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REVIEW ARTICLE

Understanding and addressing barriers to successful adenovirus-based virotherapy for ovarian cancer

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Abstract

Ovarian cancer is the leading cause of death among women with gynecological cancer, with an overall 5-year survival rate below 50% due to a lack of specific symptoms, late stage at time of diagnosis and a high rate of recurrence after standard therapy. A better understanding of heterogeneity, genetic mutations, biological behavior and immunosuppression in the tumor microenvironment have allowed the development of more effective therapies based on anti-angiogenic treatments, PARP and immune checkpoint inhibitors, adoptive cell therapies and oncolytic vectors. Oncolytic adenoviruses are commonly used platforms in cancer gene therapy that selectively replicate in tumor cells and at the same time are able to stimulate the immune system. In addition, they can be genetically modified to enhance their potency and overcome physical and immunological barriers. In this review we highlight the challenges of adenovirus-based oncolytic therapies targeting ovarian cancer and outline recent advances to improve their potential in combination with immunotherapies.

Ovarian cancer

Ovarian cancer is the seventh most common cancer and the second cause of death among women with gynecological cancer (after that of the cervix uteri) [1]. Although incidence rates have declined since the mid-80s and mortality is falling an average of 2.3% each year [2], an estimated 21,750 new cases of ovarian cancer will be diagnosed in the US and 13,940 women will die from the disease in 2020. The overall 5-year survival rate is only 47%, with more than 75% of patients diagnosed with advanced distant-stage disease (FIGO stage III/IV disease, 5-year survival rate of 29%). For the 15% of patients diagnosed with early localized disease (FIGO stage I), 5-year survival is 92% [3, 4]. Several factors add to the high morbidity and mortality rates: late stage at time of diagnosis, a high rate of recurrence and hurdles to effective therapy for patients with advanced-stage disease and for those who relapse. Although the prognosis in cases detected at an early stage is quite favorable, organ-confined stage ovarian cancer has no obvious symptoms, and current methods for ovarian cancer screening, i.e., transvaginal ultrasonography and detection of serum cancer antigen (CA125), have demonstrated poor sensitivity at this stage. Together with the fact that the use of these screening methods did not show a significant mortality reduction, there is thus no available screening routine for the general population at the present time. Novel approaches based on the detection of specific circulating tumor DNA and miRNAs are under investigation, but those methods are not yet capable of detecting asymptomatic disease [5–7]. Even though chemotherapy is successful at the time of presentation, around 70% of patients have recurrence in the first 3 year, and 15% relapse with chemoresistant disease, that is not curable [8]. Most patients die from malignant bowel obstruction, which usually affects multiple sites and cannot be subjected to surgery [9, 10].

Ovarian cancer is not a single disease, but rather a general term for a heterogeneous group of neoplasms but with loco-regional dissemination to the ovary and pelvic organs. More than 90% of malignant ovarian tumors are epithelial in origin. Epithelial ovarian cancer (EOC) is further classified into five subtypes with different cellular origin,

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pathogenesis, molecular alterations, gene expression, and prognosis [3, 11]. In particular, high-grade serous ovarian carcinoma (HGSOC) is the most aggressive and most common type of EOC, accounting for 75% of all deaths [12]. It originates in both ovarian surface epithelium and fallopian tube epithelium and typically presents as a large ovarian mass accompanied by widespread peritoneal metastasis and presence of ascites [13, 14].

In contrast to most carcinomas, dissemination of ovarian carcinoma through the vasculature is rare, which explains its confinement within the abdominal cavity. Ovarian cancer cells spread by direct extension to adjacent organs, and exfoliated tumor cells, either as single cells or as spheroids, freely disseminate throughout the peritoneal cavity by the peritoneal fluid and preferentially colonize the omentum, the diaphragm and the mesentery [15, 16]. Metastatic tumors also seed in other organs of the peritoneum, but only invade the superficial layers of tissue [17, 18]. In fact, the exfoliation capability from small masses difficults HGSOC screening [7]. Two key features of disease progression are the characteristic anoikis resistance of EOC cells and their ability to attach to the mesothelial cells covering the peritoneal organs [17, 19], a process where matrix metalloproteinases (MMP-2) and integrins (α5β1 and αVβ3) are largely involved [20–22].

Treatment guidelines for EOC have largely been driven by HGSOC, which is characterized by homologous recombination deficiency and harbor *TP53* mutations, with lower prevalence but recurrent somatic mutations in *BRCA1*, *BRCA2*, *NF1*, *RB1* and *CDK12* [23, 24], even though it has been demonstrated that response to treatment varies by gene expression profiles [25, 26].

An overview of the treatment strategies developed for EOC over the past 30 years [27, 28], from standard chemotherapy to anti-angiogenic therapy, and the latest incorporation of poly (ADP Ribose) polymerase (PARP) inhibitors and immune checkpoint inhibitors, reveals that, although at a slower rate compared to other malignancies, increasing knowledge about genetic mutations and associated biological behavior are leading to the incorporation of the standard of care into the era of targeted therapy [8, 29]. In this context, gene therapy, and in particular virotherapy [30], have explored different treatment opportunities and demonstrated encouraging preclinical results.

Adenovirus-based oncolytic virotherapy

Adenoviruses (Ad), in particular serotypes 2 and 5, are the most well-described and frequently used platforms for virotherapy applications. Their large packaging capacity, ability to infect both dividing and non-dividing cells, lack of integration into the host genome and the mild nature of illness after infection, are some of the many features that make them attractive for gene therapy [31–34]. In the context of cancer gene therapy, first clinical trials employing replication-incompetent adenoviruses demonstrated their potential to deliver and express the p53 tumor suppressor gene in ovarian cancer and other malignancies [35–41]. To overcome the limited efficacy and duration of transgene expression and at the same time diminish potential side effects, earlier efforts investigated the use of oncolytic Ads, which specifically replicate within tumor cells, ultimately killing them and spreading through the tumor and, potentially triggering the host's immune system [42, 43]. Tumor selectivity is not a natural feature of adenoviruses, but advances in the knowledge of Ad biology and recombinant tools to manipulate their viral genomes facilitated engineering advanced conditionally replicative adenoviruses (CRAd) based on two general strategies [44]. One involves the insertion of tumor-specific promoters (TSP) into the viral genome to drive the expression of E1A gene, which initiates Ad replication. The other involves deleting parts of the E1A or E1B genes to prevent replication in normal cells, but enable replication in tumor cells with malfunctioning cellular transcriptional machinery. Relevant modifications involve the deletion of the E1B-55k gene (*dl*1520/ Onyx-015) that prevents p53-mediated apoptosis and the deletion of the Rb protein binding site on E1A (*dl*922-947 and AdΔ24) [45, 46], with E1A-deletion mutants having improved oncolytic efficacy compared to E1B mutants both in vitro and in vivo [47, 48]. In addition, by taking advance of cell cycle dysregulation, early generation oncolytic Ads show encouraging results in combination with standard chemotherapy in advanced and recurrent resistant disease [49–51].

Preclinical in vivo models that both accurately recapitulate human HGSOC and allow testing of oncolytic Ad vectors and their immunobiological effects are absent [52–54]. A focus of the last decade has been the search for immunocompetent animal models and humanized models accurately representative of EOC that contribute to decipher virus/host interactions, as studies to date mostly use human xenografts in immunodeficient mice. Although ID8 is the most studied murine syngeneic EOC model and improved derivatives have been developed [55–57], full replication of human Ad is limited to certain murine cell lines [58–61]. Syrian hamsters have been reported to support Ad5 replication [62], but no EOC models are available. Patient-derived ex vivo tumor cultures of EOC have been used as an alternative to study Ad immune-modulation [63].

Restricting adenoviral infection through transductional targeting

The first clinical trials testing Ad virotherapy established insufficient targeting as one of the main reasons for failure.

The entry of Ad5 into cells requires binding of the knob domain of the fiber protein with the high-affinity coxsackievirus and adenovirus receptor (CAR) and subsequent interaction between the arginine-glycine-aspartic acid (RGD) sequence of the penton and αvβ3 and αvβ5 integrins [64, 65]. Evidence demonstrated that CAR and integrins are expressed at low levels in many epithelial ovarian cancers and those levels are inversely related to tumor grade [66–68]. To circumvent CAR-dependent cell entry and improve tumor selectivity, Ad5 fiber has been modified by inserting peptide ligands selective for tumor-associated receptors in EOC cells [69]. It was demonstrated that the addition of an RGD motif or a polylysine (pk7) motif into the knob increases Ad5 binding to integrins or heparan sulfate proteoglycans (HSPGs), respectively, achieving improved level of gene transfer into EOC cell lines and primary tumors that were resistant to Ad5 infection, without affecting fiber function [70–72]. Combining an RGD motif in both the fiber and the capsid protein IX showed greater oncolytic activity in vitro, but no benefit in vivo [73]. Of particular relevance is Ad5-Δ24RGD, which yielded positive results in vitro, complete eradication of intraperitoneal disease in a xenograft mouse model and promise in a phase I clinical trial of recurrent HGSOC [74–76]. Uusi-Kerttula et al showed moderate to up to 950-fold higher efficiency in the presence of neutralizing ovarian ascites when incorporating targeting peptides into the HI loop to either αvβ6 integrin (A20) [77] or epidermal growth factor (EGFR) (GE11) [78], both overexpressed in 30% EOC and suggested a correlation with disease progression.

Another strategy to improve adenoviral tropism via a CAR-independent pathway consists on replacing the knob of Ad5 (subgroup C) with alternative serotypes, mainly from species B and species D [79]. Chimeric Ad5/3 vector, which uses predominantly desmoglein-2 (DSG2) receptor but also CD46 receptor [80, 81], was confirmed to achieved higher infectivity in EOC cells and subcutaneous tumor xenograft after intratumoral injection than Ad5 and also the RGD variant, with a similar biodistribution profile [82, 83]. Moreover, Ad5/3-Δ24 demonstrated greater oncolytic effect compared to Ad5-Δ24RGD in cell lines, clinical tumor samples and in a intraperitoneal xenograft murine model [84, 85], showing a good safety profile and potential in a phase I clinical trial [86]. In order to achieve higher transduction efficiency by using CD46, Hulin-Curtis et al pseudotyped Ad5 with the fiber of Ad35 [87, 88] and was able to achieve higher transduction efficiency in EOC cells. Similar to Ad5-A20, a chimeric Ad5/48-A20 vector based on the low seroprevalent Ad48 was able to target primary ex vivo cultures in the presence of neutralizing ovarian ascites [77].

Restricting adenoviral replication

In order to limit Ad replication to the target tissue, transcription of adenoviral genes, generally E1, can be controlled by TSPs. Early modifications include the cyclooxygenase-2 (cox-2) promoter, the secretory leukocyte protease inhibitor (SLPI) promoter and the vascular endothelial growth factor (VEGF) promoter. While Ad5RGD-Cox-2-Δ24 caused less toxicity to nonmalignant cells, VEGF tumor-specific promoter showed greater replication and no significant differences in vivo compared to Ad5-Δ24RGD [89–91]. Similarly, Ad5/3-SLPI showed efficient viral replication and oncolysis and significantly decreased liver toxicity compared to Ad5/3Cox-2 or Ad5/3wt, although no increased survival [92]. An Ad5 vector bearing the human telomerase reverse transcriptase (hTERT) promoter together with an Ad5/3 vector bearing the multidrug resistance gene 1 (MDR1) promoter were found to significantly enhance survival in combination with chemotherapy in cisplatin-resistant xenograft models with peritoneal dissemination [93, 94]. Several groups have validated the utility of the chemokine CXCR4 receptor as well as the Survivin promoter and the Mesothelin promoter to increase selectivity, improve oncolysis and decrease liver uptake in murine xenograft EOC models when included into Ad5/3 and Ad5-RGD vectors [95–98]. Increasing evidence indicates the important role of various types of stromal cells in TME in supporting tumor progression and highlight them as attractive targets [99, 100]. In this context, Lopez et al described a stroma-targeted CRAd pseudotyped with chimeric fiber 5/3 including a SPARC promoter fragment that was effective in the remission of disseminated HGSOC in nude mice and was able to replicate in fresh tumor explants [101]. In the same way, Long et al developed an adenovirus system based on Cre/LoxP and a CD133 promoter to target $CD133^{+}$ ovarian cancer stem cells, which contribute to recurrence and chemoresistance, to increase apoptosis and suppress tumor growth [102].

Increasing potency of CRAds

Because CRAds are often unable to eliminate entire tumors by viral replication alone, they have been armed with suicide genes, transgenes that target the TME and immunomodulatory molecules to potentiate anti-tumor efficacy [103, 104]. It must be taken into consideration that the Ad genome has limited space and can accommodate up to 105% of the wildtype genome length without compromising viral assembly. In addition, the insert gene should be placed in a location that yields ideal expression and allows normal replication. The most extensively used transgenes for boosting cell killing are “suicide genes” that encode prodrug-converting enzymes and promote a “bystander

effect", by which the cytotoxic metabolites generated diffusing into neighboring cells are also killed. In EOC, results reported when using the herpes simplex virus thymidine kinase/gancyclovir (HSV-TK/GCV) suggest that, although Ad5 and Ad5/3 vectors with E1A or E1B deletions containing the TK transgene were able to suppress tumor growth in subcutaneous and intraperitoneal xenograft tumor models, the addition of ganciclovir was effective only when the oncolytic potential of the CRAds was low [105–107]. The cytosine deaminase/5-fluorocytosine (CD/5FC) system included into an Ad vector reported promising results against EOC cells [108, 109]. Other groups included a manganese superoxide dismutase (MnSO) transgene or a reverse activator of transcription-3 (STAT3) cDNA to enhance drug sensitivity in cisplatin-resistant HGSOC cells [110, 111].

The TME also consists of non-cellular components such as the extracellular matrix (ECM), ECM remodeling enzymes and growth factors, whose complex structure can act as a physical barrier that limits CRAds efficacy [112, 113]. In order to facilitate viral spread, Yang et al incorporated the tissue inhibitor of metalloproteinase 2 (TIMP2) gene into Ad5/3-CXCR4 to target angiogenesis and tumor invasion and demonstrated more efficient and specific replication and oncolysis in a HGSOC ex vivo model compared to unarmed CRAds [114].

To enhance the activity of the immune system against the tumor, cytokines or chemokines can be locally expressed by oncolytic Ads, maximizing the anti-tumor effect against the primary tumor and potentially the metastasis and minimizing systemic toxicity. Construction of Ad5/3Δ24 containing either IL-24 or ING4 revealed interference in CRAd propagation from ING4 expression but significantly enhanced oncolytic potency of CRAd-IL4 in vitro as compared to non-armed CRAd and CRAd-ING4 [115], although no improvements were seen in a subcutaneous xenograft murine model [61]. Different studies have sought to increase oncolytic activity and reduce acute immune stimulation during CRAd therapy by blocking the expression of TNF-α, β3 integrin or IL-8 [116–118], but this potentially limits the adaptive anti-tumor immune response [43, 119, 120]. Because T cells are not only generated against tumor cells but are also able to initiate a strong anti-viral response, CRAd design and route of injection will influence the balance towards an anti-viral or anti-tumor response. While no differences in anti-tumor efficacy were seen between Ad5/3-E2F-Δ24 and Ad5/3-E2F-Δ24-hTNFα-IRES-hIL2 in an immunocompromised model, the expression of the cytokines enhanced T cell recruitment and activation in an ex vivo EOC model and an immunocompetent model, demonstrating resistance to tumor recurrence [63, 121, 122]. Two oncolytic Ads based on Ad5Δ24 and Ad5/3Δ24 armed with granulocyte-macrophage colony stimulating factor (GMCSF) were capable of inducing both tumor- and virus-specific immunity, were well tolerated and demonstrated clinical benefits in some patients with refractory advanced solid tumors. In particular, administration of ONCOS-102 (Ad5/3Δ24-GMCSF) showed induction of strong tumor-specific CD8+ T cells in tumors and systemically [123–126].

Overcoming physical barriers to dissemination of oncolytic adenoviruses

While the intraperitoneal confinement of HGSOC allows for localized delivery of CRAds and potentially bypass many restrictions associated with intravenous delivery and reduce toxicity [127, 128], this route can suffer from rapid Ad clearance and poor distribution if the cavity becomes loculated (Fig. 1). Ascites, a complex heterogeneous mixture consisting mainly of tumor cells, mesothelial cells, fibroblasts, immune cells, cytokines and growth factors, accumulate due to increased permeability of afferent vessels of the peritoneal lining and reduced lymphatic flow. In addition, high-volume ascites are likely to result in tumors with poor vascular delivery [129–131]. More importantly, neutralizing antibodies against Ad5 have been found in serum from healthy patients and in the ascites of HGSOC patients [132–134]. Additionally, host Ad sensing mechanisms activate proinflammatory signaling and promote long-term local inflammation and adhesion formation after intraperitoneal delivery [135].

The interactions impacting Ad biodistribution upon intravascular delivery have been widely described. Reasons for the low efficacy of Ad5-derived vectors include clearance after opsonization by natural antibodies and complement, as well as sequestration in the liver and spleen mediated by binding with human coagulation factor 10 (FX) and other coagulation factors to HSPG abundant on hepatocytes or scavenger receptors on Kupffer cells [136–138]. Since a small portion of the Ad particles injected ip are able to enter the circulation, Ad vectors administered through this route are susceptible to clearance after interacting with blood components and residential macrophages in tissue and in the peritoneal cavity [83, 139]. It was first shown that modifications of the fiber partially ablate neutralization of the virus [77, 78, 84, 140], and later, that additional pseudotyping of the hexon or the ablation of FX binding sites in the hexon decreases liver sequestration, toxicity and vector-immune system interactions in both ip and iv approaches [88, 141, 142]. Recently, Uusi-Kerttula et al combined triple detargeting through mutations on the fiber that block binding to CAR, the penton to block binding to αvβ3/5 integrins, and the hexon for FX binding, with insertion of the A20 peptide into the HI loop [143]. Ad5NULL-A20 CRAd demonstrated high tumor selectivity, significant

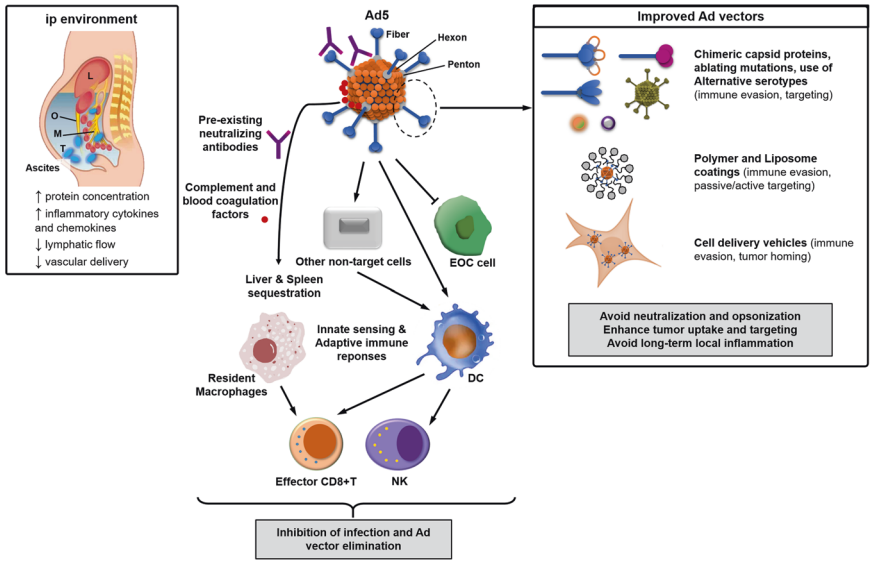

Fig. 1 Barriers to Ad vector delivery. Intraperitoneal administration of Ads into the complex biological environment of the peritoneal cavity can limit tumor transduction, favor heterogeneous distribution, rapid Ad aggregation and clearance and cytotoxicity. Genetic, chemical and physical modifications can be implemented to overcome these barriers, decrease detargeting and increase specific infection of EOC cells. L: liver, O: omentum, M: mesentery, T: tumor, ip: intraperitoneal, DC: dendritic cells, NK: natural killers.

reduction in off-target uptake and tumor-free survival in an EOC xenograft model compared to either Ad5Δ24 or Ad5Δ24.A20. In the context of Ad serotypes, Thoma et al observed that, while Ad5 induces long-term damage by promoting acute liver pathology, enlargement of the spleen and formation of severe adhesions, intraperitoneal injection of Ad11 only caused short-term mild inflammation due to macrophages controlling disproportionate inflammatory responses [135]. Based on the results obtained with Enadenotucirev (ColoAd1, chimeric Ad3/Ad11p) in colon cancer, two potent chimeric ColoAd1/Ad3 recombinant CRAds were isolated by directed evolution against platinum-resistant HGSOC that demonstrated potent and tumor selective activity in vitro and in vivo and absence of peritoneal adhesions [144]. Moreover, Enadenotucirev is currently being evaluated in a phase 1/2 trial either alone or with paclitaxel.

Another approach shields the Ad from the immune system by chemically and physically modifying the capsid with polymers and liposomes [145–147]. Since the modifications are not carried into the Ad progeny, coating the vector might be more interesting when several administrations are required. Administration of a PEGylated liposome-encapsulated Ad expressing human endostatin or complexed with multiple layers of polyethylenimine (PEI) and hyaluronic acid (HA) increased survival and demonstrated better protective activity from host immune clearance [148, 149]. In addition to ablating Ad tropism, coating the vector can increase tumor retention by passive targeting and retargeting to alternative receptors. Lanciotti et al showed five-fold increase in the tumor and 10 times reduction in the liver and spleen of an Ad coated with PEG (polyethyleneglycol) functionalized with Fibroblast Growth Factor 2 (FGF2), along with less antibody neutralization and T cell responses [150]. Morrison et al were able to protect the vector from neutralizing antibodies and demonstrated significantly lower tumor load and absence of inflammatory toxicities by coating Ad5 with poly (hydroxypropyl methacrylamide (pHPMA) and retargeting to EGFR with EGF or cetuximab [151, 152]. Thanks to their natural tumor tropism and immunomodulatory properties, mesenchymal stem cells (MSCs) of different origins have been used as carriers of oncolytic Ads [153–155], increasing targeted delivery and reducing the hepatic uptake and systemic toxicity seen in clinical trials of vectors such as Ad5-Δ24RGD [156]. Menstrual blood MSCs (MenSCs) were successfully used to deliver an Ad5/3Δ24 CRAd with microenvironment-responsive elements and the SPARC promoter without being blocked by antibodies present in the ascites, which also contain soluble factors that can serve as transcriptional enhancers [157]. Mooney et al showed that neural stem cells (NSC) carrying an oncolytic Ad bearing the survivin promoter and polylysine peptide into the fiber (CRAd-S-pk7) were able to selectively penetrate HGSOC tumor metastases, allowing replication of the virus, and synergistically reduce tumor growth in combination with cisplatin [158].

Overcoming immunosuppression

Despite the success of immunotherapy in other malignancies, such as melanoma or lung cancer, and the promising results seen in preclinical models, results obtained with immune checkpoint inhibitors, cancer vaccines or adoptive cell transfer (ACT) in EOC have not been that positive [9, 28, 159]. It has been demonstrated that the mutational profile defines immunogenicity, meaning that HGSOC tumors with disrupt DNA repair mechanisms result

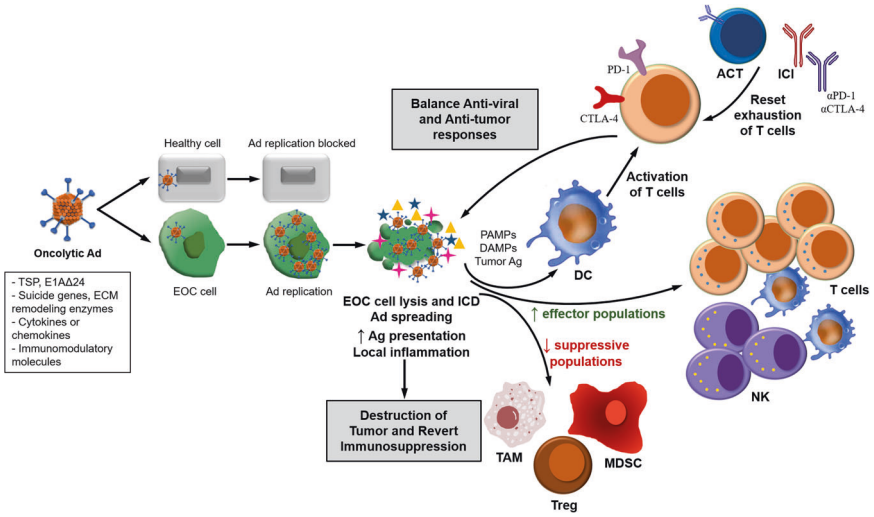

Fig. 2 Oncolytic Ads help overcoming immunosuppression and boosting anti-tumor immunity in EOC. Oncolytic Ads are genetically modified with tissue-specific promoters (TSP) and mutations on E1A to specifically replicate in and kill tumor cells. At the end of the lytic cycle, viral progeny spreads throughout the tumor, infecting and lysing surrounding cancer cells, facilitating the release of pathogen and danger associated molecular patterns (PAMPs and DAMPs) and tumor antigens (neoantigens and tumor associated antigens), together with the amplification of the expression of therapeutic transgenes in the tumor microenvironment. Oncolytic Ads promote strong antiviral innate responses and prime adaptive immune responses, facilitating the recruitment of effector cells, overcoming immunosuppression, and oncolytic Ads can be used in combination with immunotherapy approaches to prevent T cell exhaustion, successfully generating anti-tumor immunity. ECM: extracellular matrix, DC: dendritic cells, TAM: tumor-associated macrophages, Treg: regulatory T cells, MDSC: myeloid derived suppressor cells, NK: natural killers, ICI: immune checkpoint inhibitors, ACT: adoptive cell therapies.

in higher mutational burden and neoantigen presence, which in turn increases CD3+ and CD8+ TILs, PD-1/PD-L1 expression, susceptibility to immune checkpoint therapy and survival. However, only a small percentage of mutations are recognized by autologous tumor-associated T cells, and tumor heterogeneity can limit expression of neoantigens to a small proportion of tumor cells. Although EOC express known, non-mutated tumor-associated antigens (TAAs), poor immunogenicity prevents appropriate TAA-mediated tumor rejection [160, 161]. In addition, the TME in EOC is particularly immunosuppressive, not only in primary tumors but also in the ascites and in the omentum. While the omentum is a complex immunologic organ that actively and passively entraps EOC cells in its highly vascularized immunologic units known as milky spots, tumor-associated macrophages (TAMs), regulatory T cells (Treg) and myeloid derived suppressor cells (MDSCs), together with other components such as EOC-derived exosomes, cancer-associated fibroblasts (CAFs) and adipocytes, are involved in impairing both the presence and the activity of effector T cells, neutrophils and natural killer (NK) cells, and disease progression [162–165].

In order to generate a significant anti-tumor immune response, different combination strategies are under investigation based on priming T cell responses, inducing immunogenic cell death and targeting checkpoint inhibitors (PD1/PD-L1 and CTLA-4/CD80/CD86 pathways) responsible for T cell anergy and/or exhaustion [166, 167], depending on EOC immune status. In this context, oncolytic Ads represent a powerful tool to generate immunologically hot tumors, since by replicating and killing tumor cells, pathogen and danger associated molecular patterns (PAMPs and DAMPs), TAAs and tumor neoantigens are released, stimulating the immunomodulation of the TME, TIL recruitment, priming dendritic cells and tumor-specific T-cell responses and potentially generating memory responses [42, 168]. Different regimens of administration are being tested combining oncolytic Ads and CAR T therapy, adoptive TIL transfer and immune-checkpoint inhibitors to boost the anti-tumor activity [169] (Fig. 2).

In order to combine direct Ad-mediated cytotoxicity, stimulation of a proinflammatory TME and activation of endogenous T cells to kill EOC cells or CAFs, Enadenotucirev has been modified to express a bispecific T-cell engager (BiTE) against epithelial cell adhesion molecule (EpCAM) [170] or fibroblast activation protein (FAP) [171], respectively, successfully reversing TME-mediated immunosuppression in ex vivo malignant ascites. Santos et al demonstrated that an Ad5/3-Δ24 expressing TNFα and IL2 under the control of the E2F promoter was able to reduce the suppressive cytokines and increase activation of CD4+ and CD8+ TILs in ex vivo tumor cultures independently of PD-L1 tumor expression, although at a lower level compared to other malignancies, highlighting the immunosuppressive TME in EOC [63, 172]. Huang et al confirmed that an oncolytic Ad5/35 armed with a SIRPα-Fc

Table 1 Adenoviral-based therapies used in EOC preclinical trials.

Approach	Ad vectors	Strategies	Models	References
Conditional replication	Onyx-015 (*dl*1520)	E1B-55-kD deletion, 24 bp deletion in E1A	Human xenografts immunodeficient mice ip + ip*	[49, 50, 179–181]
	Onyx-015 + CDDP			
	*dl*922-947			
	*dl*922-947 + paclitaxel			
Enhancing infectivity	Ad5-RGD	Targeting to αvβ-integrins (fiber/fiber and pIX), targeting to heparan sulfate	Human xenografts immunodeficient mice sc + it*/ip + ip*	[72–74]
	Ad5-pK7			
	Ad5-RGD-pK7			
	Ad5-RGD-Δ24			
	Ad5-Δ24DoubleRGD			
	Ad5/3-Δ24	Chimeric 5/3 fiber	Human xenografts immunodeficient mice sc + it*/ip + ip*	[84, 85, 182]
	Ad5/3-Δ24 + gemcitabine/ epirubicin			
Restricting replication	Ad5-VEGF	Inclusion of a variety of tissue-specific promoters	Human xenografts immunodeficient mice sc + it*/ip + ip*	[89–94, 96–98, 101]
	Ad5-hTERT + CDDP		Ex vivo primary ovarian tumor tissue	
	Ad5-Δ24-SPARC			
	Ad5RGD-Cox-2-Δ24			
	Ad5RGD-Survivin + CDDP			
	Ad5/3-VEGF			
	Ad5/3-SLPI			
	Ad5/3-CXCR4			
	Ad5/3-Mesothelin			
	Ad5/3-Survivin			
	Ad5/3-MDR1			
	Ad5/3-Δ24-MDR1			
Increasing potency	Ad5-CD133-Cre + Ad5-LoxP-tBid	Inclusion of inducers of cell apoptosis, suicide genes, genes targeting angiogenesis, enhancers of drug sensitivity	Human xenografts immunodeficient mice sc + it*/ip + ip*	[102, 105–107, 109–111, 114, 140, 183]
	Ad5/3-CXCR4-TIMP2		Ex vivo primary ovarian tumor tissue	
	Ad5-TK + GCV			
	Ad5Δ24-TK + GCV			
	Ad5Δ24 + GCV			
	Ad5-TK-SSTR + GCV			
	Ad5RGD-TK-SSTR + GCV			
	Ad5/3-TK + GCV			
	Ad5/3-Δ24-TK + GCV			
	Ad5- MDR1-CD + 5FC			
	Ad5-STAT3 + CDDP			
	Ad5-MnSOD + CDDP			
	*dl*922-947 + TNF-α RNAi /antibodies	Reduction of acute immune stimulation	Human xenografts immunodeficient mice ip + ip*	[116, 117]
	Ad5/3-IL24	Inclusion of immunostimulatory molecules	Human xenografts immunodeficient mice sc + it*/ip + ip*	[61, 63, 121–123]

Table 1 (continued)

Approach	Ad vectors	Strategies	Models	References
	Ad5/3-ING4 Ad5/3-IL24/ING4 Ad5/3-E2F-Δ24 Ad5/3-E2F-Δ24-hTNFα-IRES-hIL2 Ad5Δ24-GMCSF ONCOS-102 (Ad5/3Δ24-GMCSF)		Ex vivo primary ovarian tumor tissue sc/ip immunocompetent Syrian hamster (pancreatic)	
Overcoming physical barriers and immunosuppression	Ad5-A20	Triple detargeting + targeting to αvβ-integrins, use of other serotypes and chimeric vectors	Human xenograft immunodeficient mice ip + ip*	[42, 77, 135, 144]
	Ad5/48-A20		Immunocompetent mice ip + ip*	
	Ad5NULL-A20		Ex vivo ascites	
	Ad11 OvAd1 (ColoAd1/Ad11p)			
	Ad-hEndo/PEG-PEI Ad-IAI.3B/PEI-HA Ad/PEG-FGF2 Ad5/pHPMA-EGF Ad5/pHPMA-cetuximab	Encapsulation with polymers and liposomes	Human xenografts immunodeficient mice sc + it*/ip + ip*	[148–152]
	AR2011/MenSCs	Use of cellular carriers	Human xenografts immunodeficient mice sc + it*/ip + ip*	[156, 157]
	CRAd-S-pk7/NSCs CRAd-S-pk7/NSCs + CDDP		Immunocompetent mice ip + ip*	
	Enadenotucirev- EpCAM BiTE Enadenotucirev-FAP BiTE	Increased Ad-mediated cytotoxicity, stimulation of a proinflammatory TME and activation of endogenous T cells	Ex vivo ascites Human xenografts immunodeficient mice sc + it*	[170, 171, 174, 178]
	Ad5/35-hTERT-CCAU-SIRPa-IgG1 Ad5/35+ +α-PD-L1-γ1		Immunocompetent mice ip + ip*	

CDDP cisplatin, *ip* intraperitoneal, *RGD* arginine-glycine-aspartic acid, *pK7* polylysine, *sc* subcutaneous, *it* intratumoral, *VEGF* vascular endothelial growth factor, *hTERT* human telomerase reverse transcriptase, *SPARC* secreted protein, acidic, rich in cysteine, *SLP* secretory leukocyte protease inhibitor, *MDR1* multidrug resistance gene 1, *tBid* truncated Bid, *TIMP2* tissue inhibitor of metalloproteinase 2, *TK* thymidine kinase, *GCV* ganciclovir, *SSTR* somatostatin receptor, *CD* cytosine deaminase, *5FC* 5-fluorocytosine, *STAT3* single transducer and activator of transcription-3, *MnSO* manganese superoxide dismutase, *TNF-α* tumor necrosis factor α, *GMCSF* granulocyte-macrophage colony stimulating factor, *PEG* polyethyleneglycol, *PEI* polyethylenimine, *HA* hyaluronic acid increased, *FGF2* Fibroblast Growth Factor 2, *pHPMA* poly hydroxypropyl methacrylamide, *EGF* epidermal growth factor, *MenSCs* Menstrual blood mesenchymal stem cells, *NSCs* neural stem cells, *EpCAM* epithelial cell adhesion molecule, *BiTE* bispecific T-cell engager, *FAP* fibroblast activation protein, *SIRPα-Fc* signal regulatory protein α. *Indicates how the model was stablished + the route of administration of the Ad vectors.

Table 2 Adenoviral-based therapies used in EOC clinical trials.

Ad vector	Modification	Participants	Response	Toxicity	Trial Phase & References
Ad5CMV-p53 + carboplatin and paclitaxel	ΔE1A p53 expression.	59 Recurrent EOC, peritoneal cancer.	p53 expression in ascitic fluid and tumor biopsies. ip infusions.	Toxicity profile similar to intraperitoneal chemotherapy.	I, NCT00002960 [37, 38]
Ad5.SSTR/TK.RGD + Ganciclovir (GCV)	ΔE1A RGD targeting to αvβ-integrins. HSV-TK and SSTR2 cassette.	12 Recurrent EOC, other gynecologic.	1 patient disease free, 5 patients with stable disease. Noninvasive imaging with SSTR. ip infusions.	Limited toxicities related to dose.	I, NCT00964756 [183, 184]
Onyx-015 (*dl*1520)	E1B-55-kd deletion.	16 Recurrent EOC.	Viral DNA found in ascites. No anti-tumor activity. ip infusions.	Common toxicity criteria grade 1-2 and abdominal pain.	I [185]
Ad5-Δ24-RGD	24 bp deletion in E1A. RGD targeting to αvβ-integrins.	21 EOC, primary peritoneal.	Good safety profile with potential anti-tumor activity. Viral DNA found in ascites, replication. Insignificant shedding in the serum, saliva, and urine. Anti-adenoviral neutralizing antibody effects. ip infusions.	Common toxicity criteria grade 1–2.	I, NCT00562003 [75, 76]
Ad5/3-Δ24	24 bp deletion in E1A. Chimeric 5/3 fiber.	10 Recurrent EOC, other gynecologic.	75% of patients had defined stable disease. Marked anti-adenoviral antibody response detectable vector and replication in ascites. ip infusions.	Mild vector-related toxicities.	I [84, 86]
Ad5-Δ24-GMCSF	24 bp deletion in E1A GMCSF expression.	4 recurrent EOC (20 total).	Well tolerated, induction of tumor-specific and virus-specific immunity. Efficacy seen in 63% patients. Intracavity injection.	None reported.	Compassionate use [123]
Ad5/3-Δ24-GMCSF + cyclophosphamide	24 bp deletion in E1A Chimeric 5/3 fiber.	GMCSF expression 4 recurrent EOC (21 total)	Biological activity of virus seen in 13 patients. Clinical benefit seen in 8 patients. Intracavity injections.	Well tolerated, no severe adverse events.	Compassionate use [124–126] I, NCT01598129
Enadenotucirev + paclitaxel	Chimeric Ad3/Ad11p.	Recruiting Recurrent platinum resistant EOC.	ip/iv		I/II, NCT02028117
ONCOS-102 + durvalumab	24 bp deletion in E1A. Chimeric 5/3 fiber. GMCSF expression.	Recruiting. Platinum-resistant ovarian, colorectal, appendiceal.	ip/iv		I/II, NCT02963831
LOAd703 + chemotherapy/gemcitabine	24 bp deletion in E1A. Chimeric 5/35 fiber. TMZ-CD40L and 41BBL expression.	Recruiting Colorectal, ovarian, pancreatic, biliary,	Intratumoral image-guided injections.		I/II, NCT03225989

EOC epithelial ovarian cancer, *ip* intraperitoneal, *iv* intravenous, *SSTR* somatostatin receptor, *GMCSF* granulocyte-macrophage colony stimulating factor, *TMZ-CD40L* trimerized, membrane-bound human CD40L, *41BBL* full-length human 4-1BB ligand.

(signal regulatory protein α) fusion gene expressed on macrophages demonstrated anti-tumor effect on a CD47-positive SKOV3 xenograft model in the presence of immune population, indicating that CD47 blockade ('don't-eat-me' signal for immune evasion) [173] successfully increased NK cells infiltration and macrophage mediated phagocytosis [174]. Instead of using antigen-dependent approaches, adoptive immunotherapy can be based on antigen-independent strategies, such as NK cells. Because NK cells in the TME are less cytotoxic and have an exhausted phenotype, different methods are being explored to potentiate their killing capacity [175]. Leung et al recently demonstrated that *dl*922-947- and Enadenotucirev-infected EOC cells were able to activate human NK cells and augment their cytotoxicity in vitro in a contact-dependent manner through different pathways [176]. Furthermore, the combination with TIGIT blockade, an inhibitory NK receptor associated with T cell exhaustion phenotypes [177], increased NK cytotoxicity. Considering that current prevention methods are limited for high-risk women with germline mutations, Li et al developed a method to genetically modify hematopoietic stem/progenitor cells (HSPC) in vivo based on an integrating Ad5/35 + + vector expressing α-PD-L1-γ1 under the control of a miRNA regulation system that is activated only when HSPCs are recruited to and differentiated by the tumor into tumor-supporting cells [178]. They stablished a feasible in situ transduction strategy and demonstrated that intratumoral expression of α-PD-L1-γ1 early during tumor development successfully reduced primary tumor growth and prevention of recurrence.

Summary and future directions

In recent years, knowledge gained in terms of biology of both oncolytic Ads and EOC has enabled the development of more selective and potent CRAds (Table 1) able to overcome obstacles encountered in clinical trials (Table 2). While oncolytic Ads are promising tools, as they are able to kill tumor cells and stimulate an adaptive anti-tumor immune response, EOC is highly complex and its immunosuppressive microenvironment and heterogeneity among primary and metastatic tumors and ascites impairs Ad efficiency as single approach therapy. In order to create a more favorable TME, oncolytic Ads are the perfect match for combination with other emerging targeted strategies, such as immune checkpoint inhibitors or CAR-T cells. New advances should focus on finding the perfect balance between Ad replication and stimulation of the immune responses and improving regimens of administration, identification of novel unique ligands that can be efficiently targeted by incorporating single-domain antibodies (sdAbs) and fully understanding host-virus interactions in complex preclinical models.

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Compliance with ethical standards

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