in comparison to monotherapy treatment modalities. This effect was correlated with reduction of intraperitoneal recruitment of granulocyte-like myeloid derived suppressor cells granulocyte-like myeloid derived suppressor cells (G-MDSCs) (CD11bLy6G<sup>high</sup>Ly6C<sup>low</sup>) and Tregs (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>) and increase of CD11c<sup>+</sup>CD86<sup>+</sup> DCs and IL-12-producing monocytes/macrophages, which was associated with higher ratios of interferon gamma (IFN-γ)-producing CD8<sup>+</sup> T-cells to Tregs. Similarly, 50% tumor-free survival was observed in CAOV2-R-bearing SCID mice treated with CXCR4-A-Fc VV and doxorubicin compared with 10% observed in mice treated with VV-Fc and doxorubicin, indicating a higher viral replication/oncolysis in immunocompromised mice and a direct effect of CXCR4 antagonist on tumor cells.

Cancer-initiating cells (CIC) have been demonstrated in clinical and preclinical studies to survive conventional chemotherapies and to give rise to more aggressive, recurrent ovarian tumors. Therefore, it is important to develop therapies that simultaneously target CIC and the ovarian TME that promotes their growth. In a preclinical study, researchers demonstrated that targeting the CXCL12/CXCR4-signaling axis through an oncolytic vaccinia virus (OVV) resulted in decreased CXCL12 and VEGF expression in ascitic fluid, reduced accumulation of neutrophils/G-MDSCs and increased DCs recruitment into the tumor site of the treated mice. It is known that CXCL12 induces intratumoral localization of CD24<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs in ovarian cancer but in this study the treatment with a CXCR4 antagonist resulted in significantly lower percentages of tumor-infiltrating Tregs compared with control. The results of this study illustrate the therapeutic efficacy of an armed oncolytic vaccinia virus to target CIC as well as reducing the tumor immunosuppressive network and induction of humoral and cellular responses.

Cancer cells are able to use immune checkpoints to avoid immune control and rejection, therefore, inhibition of these inhibitory pathways would represent a potent strategy to combat cancer. The combination of an anti-α-PD-L1 to block PD1/PD-L1 immune checkpoint binding and an oncolytic vaccinia virus to activate antitumor immune response was tested by Liu et al. In this study, a monotherapy with VV or α-PD-L1 decreased tumor burden but a dual therapy of VV with α-PD-L1 led to a significant reduction of the tumor burden and increased mice survival compared with monotherapies in murine colon and ovarian cancer models. After 13 days of treatment, the dual therapy significantly elevated CD8<sup>+</sup> T-cells expression by CD4<sup>+</sup> cells, enhanced infiltration of effector T cells, and at the same time reduced Treg cells and exhausted CD8<sup>+</sup> T-cells. The ratio of CD8<sup>+</sup> T-cells to Treg increased significantly with the dual therapy, as well as the infiltration of IFN-γ/ Foxp3<sup>+</sup>CD4<sup>+</sup>T cells over Foxp3<sup>+</sup>CD4<sup>+</sup>T cells. The levels of killer cell activity markers in the TME such as IFN-γ, granzyme B, and perforin also increased significantly. CD8<sup>+</sup>, CD4<sup>+</sup> T cells, and IFN-γ all played essential roles in the therapeutic efficacy of the dual therapy sustaining an elicited systemic immunity. These data suggest the
optimal timing is simultaneous delivery of both agents, and this supports the idea that an anti-PD-L1 approach could be incorporated into the virus without the need for delayed expression of the transgene.89

Another virus-based anticancer therapy that is being used against ovarian cancer is the reovirus which is currently undergoing phase I/II clinical trials for the treatment of ovarian cancer (Table 4). A study lead by Gujar et al101 focused on characterizing the effects of reovirus therapy on ovarian cancer and the associated immune microenvironment, as this virus had proven cell killing activity by direct oncolysis and induction of efficient antitumor immunity in lung,102 melanoma,102 and prostate cancer103 models. A study from the same authors have previously demonstrated that reovirus does not kill non-transformed, normal ovarian cells,104 making it a suitable oncolytic therapy candidate for ovarian cancer. Ovarian cancer-bearing mice had longer survival rates when treated with reovirus compared with control treated mice, accompanied by a substantial alleviation of abdominal distension and reduction of ascetic fluid volume. Additionally, tumors from reovirus-treated mice displayed intratumoral infiltration of immune cells, upregulation of IFN-γ and a favorable modulation of the frequencies of MDSC and Tregs preceding an anti-tumor immunity response. Additionally, this study demonstrated that reovirus therapy postponed peritoneal carcinomatosis when administered at the early stages of ovarian cancer, which at the moment cannot be translated fully to the clinic because most patients are diagnosed when ovarian cancer is already fully developed to its late stages.101

In a study led by Kohno et al105, the HF10 amplicon, a highly attenuated variant of the herpes simplex virus type 1 (HSV-1), containing the murine GM-CSF cytokine (mGM-CSF amplicon) had demonstrated oncosuppressive effects in a murine colorectal tumor model, therefore, they hypothesized that the same mGM-CSF amplicon would also be effective to treat disseminated ovarian cancer in a mouse model. Indeed, the treatment with mGM-CSF of orthotopic tumors of HM-1 cells (ovarian cancer cells) in B6C3F1 mice resulted in significantly prolonged mice survival than did mice treated with either vehicle or HF10 amplicon. There was also increased infiltration of CD4+ and CD8+ T immune cells into the peritumoral layer, concomitant with significant decrease of macrophages infiltration. The mGM-CSF amplicon treatment was also able to sustain an efficient memory immune response demonstrated by the capacity of splenocytes cells from mGM-CSF amplicon-treated group to induce the expression of IFN-γ and TNF-α when cocultured with untreated ovarian cancer HM1 cells, demonstrating once more the anti-tumor immune therapeutic effect of an oncolytic virus armed with an immune transgene.106

Some oncolytic viruses are currently being used to treat patients with ovarian cancer in clinical trials either at the recruiting level or in phase I/I studies (Table 4). An edmonston vaccine strain of measles virus (MV) was engineered to express the marker peptide carcinoembryonic antigen (CEA) to permit real-time monitoring of viral gene expression in tumors in a phase I clinical trial. MV attenuated strains have an excellent safety record and it was demonstrated that MV vaccine strains predominantly enter cells via the CD46 receptor, which is highly expressed in ovarian cancer cells. Patients with taxol and platinum-refractory recurrent ovarian cancer and with normal CEA levels were eligible to determine the safety and tolerability of i.p. administration of MV-CEA up to 109 TCID50. Best objective response was stable disease in 14 of 21 evaluable patients with median duration of 92.5 days and median overall survival of 12.15 months in comparison to the expected median survival of 6 months in this patient population. No evidence of induced immunosuppression was observed following measles vaccination, and all observed toxicities were grade 1 and 2, highlighting the safety of MV as an oncolytic platform and the potential of oncolytic measles therapy in recurrent ovarian cancer patients.23

Conclusion
Most ovarian cancers can be characterized by rapid proliferation, aggressive neovascularization, resistance to chemo- and radiotherapy, and marked local and systemic immunosuppression. All of these factors contribute to high tumor recurrence and current treatment inadequacies. Combining cancer immunotherapy with oncolytic viruses is one of the most promising platforms for the treatment of cancer. To date, oncolytic viruses have shown great promise in mediating direct cancer cell cytotoxicity but beyond this, OV therapies have a unique ability to elicit anti-tumor immune response. Multiple studies described in this review have demonstrated that OV can lead to increased T cell infiltration into ovarian tumors in mice and that this is associated with a favorable clinical outcome. More specifically it is known that the presence of CD8+ cytotoxic T cells delays disease progression and extends overall survival of cancer patients.

Despite significant advances in adenovirus-based therapies, there are still limitations to overcome when using adenovirus in preclinical and clinical studies. Firstly, most of the population will have been infected at some point in their lives with adenovirus, thus presenting with developed, pre-existing immunity when re-infected with this virus. The induction of antiviral immunity to adenoviruses leads to inadequate dissemination of the virus and hampers effective delivery. Secondly, the inability of adenovirus to replicate in mouse tissue presents a major hurdle to developing in vivo models for preclinical studies. The exploration of alternative viral vectors to adenovirus such as vaccinia virus and reovirus will allow for a more comprehensive understanding of how OV therapies can initiate and shape anti-tumor immune responses.

The combination of viral oncolysis and antigen-specific immunity leads to enhanced antitumor effects, culminating in antitumor immune responses that control metastatic growth. Treatment of patients with oncolytic virus will require a well-coordinated strategy that would synergistically kill tumor cells with simultaneous induction of anti-tumor immunity, reduce intratumoral recruitment of immunosuppressive elements like Tregs in favor of immunostimulatory signals (like IL-12), and enhance local tumor-specific T cell accumulation in order to sustain a durable immune response against the tumor.

Oncolytic virus based therapies hold great promise in treating a range of cancers. It will be critical for the further development and refinement of these therapies to follow new avenues beyond adenoviruses to better understand the mechanisms of action of OV-based immunotherapies, which in turn, will lead to the development of efficient combination therapies for future clinical trials.

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Conflict of interest
The authors declare no conflict of interest.
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