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Review

Immunology of Adenoviral Vectors in Cancer Therapy

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Adenoviruses are a commonly utilized virus for gene therapy platforms worldwide. Since adenovirus components are characterized as highly immunogenic, their immunogenicity inhibits the widespread use of adenoviral vectors to treat genetic disorders. However, stimulation of the immune response can be exploited for cancer immunotherapy platforms, and thus adenoviral vectors are used for therapeutic gene transfer, vaccines, and oncolytic agents in the cancer gene therapy field. It is now accepted that the generation of anti-tumor immune responses induced by oncolytic adenovirus treatments is critical for their anti-tumor efficacy. As such, in cancer immunotherapy with adenoviral vectors, a balance must be struck between induction of anti-adenoviral and anti-tumor immune responses. The recent trend in adenoviral-based cancer gene therapy is the development of adenoviral vectors to enhance immune responses and redirect them toward tumors. This review focuses on anti-adenoviral immunity and how adenovirotherapies skew the immune response toward an anti-tumor response.

INTRODUCTION

In 2018, more than 18 million people were diagnosed with cancer and 9.5 million died from cancer-related causes globally.[1] In order to combat this, new therapeutic moieties such as gene therapy are being pursued aggressively. Cancer is the most frequent disease targeted in gene therapy clinical trials, with adenovirus (Ad) vectors being the most commonly used gene therapy vector worldwide.[2] In recent years, it has become clear that these virus-based gene therapies can be effective cancer immunotherapies.[3]

Ads are approximately 90-nm non-enveloped icosahedral, linear double-stranded DNA (dsDNA) viruses. Ads provide many advantages as gene transfer and/or oncolytic vectors: they can infect both dividing and non-dividing cells; they have broad tropism and their capsids can be genetically modified to broaden or limit this tropism; and their replication machinery is well studied, allowing for genetic modification to enhance or restrict viral replication in target tissues/cells. Ad vectors can have large transgene capacity (up to 36 kb) and can be grown to high titers (1×10^{13} virus particles [vp]/mL) that meet clinical Good Manufacturing Practice (GMP) standards. Finally, Ads are highly immunogenic but result in only mild, self-limiting clinical pathologies in immune-competent individuals.

Ads have been studied for decades as gene therapy vectors, utilized in the correction of genetic disorders, as vaccines, and as oncolytic agents. A major barrier to Ad-based therapies for genetic disorders is the host immune response to adenoviral infection; however, this immune stimulation can be harnessed in the development of immunotherapies for cancer treatments.

Oncolytic Ads are engineered to specifically replicate in and lyse tumor cells, sparing healthy tissue with the added advantage of being able to deliver therapeutic transgenes. Additionally, due to their strong immunogenicity, both replicative and non-replicative Ads can be used as *in situ* cancer vaccines. Cancer-targeted Ad therapies are a robust area of preclinical and clinical studies. As of this writing, there are 55 active clinical trials for cancer treatment in which Ads are used as oncolytic or gene therapy vectors or therapeutic vaccines listed on ClincalTrials.gov (Table 1).

In this review we discuss the induction of host immune responses through Ad infection, and how this immune activation can be taken advantage of in order to stimulate an anti-tumor immune response by Ad-mediated cancer therapy.

General Immune Responses to Ad Infection

Widespread upper respiratory infections caused by adenoviral infection result in anti-Ad serotype-specific antibodies and T cell responses cross-reactive to different serotypes.[4] The seroprevalence of the most commonly used Ad serotype, serotype 5, is approximately 50% in North America and reaches nearly 100% is some regions of Africa, while the seroprevalences of Ad3 and Ad35 in the US are around 100% and 3%–22%, respectively.[5]

Ad infection stimulates a robust innate immune response due to pathogen-associated molecular patterns (PAMPs), which include portions of the viral capsid and viral nucleic acids (Figure 1). Innate responses to Ad comprise cellular components, called pattern recognition receptors (PRRs), which induce production of type I interferons (IFNs), other pro-inflammatory cytokines, and chemokines. The

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Table 1. Active Clinical Trials Using Adenoviral Vectors for Cancer Treatment

Cancer	Vector	Serotype	Vector Generation/ Purpose	Transgene	Administration	Dose	Phase	Combination Therapy	Identifier
Prostate cancer	Ad5-yCD/ mutTKSR39rep-hIL-12	Ad5	oncolytic	cytosine deaminase, HSV-tK, hIL-12	single intraprostatic	1×10^{11} vp–1×10^{12} vp	I		NCT02555397
Metastatic pancreatic cancer	Ad5-yCD/ mutTKSR39rep-hIL-12	Ad5	oncolytic	cytosine deaminase, HSV-tK, hIL-12	single i.t.	1×10^{11} vp–1×10^{12} vp	I	5-FC, standard chemotherapy	NCT03281382
Pancreatic cancer	Ad5-yCD/ mutTKSR39rep-ADP	Ad5	oncolytic	cytosine deaminase, HSV-tk, ADP			I	chemotherapy	NCT02894944
Pancreatic adenocarcinoma, ovarian, biliary, colorectal cancer	LOAd703	Ad5F35	oncolytic	CD40L and 41BBL	eight times i.t.	1×10^{11} vp–1×10^{12} vp	I/II	standard chemotherapy	NCT03225989
Pancreatic cancer	LOAd703	Ad5F35	oncolytic	CD40L and 41BBL	six times i.t.	5×10^{10} vp–5×10^{11} vp	I/II	standard chemotherapy	NCT02705196
Neuroendocrine tumors	AdVince	Ad5	oncolytic		intrahepatic artery	1×10^{10} vp–1×10^{12} vp	I/II		NCT02749331
Locally advanced solid tumors	VCN-01	Ad5	oncolytic	PH20 hyaluronidase	single i.v.		I	Abraxane/gemcitabine	NCT02045602
HNSCC	VCN-01	Ad5	oncolytic	PH20 hyaluronidase	single i.v.	3.3×10^{12} vp–1×10^{13} vp	I	durvalumab	NCT03799744
Recurrent retinoblastoma	VCN-01	Ad5	oncolytic	PH20 hyaluronidase	single intravitreal	2×10^{9} vp–2×10^{11} vp	I		NCT03284268
Locally advanced rectal cancer	Enadenotucirev	Ad11p/Ad3	oncolytic		three to eight times	1×10^{12} vp–3×10^{12} vp	I	capecitabine and radiotherapy	NCT03916510
Advanced/metastatic epithelial tumors	NG-350A	Ad11p/Ad3	oncolytic	anti-CD40 antibody	single i.t. or three times i.v.		I		NCT03852511
Advanced/metastatic epithelial tumors	NG-641	Ad11p/Ad3	oncolytic	FAP-TAc, CXCL9, CXCL10, IFN-α	single i.t. or three times i.v.		I	chemotherapy, ICB	NCT04053283
Metastatic TNBC or NSCLC	ADV/HSV-tk	Ad5	oncolytic	HSV-tk	single i.t.	5×10^{11} vp	II	SBRT, pembrolizumab	NCT03004183
Colorectal, ovarian, appendiceal cancer	ONCOS-102	Ad5/3	oncolytic	GM-CSF	six times i.p.		I/II	durvalumab	NCT02963831
Prostate cancer	ONCOS-102	Ad5/3	oncolytic	GM-CSF	four or fewer times i.t.		I/II	DCVAC/Pca, cyclophosphamide	NCT03514836
Malignant pleural mesothelioma	ONCOS-102	Ad5/3	oncolytic	GM-CSF	four times i.t./two cycles		I/II	chemotherapy	NCT02879669
Melanoma	ONCOS-102	Ad5/3	oncolytic	GM-CSF	three to four times i.t.	3×10^{11} vp	I	cyclophosphamide, pebrolizumab	NCT03003676
Brain cancer	DNX-2401	Ad5	oncolytic		single i.t.	5×10^{8} vp–5×10^{10} vp	II	pembrolizumab	NCT02798406
Brainstem glioma	DNX-2401	Ad5	oncolytic		cerebellar peduncle		I		NCT03178032
Recurrent glioma	DNX-2401	Ad5	oncolytic		single intra-arterial		I	conventional surgery	NCT03896568
Glioblastoma	DNX-2440	Ad5	oncolytic	OX40L	single i.t.		I		NCT03714334

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Table 1. *Continued*

Cancer	Vector	Serotype	Vector Generation/ Purpose	Transgene	Administration	Dose	Phase	Combination Therapy	Identifier
HCC	H101	Ad5	oncolytic		hepatic artery		III	HAIC	NCT03780049
Melanoma	OBP-301 (telomelysin)	Ad5	oncolytic		13 or fewer times i.t.	1×10^{12} vp	II		NCT03190824
Recurrent prostate cancer	Ad-PSA	Ad5	vaccine	PSA	four times s.c., 30-day interval	1×10^{8} PFU	II	ADT	NCT00583752
Hormone refractory prostate cancer	Ad-PSA	Ad5	vaccine	PSA	three times s.c., 30-day interval	1×10^{8} PFU	II		NCT00583024
Advanced/metastatic solid tumors	AdMA3	Ad5	vaccine	MAGE-A3	single i.m. priming	1×10^{10} PFU	I/II	MG1MA3 (Maraba virus-MAGE-A3)	NCT02285816
NSCLC	Ad-MAGEA3	Ad5	vaccine	MAGE-A3	single i.m. priming	2×10^{11} vp	I/II	MG-MAGEA3, pembrolizumab	NCT02879760
Melanoma	Ad-MAGEA3	Ad5	vaccine	MAGE-A3	single i.m. priming		I	MG1-MAGEA3, cyclophosphamide, pembrolizumab	NCT03773744
HPV-associated cancers	Ad-E6E7	Ad5	vaccine	HPV E6/E7	single i.m. priming		I	MG1-E6E7, atezolizumab	NCT03618953
Colorectal cancer	Ad-CEA	Ad5	vaccine	CEA	once i.m./six cycles		II	standard chemotherapy	NCT03050814
Prostate cancer	ChAdOx1.5T4	chimpanzee Ad Ox1	vaccine	5T4	once or twice i.m.	2.5×10^{10} vp	I/II	MVA.5T4 (modified vaccinia), nivolumab	NCT03815942
Prostate cancer	ChAdOx1.5T4	chimpanzee Ad Ox1	vaccine	5T4	once i.m.		I	MVA.5T4 (modified vaccinia), cyclophosphamide	NCT02390063
Pancreatic cancer	ETBX-011	Ad5	vaccine	CEA			I/II	SBRT, chemotherapy, avelumab, bevacizumab, ALT-803 (IL-15), GI-4000, haNK	NCT03329248
Pancreatic cancer	ETBX-011	Ad5	vaccine	CEA			I/II	SBRT, chemotherapy, aldoxorubicine HCl, avelumab, bevacizumab, ALT-803 (IL-15), GI-4000, haNK	NCT03387098
TNBC	ETBX-011, ETBX-051, ETBX-061	Ad5	vaccine	CEA, brachyury, MUC1			I/II	SBRT, chemotherapy, aldoxorubicin HCl, avelumab, bevacizumab, ALT-803 (IL-15), GI-4000, GI-6207, GI-6301, haNK	NCT03387085
Squamous cell carcinoma	ETBX-011, ETBX-051, ETBX-061, ETBX-021	Ad5	vaccine	CEA, brachyury, MUC1, HER2			I/II	SBRT, chemotherapy, avelumab, bevacizumab, necitumumab, ALT-803 (IL-15), GI-4000, GI-6207, GI-6301, haNK	NCT03387111
Prostate cancer	ETBX-071, ETBX-061, ETBX-051	Ad5	vaccine	PSA, MUC1, brachyury	three times s.c./three cycles	5×10^{10} vp–5×10^{11} vp	I		NCT03481816
Prostate, lung, breast, colon cancer	ETBX-051, ETBX-061, ETBX-011	Ad5	vaccine	brachyury, MUC1, CEA	three times s.c./cycle		I		NCT03384316

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Table 1. *Continued*

Cancer	Vector	Serotype	Vector Generation/ Purpose	Transgene	Administration	Dose	Phase	Combination Therapy	Identifier
Localized prostate cancer	Ad5-SGE-REIC/Dkk3	Ad5	gene therapy	REIC/Dkk3	four or fewer times i.t.		I/II		NCT01931046
Malignant pleural mesothelioma	MTG201	Ad5	gene therapy	REIC/Dkk3	four times i.t.	3×10^{12} vp	II	nivolumab	NCT04013334
Recurrent HNSCC	Ad-P53	Ad5	gene therapy	P53	three times i.t./cycle		II	nivolumab	NCT02842125
Recurrent HNSCC	Ad-P53	Ad5	gene therapy	P53	three times i.t./cycle		II	nivolumab	NCT03544723
Recurrent HNSCC	Ad/PNP + fludarabine	Ad5	gene therapy	PNP	three times i.t./cycle		I/II		NCT03754933
Pleural mesothelioma, metastatic pleural effusions	BG00001	Ad5	gene therapy	IFN-β	twice intrapleurally	1.5×10^{12} vp–2×10^{12} vp	I		NCT00299962
Pediatric brain tumor	Ad-RTS-hIL-12	Ad5	gene therapy	IL12	single i.t.	2×10^{11} vp	I	veledimex	NCT03330197
GBM, oligoastrocytoma	Ad-RTS-hIL-12	Ad5	gene therapy	IL12	single i.t.	2×10^{11} vp–1×10^{12} vp	I	veledimex	NCT02026271
Prostate cancer	AdV/HSV-tk	Ad5	gene therapy	HSV-tk	single intraprostatic		I/II	brachytherapy	NCT01913106
HCC	AdV/HSV-tk	Ad5	gene therapy	HSV-tk	once i.p. + twice celiac/ superior mesenteric artery	1×10^{12} vp	III	liver transplantation	NCT03313596
Pancreatic adenocarcinoma	AdV/tk (GMCI)	Ad5	gene therapy	HSV-tk	i.t.		II	standard chemotherapy, radiation and surgery	NCT02446093
Lung cancer, metastatic uveal melanoma	ADV/HSV-tk	Ad5	gene therapy	HSV-tk	single i.t.	5×10^{11} vp	II	SBRT, nivolumab	NCT02831933
GBM, astrocytoma	ADV/HSV-tk	Ad5	gene therapy	HSV-tk	single i.t.		I/II	radiotherapy and standard chemotherapy	NCT03603405
GBM, astrocytoma	ADV/HSV-tk	Ad5	gene therapy	HSV-tk	single i.t.		I/II	radiotherapy	NCT03596086
Malignant pleural mesothelioma	rAD-IFN	Ad5	gene therapy	IFN-α	single intrapleural		III	chemotherapy	NCT03710876
Bladder cancer	rAd-IFN (INSTILADRIN)	Ad5	gene therapy	IFN-α	intravesical into bladder		III		NCT02773849
Glioma, GBM	Ad-hCMV-TK, Ad-hCMV-Flt3L	Ad5	gene therapy	HSV-tk, Flt3L	single peritumoral region post-resection	1×10^{9} vp–1×10^{11} vp (each)	I	standard chemotherapy and radiation	NCT01811992

hIL-12, human interleukin-12; HSV-tk, herpes simplex virus thymidine kinase; vp, viral particle; i.t., intratumoral(ly); 5-FC, 5-fluorocytosine; ADP, adenovirus death protein; i.v., intravenous(ly); HNSCC, head and neck squamous cell carcinoma; ICB, immune checkpoint blockade; FAP-TAc, fibroblast activation protein-targeting bispecific T cell activator; CXCL, chemokine ligand; IFN-α, interferon alpha; TNBC, triple-negative breast cancer; NSCLC, non-small cell lung cancer; SBRT, stereotactic body radiation therapy; GM-CSF, granulocyte-macrophage colony-stimulating factor; i.p., intraperitoneal(ly); DCVAC/Pca, autologous mature dendritic cells (DCs) pulsed with killed LNCaP prostate cancer cells; HCC, hepatocellular carcinoma; HAIC, hepatic artery infusion chemotherapy; PSA, prostate-specific antigen; PFU, plaque-forming unit; ADT, androgen deprivation therapy; s.c., subcutaneous(ly); i.m., intramuscular(ly); HPV, human papilloma virus; CEA, carcinoembryonic antigen; 5T4, oncofetal antigen 5T4; MUC1, mucin 1; REIC/Dkk3, reduced expression in immortalized cells/ dickkopf-3; PNP, *Escherichia coli* purine nucleoside phosphorylase; IFN-β, interferon beta; GBM, glioblastoma.

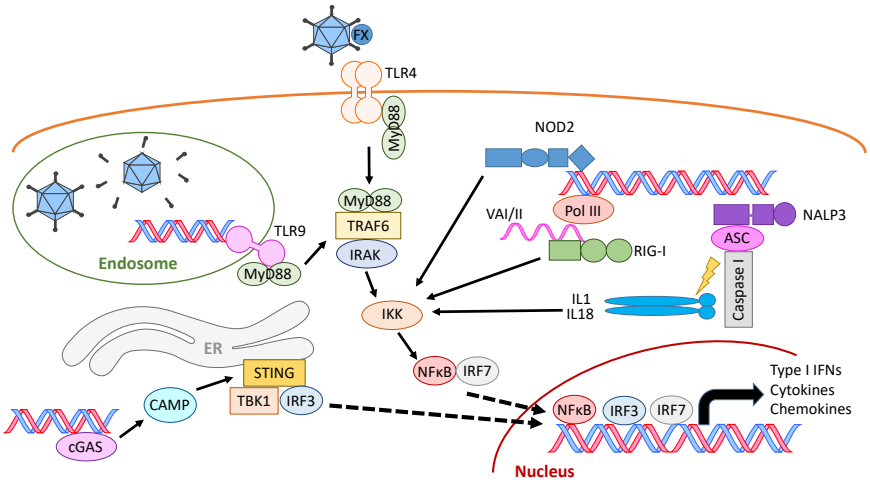

Figure 1. Pattern Recognition Receptors
The anti-adenoviral immune response is triggered by recognition of pathogen-associated molecular patterns (PAMPs) by PRRs. PAMPs are adenoviral capsid proteins or viral nucleic acids. PRR activation induces a signaling cascade that culminates in transcription factor (i.e., NF-κB, IRF3, IRF7)-mediated expression of type I interferons (IFNs), proinflammatory cytokines, and chemokines. Prior to viral entry, Toll-like receptor 4 (TLR4) recognizes the adenoviral capsid and blood coagulation factors complexed with the viral particle (in the murine system). After viral entry and viral uncoating within endosomes, TLR9 recognizes viral dsDNA. NOD-like receptors (NLRs) such as NOD2 and NALP3 recognize cytosolic dsDNA to activate the inflammasome. RIG-1 like receptors (RLRs) recognize cytosolic viral RNAs, including RNAs converted from cytosolic DNA through polymerase III, to induce type I IFNs. Cytosolic DNA is also recognized by the cyclic GMP-AMP synthase/stimulator of IFN genes (cGAS/STING) pathway to activate the NF-κB/IRF3/IRF7 signaling pathway.

initial interaction of Ad fiber and knob capsid proteins with the coxsackie and Ad receptor (CAR) (in the case of the most commonly used serotype 5 Ads[6]) and αv integrins,[7] respectively, induce a signaling cascade resulting in nuclear factor-κB (NF-κB)-mediated expression of chemokines and interleukin (IL)-1. Work in immunocompetent murine models has demonstrated that IL-1α is a key mediator of host antiviral immune responses after engagement of the Ad RGD motif with macrophage β_3 integrins.[7] After viral entry, PRRs such as Toll-like receptor (TLR)9[8] in endosomes, cytosolic sensors such as DNA-dependent activator of IFN-regulatory factors (DAIs),[9] or cytosolic inflammasome (NALP3)[10] recognize viral DNA and stimulate the IFN response.[11] This IFN response functions in an autocrine/paracrine manner to eliminate virions from within the cell[12] and block the replication of Ads by inhibiting E1A transcription.[13]

TLRs respond to PAMPS to induce an inflammatory immune cascade and are a class of PRRs expressed in immune cells such as macrophages and dendritic cells (DCs).[14] TLRs are located within the cellular membrane and within endosomes where they can detect viral capsid proteins (e.g., TLR4) or nucleic acids (e.g., TLR9). Since Ad entry is generally through endosomes, Ad dsDNA is recognized by endosomal TLR9, which detects unmethylated CpG motifs.[8,15,16] Activation of TLR9 results in myeloid differentiation primary response gene 88 (MyD88)-dependent signaling, together with IL-1R-associated kinase (IRAK)-1 and TNF receptor-associated factor 6 (TRAF6), which together activate transcription factors such as NF-κB.[17] This signaling cascade results in downstream expression of cytokines and chemokines to augment the innate immune response and induce the adaptive response.[18] Activation of TLR9 in plasmacytoid DCs causes IFN regulatory factor (IRF)7 transcription factor to complex with MyD88/TRAF6 in the cytoplasm. Following activation, IRF7 translocates to the nucleus, whereupon it induces expression of type I IFNs.[19,20] Doronin et al.[21] also demonstrated in the murine system that replication incompetent Ad vectors can induce IL-1β, IL-6, and MIP1α activation via TLR4/MyD88/TRAF6 signaling in a coagulation factor X (FX)-dependent manner. Contrastingly, human FX-bound Ad5 did not induce inflammatory cytokine expression through TLR4 in human monocytes, macrophages, or DCs.[22]

Another class of PRR are nucleotide-binding oligomerization domain-like receptors (NOD-like receptors [NLRs]). NLRs are cytosolic DNA sensors found in immune cells and others such as epithelial cells[23] and are important components of the inflammasome together with apoptosis speck protein (ASC) and caspases, which induce IL-1β and IL-18 via NF-κB signaling resulting in pro-inflammatory cell death or pyroptosis.[24] Muruve et al.[10] showed in a murine model that processed cytoplasmic Ad DNA activates NALP3 and, via ASC, drives caspase-dependent expression of IL-1β. Replication incompetent Ad vectors can also induce NALP3 and subsequent inflammatory cytokine expression not only in immune cells but also in human skin explants.[25] Another study confirmed that NOD2, an NLR that recognizes cytosolic muramyl dipeptide (MDP), contributes to the innate response to replication incompetent Ad independently of MyD88, as NOD2/MyD88 knockout mice showed further reduction in inflammatory cytokine production compared to either deficiency alone.[26]

RIG-1-like receptors (RLRs) are another family of PRRs that recognize cytoplasmic viral RNAs; Mda5 recognizes double-stranded RNA (dsRNA) while retinoic acid-inducible gene (RIG)-1 recognizes single-stranded RNA (ssRNA). Similar to TLRs, the RIG-1 signaling cascade activates NF-κB to mediate expression of type I IFNs.[27] RIG-1 can recognize small non-coding virus-associated (VA) RNAs expressed by Ads; VA RNA I (VAI) and VAII.[28] Another study showed that cytosolic Ad DNA can be converted into ssRNA through RNA polymerase III, and that this ssRNA activates the RIG-I pathway.[29]

Another mechanism by which cells induce anti-viral inflammatory responses to Ad infections is by the DNA sensing cyclic guanosine

monophosphate (GMP)-AMP synthase/stimulator of interferon genes (cGAS/STING) pathway, as demonstrated in murine endothelial cells and macrophages.[30] cGAS is a cytosolic protein expressed in both immune and non-immune cells.[30,31] cGAS binds dsDNA, after which it dimerizes to catalyze cyclic GMP-AMP (cGAMP), which in turn binds STING to activate the transcription factors NF-κB and IRF3/7, ultimately resulting in IFN-β production.[31] Lam et al.[30] demonstrated that the cGAS/STING pathway is activated in murine macrophage and endothelial cells following infection with a replication-deficient Ad vector, confirming that cGAS is a PRR to Ad. Lam and Falck-Pedersen[32] subsequently showed that the cGAS/STING pathway is activated after Ad infection in some human cancer cell lines, such as HeLa, but not in others, for example A549.

Ads not only induce robust innate responses but also elicit strong adaptive responses.[33] As mentioned above, most people have some level of preexisting immunity to Ads due to common ocular, respiratory, and gastric Ad infections. In the humoral response, neutralizing antibodies (NAbs) against Ad capsid proteins, mainly hexon,[34–36] are generated and result in serotype-specific reduction of Ad infectivity.[37] The presence of NAbs is a major impediment to systemically administered Ad-based gene therapies. In addition to the pre-existing anti-Ad humoral response, virus-specific T cells also play an important role in hindering Ad infections. Unlike NAbs, Ad-specific T cell responses can cross-react to different serotypes[38] and are mainly primed against conserved regions of hexon.[39] Additionally, it has been demonstrated *in vitro* that Ad-specific T cells are predominantly effector memory T cells and are therefore able to readily lyse cell-containing viral antigens.[40]

Thus, humans have evolved numerous mechanisms by which Ad infection can be cleared. Therefore, it is important to consider the anti-viral immune responses when designing Ad-based therapeutics, as Ad vectors can be readily eliminated. In most ongoing clinical trials for cancer, the Ad vectors used are based on Ad5 (Table 1). A recent clinical study in China confirmed that there is no difference in the seroprevalence of Ad5 in healthy individuals and cancer patients.[41] Due to the use of Ad5, most Ad vectors are directly injected into the tumor mass because intratumoral administration attenuates (1) the systemic circulation of Ad vectors, (2) its subsequent sequestering by Kupffer cells and hepatocytes in the liver, and (3) its inactivation by neutralizing antibodies or (4) the complement system.[4,42,43] Furthermore, this delivery method ensures that a high proportion of the vector dose is available to transduce target tumor cells with limited toxicity to normal tissue (e.g., liver).[44] However, one drawback of intratumoral injection is that the lytic effect is limited to the treated tumor sites. In Ad-based cancer therapy, the dogma until recently was to develop oncolytic Ad vectors designed to replicate within and lyse tumor cells as efficiently as possible to induce an anti-tumor effect before immune-mediated viral clearance. Recently, however, multiple investigators are exploring the possibility of harnessing the strong anti-Ad immune responses and co-opting them into anti-tumor immune responses.

Immune Responses to Adenoviral Vectors in Cancer

Similar to other types of oncolytic virotherapy, one of the biggest advantages of an oncolytic Ad (OAd) is that viral tumor cell lysis not only de-bulks the tumor, but it also elicits powerful anti-viral and anti-tumor immune responses. Indeed, the initial phase of tumor cell lysis releases tumor-associated antigens (TAAs), and the presence of the OAd recruits, stimulates, and matures professional antigen-presenting cells (APCs) via PRRs, thus facilitating epitope spreading.[45,46] This immune stimulation activates not only an anti-viral response, but also immune responses to TAAs, potentiating systemic anti-tumor responses against uninfected metastases. This abscopal effect was demonstrated by the first oncolytic virus to gain approval by the US Food and Drug Administration (FDA), an oncolytic herpes simplex virus expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) (talimogene laherparepvec [TVEC]), which reduced not only the treated tumor but also, in some cases, untreated tumor lesions.[47]

In fact, the way cancer cells die can be a source for stimulation of the adaptive immune response, a phenomenon called immunogenic cancer cell death (ICD).[48] ICD is defined as the stimulation of the suppressed anti-tumor immune system by exposure to damage-associated molecular patterns (DAMPs) in the tumor microenvironment (TME) that can induce anti-tumor immunological memory.[49] Viral induction of ICD promotes anti-tumor response via recruitment and activation of APCs and subsequent activation of T cells, resulting in long-lasting memory.[50] There is evidence that immunological memory responses against TAAs may be important for long-term anti-tumor responses in patients.[51,52] Furthermore, anti-tumor immunity is more important than direct oncolysis, as it allows for the generation of tumor-specific memory T cells. Li et al.[53] demonstrated in a Syrian hamster model that although OAd replication is enhanced in tumors depleted of T cells, the overall anti-tumor effect is diminished.

For ICD to occur, (1) an immune signal must be present (e.g., DAMPs and viral pathogen-associated molecular patterns [PAMPs]); (2) pro-inflammatory cytokines must stimulate and mature APCs to take up and present TAAs; and (3) cross-priming of cytolytic T cells. Key DAMPs include cell-surface calreticulin, extracellular ATP, and high mobility group box 1 (HMGBI).[54] While OAd induction of ICD has not yet been confirmed, OAds are known to induce anti-tumor adaptive immune responses.[55] Di Somma et al.[56] demonstrated that an OAd *dl*922-947 induces hallmarks of ICD, including surface calreticulin, HMGB1, and ATP release in infected human malignant pleural mesothelioma cell lines, resulting in tumor control and prolonged survival in xenograft models. Their data suggest that cancer cells themselves recognize Ad infection, thereby inducing pro-inflammatory cytokines. The group B OAd, enadenotucirev, also elicits exposure of calreticulin and release of ATP, HMGBI, and heat shock protein (HSP)70 and stimulates DCs and $CD4^+$ T cells in *in vitro* tumor models. Furthermore, enadenotucirev, which is currently being evaluated in a phase I clinical trial in locally advanced rectal cancer (ClinicalTrials.gov: NCT03916510) (Table 1), was shown to mediate

TNF-α, IL-6, and HMGB1 expression in human colorectal cancer biopsy *ex vivo* cultures.[57]

Since cGAS/STING is ubiquitously expressed in immune and non-immune cells, including cancer cells, recent reports showed that cGAS/STING in cancer cells could recognize viral infections by Ad,[32] Herpes simplex virus (HSV),[58] and vaccinia virus (VV),[59] leading to type I IFN expression. To trigger these responses, direct agonists of STING have garnered substantial interest as a therapeutic target. In a syngeneic murine melanoma model, intratumoral administration of synthetic cyclic dinucleotides (CDNs) were shown to activate STING and resulted in significant primary tumor regression, rejection of metastatic tumors, and the formation of immunological memory to reject tumor rechallenge.[60] In contrast to cGAS/STING, TLR9 expression is limited in immune cells (e.g., DCs), and these immune cells are infrequent within tumor sites. Thus, stimulation of the cGAS/STING pathway through oncolytic viruses (OVs), including OAd, may be a critical way to initiate IFN signaling in solid tumors, and its mechanisms should be elucidated further in future studies.

How we study the therapeutic benefit of Ad vectors for cancer immunotherapy is another important issue. Human cell-based *in vitro* studies provide a platform for mechanistic elucidation but cannot evaluate immune responses as a whole. The standard *in vivo* models have been human tumor xenografts into immunodeficient mice due to the limited replication capacity of human Ad-based vectors in murine cells. These models allow for evaluation of direct Ad oncolysis; however, the ability to study the induction of anti-tumor immunity through oncolysis as well as immunomodulatory molecules expressed by OAd ("Armed" OAd) is obviously lacking.

Another option is to use semi-permissive animal models for human Ads such as cotton rats[61] and Syrian hamsters.[62] While these animal models do allow for some amount of oncolytic replication and viral dissemination, the amount of infectious Ad released by rat and hamster cells is orders of magnitude reduced compared to human cells.[63] However, syngeneic tumor models in semi-permissive hosts allow for the evaluation of rodent immune responses.[64,65] One caveat is that these responses may not translate to humans because there are significant differences between mice (rodents) and humans in terms of immune system development, activation, and response to challenge in both the innate and adaptive arms.[66]

Currently the most advanced models are humanized mouse models reconstituting human innate and adaptive immune cells.[67,68] While these models are expensive, time-consuming, and difficult to generate in large numbers, they do allow for the use of human tumor cells with full Ad replication potential and the ability to study human immune responses to therapy *in vivo*.[69]

Ads as Onco-immunotherapies

While Ads can induce significant anti-Ad immune responses in healthy tissue, clinical trials have demonstrated that the immune responses induced by Ad infection are not capable of eradicating bulky or metastasized tumors, likely due to the highly immunosuppressive TME. Arming Ads with molecules to disrupt the TME and/or stimulate the suppressed immune cells can enhance tumor cell killing and induce systemic immunological memory.[70] To increase the efficacy of OAd therapy, Ads have been armed with a variety of immunostimulatory transgenes (cytokines, chemokines, co-stimulatory molecules) and immune-targeting molecules (bi-specific T cell engagers) to further activate both innate and adaptive immune cells.[71]

In order to increase recruitment of immune cells, Ad vectors can be modified to express ligands for innate immune receptors. OAd engineered with a CpG-rich motif into the vector backbone demonstrated a TLR9-dependent anti-tumor effect mediated by increased tumor $CD8^+$ T cell infiltration in a syngeneic B16-ovalbumin (OVA) model.[72] In contrast to utilizing specific receptor agonists, one study demonstrated that Ad-MyD88 overexpression in murine DCs results in a T helper 1 (Th1) inflammatory transcriptional signature and enhances adaptive immune responses *in vivo*. Ad-MyD88 also elicited natural killer (NK) and T cell-mediated systemic suppression of tumor growth in a syngeneic colorectal cancer murine model.[73]

Cytokines are important cell signaling molecules and immunomodulatory agents. TVEC, which has been approved by the FDA for the treatment of melanoma, is an oncolytic HSV that expresses the cytokine GM-CSF. GM-CSF promotes maturation of macrophages and DCs for antigen presentation and initiation of cellular immune responses. Building on this strategy, Pesonen and colleagues[74,75] have developed an OAd-expressing GM-CSF that is currently in phase I/II trials and has demonstrated induction of systemic $CD8^+$ T cell responses in patients with mesothelioma and ovarian cancer, among others. Another OAd-expressing GM-CSF, CG0070, is currently in a phase II clinical trial in patients with high-grade non-muscle invasive bladder cancer. An interim report from the trial demonstrated close to 50% complete response rate at 6 months.[76] Patients with advanced solid tumors treated with OAd-expressing GM-CSF demonstrated an increase in survivin-specific T cells in all patients analyzed, suggesting that OAd-GM-CSF generated systemic anti-tumor responses.[77] Another clinical trial currently underway is evaluating the combination of two replication-deficient Ad vectors expressing HSV-thymidine kinase (tk) and Flt3L in glioma patients. Ad-HSV-tk results in the death of infected tumor cells when the prodrug ganciclovir is given, releasing TAAs to be taken up and cross-presented by DCs. Flt3L is a potent inducer of DCs, allowing them to infiltrate and expand within the TME[78] When combined, Ad-HSV-tk and Ad-Flt3L eradicated tumors in a murine syngeneic model of glioblastoma.[79] Preliminary results of the current phase I trial suggest tumor infiltration by inflammatory cells and a potentially significant survival advantage (P.R. Lowenstein et al., 2019, J. Clin. Ecol., abstract).

Numerous other cytokine-armed Ads are being investigated in preclinical and clinical studies.[80] Of particular interest are those exploring the use of IL-12, a cytokine produced by APCs to stimulate NK and T cells. Ads armed with IL-12 have shown an enhanced anti-

tumor effect and immune stimulation in murine[81] and Syrian hamster models,[82] and in clinical trials.[83] Oncolytic and replication-deficient Ad vectors expressing IL-12 are currently in phase I clinical trials for prostate and pancreatic cancer, pediatric brain tumors, glioblastomas (GBMs), and oligoastrocytomas (ClinicalTrials.gov: NCT02555397, NCT03281382, NCT03330197, NCT02026271).

In addition to cytokines, Ads expressing chemokines can promote APCs and cytotoxic immune infiltration into tumors. In murine models of mammary adenocarcinoma and lymphoma, Ad-RANTES-E1A eradicated established tumors and inhibited metastases by recruiting DCs, macrophages, NK cells, and $CD8^+$ T cells into the immunologically "cold" tumors.[84]

The TME is complex and can have varying degrees of immunosuppression. TMEs can be broadly assigned to three classes according to the presence or absence of immune infiltrate.[85] Tumors can lack immune infiltrate (cold tumors) or can have immune cells within the tumor mass or even contain tertiary lymphoid structures, but due to inhibitory signals they cannot function ("hot" or "warm" tumors). Therefore, simply increasing the infiltration of APCs and/or cytotoxic cells into the tumor may not be enough to have an efficacious immune-mediated eradication of the tumor.

Several strategies to overcome this obstacle are being explored. The first is to utilize Ad vectors to express costimulatory molecules in infected tumor cells. CD40L promotes DC maturation and Th1 immune responses[86] and has shown promise both in preclinical models[87] and clinical trials. Indeed, in one clinical trial three out of five bladder cancer patients showed no detectable tumor cells in the bladder following treatment with replication defective Ad-CD40L, although disseminated malignant cells could still be found.[88,89] To further enhance the stimulatory effect of Ad-CD40L, a new OAd (LOAd703) has been developed to co-express CD40L and 41BBL, another costimulatory molecule. T cell costimulatory molecules are required for full activation of after the T cell receptor (TCR) is triggered by major histocompatibility complex (MHC)-presented peptides,[90] and 41BB signaling has been implicated in promoting effector CD8 T cell survival and tumor clearance.[91] LOAd703 is currently in phase I/II clinical trials in pancreatic and ovarian cancer patients (ClinicalTrials.gov: NCT02894944, NCT02705196). Using armed OAds to combine direct lysis of tumors cells, and subsequent release of TAAs, with expression of immunostimulatory transgenes may aid in the induction of tumor-specific immune responses. Results of a phase I trial in patients with advanced solid tumors show an increase in survivin (TAA)-specific T cell responses in four out of eight evaluable patients after treatment with an OAd-expressing CD40L.[92]

Due to their tumor selectivity and immunogenicity, OAds function as *in situ* cancer vaccines. As such, developing therapeutic viral cancer vaccines is one area of intense research. Notably, robust anti-Ad immune responses may hinder Ad-based vaccines in some cases, as occurred in the HIV STEP trial. During this trial, an Ad5-based vector expressing HIV-1 gag/pol/nef did not prevent HIV-1 infection; instead, Ad5-seropositive men treated with the vaccine showed a higher incidence of HIV infection compared to placebo recipients.[93] The immune response to the Ad vector may have been stronger than anti-HIV development, resulting in reduced efficacy. As Ad vaccine can induce robust anti-Ad immune responses, subsequent administration of a distinct virus that is not cleared by the anti-Ad response may be required for effective vaccination.

In Syrian hamster models of pancreatic and kidney cancer, a treatment protocol of three low doses of OAd followed by three low doses of VV resulted in tumor clearance in 62% of animals. However, reversing the order of the viruses or depleting the $CD3^+$ T cells reduced the efficacy.[94] In a Syrian hamster model of pancreatic ductal adenocarcinoma (PDAC), OAd-expressing oncostatin M (OSM), an immunostimulatory cytokine, caused severe toxicity. However, when the dose was decreased and combined with a subsequent administration of an oncolytic Newcastle disease virus (NDV) also expressing OSM, treatment significantly reduced tumors with an improved safety profile.[95] In a similar cancer vaccine regimen, prime-boost vaccination, an Ad vector expressing a tumor antigen is given to a patient before a second distinct virus also expressing TAAs. For example, one report showed T cell-mediated tumor regression and protection from re-challenge following a replication-defective Ad vector expressing MAGEA3 given prior to a Maraba virus (MG1) booster that also expressed MAGEA3. Ad inoculation was vital to the success of this regimen in a murine prostate adenocarcinoma syngeneic model, as without it the anti-MG1 response predominated, negating the anti-tumor response.[96] This approach has been studied for human papilloma virus (HPV) antigens[97] and is currently undergoing phase I/II clinical trials (ClinicalTrials.gov: NCT02285816, NCT02879760, NCT03773744, NCT03618953) (Table 1).

As discussed above, ICD induced by OVs can promote anti-tumor responses by activating tumor-directed T cells that can kill tumor cells bearing MHC-presented peptide and stimulate a memory response important for long-term tumor control.[50] However, most TAAs are self-antigens and therefore less immunogenic. Additionally, tumor cells often downregulate MHC expression and the ability to present antigens in a way that exposes them to TCR recognition. Recently, Niemann et al.[98] engineered a bifunctional adaptor molecule made up of a single-chain variable fragment (scFv) targeting a tumor-specific surface antigen (polysialic acid) linked to the Ad5 hexon DE1 domain, which can bind anti-Ad-specific antibodies and retarget them to tumor cells. Systemic infusion of the adaptor molecule in Ad-immunized mice reduced tumor burden, and this anti-tumor activity was mediated by NK and $CD8^+$ T cells. When combined with OAd therapy, this adaptor molecule further enhanced OAd anti-tumor efficacy.

Bispecific T cell engagers (BiTEs) are engineered molecules made up of a CD3 scFv linked to an scFv to a tumor-specific antigen. In the presence of these molecules, tumor-infiltrating T cells can target antigens expressed on cancer cells even in the absence of MHC.

The FDA approved a CD19-directed BiTE for the treatment of relapsed or refractory B-ALL,[99] and OAd-expressing BiTE molecules are currently being investigated. A BiTE molecule generated *in situ* from an oncolytic vector has the advantages of being constantly produced in the tumor area, thus avoiding the burden, potentially as well as the toxicity, of constant infusions of the BiTE molecule into patients. OAd-expressing EGFR.BiTE demonstrated infiltration and proliferation of adoptively transferred T cells in subcutaneous colorectal murine models,[100] an effect that was further enhanced by administration of chimeric antigen receptor-modified T (CAR-T) cells targeting folate receptor alpha (FR-α).[101]

Recently, the field of oncolytic virotherapy has shifted from engineering vectors with enhanced lytic effects to vectors that take advantage of the viral immune response and even further potentiate strong immune responses. The key will be to find the balance between triggering an inflammatory response against the tumor and avoiding anti-viral immunity that would clear virus and reduce the lytic effect. However, as Li et al.[53] demonstrated, the balance should tip in favor of the anti-tumor immune response, as it is more important than direct viral-mediated oncolysis.

Concluding Remarks and Future Outlook

Despite some successes, in recent years it has become clear that oncolytic virotherapy as a monotherapy may be insufficient to cure advanced metastatic tumors. According to Peters, Grandi, and Nigim in the Cancers Molecular Therapy 2018 Meeting report, the biggest shift in the OV field is success of immune checkpoint blockade (ICB). As we have discussed, Ad vectors can recruit and activate immune responses within tumors. As we discussed above, OAds (as well as other OVs) can turn cold tumors hot, and maintaining these active responses can be achieved in part by inhibiting the tumor-mediated suppression of immune effector cells via antibodies against these immune checkpoints such as programmed death receptor 1 (PD-1), programmed death ligand 1 (PDL1), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). Combining ICB with OAd therapy could result in synergistic anti-tumor effects, with the additional potential to enhance the safety profile of Ad treatment regimens, since lower doses of viral vectors could be used due to the amplified therapeutic response. Preclinical data combining OAds with ICB are promising[102–104] and clinical trials investigating combinatorial treatments are currently ongoing (Table 1). Combination of ONCOS-102 (OAd-GM-SCF), which induced $CD8^+$ T cell tumor infiltration in a phase I clinical trial (ClinicalTrials.gov: NCT01598129), and pembrolizumab (anti-PD-1) showed synergistic anti-tumor effects in a humanized murine melanoma model.[105]

ICB requires the presence of adaptive tumor-directed T cells, which can be excluded from immunologically cold tumors. Adoptively transferred CAR-T cells can systemically target and kill targeted cells (turning cold tumors to warm/hot tumors), but, similar to endogenous T cells, they are still susceptible to inhibitory effects of tumors, including immune checkpoints. We have shown that our oncolytic Ad vector platform locally expressing anti-PDL1 mini-antibody (CAd*PDL1*) synergizes with adoptively transferred HER2-specific CAR-T cells in ovarian and prostate xenograft models and that anti-PDL1 expressed locally from the infected tumor caused less toxicity than did systemically administered anti-PDL1 antibody.[106] Evaluating this regimen compared to systemic ICB in a preclinical model (i.e., humanized mice) and potentially in clinical trials will be valuable to address not only safety, but also whether systemic ICB functions primarily at the tumor site or within lymph nodes during OAd-mediated adaptive immune developments. Furthermore, we showed that addition of IL-12p70 expression into our system (CAd*IL12_PDL1*) combined with HER2-specific CAR-T cells was able to control both primary and metastasized tumors, resulting in 100% survival at 120 days compared to median survival of only 13 days for untreated animals in an orthotopic xenograft model of head and neck squamous cell carcinoma.[107] These studies validate that combination of OAd with CAR-T cells is warranted for further evaluation of efficacy and safety in clinical studies. As evidenced by the numerous ongoing clinical trials using Ad vectors as therapeutic platforms for cancer treatment, Ad-mediated onco-immunotherapy is a promising modality for combating human cancers.

AUTHOR CONTRIBUTIONS

A.R.S. and M.S. conceived of and wrote the manuscript and approved it for publication.

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