



Factors Which Contribute to the Immunogenicity of Non-replicating Adenoviral Vected Vaccines

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OPEN ACCESS

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Specialty section:

This article was submitted to
Vaccines and Molecular Therapeutics,
a section of the journal
Frontiers in Immunology

Received: 30 January 2020

Accepted: 20 April 2020

Published: 19 May 2020

Citation:

Coughlan L (2020) Factors Which
Contribute to the Immunogenicity of
Non-replicating Adenoviral Vected
Vaccines. *Front. Immunol.* 11:909.
doi: 10.3389/fimmu.2020.00909

Adenoviral vectors are a safe and potentially immunogenic vaccine delivery platform. Non-replicating Ad vectors possess several attributes which make them attractive vaccines for infectious disease, including their capacity for high titer growth, ease of manipulation, safety, and immunogenicity in clinical studies, as well as their compatibility with clinical manufacturing and thermo-stabilization procedures. In general, Ad vectors are immunogenic vaccines, which elicit robust transgene antigen-specific cellular (namely CD8⁺ T cells) and/or humoral immune responses. A large number of adenoviruses isolated from humans and non-human primates, which have low seroprevalence in humans, have been vectorized and tested as vaccines in animal models and humans. However, a distinct hierarchy of immunological potency has been identified between diverse Ad vectors, which unfortunately limits the potential use of many vectors which have otherwise desirable manufacturing characteristics. The precise mechanistic factors which underlie the profound disparities in immunogenicity are not clearly defined and are the subject of ongoing, detailed investigation. It has been suggested that a combination of factors contribute to the potent immunogenicity of particular Ad vectors, including the magnitude and duration of vaccine antigen expression following immunization. Furthermore, the excessive induction of Type I interferons by some Ad vectors has been suggested to impair transgene expression levels, dampening subsequent immune responses. Therefore, the induction of balanced, but not excessive stimulation of innate signaling is optimal. Entry factor binding or receptor usage of distinct Ad vectors can also affect their *in vivo* tropism following administration by different routes. The abundance and accessibility of innate immune cells and/or antigen-presenting cells at the site of injection contributes to early innate immune responses to Ad vaccination, affecting the outcome of the adaptive immune response. Although a significant amount of information exists regarding the tropism determinants of the common human adenovirus type-5 vector, very little is known about the receptor usage and tropism of rare species or non-human Ad vectors. Increased understanding of how different facets of the host response to Ad vectors contribute to their immunological potency will be essential for the development of optimized and customized Ad vaccine platforms for specific diseases.

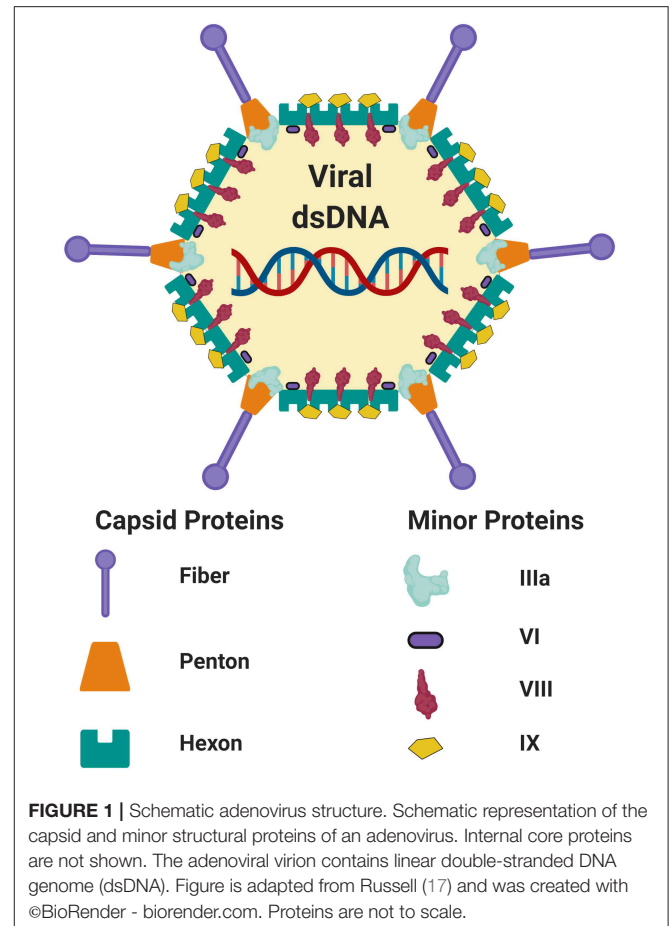
Keywords: adenovirus, adenoviral, vector, vaccine, immunogenicity

INTRODUCTION

Use of Adenoviral Vectors as Vaccines for Infectious Disease

Adenoviruses (Ad) represent a promising vector platform for the development of vaccines for infectious disease, largely due to their safety and ability to stimulate robust cellular and/or humoral immune responses in multiple species (1–8), as compared with other genetic vaccine platforms (5, 9–12). Adenoviruses derived from humans and non-human primates (NHP) belong to the family *Adenoviridae* and the genus *Mastadenoviridae*, and are further subdivided into species A–G (i.e., for species A viruses, these are denoted HAdV-A followed by the type number). Accounting for the inclusion of many Ad recombinants (13, 14), ~103 human Ads (<http://hadv.wg.gmu.edu/>) and >200 non-human Ad serotypes have been identified to date. Adenoviruses are non-enveloped viruses which contain a double-stranded DNA genome. The virion exterior is composed of three major structural proteins, the fiber, the penton base and the hexon [(15–18); **Figure 1**]. Recombinant Ad (rAd) vectors can easily be rendered replication-incompetent (non-replicating) through deletion of the essential viral gene E1 from their genome and can be vectorized for easy manipulation (16, 19–21). Further improvements to these first-generation Ad vectors have been developed in which the E3 region is also deleted, to accommodate a larger heterologous transgene capacity of ~7.5 kbp. Ad vectors display a number of desirable characteristics which make them particularly well-suited to prophylactic vaccine applications. Their genome is stable and easy to manipulate, they can be amplified and produced to high titers using various complementing cell lines which adhere with good clinical practice (GCP) procedures (22), and they have an outstanding track-record as safe and immunogenic vaccines in numerous human clinical trials (1–7, 23–25). Historically, the most commonly used rAd has been *Human mastadenovirus C*, Human Adenovirus Type-5 (HAdV-C5, referred to *Ad5 throughout this manuscript*). However, despite its well-characterized biology and robust immunogenicity, high seroprevalence has limited its widespread use in humans and has prompted the development and investigation of novel Ad species, either rare species human Ads (6, 20, 26) or those derived from NHPs (5, 8, 27), many of which have very low seroprevalence (27–31). For the purpose of this review, the nomenclature of Ad types will use reference to the vertebrate species from which the vector was derived (i.e., H for human or Ch for chimpanzee) followed by the virus type number, as outlined in **Table 1**. Human Ad vectors will include their assigned adenovirus species group (i.e., A–G). This nomenclature has been proposed to ICTV by Dr. Don Seto, George Mason University and Dr. James Chodosh, Harvard University (*personal communication*).

The use of non-replicating viral vectors as a vaccine platform has several advantages over other vaccine formulations (i.e., recombinant protein, inactivated particles). Viral vectored vaccines retain some characteristics of a live attenuated vaccine in terms of their ability to enter target cells, engage intracellular trafficking pathways to deliver their genome and facilitate antigen (Ag) expression and subsequent Ag-presentation *in vivo*, but



possess additional safety features. Furthermore, in order to drive the expression of substantial quantities of transcripts which correspond to the encoded vaccine Ag, non-replicating Ad vectors make use of powerful exogenous promoters, such as the cytomegalovirus (CMV) promoter (32). Unlike recombinant protein or inactivated vaccines in which antigen quantity is limited to the input vaccine dose, the use of exogenous promoters facilitates more sustained transgene antigen expression *in vivo*.

In general, Ad vectors are well-established to stimulate CD8⁺ T cell responses directed toward transgene Ag, with selected Ad types confirmed to elicit robust cellular immunity in both animal models (8, 32–36) and humans [(1, 3, 6, 23, 33, 37); see **Table 2**]. Memory CD8⁺ T cell responses elicited following vaccination with Ad vectors exhibit an extended contraction phase (38). Importantly, the persistent Ag expression following immunization with Ad vaccines enables the induction of sustained immune responses (5, 36, 39–41), making them very attractive vaccine vectors for conferring long-lasting immunity. It is believed that the prolonged expression of vaccine Ag facilitates the maintenance of effector CD8⁺ T cells while simultaneously permitting their differentiation into central memory populations (36). Improved understanding into how Ad vectors prime and maintain such long-lived responses will be crucial not only in designing improved Ad vaccines, but also other vaccine

TABLE 1 | Nomenclature of adenoviruses discussed in this review.

Ad name (<i>this review</i>)	Description (vertebrate species, type)	Species/Group classification	Alternative names reported in the literature
HAdV-B3	Human adenovirus type 3	B	Ad3, rAd3
*HAdV-B35	Human adenovirus type 35	B	Ad35, rAd35
HAdV-C2	Human adenovirus type 2	C	Ad2, rAd2
*HAdV-C5	Human adenovirus type 5	C	Ad5, rAd5, AdHu5
*HAdV-C6	Human adenovirus type 6	C	Ad6, rAd6, AdHu6
HAdV-D11	Human adenovirus type 11	D	Ad11, rAd11
*HAdV-D26	Human adenovirus type 26	D	Ad26, rAd26
HAdV-D28	Human adenovirus type 28	D	Ad28, rAd28
*HAdV-E4	Human adenovirus type 4	E	Ad4, rAd4
ChAdV-1	Chimpanzee adenovirus type 1	B2	ChAd1, AdC1, SAd21
*ChAdV-3	Chimpanzee adenovirus type 3	C	ChAd3
ChAdV-7	Chimpanzee adenovirus type 7	E	AdC7, ChAd7, SAd24, Pan7
*ChAdV-63	Chimpanzee adenovirus type 63	E	ChAd63
ChAdV-68	Chimpanzee adenovirus type 68	E	AdC68, ChAd68, SAd25, Pan9, ChAdOx2
*ChAdOx1	Chimpanzee adenovirus type Y25	E	Y25
SAdV-11	Simian adenovirus type 11	Yet undefined	SAd11, sAd11
SAdV-16	Simian adenovirus type 16	Yet undefined	SAd16, sAd16
SAdV-23	Simian adenovirus type 23	E	ChAdV-6, AdC6, ChAd6, Pan6
*PanAdV-3	Pan (<i>paniscus</i>) adenovirus type 3	C	PanAd3

Adenovirus classification for human and non-human Ad vectors referred to in this review. The nomenclature of Ad vectors derived from non-human primates, including chimpanzees, is not standardized, resulting in the confusing use of multiple names assigned by individuals who vectorized these constructs. In this review text, we propose to follow current standards for human Ad vectors, such as HAdV-C5, as outlined by ICTV, for descriptions of Ad vectors derived from chimpanzees or non-human primates. Abbreviated "alternative" names are used in **Table 2** due to space constraints. H, human; Ch, chimpanzee; S, simian. *Vectors used in human clinical trial.

platforms which are optimized for diverse disease targets. However, the precise factors which contribute to the robust immunogenicity associated with particular Ad-vectored vaccines are currently unclear.

It is widely appreciated in the Ad vaccine field that Ad vectors can act as a "self-adjuvant," allowing the stimulation of multiple innate immune signaling pathways upon viral entry, which can augment the immunogenicity of the encoded Ag (although conversely, stimulation of certain signaling pathways can also be detrimental to their immunogenicity, as discussed below). Although we have some understanding of how individual pathways work *in vitro* in defined cell types (i.e., dendritic cells, macrophages), understanding how these pathways intersect, or cooperate in the development of protective immunity *in vivo*, is complex and our understanding is incomplete. Additionally, it is apparent that there is a clear hierarchy of immunological potency when evaluating distinct Ad species and serotypes as vaccine vectors in animal models. Although a few selected vectors display robust immunogenicity *in vivo* which is comparable to that of Ad5, most are less immunogenic (5, 8, 15, 29, 31), and there are considerable differences in the phenotype and functionality of immune response elicited (8, 42, 43). In recent years, investigators have begun to identify several crucial factors which could contribute to these profound disparities in immunological potency. It is now believed that differences in (i) cellular receptor and/or co-receptor usage, viral entry, trafficking, endosomal escape, and *in vivo* tropism can contribute to the (ii) differential activation of innate immune signaling which

influences subsequent immune responses (44). In addition, it is apparent that the (iii) magnitude and persistence of transgene expression can also shape the ensuing immune response (33) and all of these factors are in turn affected by the (iv) vaccine dose (8) and route of administration (45). Increased understanding of, and implementation of efforts to overcome these striking differences in immunological potency or quality, will absolutely be required for the development of optimal Ad vectors for clinical use.

Viral Entry and Cellular Tropism

Entry in non-immune cells

As a result of extensive study over the past few decades, we have a clear understanding of the *in vitro* tropism determinants of vectors derived from species C HAdVs (i.e., HAdV-C5/Ad5) (18). The classical entry pathway of rAd5-based vectors in non-immune cells is mediated by binding of the fiber knob domain (**Figure 2**) to the Coxsackie and Adenovirus receptor (CAR). Following this "docking" interaction, viral internalization is facilitated through interactions between the arginine-glycine-aspartate (RGD) motif within the viral penton base and cellular integrins (namely $\alpha v\beta 3$ and $\alpha v\beta 5$) on the surface of cells (46, 47). Adenovirus capsid disassembly then proceeds in a systematic and stepwise process. Initial binding to CAR is a motile interaction, whereas the subsequent interaction with immobile αv integrins results in the ripping or shedding of fibers from the virion, initiating partial disassembly of the virion at the plasma membrane (48, 49). It was previously believed

TABLE 2 | Summary of the comparative immunogenicity of diverse AdV vaccines.

A. Ad vectors compared	B. Vaccine antigen	C. Parameters used to define hierarchy of immunogenicity	Optimal vector	PMID
SPECIES: MICE				
Human Ads B: Ad34, Ad35 C: Ad5, Ad6 D: Ad24	HIV-1 <i>gag</i>	Maintenance of Gag⁺CD8⁺IFNγ⁺ responses with dose de-escalation (10¹⁰-10⁶ vp): Ad5, Ad6 > Ad24 > Ad35 > Ad34 (<i>Balb/c mice</i> , intramuscular immunization) <ul style="list-style-type: none"> Ad5 and Ad6 were capable of eliciting T cell responses at a vaccine dose of 10⁶ viral particles (vp). Ad24 = 10⁸ vp, Ad35 = 10⁹ vp, and Ad34 = 10¹⁰ vp. 	Ad5, Ad6	22218691
Non-human Ads B: ChAd30 C: ChAd3, PanAd3, PanAd1, PanAd2, ChAd11, ChAd19, ChAd20, ChAd24, ChAd31 (listed in order of immunogenicity) E: ChAd63, ChAd83, ChAd6, ChAd9, ChAd10, ChAd43, ChAd55, ChAd147, ChAd4, ChAd5, ChAd7, ChAd16, ChAd38, ChAd146, ChAd149, ChAd150 (listed in order of immunogenicity)		Maintenance of Gag⁺CD8⁺IFNγ⁺ responses with dose de-escalation (10¹⁰-10⁶ vp): ChAd3, PanAd3 > ChAd63 > PanAd1 > as listed in column A (<i>Balb/c mice</i> , intramuscular immunization) <ul style="list-style-type: none"> 26 chimpanzee adenoviral isolates were screened for immunological potency. Group C Ad vectors were most potently immunogenic, followed by Group E. Group B ChAd30 was weakly immunogenic. ChAd3 and PanAd3 were capable of eliciting T cell responses at a dose of 10⁶ viral particles (vp), ranking them as comparable with the immunogenicity of Ad5. ChAd63 also elicited T cell responses at 3 × 10⁶ vp making it only slightly less immunogenic than ChAd3 and PanAd3. 	ChAd3, PanAd3, ChAd63	
Human Ads B: Ad35 C: Ad5 D: Ad28 Non-human Ads C: ChAd3 E: ChAd63 ND: SAd11, SAd16	SIV <i>gag</i>	Magnitude and protective efficacy of CD8⁺ with dose de-escalation (10⁹-10⁷ particle units PU): Efficacy ranking: Ad5+ChAd3 > Ad28+SAd11 > ChAd63 > SAd16 > Ad35 (<i>C57BL/6 mice</i> , sub-cutaneous immunization) <ul style="list-style-type: none"> Study performed a dose titration of vaccines with detailed phenotyping of T cell response at peak and memory timepoints. Ad5, Ad28, SAd11, and ChAd3 were comparable in conferring CD8⁺ mediated protection in challenge model using <i>gag</i>-expressing <i>Listeria monocytogenes</i>. 	Ad5, Ad28	23390298
Human Ads B: Ad35 C: Ad5 D: Ad28 Non-human Ads C: ChAd3 E: ChAd63 ND: SAd11, SAd16	SIV <i>gag</i> <i>eGFP</i>	Memory (D70) Gag Tetramer⁺CD8⁺ at 10⁸ PU and protection from challenge: Ad5, ChAd3 > ChAd63 (<i>C57BL/6 mice</i> , sub-cutaneous immunization) <ul style="list-style-type: none"> The protective efficacy of Ad vectors in a murine challenge model of <i>gag</i>-expressing <i>Listeria monocytogenes</i> was tested. Ad5 and ChAd3 were most potently protective, followed by ChAd63. Vaccination with Ads expressing reporter eGFP resulted in higher frequencies of eGFP⁺ CD11c⁺ dendritic cells in draining lymph nodes (dLNs) for Ad5, ChAd3 > ChAd63. Transcriptional profiling of dLNs revealed that the most potently protective vectors, Ad5 and ChAd3, exhibited weak induction of IFN-stimulated genes, unlike other Ad vectors (8–24 h). More pronounced upregulation of IFN-responsive gene modules in dLNs at 24 h were associated with reducing Ad transgene expression levels and thereby limiting their immunological potency. 	Ad5 ChAd3, ChAd63	25642773
Human Ads B: Ad11, Ad35, Ad50 C: Ad5 D: Ad26, Ad48, Ad49	SIV <i>gag</i>	Maintenance of Gag⁺CD8⁺IFNγ⁺ responses with dose de-escalation (10¹⁰-10⁷ vp): Ad5 > Ad26 (<i>C57BL/6 mice</i> , intramuscular immunization) <ul style="list-style-type: none"> A head-to-head comparison of species B and D Ad-based vaccine vectors was performed compared with species C Ad5. At a dose of 10⁷ vp, Ad5 and Ad26 were more potent than all other Ad vectors, but only Ad5 was immunogenic at a dose of 10⁶ vp. 	Ad5, Ad26	17329340

(Continued)

TABLE 2 | Continued

A. Ad vectors compared	B. Vaccine antigen	C. Parameters used to define hierarchy of immunogenicity	Optimal vector	PMID
Human Ads C: Ad5 D: Ad26	HIV-1 <i>gag</i>	Magnitude of Gag⁺CD8⁺IFNγ⁺ responses at 10⁹ or 10⁸ vp: Ad5 > Ad26, AdC6, AdC7 (<i>Balb/c mice</i> , intramuscular immunization) <ul style="list-style-type: none"> At 10¹⁰ vp gag-specific CD8⁺ responses were comparable for Ad5, Ad26, AdC6, and AdC7. At 10⁹ vp, Ad5 was superior to all. 	Ad5, Ad26	20686035
Non-human Ads E: AdC6 (SAdV-23), AdC7 (SAdV-24)	Rabies <i>glycoprotein</i>	Induction of NAbs to Rabies GP and Protective Efficacy at 10¹¹-10⁹ vp: Ad5 > Ad26, AdC6, AdC7 (<i>ICR outbred mice</i> , intramuscular immunization) <ul style="list-style-type: none"> At all doses neutralizing antibodies considered protective according to comparison with WHO standard serum samples (>0.5 IU) were induced, but Ad5 was the best. Ad5 had superior protective efficacy from challenge with rabies virus strain CVS-24 at all doses, survival with other Ads was reduced, even at 10¹¹ vp. Ad26 displayed 60% survival, AdC6 was ~50% survival at 10¹¹ vp. 	Ad26, AdC6	

We are only including murine studies that performed a head-to-head comparison of vector immunogenicity for Ad vaccines derived from more than three Ad species groups. Please note, vector type names are listed as shortened versions due to space constraints within the Table. IFN γ , interferon gamma; vp, viral particle; PU, particle unit; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; eGFP, enhanced green fluorescent protein; dLN, draining lymph node; GP, glycoprotein. Abbreviated "alternative" names for Ad vectors are used in this table due to space constraints. See **Table 1** for their standardized nomenclature.

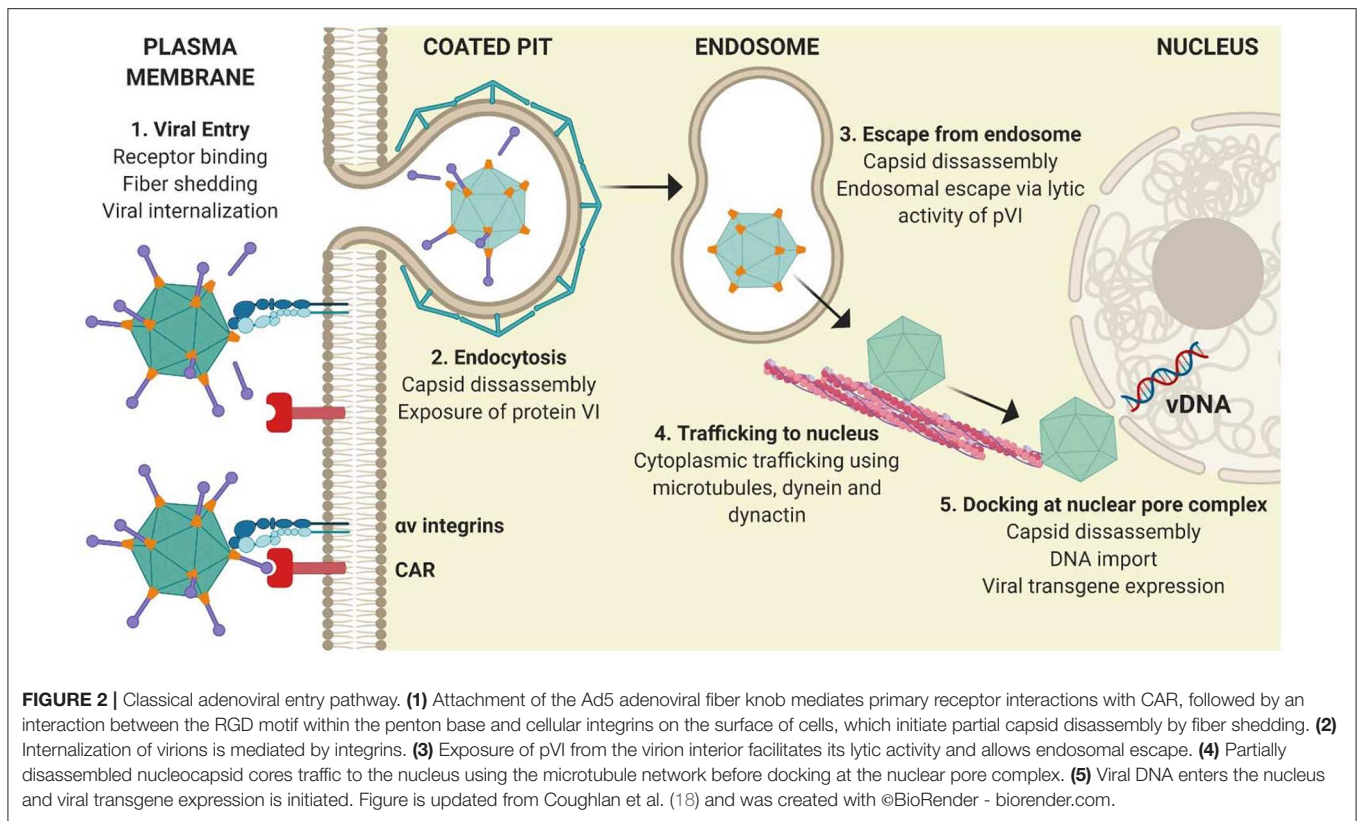
that endosomal escape was pH-dependent (50). However, it has subsequently been demonstrated that exposure of protein VI from the capsid interior (51) at the cell surface, as a result of mechanical strain induced by the antagonistic CAR:integrin interaction (48), facilitates access to the cytoplasm through the action of its pH-independent membrane lytic activity (52–54). Following endosomal escape, the virion is transported to the nuclear pore complex via the microtubule network (50, 52). Once the virion has docked at the nuclear pore complex, interactions with cellular proteins trigger further capsid disassembly and allow the viral DNA to extrude into the nucleus for subsequent gene expression (18).

However, unlike Ad5, adenoviral types derived from species B or D viruses such as HAdV-B35, HAdV-B3, HAdV-D11, or HAdV-D26 can use alternative binding/entry factors or receptors to CAR, such as CD46 (55–57), desmoglein-2 (DSG-2) (58), or sialic acid (59). The post-entry steps of these rare species Ad viruses in diverse cell types are not as well-characterized as the CAR-mediated entry of Ad5. However, it is considered that the use of alternative entry pathways or different receptors can not only result in differences in endosomal escape, trafficking to the nucleus and subsequent transgene expression (60), but can also impact on the *in vivo* tropism of the vector following different routes of vaccine administration (i.e., intramuscular vs. intranasal). As a result, triggering of innate immune signaling pathways may also differ at each step of the entry process (33, 61). Less efficient trafficking pathways could result in weak or limited induction of cytokines/chemokines (62), or increased uptake in cell types which result in vector degradation with minimal transgene expression (63–66). Consequently, such differences between diverse Ad vectors can impact on the magnitude and phenotype of the ensuing adaptive immune response when they are used as a vaccine platform (8, 33, 44).

Entry in immune cells

Macrophages. In addition to the classical *in vitro* entry pathways described above, Ad vectors can infect mononuclear phagocytes efficiently both *in vitro* and *in vivo*, independently of their described surface receptors (i.e., CAR, DSG-2) (67), which are absent on murine macrophages. *In vivo* interactions with tissue resident macrophages, such as Kupffer cells in the liver or alveolar macrophages in the lung, can result in scavenging and degradation of significant amounts of input Ad vector (64–66). Not only can these interactions result in limited transgene expression which could affect the therapeutic efficacy, but the phagocytosis of Ad particles can trigger inflammatory responses (68, 69), leading to undesirable off-target toxicity (70). This is a particularly important consideration for therapeutic applications which require systemic administration or are designed for use in immunocompromised individuals (i.e., oncolytic viral therapy for disseminated metastases) (18). As a result, efforts have been made to characterize the mechanisms of Ad viral entry in macrophages and to better understand how these interactions contribute to the induction of inflammatory responses within defined anatomical compartments following different routes of administration (i.e., intramuscular, intranasal vs. intravenous).

Opsonization of Ad viral particles by complement or antibodies (natural or anti-viral) can bridge entry into macrophages by engaging Fc-receptors (FcRs) or complement receptors (71–73). Additionally, a role for scavenging receptors in facilitating viral entry into murine macrophages, both *in vitro* and *in vivo*, has been outlined (62, 67, 74). Scavenging receptors are a heterogeneous and structurally diverse family of receptors capable of interacting with endogenous proteins and lipids, microbial ligands, and non-opsonized particles, including viruses (75). In addition to contributing to the clearance of particulate Ag, scavenging receptors have been

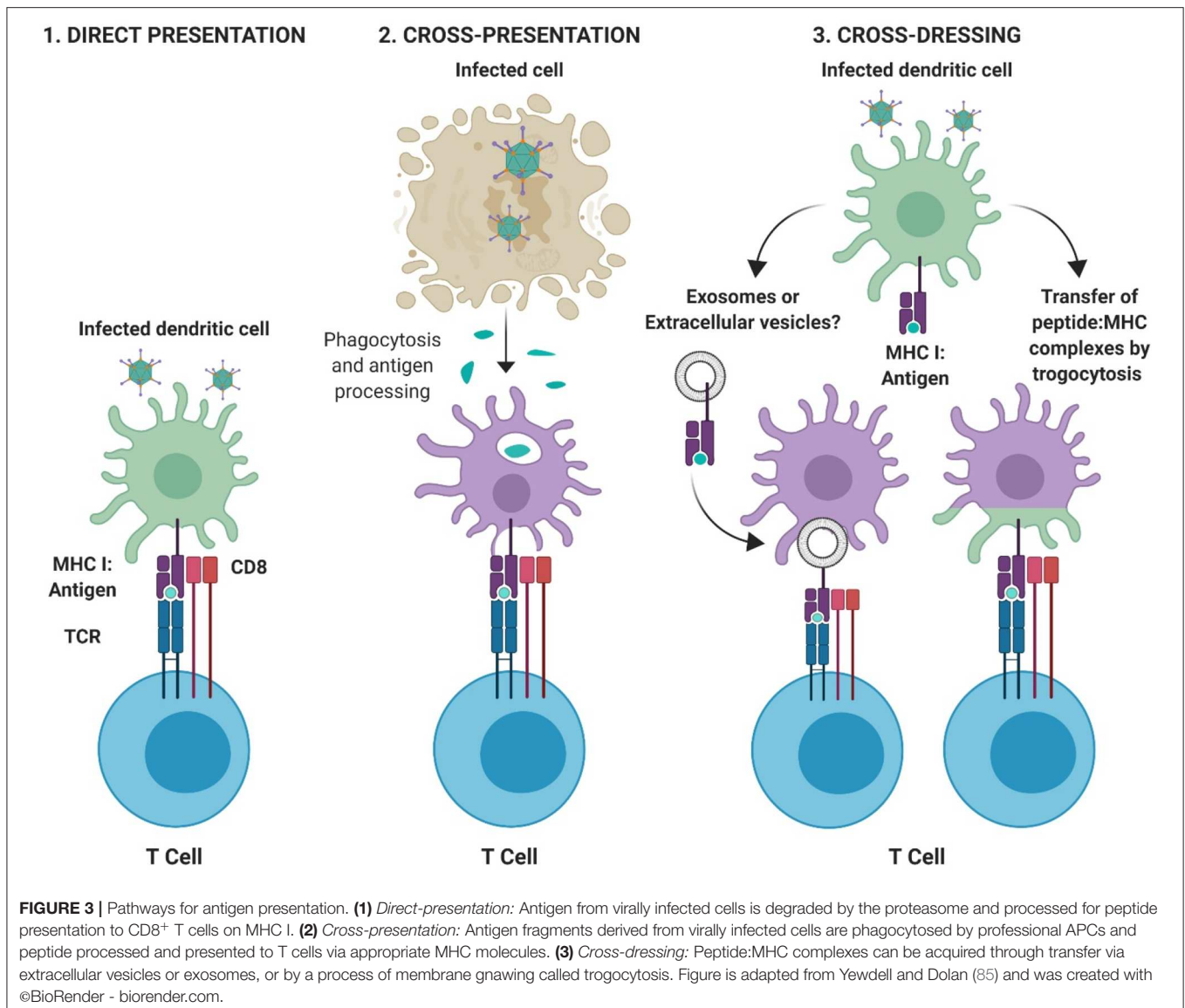


implicated in innate immune sensing, due to their ability to recognize pathogen-associated molecular patterns (PAMPs). Murine SR-A1 (74), SR-AII (76), and MARCO (SR-A6) (67) have been described as receptors for rAd vectors, and MARCO⁺ marginal zone macrophages in the spleen have been shown to accumulate Ad5-based vectors following intravenous (*i.v.*) delivery in mice (28, 77). The fiber knob (*i.e.*, SR-A1), or hexon protein has been implicated in mediating these interactions (*i.e.*, SR-AII and SR-A6). Interestingly, SR-A6 (MARCO) was shown to not only facilitate entry and efficient gene transduction with Ad5, but also with HAdV-C2, HAdV-B35, and HAdV-D26 (67). The mechanism of interaction between Ad and SR-AII/SR-A6 was proposed to involve the negative charge conferred by specific hypervariable regions (HVRs) of the viral hexon, namely HVR1. In support of this, preferential scavenging of negatively charged particles has previously been shown to contribute to the differential recognition of Ad vectors by macrophages *in vivo* (76, 78, 79).

Dendritic cells. Dendritic cells (DCs) are a specialized subset of professional Ag presenting cells which are central to the development of protective immunity. DCs can process Ag using several methods; (i) direct presentation, in which an infected DC presents peptide:major histocompatibility complex class (MHC) complexes directly to T cells, (ii) cross-presentation in which Ag derived from other infected cells is phagocytosed by DCs, processed and then presented to T cells, and (iii) cross-dressing (80), in which peptide:MHC complexes are acquired from other

professional or non-professional antigen presenting cells (APCs) and are transferred to the DC through a process of trogocytosis, in which fragments of the plasma membrane containing the MHC complex merges with the recipient cell. Cross-dressing is also hypothesized to occur through the intercellular transfer of pre-formed peptide:MHC complexes by extracellular vesicles, such as exosomes [(81–84); **Figure 3**].

A critical role for DCs in the robust induction of CD8⁺ T cell responses following immunization with rAd vectors has been demonstrated (86, 87). It has been shown that CD8⁺, but not CD4⁺ T cell responses elicited by Ad vaccines, are dependent on cross-presentation by specific sub-populations of DCs, including CD8 α ⁺ DCs (33). In support of this, Ag-specific CD8⁺ T cell responses in BATF3-deficient mice (*Batf3*^{-/-}), which preclude the development of the latter DC population (CD8 α ⁺ DCs), were shown to be ~80% lower following immunization with several Ad vectors (33). DCs infected with Ads upregulate MHC, as well as co-stimulatory molecules including CD40, CD80, or CD86, leading to the activation or maturation of DCs (88), in a manner which is believed to be dependent on nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling (89). The maturation of murine DCs has been proposed to be mediated by the fiber knob domain of Ad5 (90). *In vivo* experiments in mice have shown that uptake of rAd5-based vaccines in draining lymph nodes (dLNs) following intramuscular (*i.m.*) or subcutaneous (*s.c.*) vaccination is highest in CD11c⁺ CD8⁻ B220⁻ DCs, although CD11c⁺ CD8⁺ B220⁻ DCs were the most potent for eliciting naïve Ag-specific T cell proliferation (86). As



previous studies have shown that targeting vaccine Ag to defined populations of DCs can improve immunity and vaccine efficacy (91, 92), efforts are ongoing to better understand the precise interactions between diverse Ad types and DC subpopulations *in vivo*, as well as how we can engineer Ad vectors which are targeted to specific receptors on the surface of DCs (93, 94).

Receptors on the surface of dendritic cells (DCs) can permit entry of Ads independently of the classical CAR receptor, which is largely absent on DCs (95). Receptors proposed to be involved in Ad viral entry into human or murine DCs include Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (95), CD46 (61, 96, 97), or CD80/CD86 (98). A vector based on chimpanzee Ad vector 1, ChAdV-1, was shown to efficiently transduce CD46-expressing murine DCs *in vitro* (97). Recombinant Ad5 vectors pseudotyped with the fiber knob from Ad3 (99) or porcine adenovirus type 4 (100), can increase entry into human DCs, via CD80/CD86

and surface glycans, respectively. Similarly, pseudotyping rAd5 with the fibers from species B, HAdV-B16 or species D, HAdV-D37 displayed increased entry into murine DCs compared with unmodified rAd5 (101). Interestingly, in the latter study, increased entry into DCs was not associated with improved cellular immunity following subcutaneous immunization. In support of this, species B Ad vectors, including Ad35, were long considered to hold great potential as potentially immunogenic vaccine vectors due to their increased ability to target both myeloid and plasmacytoid human DCs via CD46. However, these vectors were subsequently shown to be some of the least potent Ad vaccines *in vivo* (5, 8). These observations and findings are important in highlighting that multiple parameters, such as post-entry intracellular trafficking kinetics or differential activation of innate immune signaling pathways, not just viral tropism, likely play key roles in the induction of robust immunogenicity.

Innate Immune Responses to Ad Vectors

The ability of diverse rAd vaccines to elicit robust cellular immune responses and confer protective immunity in animal models makes them an attractive vector platform for vaccine development (Table 2). In addition to this, their safety in clinical applications, compatibility with clinical-grade manufacturing and scale-up (102, 103) and their suitability for long-term storage (104), or thermo-stabilization (105–107) and stockpiling for cold-chain free storage, has solidified their appeal as vaccines for major infectious diseases (108). It is this clear translational potential which has emphasized the importance of improving our understanding of the mechanisms which underlie differences in the immunological potency of diverse Ad types. Ad vectors are capable of triggering multiple innate immune sensors at several steps in the viral entry pathway (109, 110), in a process which does not require viral replication or gene expression (111). Viral penton RGD:cellular integrin-mediated internalization and subsequent escape from the endosome is considered to be a crucial step in activating many innate immune responses to Ad vaccines (112). It is considered that preferential stimulation (or avoidance) of defined innate immune signaling pathways could impact on the downstream immunogenicity of distinct Ad vectors. With regard to assessing the differential stimulation of innate immune signaling pathways, this is complicated by the fact that many vectors have not been compared side-by-side, and published data proposing roles for these pathways in the immunological potency of different Ad vectors are often contradictory. However, a number of pathways which have been implicated in innate immune sensing of Ad vectors and the caveats associated, are described below.

TLRs

Recombinant Ad vectors contain PAMPs which can be sensed by cell-surface or endosomal pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs). TLRs implicated in the sensing of Ad vectors include TLR2 (113), TLR4 (114), and endosomally located TLR9 (61, 113), which can trigger the down-stream activation and transcription of anti-viral genes including NF- κ B, mitogen-activated protein kinases (MAPK) and interferon-regulatory factors (IRFs). The intracellular adaptor protein MyD88 has been reported to play a major role in the induction of Ag-specific cellular immune responses following TLR-mediated sensing of Ad vaccines (109, 113). Importantly, the ability to engage multiple MyD88-dependent signaling pathways simultaneously, is believed to contribute to the robust immunogenicity associated with Ad vaccines. However, their immunological potency is also attributed to the fact that innate immune activation by recombinant Ad vaccines can occur not only via TLR-dependent mechanisms, but also through numerous TLR-independent pathways (86, 109, 110, 113, 114).

cGAS/STING

The viral DNA itself can play a crucial role in triggering innate immune responses. In recent years it has been demonstrated that following rupture of the endosomal membrane, Ad viral DNA can also be sensed by the cytosolic DNA sensor cGAS

(115, 116). The engagement of cGAS triggers a signaling cascade involving the adaptor STING (117) and activation of the kinase TBK1, which initiate the induction of IRF3-responsive genes (115), such as Type I interferons (IFNs). It has been shown that the absence of cGAS or STING results in reduced activation of early innate immunity (i.e., IFN- β , cytokines, chemokines) but does not impact adaptive anti-vector immune responses in mice. However, the latter studies were performed in the context of *i.v.* delivery and anti-vector, not transgene-specific immunity (116), and as such, the relative importance of DNA sensor pathways in the immunogenicity of Ads as vaccine vectors is less clear. It has recently been suggested that Ag expression is a more crucial predictor of Ag-specific memory T cells (33), as abrogation of STING and Type I IFN responses during Ad vaccination in mice merely altered the early kinetics of CD8⁺ T cells, but did not impair the magnitude of T cell memory responses (33). In the latter study, it was shown that STING could act as a dominant innate PRR sensor for many Ad vectors. Interestingly, abrogation of STING accelerated the kinetics of Ag-specific T cell responses following vaccination with ChAdV-63, a chimpanzee Ad vector, but was dispensable for the early induction of CD8⁺ T cell responses for Ad5 and rare species HAAdV-D28-based vaccines (33). This supports the idea that a complex interplay between multiple PRR-mediated signaling pathways exists, and that different Ad vectors are differentially impacted by these pathways. Our understanding of this is confounded by differences in receptor usage, *in vivo* tropism, engagement of PRRs in diverse hematopoietic and non-hematopoietic cells, and by differences in putative PAMPs on diverse Ad particles.

The NLRP3 inflammasome

In addition to inducing the expression of anti-viral genes, infection with Ad vectors also triggers pro-inflammatory responses through cytosolic DNA-sensing mechanisms which are independent of TLR9 and IRFs (111). In macrophages, recognition of Ad viral DNA has been shown to be mediated by the innate cytosolic molecular complex, or inflammasome, in a process involving NLRP3 and (ASC), which is independent of viral gene expression or replication (117). The multi-protein inflammasome complex mediates caspase-1 activity, resulting in the processing of pro-interleukin-1 β into its active and secreted form. IL-1 β subsequently induces signaling cascades of pro-inflammatory cytokines and chemokines through the IL-1RI both *in vitro* and *in vivo* in response to Ad infection. However, alternative and contradictory mechanisms of immune activation in macrophages by Ads have also been identified which are independent of the NLRP3 inflammasome and its components. Di Paolo et al. showed that direct interactions between the RGD motif within the penton base of the Ad virion and the β 3 subunit of integrins on the surface of macrophages were responsible for activating IL-1 α . The authors also proposed that IL-1 α , not IL-1 β , was the predominant activator of innate immune responses to Ad5 *in vivo* (77).

One very important caveat which complicates our ability to systematically investigate how innate immune responses contribute to downstream adaptive immunity to Ad vaccines, is that many studies are performed *in vitro*, using defined

non-immune epithelial/endothelial cells or cultured immune cells including macrophages or dendritic cells (110, 115, 118). These cell type-specific findings often contradict subsequent *in vivo* studies using transgenic mice in which these “critical” mediators of innate immunity are knocked-out (77, 116). For example, despite numerous reports describing an important role for TLR9 *in vitro*, comparisons of wildtype and TLR9^{-/-} mice have demonstrated that the impact of TLR9 in innate immune sensing of Ad particles *in vivo* is minimal (77), at least for *i.v.* administration of Ad5. Similarly, although cGAS or STING were shown to be pivotal in *early* immune sensing of Ad *in vivo*, studies using cGAS^{-/-} or STING^{-/-} mice showed that these molecular effectors have little impact on subsequent adaptive immunity and antibody production (116). These discrepancies are obviously further complicated by the known capacity of Ad vectors to engage multiple innate signaling pathways simultaneously, rendering individual pathways at least partially redundant *in vivo* (110). In addition to this, differences in the route of Ad vector delivery (i.e., *i.m.*, *i.v.*, or intranasal) and access to different cell types, the multiplicity of infection (MOI) or injected dose, the timing or method of analysis reported in published work and the use of non-human or rare species Ad vectors with differential receptor usage, also limits our ability to fully dissect out the key contributing pathways (118, 119). Collectively, these factors highlight the many challenges facing the field and explain why we currently lack consensus on precisely which innate signaling pathways could contribute to protective immunity following vaccination with diverse Ad vectors.

Stimulation of type I IFNs

It has been proposed that *minimal* induction of Type I IFNs (44, 102, 120), in conjunction with sustained transgene expression (33), are hallmarks of potently immunogenic Ad vaccine vectors *in vivo*. Excessive stimulation of Type I IFN pathways at early time-points following immunization has been shown to lead to decreased transgene expression and subsequently reduced Ag-specific antibody (Ab) responses, following immunization with a chimpanzee Ad vector, ChAdV-68 (120). The authors demonstrated that these effects could be reversed by immunizing mice which have a defective type I IFN receptor IFNAR^{-/-}, resulting in an increased Ab response, thereby confirming that Type I IFN stimulation can have a detrimental impact on humoral immunity directed toward the Ad-encoded transgene Ag (120). In support of these findings, Quinn et al. also showed that abrogation of Type I IFN and STING could increase transgene expression from rAd vaccines, and that the development of protective cellular immunity correlated with this increased transgene expression (33). Following immunization with rare species and non-human Ad vectors, the authors used a systems biology-based, gene expression analysis approach at several time-points to confirm the differential modulation of IFN responsive genes. They determined that Type I and Type II IFNs were upregulated at 8 h post-immunization, which was followed by the induction of ISGs by 24 h. Interestingly, the most protective rAds identified in their study (i.e., Ad5 and chimpanzee Ad, ChAdV-3) exhibited the weakest transcriptional activation of these pathways. However,

only the impact of innate gene activation on CD8⁺ T cell responses, but not transgene-specific Abs, was investigated (33). Nonetheless, collectively, these studies support the concept of robust, persistent Ag expression combined with low innate gene stimulation in contributing to the potency of rAd vaccines (33, 102). This is also supported by the knowledge that relative to rare species human and non-human Ad vectors (33, 120), immunization with the potently immunogenic Ad5 vector is well-established to result in robust, persistent Ag expression (33, 36, 41, 121, 122), while triggering minimal Type I IFN responses *in vivo*. Future studies which aim to comprehensively characterize the contribution of early innate immune activation and correlate this with the downstream immunological potency and efficacy of lead Ad vaccine platforms will be required.

Magnitude and Persistence of Antigen Expression

Non-replicating Ad5-based vectors are well-established for their ability to confer robust transgene expression following immunization (36). Furthermore, low level transgene expression can persist long-term (41, 122), with transcriptionally active Ad vector genomes being maintained in muscle at the injection site, or within draining lymph nodes (36, 122), depending on the route of administration. As previously outlined, it has been proposed that it is this magnitude and persistence of transgene Ag expression which is crucial for the induction of robust and protective T cell responses following Ad vaccination (33). As described above, Quinn et al. demonstrated that strong activation of innate immune gene expression profiles in the draining lymph nodes (dLNs) correlated with limiting Ad-mediated transgene expression for many rare or non-human Ad types (33). The Ad vectors with the highest levels of transgene Ad expression in dLNs, Ad5, and chimpanzee Ad vector ChAdV-3, also had the most attenuated IFN induction. The latter vectors were both potently immunogenic following immunization in mice. In agreement with this, similar comparative immunogenicity studies in mice have shown that transgene expression levels within muscle and dLNs are lower following immunization with a chimpanzee Ad vector, ChAdOx1, when compared with Ad5, and that this translates to superior immunogenicity observed with Ad5 (123).

To directly address the question of how persistence of Ag contributes to the induction of the robust immunogenicity of Ad vaccines, Finn et al. constructed an Ad vaccine with a doxycycline-regulated transgene expression cassette (121). By switching off transgene expression at early vs. late time-points post-immunization, the authors confirmed the importance of presence of Ag in expanding and maintaining memory T cell responses up to D30, but showed that the maintenance of memory responses at later time-points (D60) is independent of transgene expression.

As discussed above, it is apparent that a combination of multiple parameters influences the extent of transgene expression. These factors include the receptor usage and cellular tropism of each Ad vector, the presence and accessibility of specific cell types at the site of injection, in addition to differences in the induction of early innate immunity by diverse Ad

vectors. Collectively, these parameters shape subsequent adaptive immune responses.

Route of Administration

The successful transduction of cells at the site of vaccine administration, and subsequent engagement of defined and desirable PRRs which result in robust transgene expression, depend on the cell type and the specific Ad vector being studied. With this in mind, much of the evidence to date has focused on characterizing the induction of innate immune responses *in vitro* in APCs such as DCs or macrophages, which play important roles in initiating anti-viral immune responses. It is considered that inflammation induced at the injection site, can lead to an influx of APCs (monocytes or DCs) which carry Ag to the dLNs (124). Immature DCs residing at the site of vaccination respond to innate immune signals (i.e., stimulation of TLR pathways) by undergoing maturation, upregulating co-stimulatory molecules and migrating to dLNs where they present Ag to naïve T cells (124). However, non-professional APCs expressing MHC I (125, 126), such as tissue-specific epithelial or endothelial cells, could also contribute to the immune sensing of Ad vectors at the site of injection (39). Therefore, it is clear that the route of vaccine administration (45, 127, 128), the abundance and accessibility of cell types at those locations, as well as the surface expression of suitable entry receptors, will profoundly affect the immunological potency and protective efficacy of a chosen Ad vector.

As a result of the long history of experimental use of Ad vectors as oncolytic agents aimed at treating disseminated metastases, a significant amount of information exists in the literature regarding interactions between Ad vectors, immune cells (28, 77, 129), parenchymal cells (18, 63, 113, 130–134), and stromal cells (130) following *i.v.* delivery of Ad vectors (18, 112, 135). However, *i.v.* vaccination would be impractical for widespread population use and so immunization by *i.m.* or *i.n.* administration is preferable, particularly for vaccination against respiratory pathogens. Unfortunately, less is known about the precise interactions which occur at the site of injection or within dLNs *in vivo* following *i.m.* or *i.n.* immunization with Ads in animal models, and these questions are even more difficult to address in humans, without the use of invasive procedures (such as fine needle aspirates of lymph nodes) (136).

Intramuscular delivery

The cell types present when vaccine is administered *i.m.* include myocytes, skeletal muscle cells, fibroblasts and endothelial cells, with APCs such as DCs or macrophages representing a minority when compared with the abundance of murine skeletal muscle cells (87). Early studies by Mercier et al. demonstrated that transduction of different cell types can modulate the outcome and phenotype of the humoral immune response following Ad vaccination. The authors transduced DCs (professional APCs), myoblasts (progenitor cells which give rise to muscle cells) and endothelial cells *ex vivo* with Ad5 expressing a model antigen β -galactosidase (β -gal) (87), and vaccinated mice *i.m.* with the Ad-transduced cells. The authors found that all transduced cell types elicited humoral immune responses to the β -gal transgene to a similar extent (albeit with differences in their temporal

kinetics), but that the IgG isotype subclass profile differed. Injection of transduced DCs or endothelial cells resulted in production of Ag-specific Abs which were exclusively IgG_{2a}, whereas vaccination with Ad-transduced myoblasts elicited a more balanced Ab response with equivalent IgG₁:IgG_{2a}. Interestingly, only mice immunized with Ad-transduced DCs elicited robust CD8⁺ T cell responses, as did vaccination with control virus Ad- β -gal, suggesting that Ad interactions with different cell types *in vivo* could influence divergent arms of the adaptive immune response.

Bassett et al. also demonstrated that Ag presentation by non-lymphoid cells, in cooperation with hematopoietic APCs, contributes to the kinetics of primary CD8⁺ T cell expansion, the maintenance of memory responses and to the functional phenotype of the cellular immune response following Ad vaccination (39). Through a series of investigations, the authors showed that although dLNs act as the site of immunological priming in response to Ad vaccination, primary expansion of the Ag-specific CD8⁺ T cell response requires a source of sustained Ag expression outside of dLNs (39). They hypothesized that this cell type was of non-hematopoietic origin, due to their prior findings that a radioresistant population of cells was capable of priming CD8⁺ T cell responses in mice leukopenic mice (137). Therefore, it is feasible that several modes of Ag presentation take place following *i.m.* immunization with Ad vectors, all of which contribute to different facets of the ensuing immune response. These interactions are summarized in **Figure 4**, showing that Ag presentation could take place not only within dLNs, but also at the site of injection, with or without the involvement of professional APCs.

It is important to note that the majority of detailed mechanistic studies into the immunogenicity of Ad vectors have been performed using Ad5-based vaccines and as such, similar information regarding the *in vivo* cellular tropism of rare species or non-human Ad vectors, is much more limited (123). It will therefore be crucial to outline the precise factors which confer a hierarchy of potency between Ad vectors in the future, as many Ad vectors are already under clinical investigation, or are advancing rapidly into clinical trials. This improved knowledge would allow us to engineer optimal platform vectors which combine multiple attributes associated with potent immunogenicity and long-lived protective efficacy.

Aerosolized or intranasal delivery

Mucosal *i.n.* or aerosolized (*a.e.*) delivery of Ad vectors is particularly attractive for the development of vaccines against respiratory pathogens (108, 139, 140). Ad vectors are capable of eliciting both humoral and cellular immune responses following *i.n.* (4, 140–142) and *a.e.* (143) vaccination in animal models, both in the bronchoalveolar lavage (BAL) and within the lung interstitium (15, 144). Many wildtype adenoviruses are common respiratory pathogens (i.e., HAdV-C5, HAdV-E4), highlighting their potential suitability for targeting vaccine Ag to mucosal surfaces within the respiratory tract. However, several chimpanzee Ad vectors, which are not associated with respiratory infections, have displayed superior immunogenicity to Ad5 when

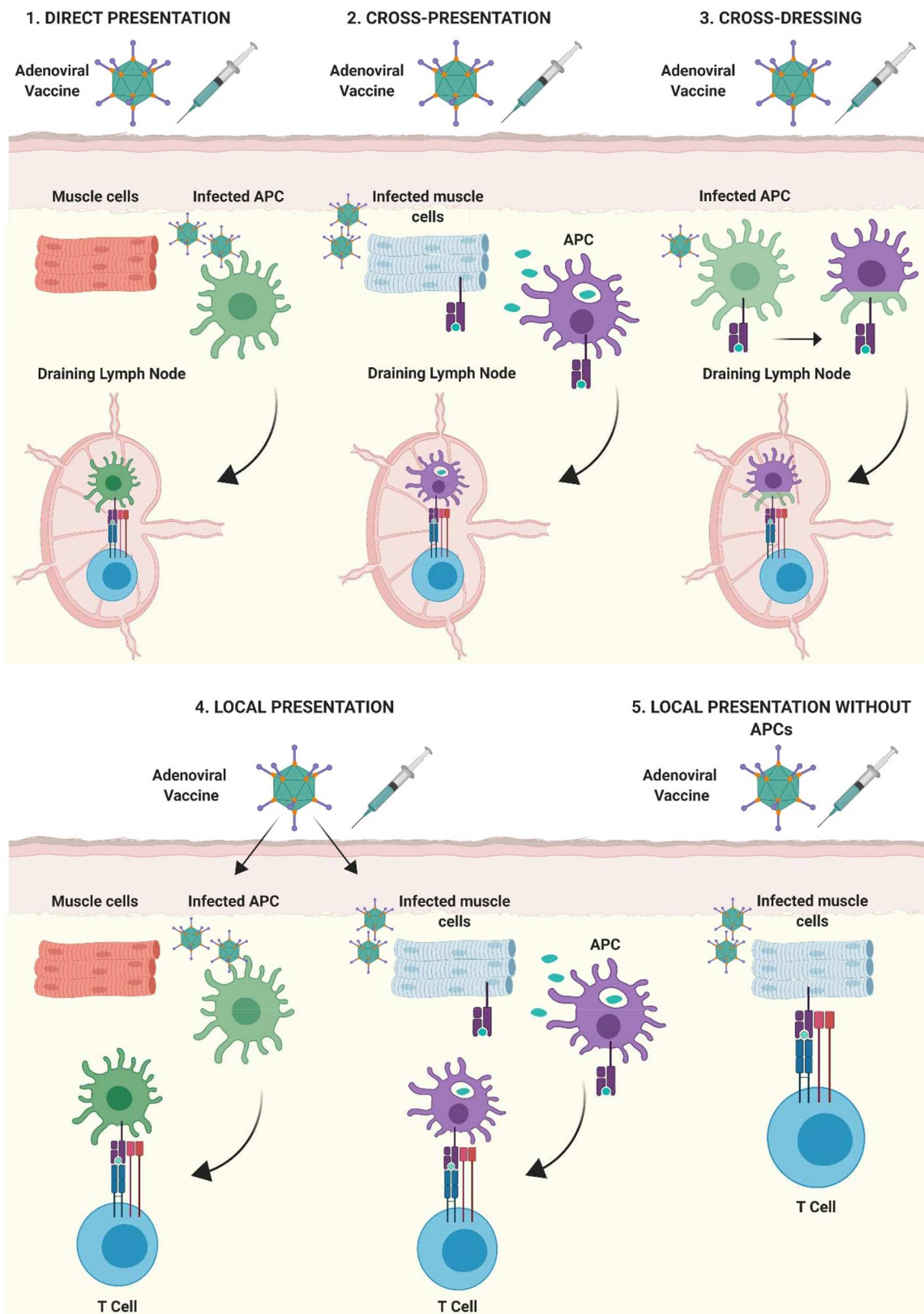


FIGURE 4 | Mechanisms of antigen presentation after intramuscular immunization with adenoviral vectored vaccines. **(1) Direct-presentation:** Adenoviral vaccine transduces APCs at the site of injection. APCs migrate to draining lymph nodes (dLNs) where they present processed vaccine antigen to T cells.

(Continued)

FIGURE 4 | (2) Cross-presentation: Vaccine antigen debris from Ad vaccine transduced cells is phagocytosed by professional APCs at the site of injection, transferred to dLNs by APCs and presented to lymphocytes. **(3) Cross-dressing:** Peptide:MHC complexes from Ad-transduced APCs may be transferred to naïve APCs by a process of membrane gnawing called trogocytosis. **(4)** APCs present at, or infiltrating into the site of injection, can present antigen directly to T lymphocytes. **(5)** Non-professional APCs such as parenchymal cells at the site of injection (muscle cells shown as an example) can present antigenic peptide on MHC I to infiltrating CD8⁺ T lymphocytes, outside of secondary lymphoid organs. Figure is updated from Coughlan et al. (108) and Holst and Thomsen (138) and was created with ©BioRender - biorender.com.

administered by the *i.n.* route in mice, including a ChAdV-68-based vaccine against pulmonary tuberculosis (TB) (144) and a ChAdV-7 vaccine against *Pseudomonas aeruginosa* (145).

A major limitation in the application of rAd vaccines to mucosal respiratory surfaces may be the rapid scavenging and degradation of Ad vaccine vector particles by tissue resident alveolar macrophages in the lung, as it has previously been estimated that ~70% of Ad vector genomes are lost within the first 24h following lung delivery in mice (intratracheal) (66). Furthermore, alveolar macrophages were found to be the predominant cell type responsible for initial inflammatory cytokine induction (68). However, depending on the balance of innate immune stimulation and the retention of a certain level of transgene expression within the respiratory tract, these interactions could be beneficial for the induction of subsequent adaptive immune responses.

Alveolar macrophages may play a role in trafficking to dLNs to facilitate Ag priming, or the inflammatory cytokines they release could signal the recruitment of lymphoid cells which could further potentiate immune responses to Ad-encoded transgene Ag. The many studies which have confirmed the induction of protective immunity following *i.n.* vaccination with Ad vectors support this possibility, but the precise mechanistic factors which underlie these effects and the specific cell types which contribute to vaccine efficacy are not extensively described. As differences in the phenotype and trafficking potential of CD8⁺ T cells has been observed when comparing *i.m.* vs. *i.n.* vaccination of mice with Ad vectors, it will be important to identify the optimal Ad vaccine platform which elicits the correct correlates of protection for a specific disease target, before deciding on the ideal route of vaccine administration (128).

Structural Basis for Immune Activation by Ad Particles

The ability to engage and activate multiple, diverse innate immune signaling pathways simultaneously (excluding Type I IFN) can allow rAd vectors to act as an effective self-adjunct, relative to other vaccination platforms (109, 110, 146). These attributes suggest that Ad virions themselves possess several PAMPs. In fact, it is considered that the native receptor determinants or entry factors of a particular Ad vector may, at least in part, regulate innate immune activation. In support of this, binding of the fiber knob domain to CAR is sufficient to activate p38MAPK, p44/42MAPK (ERK1/2), and NF- κ B pathways (147), resulting in the transcription of pro-inflammatory cytokines both *in vitro* and *in vivo* (148). Similarly, Ad vectors which are capable of using CD46 as a receptor have been shown to display preferential activation of TLR9 to CAR-binding Ad vectors *in vitro* (61). However, CD46 distribution is

limited to the expression in the testes in mice, rats and guinea pigs, in contrast to its widespread expression in humans (149). Therefore, this likely has little contribution to the immunological potency of CD46-using Ad vectors in murine models.

The fiber knob domain of Ad5-based vectors has been shown to contribute to the maturation of murine DCs, as recombinant knob protein, full-length fiber protein and penton capsomers (penton plus fiber), but not hexon or penton alone, were capable of exerting this effect (90). In support of this, Teigler et al. previously suggested that fiber-receptor interactions were important for triggering innate immune responses to rare species Ad vectors (26). Furthermore, studies using HAdV-B35 vectors pseudotyped with the fiber knob from Ad5 were shown to be more immunogenic in mice and NHP, suggesting that the fiber knob domain could contribute substantially to immunological potency (150). However, similar studies in which the entire fiber and penton RGD motif from rAd5 were swapped into chimpanzee Ad vector ChAdOx1, failed to increase the immunogenicity of ChAdOx1 relative to Ad5, suggesting that factors beyond fiber/penton capsomer interactions confer immune responses to ChAdOx1 (123).

More recently it has been shown that a member of the vitamin-K dependent protein family, growth arrest specific protein (Gas6), can bind differentially to the fiber of rAd vectors (151), interacting with a putative epitope which is outside of the fiber knob domain, such as the fiber shaft. Interestingly, Gas6 bound efficiently to the fiber of CAR-binding Ad5, without affecting its ability to enter cells, but significantly reduced the induction of type I IFNs, resulting in prolonged transgene expression. Conversely, Gas6 failed to bind to the fibers of non-Ad5 and non-CAR binding viruses, such as a species D Ad vectors HAdV-D28, which has been shown to display reduced immunogenicity in mice when compared with rAd5 (152). In support of this, HAdV-D28 has been shown to elicit robust IFN- α and had increased stimulation of IFN-responsive genes when compared with Ad5 (44). Importantly, immunization of IFNRI^{-/-} mice with HAdV-D28 resulted in robust cellular immune responses, comparable to Ad5 (44). These data support the hypothesis by several other groups that Ad vectors which trigger robust type I IFN responses exhibit reduced transgene expression and impaired vaccine immunogenicity (33, 120). Therefore, one strategy to overcome stimulation of IFN- α could be to identify the precise amino acid binding epitopes for Gas6 and genetically engineer this region into the fiber of non-Gas6 binding Ad vectors in an effort to increase their immunogenicity (151).

In addition to interactions with CAR, interactions between the RGD motif within the penton base and cellular integrins can contribute to the induction of innate immune signaling pathways both *in vitro* (153) and *in vivo* (77). Following *i.v.* administration

of Ad5, interactions between the $\beta 3$ subunit of cellular integrins and the RGD motif on MARCO⁺ marginal zone macrophages in the spleen were required for triggering IL-1 α -dependent innate immune signaling in mice (77). However, it is unclear how these interactions affect immune responses following immunization by other routes of administration. Finally, the Ad virion itself and its major capsid protein, the hexon, has been described as a potent adjuvant, capable of activating CD4⁺ and CD8⁺ cellular immune responses to a model immunogen mixture in mice (146). Although the precise mechanism underlying this effect has not been identified, subsequent studies have suggested that the HVR regions, particularly HVR1, are involved in enhancing interactions with scavenging macrophages (76, 78).

Novel Approaches to Increasing the Immunological Potency of Ad Vectors

One approach to identifying lead vaccine candidates is to perform head-to-head comparisons of immunogenicity in animal models to identify the most immunogenic vectors, followed by detailed and systematic experimental studies to try to identify the underlying mechanisms which contribute to immunological potency (i.e., transgene expression magnitude and duration, innate immune stimulation). However, in the interim, efforts are ongoing to try to maximize immune recognition of the Ad encoded Ag, in an effort to compensate for the reduced immunogenicity associated with certain Ad vectors which are otherwise an ideal platform (i.e., low seroprevalence, high titer growth, stability). Furthermore, this type of modification could permit dose-sparing, which would have cost saving effects, as well as minimizing any vector-induced reactogenicity, without compromising on immunogenicity. Such approaches include the use of molecular or genetic adjuvants, namely in the form of fusion proteins with the vaccine antigen of interest, which help to potentiate immunogenicity by enhancing Ag presentation or dissemination [reviewed in detail by Neukirch et al. (154)].

One such approach which has previously been described includes the generation of fusion-Ag cassettes which enhance MHC presentation. This can be achieved by fusing vaccine Ag to β -microglobulin for enhanced MHC I presentation (155), or to the invariant chain of MHC II (156–159). These approaches were capable of enhancing antigen-specific CD8⁺ T cells responses in mice following immunization with an Ad vector encoding the Ii or $\beta 2$ -microglobulin fusion antigen. In a separate approach, we recently have shown that encoding vaccine Ag cassettes in which a model antigen, enhanced green fluorescent protein (EGFP), is fused to a protein domain known to tether proteins to the surface of extracellular vesicles (EVs), can dramatically improve the humoral immunogenicity of both Ad5 and chimpanzee Ad vector ChAdOx1 in mice following *i.m.* and *i.n.* vaccination. EVs play important roles in regulating immune responses and are conveyors of cellular communication signals (15). As EVs are frequently implicated in host-pathogen interactions and have been shown to transfer antigenic material to APCs (81, 82), we reasoned that directed targeting of a vaccine Ag to their surface could potentiate immune responses. Although cellular immunity was largely unaffected, Ag-specific humoral immune responses were increased up to 400-fold when compared with

unmodified EGFP (15). The choice of molecular or genetic adjuvant will depend on the predicted correlates of protection for a specific disease target: in certain cases, a robust humoral immune response will be more important than a cellular immune response. Further to this, some adjuvanting technologies will work for one Ag but not for another, and structural constraints may limit the ability to modify complex, multimeric Ags. This will require the optimization and selection of different components to be combined and assembled into customized Ad vectors which are tailored to specific pathogens.

Clinical Evaluation of Ad Vaccines for Infectious Disease; Challenges; and Advances

A number of promising animal studies solidified the appeal of Ad5 as a platform vaccine vector for infectious diseases. In particular, a study by Sullivan et al. demonstrated that a single-shot, low dose (1×10^{10} vp) immunization with Ad5 expressing Ebola virus glycoprotein (GP) could provide 100% protection from challenge in NHPs (160). Similar studies using Ad5 based vectors expressing SIV gag demonstrated its superiority to plasmid DNA or modified vaccinia Ankara (MVA) in attenuating viremia following virus challenge (12). On the basis of these promising early results, a multicenter Phase II clinical trial called the Merck STEP study was initiated, in which a vaccine composed of a mixture of Ad5 vectors expressing HIV-1 *gag*, *pol*, and *nef* genes was administered to participants at high risk for HIV-1 acquisition (161, 162). Phase I safety and immunogenicity studies in healthy, HIV-seronegative adults showed that this vaccine could elicit antigen-specific IFN- γ ELISpot responses in both Ad5 seronegative and seropositive individuals (163). However, the Phase II study was terminated prematurely due to futility and failure to meet pre-defined endpoints: an interim analysis determined that the vaccine would not be efficacious in preventing HIV-1 infection, or in reducing viral-load setpoint in seroconverters, despite eliciting T cell responses in most participants (162, 163). Subsequent to this, a *post-hoc* analysis of the study suggested an association between vaccination with the Ad5 vaccine and increased acquisition of HIV-1 (161). On multivariate analyses, this increase was largely restricted to a defined sub-set of participants: uncircumcised men with high baseline antibody titers against Ad5. Several hypotheses were proposed to explain the increase in HIV-1 acquisition, including the formation of immune complexes (IC) between anti-Ad5 antibodies and DCs, which were shown to enhance HIV-1 infection of T cells in DC-T cell co-cultures *in vitro* (164). An alternative hypothesis suggested that Ad5 immunization induced the expansion of Ad-specific memory CD4⁺ T cells which upregulate CCR5 expression and/or homing markers for mucosal sites, thereby increasing the pool of HIV-1 susceptible cells at the site of infection (165). Although the latter hypothesis has been challenged (166), the precise mechanisms underlying the increased acquisition of HIV-1 in the Merck Step trial remain inconclusive (166). However, it is important to note that the effects were shown to wane over time (167). Nonetheless,

this outcome led to a dampening in enthusiasm for the broad application of Ad5-based vaccines for major infectious diseases, prompting the investigation of novel rare species human or non-human Ad viruses as alternative vaccine platforms.

Advances

Several rare species or non-human Ad vaccines are now leading the way in human clinical trials, namely species C vector HAdV-C6 and species D vector HAdV-26, as well as chimpanzee Ad vectors ChAdV-3, PanAdV-3, ChAdV-63, and ChAdOx1, which cluster phylogenetically with species C or E human Ads (see **Table 1**). Many of these vector candidates had previously been identified in animal studies as being potently immunogenic, and in some cases were comparable to the potency of Ad5 [(5, 168); **Table 2**]. In particular, HAdV-C6 and ChAdV-3 appear to possess attributes which make them an attractive platform vector (22%, 12% seroprevalence, respectively) (2, 5), and as a result, have been developed for clinical testing as vaccines against major global pathogens, hepatitis C virus (HCV) (2) and HIV-1 (169). Both HAdV-C6 and ChAdV-3 were shown to be safe and immunogenic in humans when used as a vaccine to elicit immunity against HCV, although HAdV-C6 appeared to be superior in its ability to elicit cellular immune responses with increased magnitude and breadth at lower doses (i.e., 5×10^8 vp) (2). With regard to ChAdV-3, part of its appeal includes its ability to elicit long-lived cellular and humoral immune responses directed toward the encoded vaccine antigen. Immunization of NHPs with ChAdV-3 expressing HIV *gag* resulted in cellular immune responses which persisted for more than 5 years (5). A heterologous boost with PanAdV-3 at week 299 facilitated an expansion of *gag*-specific IFN- γ secreting T cells, in addition to boosting antibodies to HIV-1 *gag*. As such, there is broad interest in using these rare vectors in heterologous prime:boost vaccination regimens in an effort to confer long-lived protective immunity against challenging pathogens. In support of this, a Phase I clinical trial to test the HAdV-C6 and ChAdV-3 vectors expressing HCV antigens demonstrated that heterologous boost immunizations resulted in long-lived, polyfunctional effector, and central memory T cell responses which were sustained for up to 1 year in humans (2).

PanAdV-3 expressing Respiratory Syncytial Virus (RSV) fusion (F), nucleocapsid (N), and matrix (M2-1) antigens has also been tested in humans following *i.n.* and *i.m.* administration (37). Neutralizing antibodies to RSV F were increased following *i.m.*, but not *i.n.* prime immunization. Similar trends were observed for antigen-specific T cell responses to the vaccine inserts, although increases were minimal following PanAdV-3 *i.m.* prime only. This vaccine was also evaluated in older adults (60–75 years) who are at increased risk of severe RSV disease, with similar results (170). Immune responses elicited by PanAdV-3 were improved upon boosting with an MVA vector which also encoded the RSV transgene antigen. ChAdV-63, also identified as a clinically viable Ad vector with low seroprevalence which displayed protective efficacy in animal studies (**Table 2**), has been shown to be safe and immunogenic in children and adults (171–174). When ChAdV-63 has been used in a prime:boost vaccination regimen with MVA expressing

malaria antigens, promising efficacy was observed in UK and Kenyan adults (175, 176), but has recently been associated with disappointing efficacy in field trials in young children in Burkina Faso, a highly endemic malaria transmission region (177). ChAdOx-1 is a species E chimpanzee Ad vector developed by the Jenner Institute at University of Oxford which has been tested clinically as a vaccine for influenza virus as a standalone vector or for use in prime:boost with MVA (1, 3), and for several other infectious disease targets such as Chikungunya Fever (NCT03590392), malaria (NCT03203421), and tuberculosis (NCT01829490). Numerous additional trials are currently ongoing or actively recruiting participants, including a recently initiated study to test a novel vaccine for COVID-19 (NCT04324606).

In addition to vectors derived from NHPs, promising advances have been the development of HAdV-D26 vectors. With a number of clinical trials registered on ClinicalTrials.gov, this platform has advanced into clinical studies as a vaccine against Ebola virus (178–180), RSV (181), HIV-1 (6, 182, 183), and has also very recently been announced as a candidate vectored vaccine against COVID-19. The first-in-human testing of HAdV-D26 expressing HIV-1 Env demonstrated that the vaccine was safe and well-tolerated and elicited Env-specific antibodies and antigen-specific ELISpot responses in all participants (182). Although HIV-1 specific neutralizing antibodies were not detected, the study reported multi-functional readouts for the non-neutralizing antibodies elicited, including effector functions such as antibody-dependent cell-mediated phagocytosis (ADCP), antibody-dependent cell-mediated virus inhibition (ADCVI) (183). This vaccine platform was subsequently improved upon by encoding polyvalent “mosaic” HIV-1 antigens, Env, Gag and Pol, representing computationally optimized sequences aimed at maximizing recognition of T cell epitopes (184). Evaluation of the HAdV-D26 platform in various prime:boost regimens is ongoing and preliminary data suggest that it is immunogenic, capable of eliciting Env-specific antibodies which exhibit ADCP and cellular immune responses out to week 50. Importantly, these assays were found to be correlates of protection in a parallel SHIV challenge model in rhesus monkeys (*Macaca mulatta*) (6). The HAdV-D26 mosaic HIV-1 vaccine is currently in Phase IIb efficacy studies in sub-saharan Africa (NCT03060629).

In Conclusion

Based on the literature, it appears that Ad vectors derived from species C, D, or E are most likely to be immunogenic vectors. Requirements for selecting specific vectors will vary depending on whether the required application is as a stand-alone vaccine or as part of a prime:boost regimen. For standalone vaccination regimens aimed at eliciting a rapid, protective response during an emerging pandemic, the magnitude of response to an identified correlate of protection following a single shot is crucial. Secondary considerations for an evolving pandemic scenario would be rapid immunogenicity at a low dose, and the capacity for lyophilization or stabilization, to facilitate dose-sparing, vaccine cost-effectiveness and pandemic preparedness. In this regard, of the Ad vectors evaluated to date, ChAdV-3 and HAdV-C6 appear promising. For protection against more complex

pathogens which require long-lived polyfunctional responses, or sustained humoral immunity with extensive breadth (i.e., universal influenza vaccine, HIV-1 or HCV), heterologous prime:boost vaccination regimens should be evaluated using diverse Ad vectors, or Ads in combination with MVA or protein based immunogens at different intervals. It is difficult to predict which Ad vectors should take precedence as the encoded antigen will need to be tailored to elicit the correct phenotype of immunity against a defined correlate of protection for each specific disease target. However, underpinning the evaluation of all Ad-based vaccines in pre-clinical animal studies, should be the inclusion of species C Ad5 vector controls to represent a benchmark of immunological potency. In addition, Ad vaccine candidates should be compared at several doses to evaluate the maintenance of vaccine potency upon dose de-escalation. The field should also make efforts to improve our understanding of the basic biology of many of these novel Ad vectors, as insights into the receptor usage, interactions of Ad vectors with different cell types following immunization and subsequent stimulation of differential innate signaling pathways will all impact on their downstream immunogenicity and ability to confer protective efficacy. Unfortunately, one major challenge in performing these types of head-to-head comparisons is the lack of widespread availability of many of these rare species or non-human Ad vectors to academic investigators, as many of these are being developed by large pharmaceutical companies.

SUMMARY

It is clear that a hierarchy exists in the immunological potency observed between rare species human and non-human Ad

vectors in various animal species. As outlined above, Ad vector immunogenicity is most likely dependent on a complex combination of factors, rather than any particular factor in isolation. An ideal Ad vaccine platform will combine the following attributes; (i) low seroprevalence in humans, (ii) robust immunogenicity with potential for dose-sparing, and (iii) suitable manufacturing characteristics (i.e., growth to high titers, genome stability). Better understanding of the mechanisms which define effective vaccines, will enable us to design and customize improvements to existing Ad vaccine vectors, enhancing their potential for future clinical use.

AUTHOR CONTRIBUTIONS

LC: writing and editing—original and final draft.

FUNDING

LC was supported in part by funding from NIH/NIAID 1R21AI146529-01, CEIRS (HHSN272201400008C, Option 17C) and the 2018 *Pichichero Family Foundation Research Development Vaccines for Children Initiative Award in Pediatric Infectious Diseases* from the Pediatric Infectious Disease Society (PIDS).

ACKNOWLEDGMENTS

Thanks to Prof. Peter Palese, ISMMS, for helpful and constructive discussion of this review. Figures were created with ©BioRender - biorender.com.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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