Human adenovirus type 26 basic biology and its usage as vaccine vector

Running head: Human adenovirus type 26 as vaccine vector

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Summary

Due to their nature, adenoviruses have been recognized as promising candidates for vaccine vector development. Since they mimic natural infection, recombinant adenovirus vectors have been proven as ideal shuttles to deliver foreign proteins aiming at inducing both humoral and cellular immune response. In addition, a potent adjuvant effect can be exerted due to the adenovirus inherent stimulation of various elements of innate and adaptive immunity. Due to its low seroprevalence in humans as well as induction of favourable immune response to transgene, human adenovirus type 26 (HAdV-D26) has been recognized as a promising platform for vaccine vector development and is studied in number of completed or ongoing clinical studies. Very recently HAdV-D26 based Ebola and Covid-19 vaccines were approved for medical use. In this review, current state of the art regarding HAdV-D26 usage as vaccine vector will be discussed.

1. Introduction

Adenovirus (AdV)-based vectors are currently leading vectors used in cancer gene therapy and vaccination clinical trials (https://clinicaltrials.gov/). AdV-based vectors are being investigated as vaccines targeting a broad range of pathogens and are particularly used in disease areas where classical vaccination strategies have proven difficult, technically impossible or in the area of infectious diseases where protection is associated with cellular responses. Adenovirus vectored vaccines are capable of inducing cross-protective immunity and activate T cell response, thus may be robust enough to induce protective immunity in the elderly and immunocompromised persons. In addition, adenovirus vector platform allows design of vaccine against pathogen whose biology is poorly understood. Very important milestone has been achieved when European Medicines Agency recently approved three AdV-based vector vaccines, namely Ad26.ZEBOV [1] against Ebola and Ad26.COV2.S [2] against Covid-19, both based on HAdV26, and ChAdOx1-S [3] against Covid-19, based on simian adenovirus.

2. Adenoviruses at glance

Human adenoviruses (HAdVs) are non-enveloped double stranded DNA viruses with icosahedral capsid of approximately 90 nm in diameter and mass of 150 megadaltons [4]. Major building blocks of HAdV capsid are hexon and penton. There are 240 copies of the hexon trimer, and 12 pentons comprising extended fiber protein non-covalently attached to the penton base protein [5]. Today, we distinguish between 104 human types classified into 7 groups from A-G, according to hemagglutination and serum neutralization reactions, or by genomic sequencing and bioinformatics [6]. Naturally present adenoviruses can cause mild health problems like acute respiratory, gastrointestinal and ocular infections, but have no oncogenic potential in humans. Due to the good knowledge of their molecular biology, HAdVs have been recognized as favourable vectors for gene transfer. Besides being vectors for gene transfer, HAdVs are used also as vectors for vaccination. Currently, there are many active clinical studies investigating usage of adenovirus vectors in vaccination approach for treating both infectious diseases and cancer (https://clinicaltrials.gov/).

Adenovirus infection starts with binding to cellular receptors usually via the knob portion of the fiber protein. Receptors that mediate cellular attachment of human adenoviruses have been reviewed recently [7]. The Coxsackie and Adenovirus Receptor (CAR) is responsible for the attachment of all adenovirus types, for most of them as a primary receptor, except those from group B. Members of group B can use CD46, which is expressed both apically and basolaterally on cells, or desmoglein-2 (DSG2) as a receptor. Molecules used as receptors by group D adenoviruses are less well defined and include CAR, CD46 and sialic acid. After the initial interaction with the primary receptor, internalization of viral particle proceeds via endocytosis triggered by binding of an exposed RGD motif on the adenovirus penton base to αv integrins (αvβ3, αvβ5, αvβ1, α5β1, and α3β1) at the cell surface [8-10]. In the case of human adenovirus type 5 (HAdV-C5), uptake occurs via dynamin and clathrin-dependent receptor-mediated endocytosis [11], while HAdVs belonging to group B use macropinocytosis as an infectious pathway [12, 13]. It has been reported that HAdVs can also use lipid rafts and caveolae as routes of entry in plasmocytic cell lines [14] and human corneal cells [15]. Following liberation from the endosome, human adenovirus encounters complex networks of organelles and proteins in cytoplasm which impair diffusive mobility. For this reason, adenovirus intracellular trafficking cannot rely on simple diffusion, but rather employs active transport mechanisms. By interacting with cytoplasmic dynein [16] or kinesin [17] and microtubules, adenovirus moves toward the nucleus, where the capsid docks to the nuclear pore complex (NPC) protein Nup214, and attaches the highly mobile nuclear histone H1 to acidic clusters of the major capsid protein hexon [18]. After entering the nucleus AdV DNA persist episomal without integration into the host genome. Adenoviral genes are divided into early and late. Early genes include six transcription units: E1A, E1B, E2A, E2B, E3, and E4, whose protein products allow AdV DNA to replicate. Late genes (L1-5) encode structural AdV proteins as well as proteins involved in the assembly of new adenoviral particles. In addition to the early and late genes, there are also intermediate genes (IVa2, IX, VAI, VAII) whose products help in the transcription of late genes and the assembly of new adenoviral particles [19]. Representative scheme of adenovirus cell entry pathway is depicted on figure 1.

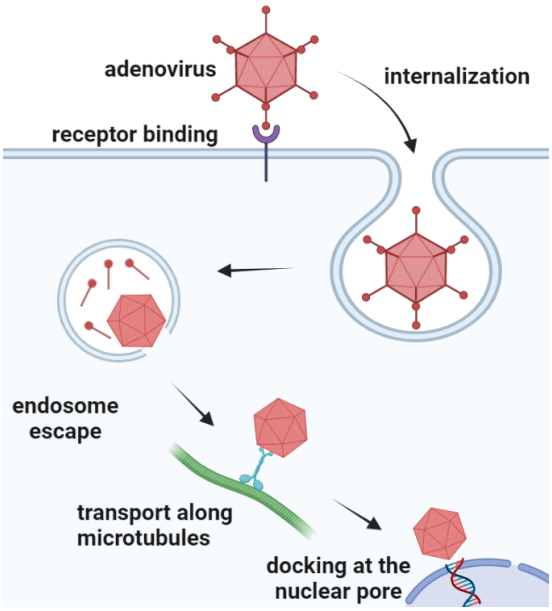

Figure 1. Scheme of adenovirus cell entry. After binding to the corresponding receptor, adenovirus enters the cell by using process of endocytosis. After being engulfed within endocytic vesicle, adenovirus escapes from the endosome, attaches to the microtubules and translocate to the nucleus. After docking to the nucleus, adenovirus DNA enters the nucleus where is stay episomaly. Created in BioRender.com.

Due to the extensive knowledge of their molecular biology as well as development of many methods for manipulating the viral genome, adenoviruses became attractive candidates for vector design, both vectors for gene transfer and vaccine vectors. To create space for inserting foreign DNA, for example antigen of interest, one can literally delete almost the entire genome of adenovirus and obtain a recombinant AdV vector that can accommodate up to 37 kb of a foreign DNA. One differentiates between conditionally replicative AdV vectors, also known as oncolytic AdV, and replication incompetent AdV, which include first- and second-generation AdV vectors that lack one or more early AdV genes, and high-capacity AdV vectors. State-of-the-art regarding human adenovirus vectorology has been reviewed recently [20].

Adenoviruses are highly immunogenic and following infection can induce both innate and adaptive immune responses in mammalian hosts. Host immune system responds to the presence of adenovirus capsid, DNA or infection itself which are recognized as a pathogen-associated molecular pattern (PAMPs). Adenovirus related PAMPs can be recognized during almost all steps of adenovirus infection pathway from binding and endocytosis to intracellular trafficking. Adenovirus infection induces production of numerous chemokines and cytokines that modulate the initiation of inflammation. In addition, responses to adenovirus vectors can be induced as a consequence of interactions with the cell surface receptors. For example, interaction of HAdV-C5 fiber with CAR promotes transcription of the chemokines interleukin-8, GRO (growth-

regulated oncogene) -α, GRO (growth-regulated oncogene) -γ, RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted), and interferon-inducible protein 10 [21]. Since virtually every individual will be infected by adenovirus at some point in their life, often at an early age, most populations display pre-existing immunity to the most common adenovirus types. As a consequence, the prevalence of neutralizing antibodies against common types, for example HAdV-C5, is high in humans [22-24]. Adenovirus neutralizing antibodies are mostly targeted against the surface loops of the hexon capsid protein, but also against the penton base and fiber knob [25-27]. In the context of adenoviruses being used as vaccine vectors, vector-specific antibodies may impede the induction of immune responses to the vaccine-encoded antigens, as they may reduce the dose and time of exposure of the target cells to the vaccinated antigens. These features have forced the development of new strategies, including the search for other types of adenoviruses that occur at low prevalence in human populations. Among different rare human adenoviruses, HAdV-D26 has been proven to be a very promising candidate. On the other hand, induction of innate immune may be advantageous for adenovirus based vaccine vectors by providing the characteristics of a natural adjuvant. Mode of action of adenovirus based vaccine vector is shown on figure 2.

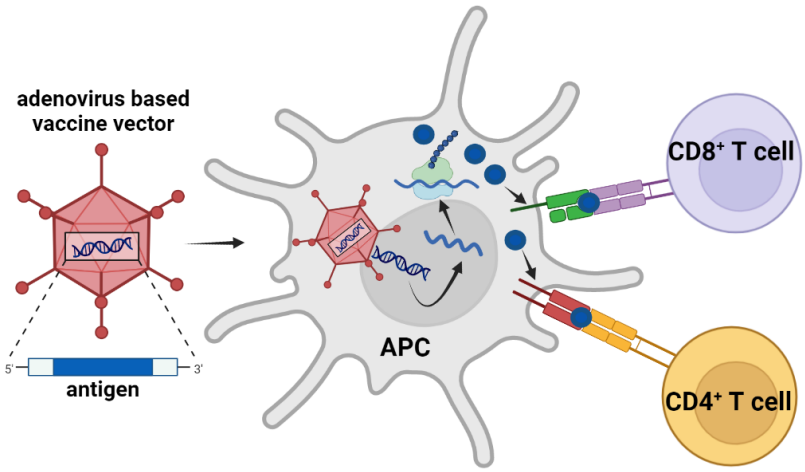

Figure 2. Mode of action of adenovirus based vaccine vector. After adenovirus based vaccine vector encoding for the antigen of interest infect the cell, synthesis of antigen (dark blue dot) begins. Infected dendritic cell displays antigen of interest on its surface. Subsequently, this antigen can be presented to $CD4^+$ and $CD8^+$ T cells, ultimately leading to the formation of specific cytotoxic T lymphocytes and antibody-secreting plasma cells, but also antigen-specific T and B. Created in BioRender.com.

3. Human adenovirus type 26 basic biology

Data describing HAdV-D26 basic biology, namely receptor usage and intracellular trafficking are still under debate. HAdV-D26 was firstly isolated from an anal specimen

collected from a 9-months-old child [28], however, the exact tropism of this virus is still unknown. There are several reports describing HAdV-D26 receptor/s, with rather ambiguous conclusions. By using B16F10-CD46 cell clones (murine melanoma cell line stably transfected with CD46) it was reported that adenoviruses from group D, namely HAdV-D26, human adenovirus type 48 (HAdV-D48) and type 49 (HAdV-D49) can use CD46 to facilitate cellular entry. The HAdV vectors from group D, however, appeared less efficient than those from group B at transducing B16F10-CD46 cells. The authors did not exclude the possibility that the HAdV vectors from group D, including HAdV-D26, may also utilize other receptors in addition to CD46 [29]. It was shown that HAdV-D26 transduction in human peripheral blood mononuclear cells is CD46-dependent and is efficiently blocked by anti-CD46 but not anti-CAR antibodies, demonstrating that HAdV-D26 utilizes CD46 as a primary cellular receptor in those cells [30]. When CHO-CAR (Chinese hamster ovary cells stably transfected with CAR) cells were infected at higher dose HAdV-D26 showed comparable infectivity to HAdV-C5 indicting that HAdV-D26 might use CAR for cell entry. However, the more pronounced transduction of CAR-negative cells at high vector doses and upon prolonged incubation indicated that HAdV-D26 more readily enters cells upon binding to alternative receptors, such as integrins [31]. Structural and biological analysis of the receptor binding fiber-knob protein of HAdV-D26, reporting crystal structures, and modelling putative interactions with CD46 and CAR provided evidence of a low affinity interaction with CAR, suggesting affinity is attenuated through extended, semi-flexible loop structures, providing steric hindrance. Conversely, *in silico* and *in vitro* experiments were unable to provide evidence of interaction between HAdV-D26 fiber-knob with CD46 [32]. Later on, same authors established sialic acid as a primary entry receptor used by HAdV26. They demonstrated that the removal of cell surface sialic acid inhibits HAdV-D26 infection, and provided a high-resolution crystal structure of HAdV-D26 fiber-knob in complex with sialic acid [33]. More recent study proposed that in human epithelial cells HAdV-D26 engage CD46 through a nonconventional interaction involving the hexon instead of the fiber [34]. It has been reported that HAdV-D26 can functionally interact with CD46 for *in vitro* and *in vivo* infection when CD46 is ectopically expressed in cells or in mice, underlying use of CD46 by HAdV-D26 under certain situations [35]. Another molecule has been proposed as a facilitator for HAdV-D26 entry into the macrophages. Namely, it has been shown that scavenger receptor SR-A6 allows binding and entry of HAdV26 in murine alveolar macrophage-like MPI cells [36].

The more pronounced transduction of CAR-negative cells at high vector doses and upon prolonged incubation suggested that HAdV-D26 more readily enters cells upon binding to alternative receptors, such as integrins [31]. Role of integrins in HAdV-D26 cell entry was examined also by Nestić et al [37]. By performing different gain- and loss-of-function studies, we found that αvβ3 integrin is required for efficient infection of epithelial cells by HAdV-D26, while CAR and CD46 did not influence the transduction efficiency of HAdV-D26. Additionally, we observed that HAdV-D26 colocalizes with αvβ3 integrin and that increased αvβ3 integrin enhances internalization of HAdV-D26, thus leading us to conclude that HAdV-D26 uses αvβ3 integrin as a receptor for infecting epithelial cells

[37]. Resume of the molecules that have been reported as HAdV-D26 receptor, as well as cells that were used as models in corresponding experiments, is shown in Figure 3.

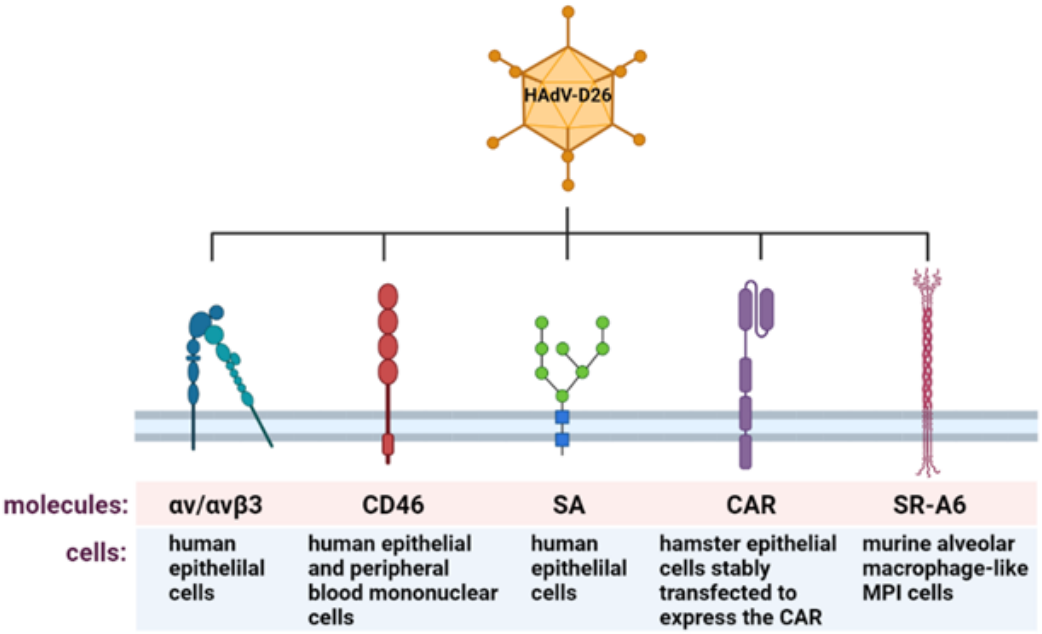

Figure 3. Illustration of receptors and molecules involved in HAdV-D26 binding and/or cell entry. This presentation is summarized from studies reporting *in vitro* analysis of HAdV-D26 receptor usage. αv/αvβ3, αv/αvβ3 integrin [37]; CD46, membrane cofactor protein [29, 30, 34]; SA, sialic acid [33]; CAR, coxsackie and adenovirus receptor [31]; SR-A6, scavenger receptor [36]. Created in BioRender.com.

It is clear that adenovirus intracellular trafficking is determined by the initial interaction with the receptor [38]. There is only one report regarding HAdV-D26 intracellular trafficking. It has been shown that while HAdV-C5 traffics rapidly to the nucleus following infection, HAdV type 35 (HAdV-B35) and HAdV-D26 accumulate in late endosomes between 2-8 hours post-infection. Authors also reported that innate immune cytokine elicitation by all HAdV types was abrogated by blockade of endosomal acidification, Cathepsin B and Caspase-1. This indicatates that virus interactions with acid-dependent sensors, such as Toll-like receptor- and cathepsin-dependent inflammasome activation in late endosomes, may trigger innate immunity. It was suggested that a mechanism by which AdV vectors from various types differentially trigger innate antiviral pathways involves distinct intracellular trafficking to late endosomes [39]. By studying HAdV uptake and induction of innate response in human phagocytes very recently was shown that lactoferrin binds HAdV-D26 with affinities in the micromolar range and retargets it to Toll-like receptor 4 complexes [40]. From earlier studies it was known that HAdV-D26 does not bind coagulation factor(F)X [41].

In order to understand better the molecular basis governing distinct biological properties of HAdV-D26, its structure at 3.7 Å resolution by cryo-electron microscopy has been determined. The amino acid sequence identity of various corresponding capsid proteins in HAdV-D26 and HAdV-C5 varies between 47 and 77%. Despite the sequence differences and the fact that they come from different groups (C and D), the overall structure and the organization of the HAdV-D26 capsid are mostly similar to those of HAdV-C5. The most obvious difference between HAdV-C5 and HAdV-D26 is seen in fiber protein. HAdV-D26 has a relatively short fiber with 7 to 8 shaft repeats, compared to ~22 repeats in the case of HAdV-C5 [42]. HAdV-D26 fiber length, i.e. shortness, could be the reason for its rather ambiguous receptor usage.

4. Human adenovirus type 26 as a vaccine vector

Main reason why HAdV-D26 became investigated as a vaccine vector was low frequency of HAdV-D26 neutralizing antibodies in various populations compared to HAdV-C5 [43], i.e. its low seroprevalence. Construction of recombinant vector based on HAdV-D26 aimed at vaccination was reported almost 15 years ago. HAdV-D26 vector proved to be the most immunogenic among the rare type recombinant HAdV vectors studied and one of its advantages is that is can be grown to high titers in HAdV-C5 E1-complementing cell lines [29].

HAdV-D26 elicits broad and diverse antigen-specific humoral and cellular immune responses in humans, can be used repeatedly and the humoral immune responses could be boosted in the face of anti-vector immunity. Vaccination of rhesus monkeys with HAdV-D26 induced substantially higher levels of antiviral and proinflammatory cytokines than vaccination with HAdV-C5 on day 1 following immunization. These differences in innate triggering result in markedly different immunologic milieus for the subsequent generation of adaptive immune responses by HAdV-D26-based vaccine vectors [44]. Additionally, while memory T cells elicited by HAdV5 vectors are high in magnitude, they exhibit functional exhaustion and decreased anamnestic potential following secondary antigen challenge compared with HAdV-D26 vectors [45].

Vaccines are sensitive biological substances that can lose their potency and effectiveness if not handled as recommended by the manufacturer. The stability profile of a vaccine has important implications for storage, cold chain management and field deployment. Due to its stability HAdV-D26-based vaccine vectors could be a vaccine of choice where cold chain maintenance is challenging owing to infrastructure and resource limitations [46].

Adenovirus-based vectors are investigated as vaccines targeting viral, bacterial, and protozoan pathogens. HAdV-D26-based vaccine vectors which have been evaluated as interventions against diseases caused by human immunodeficiency virus (HIV), respiratory syncytial virus (RSV), ebola virus, zika virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Graphical summary of the current state of the

art regarding these vaccines is shown in Figure 4. Detailed overview will be given in the following chapters.

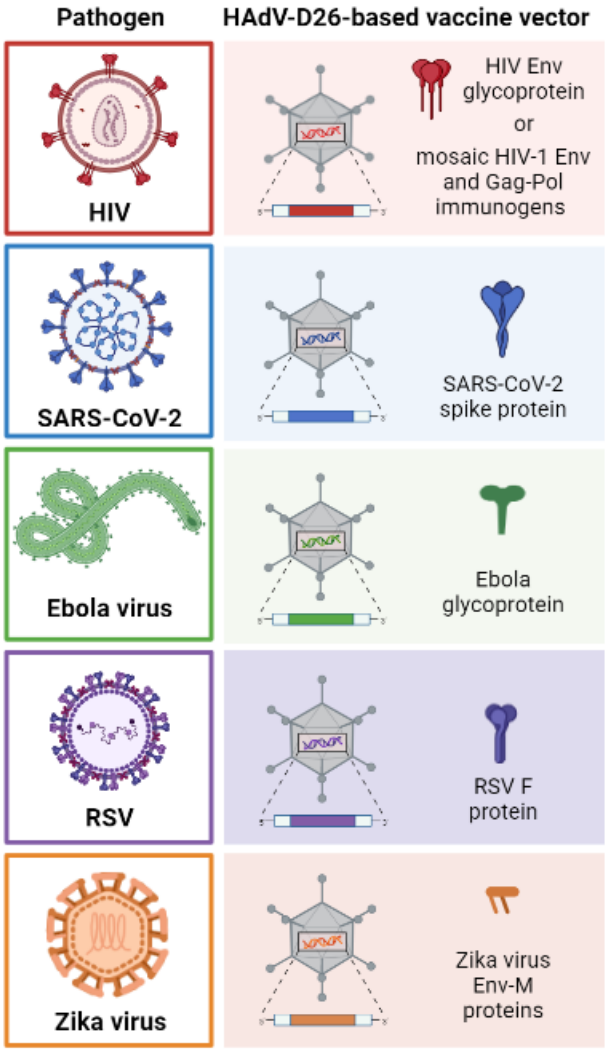

Figure 4. Summary of the HAdV-D26-based vaccine vectors. On the right hand of the panel antigens used in the corresponding vaccine vector are presented. Created in BioRender.com.

4.1 HAdV-D26 HIV vaccine

A first-in-human evaluation of the safety and immunogenicity of a recombinant HAdV-D26-based vaccine expressing clade A HIV-1 envelope protein (Env) demonstrated that this vector elicited broad and diverse antigen-specific humoral and cellular immune responses in humans [47, 48]. Single intramuscular administration of HAdV-D26-vectored HIV-1 Env vaccine induced both systemic and mucosal immune responses in humans. Induction of antigen-specific humoral and cellular mucosal immunity was not accompanied by a detectable increase in mucosal inflammation [49].

Robust protection against acquisition of neutralization-resistant virus challenges in rhesus monkeys has been demonstrated by HAdV26/Env vaccines, i.e. HAdV-D26-based vector expressing SIVsmE543 Env, Gag, and Pol priming followed by purified Env glycoprotein boosting [50]. It has been also shown that IgG and IgA responses following intramuscular immunization of rhesus monkeys with HAdV26/Env regimens were correlated in terms of the magnitude of the responses and in terms of the antibody specificities against HIV-1 epitopes, both in peripheral blood and mucosal compartments [51]. Data obtained by HAdV-D26-based HIV-1 Env vaccine were clearly different from previously conducted STEP study (also known as HVTN 502 and Merck V520-023) with HAdV-C5-based vaccine [52] indicating that HAdV-D26 has the advantage over HAdV-C5 when vaccination against HIV-1 is considered.

In order to increase the breadth of HIV vaccine-elicited immunity, optimised bivalent global mosaic antigens have been designed bioinformatically. HAdV-D26 vectors expressing mosaic HIV-1 Env and Gag-Pol immunogens, HAdV26.Mos.HIV, induced robust immune responses in humans and rhesus monkeys [53]. Preclinical data obtained in rhesus monkeys have also shown partial protective efficacy of the short regimens of a mosaic HAdV26.Mos.HIV prophylactic vaccine combined with aluminium phosphate-adjuvanted clade C gp140 protein. Such shortened regimens would be valuable to increase vaccine delivery at the community level, particularly in resource-limited settings [54]. Further work aimed at enhancing the breadth of the HIV-1 vaccine response has led to development of HAdV-D26-based tetravalent vaccine candidate, HAdV26.Mos4.HIV, which was generally safe, well-tolerated, and found to elicit higher immune responses than the trivalent regimen. These immune responses persisted until the end of follow-up at 72 weeks [55].

Live, attenuated HAdV-D26-based vectors that express HIV-1 antigens have been investigated in order to obtain durable immune responses against HIV-1. The highly attenuated rcHAdV26.MOS1.HIV-Env vaccine was well tolerated in healthy, HIV-1-uninfected adults, though the single dose was poorly immunogenic, suggesting the replicative capacity of the vector was too attenuated. There was no evidence of shedding of infectious virus or secondary vaccine transmission following the isolation period, proposing the use of less attenuated viral vectors in future studies of live, oral HIV-1 vaccines [56].

4.2 HAdV-D26 RSV vaccine

A licensed vaccine against RSV, which can cause severe respiratory disease, is still not available. Intramuscular immunization with HAdV26 vector containing the RSV fusion protein (F) transgene induced a strong, Th1 skewed immune response in mice and protective and safe immunity against RSV challenge in cotton rats [57]. An HAdV26 vector encoding the RSV F protein stabilized in its prefusion conformation, Ad26.RSV.preF, induced superior, Th1-biased IgG2a-dominated humoral responses both in adult and neonatal mice, thus further supporting clinical development of Ad26.RSV.preF for use in infants [58]. Experimental Ad26.RSV.preF was evaluated also

as a vaccine for older adults in which this vector had an acceptable safety profile and elicited sustained humoral and cellular immune responses after a single immunization [59] thus could potentially protect against natural RSV infection and disease [60].

Two homologous and one heterologous intramuscular prime-boost vaccination regimens using HAdV26 and HAdV35 expressing a prototype antigen based on the RSV wild-type F protein induced substantial boostable antibody responses that recognized the F protein in pre- and post-fusion conformation in adult RSV-naive cynomolgus macaques. This immune response neutralized multiple strains of RSV and persisted for at least 80 weeks. Indicating that intramuscular immunization with HAd26 and HAdV35 vectors could be a promising approach for the development of an optimized RSV vaccine expected to induce long-lasting humoral and cellular immune responses [61].

Since RSV and influenza infection overlap, one study assessed co-administration of the investigational vaccine, Ad26.RSV.preF with a seasonal influenza vaccine in older adults. Coadministration of Fluarix with Ad26.RSV.preF vaccine had an acceptable safety profile and showed no evidence of interference in immune response proposing compatibility of simultaneous seasonal vaccination with both vaccines [62].

4.3 HAdV-D26 Ebola virus vaccine

Both short- and long-term protective immunity is desired when it comes to Ebola virus vaccination. Partial protection against Ebola virus by a single-shot of HAdV-D26 vaccine, coding for EBOV GP (ebola virus glycoprotein) antigen, and complete protection when this vaccine was boosted by HAdV-B35 coding for EBOV GP antigen, 1 month later, has been demonstrated. Increases in efficacy were paralleled by substantial increases in T- and B-cell responses to EBOV GP warranting further development of HAdV-D26 as a candidate vaccine for Ebola virus [63].

HAdV-D26 coding for glycoproteins from two Ebola species (Ebola Zaire and Ebola Sudan/Gulu,), two Marburg strains (Marburg Angola and Marburg Ravn) and one more distant non-lethal Ebola Ivory Coast species for broadest coverage induced a potent cellular and humoral immune response in mice after single vaccination in a dose dependent manner that was cross-reactive within the Ebola and Marburg lineages. Thus, combination of the five selected filovirus glycoproteins in one multivalent vaccine potentially elicited protective immunity in man against all major filovirus strains that have caused lethal outbreaks in the last decades [64]. In addition, HAdV-D26 demonstrated its feasibility to be part of a multivalent filovirus vaccine that can protect against lethal infection by multiple members of the filovirus family [65].

Heterologous and homologous vaccination with HAdV26.ZEBOV and MVA-BN-Filo did not have any vaccine-related serious adverse events [1], was well tolerated and highly immunogenic against Ebola virus glycoprotein in healthy volunteers [66, 67]. Immunological memory induced by HAdV26.ZEBOV vaccination was rapidly recalled by booster vaccination, suggesting HAdV-D26 booster doses could be considered for individuals at risk of Ebola virus infection, who previously received the two-dose regimen

[68]. Humoral and cellular immune responses induced by two-dose heterologous regimen with HAdV26.ZEBOV and MVA-BN-Filo were persisting for 1 year after vaccination [69].

All together the efforts to develop efficient vaccine against Ebola virus yielded success when HAdV26.ZEBOV/MVA-BN-Filo recently received regulatory approval in the European Union (https://www.ema.europa.eu/en/news/new-vaccine-prevention-ebola-virus-disease-recommended-approval-european-union; accessed on 15 August 2021).

4.4 HAdV-D26 Zika vaccine

The immunogenicity and protective efficacy of HAdV-D26 that encodes the Zika virus M-Env antigens, Ad26.ZIKV.M-Env, was evaluated in mice and non-human primates. Ad26.ZIKV.M-Env induced strong and durable cellular and humoral immune responses in preclinical models. Humoral responses were characterized by Env-binding and Zika virus neutralizing antibody responses while cellular responses were characterized by Zika virus reactive CD4+ and CD8+ T cells. Importantly, a single immunization with a very low dose of Ad26.ZIKV.M-Env protected mice from Zika virus challenge. In non-human primates, a single immunization of Ad26.ZIKV.M-Env also induced Env-binding and Zika virus neutralizing antibodies and Env and M specific cellular immune responses that associated with complete protection against viremia from Zika virus challenge as measured in plasma and other body fluids [70]. Safety and immunogenicity of Ad26.ZIKV.001, a prophylactic Zika virus vaccine candidate, has been assessed in phase 1 randomized, double-blind, placebo-controlled clinical study. All regimens were well tolerated and antigen specific cellular responses were induced, thus making Ad26.ZIKV.001 a promising candidate for further development if needed [71].

4.5 HAdV-D26 SARS-COV-2 vaccine

Currently ongoing Covid-19 pandemic urged development of new vaccination tools. HAdV-D26-based vector vaccine against SARS-CoV-2 was a logical choice and immunogenicity and protective efficacy of a single dose of HAdV-D26-based vector vaccines expressing the SARS-CoV-2 spike (S) protein has been shown. The HAdV-D26 vaccine induced robust neutralizing antibody responses and provided complete or near-complete protection in broncho alveolar lavage and nasal swabs after SARS-CoV-2 challenge demonstrating robust single-shot vaccine protection against SARS-CoV-2 in non-human primates [72] and hamsters [73].

It is known that design of antigen to be used in vaccine can be of crucial importance. Initial data demonstrated that the introduction of stabilizing substitutions in the hinge region of S protein increased the ratio of neutralizing versus non-neutralizing antibody binding, suggestive for a prefusion conformation of the S protein. Ad26.COV2.S, HAdV-D26 vector encoding for a membrane-bound stabilized S protein with a wild-type signal peptide, elicited potent neutralizing humoral immunity and cellular immunity that was polarized towards Th1 IFN-γ [74] and type 1 helper T cells [2]. Data collected during phase 1 to 3 clinical trials showed that a single dose of Ad26.COV2.S induced rapid

binding and neutralization antibody responses as well as cellular immune responses [75] thus protected against symptomatic Covid-19 and asymptomatic SARS-CoV-2 infection and was effective against severe-critical disease, including hospitalization and death [76].

Ad26.COV2.S vaccine elicited durable humoral and cellular immune responses with minimal decreases for at least 8 months after immunization [77]. Lower dose of Ad26.COV2.S provided robust protection in bronchoalveolar lavage, whereas higher doses were required for protection in nasal swabs of rhesus macaques indicating that higher vaccine dose may be required for protection in the upper respiratory tract [78].

When Ad26.COV2.S vaccine was assed against different SARS-CoV-2 variants, it was reported that neutralizing antibody responses were reduced against the B.1.351 and P.1 variants, but functional non-neutralizing antibody responses and T cell responses were largely preserved against WA1/2020, B.1.1.7, B.1.351, P.1 and CAL.20C SARS-CoV-2 variants [79, 80]. While a two-dose Ad26.COV2.S regimen induced higher peak binding and neutralizing antibody responses against G614 spike SARS-CoV-2 virus variant in both Syrian hamster model [81] and nonhuman primates, neutralization was reduced for B.1.351 lineages and maintained for the B.1.1.7 lineage independent of Ad26.COV2.S vaccine regimen [82]. By analysing responses from non-human primates both before and after immunization with DNA or HAdV-D26 vectored vaccines, patterns of cross reactivity that mirror those induced by SARS-CoV-2 infection were identified, highlighting the similarities between infection and HAdV-D26 vaccine induced humoral immunity for SARS-CoV-2 and cross-reactivity of these responses to other coronaviruses [83].

Finally, two HAdV-D26-based vaccines against SARS-CoV-2 found their way to the market, and are currently used worldwide: rAd26-S as part of Sputnik V vaccine [84, 85] and Ad26.COV2.S as COVID-19 Vaccine Janssen [2, 76].

4.6 HAdV-D26 as therapeutic vaccine

Except for prophylactic purposes, HAdV-D26-based vaccine was evaluated also as therapeutic approach to target the viral reservoir in HIV-1-infected individuals and as an intervention against cervical cancer caused by human papillomavirus (HPV).

Therapeutic vaccination with HAdV-D26/MVA (HAdV-D26 prime, modified vaccinia Ankara (MVA) boost) and stimulation of Toll-like receptor 7 improved virologic control and delayed viral rebound following discontinuation of antiretroviral therapy in SIV-infected rhesus monkeys that began antiretroviral therapy during acute infection. The breadth of cellular immune responses correlated inversely with set point viral loads and correlated directly with time to viral rebound. These data demonstrate the potential of therapeutic vaccination combined with innate immune stimulation as a strategy aimed at a functional cure for HIV-1 infection [86].

Immunogenicity of HAdV-D26 expressing a fusion of human papillomavirus (HPV) type 16 (HPV16) E6 and E7 oncoproteins evaluated in mice after intramuscular and/or

intravaginal immunization showed induction and trafficking of HPV-specific CD8+ T cells producing IFN-γ and TNF-α to the cervicovaginal tract. This study prompted further evaluation of immunization with AdV vectors expressing modified E6 and E7 antigens for therapeutic vaccination against persistent HPV infection and cervical intraepithelial neoplasia [87]. HAdV-D26 expressing HPV16- and HPV18-specific antigens consisting of fusion proteins of E2, E6 and E7 also demonstrated as a promising vaccine for every disease stage, from incident and persistent infections where E2 is predominantly expressed, to late stages where E6 and E7 expression are upregulated [88].

5. Future of human adenovirus type 26

So far in this review only replication incompetent HAdV-D26 vectors were discussed. However, replication-competent HAdV-based vaccine vectors may theoretically provide immunological advantages over replication-incompetent ones, but they also raise additional potential clinical and regulatory issues. Replication-competent HAdV-D26 vectors, rcAd26, were created by adding the E1 region back into a replication-incompetent HAd26 vector backbone with the E3 or E3/E4 regions deleted. Attenuation of rcAd26 vaccines occurred in a stepwise fashion, with E3 deletion, E4 deletion, and transgene insertion all contributing to reduced replicative capacity compared to that with the wild-type HAdV-D26 vector. The rcAd26 vector with E3 and E4 deleted and containing HIV-1 Env transgene exhibited 2.7- to 4.4-log-lower replicative capacity than that of the wild-type HAdV-D26 *in vitro*. This rcAd26 vector is currently being evaluated in a phase 1 clinical trial. Attenuation as a result of vectorization and transgene insertion has implications for the clinical development of replication-competent vaccine vectors [89].

Except as vaccine vector, HAdV-D26 could be used as oncolytic agent. It has been shown that together with some other adenoviruses belonging to group D, HAdV-D26 has ability to replicate and kill multiple myeloma [90] and particularly B-cell carcinoma [91]. For the time being, development of oncolytic HAdV-D26 has not been further persuaded, however one can expect that HAdV-D26 will soon find its way also in cancer treatment.

Altogether, there is no doubt that HAdV-D26 presents promising platform for vaccine development. New knowledge that will be gathered regarding basic biology of this virus will most probably prompt usage of HAdV-D26-based vectors also for other therapeutic applications. Therefore, bright future awaits HAdV-D26.

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