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GENERAL INTRODUCTION

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VACCINES

Vaccines have made an enormous contribution to human health since their first introduction, approximately 200 years ago [1, 2]. According to the World Health Organization (WHO), vaccines prevent more than 2.5 million deaths annually [3, 4]. In developed countries, a striking 100% decrease in the number of cases and deaths due to vaccine-preventable diseases such as diphtheria, smallpox, polio, measles, mumps and rubella has accompanied routine mass immunization programs [3, 5, 6]. Worldwide, vaccination has resulted in the eradication of smallpox [7] and near eradication of polio [8, 9]. Disease prevention and eradication have a significant socio-economic impact, with an estimated 280 billion US dollars of social and economic benefits from vaccination for the period 2001-2020 worldwide [10, 11]. Importantly, averting death and medical impoverishment greatly reduces inequity worldwide and promotes social and economic growth, especially in developing regions [12, 13].

Vaccines function by stimulating the host to generate an immune response against a specific pathogen without causing disease. In this way, the immune system is prepared to protect the host when the real pathogen is encountered. Classically vaccines consisted of inactivated or weakened pathogens or toxins generated by virulence attenuation (e.g. through cell-culture passaging) and chemical inactivation (e.g. formaldehyde) [1, 2]. However, new strategies for the development of preventive or therapeutic vaccines are necessary for communicable and non-communicable diseases which have proven difficult to target using these classical techniques. These include pathogens such as respiratory syncytial virus (RSV), human immunodeficiency virus (HIV) and cancers [14, 15]. RSV is a leading cause of severe respiratory infection and hospitalization in infants and young children [16], yet vaccine development using conventional technologies has been unsuccessful to date. In the 1960s, children vaccinated with a formalin-inactivated RSV vaccine experienced enhanced RSV disease after exposure to wild-type RSV [17]. More recent attempts to develop live-attenuated RSV vaccines have also faced challenges with respect to finding the right balance between attenuation, immunogenicity and safety [18-20].

New technologies that are being investigated for vaccine generation include novel adjuvants, reverse vaccinology, structural vaccinology, bioconjugation and recombinant DNA [14, 15]. Recombinant DNA technology has revolutionized vaccine development. Recombinant subunit or DNA vaccines can now be produced safely in large quantities without culture contamination of (highly) infectious pathogens. The highly successful virus-like particle (VLP) vaccines against human papilloma virus and hepatitis B [21, 22] have been generated using the recombinant subunit technology. The concept of DNA or genetic vaccines has been widely tested against various infectious diseases and cancers [23]. Genetic vaccines are engineered to encode an antigen. The genetic material enters the host cell, utilizes the cellular machinery to express the transgene which then promotes host production of potent antigen-specific B- and T-cell immune responses

against the encoded antigen. There are several types of genetic vaccines: 'naked' DNA (plasmid) or RNA (mRNA) vaccine platforms [24-26], virally derived self-replicating RNA encoding both the antigen and proteins [27-29], and vectored vaccines using viral vectors as vehicles [30-32].

Viral vectors may offer many advantages over other vaccine platforms. As particles they are typically highly stable and can be produced at high titer yields in producer cell lines. They exhibit an acceptable safety profile and are highly efficient at transducing host cells inducing strong B- and T-cell immune responses against the encoded antigen without the need for an adjuvant [30]. Importantly, viral vectors can be modified to encode single or multiple antigens for use as preventive or therapeutic vaccines [33]. Viruses 'vectorized' for vaccine purposes include modified vaccinia Ankara (MVA), cytomegalovirus (CMV), vesicular stomatitis virus (VSV), yellow fever and adenoviruses (AdV) [30-32, 34, 35]. AdV vector vaccines are being extensively tested in humans for the prevention of numerous infectious diseases such as HIV or treatment of cancer. This thesis focuses on advancing the design of adenovirus vectored vaccines against infectious diseases.

Adenoviridae

AdV are members of the *Adenoviridae* family and can be further divided into genera *Mastadenvirus (e.g. human AdV [HAdV]), Atadenovirus* (e.g. ovine AdV), *Aviadenovirus* (e.g. fowl AdV), *Ichtadenovirus* (e.g. sturgeon AdV) and *Siadenovirus* (e.g. frog AdV) [36-38]. The discovery of HAdV dates back to 1953 when Rowe and Hilleman & Werner almost simultaneously isolated a 'new cytopathogenic agent' from the adenoid tissue of individuals presenting with respiratory illness [39, 40]. Today, over 65 different HAdV types have been identified and classified into seven species (A-G) [41]. HAdV classification was historically based on their ability to agglutinate erythrocytes and oncogenicity in rodents. More recently, viral genomic sequence analysis has become an important tool in the classification of AdVs [42, 43] and resulted in inclusion of non-human primate AdV to the HAdV taxonomy due to their high degree of sequence homology [44]. The existence of a large group of human and non-human primate AdVs provides the opportunity to choose AdV types for with beneficial characteristics for vectorization and vaccination of humans; such as chimp AdV type 3 (ChAdV3) or HAdV type 26 (HAdV26).

WILD-TYPE ADENOVIRUS INFECTION IN HUMANS

In healthy humans, HAdV usually cause mild and self-limiting upper respiratory infections and are one of the causes of the 'common cold'. By age 10, most children have had multiple respiratory AdV infections [45]. In rare cases, some HAdV types can cause other clinical diseases such as conjunctivitis (e.g. HAdV8, HAdV19), gastroenteritis (e.g. HAdV40, HAdV41), and pneumonia (e.g. HAdV4, HAdV7) [45]. In crowded conditions such as military training camps, serious respiratory illness caused by HAdV4 (species E) and HAdV7 (species B) has been reported [46]. Due to this high disease burden, a highly

effective oral HAdV4 and HAdV7 vaccine has been administered to millions of American military recruits since 1971 [47]. A halt in the vaccine program was followed by an increase in HAdV4 and HAdV7-associated respiratory illnesses among recruits. Resumption of the vaccine program in 2011 was associated with a rapid decline in disease cases [48]. HAdV infection is most severe in immunocompromised individuals who may develop significant and potentially fatal clinical disease [49]. Currently, there are no HAdV-specific antiviral drugs available. HAdV infection is sometimes treated with broad-spectrum antivirals such as the nucleoside analogues cidofovir and ribavirin, although with varied success [50, 51].

ADENOVIRUS CAPSID ORGANIZATION

The non-enveloped AdV ~90nm capsid is highly structured and organized in an icosahedron [52-55]. The AdV icosahedron consists of three major capsid proteins; hexon, penton, and fiber, and four minor 'cement' proteins; IIIa, VI, VIII and IX. Six additional non-structural proteins packaged together with the double stranded DNA (dsDNA) genome are protein V, VII, μ, IVa2, terminal protein (TP, covalently linked to the '5 DNA termini), and adenovirus protease (AVP) [56-59] (**Figure 1**). The exact fold, symmetry and arrangement of the AdV capsid proteins have been determined in great detail [53, 55].

Major capsid proteins

The major capsid protein hexon monomers contain a conserved base and surface-exposed hypervariable regions (HVR1-7) [43, 60]. Per capsid, 720 hexon monomers self-associate into a trimeric hexagon and make up the main structure of the AdV icosahedron. Five peripentonal (i.e. associated with penton) hexon trimers are directly associated with five penton monomers at the 12 capsid vertices. The penton protein is highly conserved amongst AdV types aside from the Arg-Gly-Asp (RGD) motif region [61-64]. All HAdV, except the enterotropic species F types 40 and 41, contain a RGD motif surrounded by long (e.g. species C HAdV2) or short linker peptides (e.g. species A HAdV12) [61]. Five penton monomers assemble into a pentameric pore-like ring structure allowing fiber trimers to bind on the top of the penton central pore [61, 62]. The fiber consists of three distinct domains; top C-terminal knob domain, the central flexible β-sheets shaft region and a highly conserved N-terminal tail which interacts with the penton [62, 65].

Minor capsid proteins

The major capsid proteins are tethered in place by minor capsid proteins [52, 66]. Protein IX, exclusive to the *Mastadenovirus* genus, with 240 copies per capsid, is imperative for stabilizing the hexon-hexon interactions [58]. Protein IX is a flexible protein, arranged in a triskelion conformation in the hexon grooves [52, 66, 67]. The highly conserved N-terminus of protein IX is essential for the capsid-cementing properties. The central domain facilitates self-association, while the variable C-terminus of the protein protrudes

through the hexon cavities exposing its tail to the outer surface [68, 69]. AdV can be generated without protein IX, although at a cost of reduced thermostability [70] and impaired packaging of full-length genomes [71].

Protein IIIa (pIIIa) is highly conserved in all AdV and is essential for assembly of the capsid [72]. Sixty pIIIa monomers form an antiparallel four-helix bundle structure with a long-extended N-terminus reaching all the way toward the penton vertices. During capsid assembly premature IIIa is incorporated into the capsid and cleaved by AVP at its C-terminus to give rise to mature IIIa protein [73]. The minor capsid proteins VI and VIII are predominantly centered directly below the capsid vertices, fortifying the capsid from inside out [52, 66]. Protein V, strictly a core protein, interacts with VI and VIII, most probably tethering the dsDNA genome in place and contributing to the overall structure of the icosahedron [61].
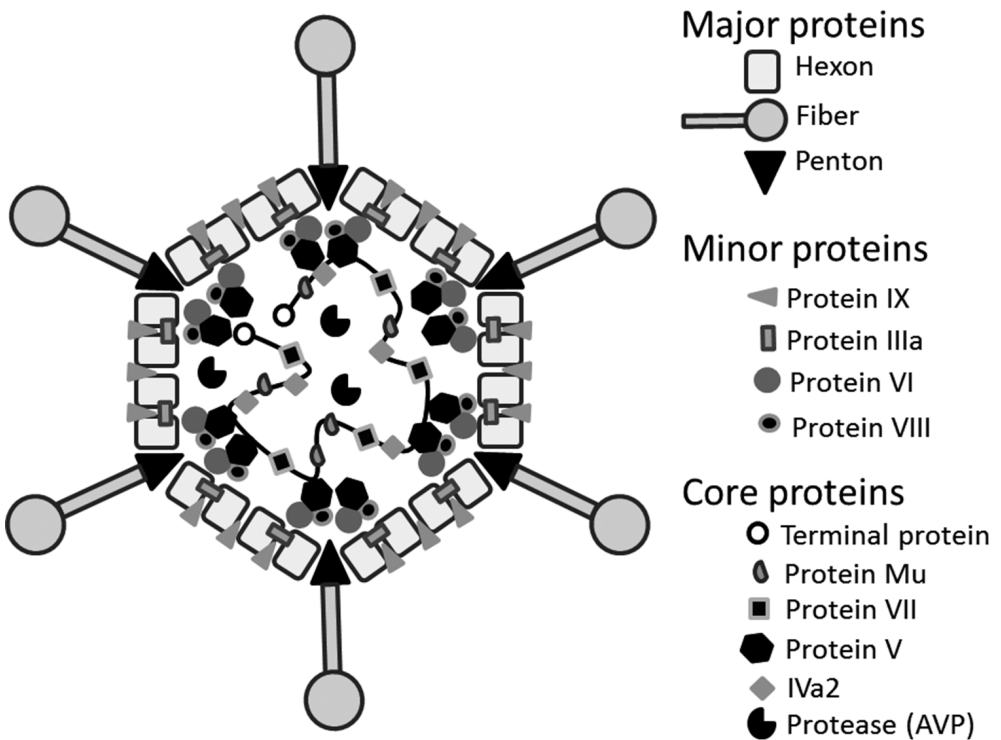
Figure 1. AdV capsid organization (adapted from [74]). Schematic representation of the organization of an AdV capsid. The outer capsid shell consists of the major capsid proteins hexon, fiber and penton base. The minor capsid proteins bolster the capsid by organizing an outer protein cage (protein IX and protein IIIa) and an inner protein cage (protein VI and VIII). Non-structural core proteins, protein V, VII, μ, IVa2, terminal protein, and adenovirus protease (AVP) are packaged with the dsDNA protein.

ADENOVIRUS CELL ENTRY

AdV can efficiently enter a wide range of dividing and non-dividing cells. AdV cell-entry is initiated by capsid protein attachment to cellular receptors, followed by internalization and transport to the nucleus (**Figure 2**). Initial attachment is facilitated by the interaction of the fiber protein with primary cellular receptors. Different AdV types interact with different cellular receptors which determines their tissue specificity, or tropism. The fiber of AdV species A, C, D, E and F can interact with the coxsackie and adenovirus receptor (CAR) a member of the immunoglobulin superfamily which forms intercellular homodimers in epithelial cell junctions in a number of tissues such as the prostate and heart [75]. Some AdV species B (e.g. HAdV35) fibers interact exclusively with CD46 receptor, a member of the complement activation family expressed on all nucleated cells [76], while others (e.g. HAdV3), bind to additional receptors such as desmoglein, a cell adhesion molecule [77]. HAdV species D which contains the majority of identified HAdV types, is the least well-characterized group with respect to cellular receptor interactions [75]. The fiber of some species D (e.g. HAdV9) interacts with the CAR receptor [78], whereas others interact with sialic acid-containing glycoproteins (expressed on all cells) [79] and CD46 (e.g. HAdV37) [80].

After the attachment to the cell surface, the AdV penton interacts with the cell adhesion molecules integrins, triggering virus internalization by clathrin-dependent,
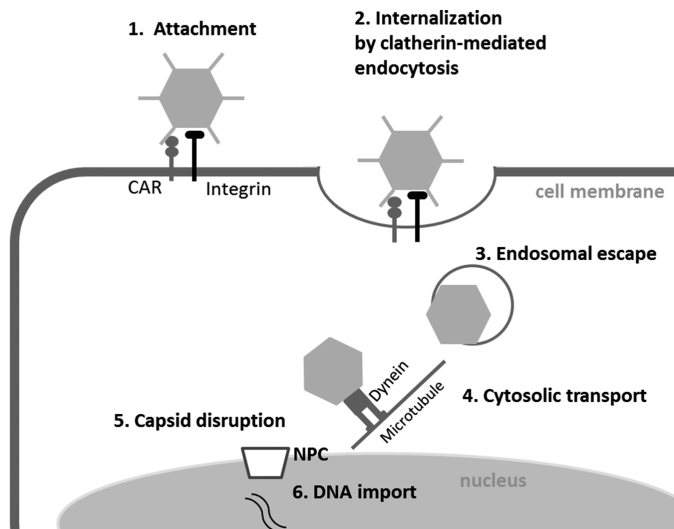
Figure 2. AdV cell entry. Schematic outline of an HAdV cell entry mechanism. Cell attachment takes place by AdV capsid binding to the primary cellular (e.g. CAR) and secondary cellular (integrins) receptors. The AdV is subsequently internalized in a process termed clathrin-mediated endocytosis. In a pH dependent step AdV escapes the endosome and is transported over the microtubule by dynein. In the final step the capsid disrupts releasing the double stranded DNA genome which is transported through the nuclear pore complex (NPC) into the nucleus (original drawing).

receptor-mediated endocytosis [81, 82]. The AdV integrin interaction is largely mediated by the penton RGD-motif that binds integrins such as αvβ3 and αvβ5 [83]. The AdV particles are endocytosed together with their integrin receptors. Release of the particles into the cytoplasm is believed to be triggered by the acidic environment in the endosome, although the underlying mechanism is poorly understood [84, 85]. In the cytoplasm, AdV particles bind the dynein complex through the hexon protein [86] and are trafficked along cellular microtubules towards the nucleus [87]. The AdV particle subsequently docks at the nuclear pore complex where particle disassembly takes place to allow translocation of the AdV genome into the nucleus [88].

ADENOVIRAL GENOME

Knowledge of the AdV genome has enabled AdV vector engineering. AdV contains a ~36 kbp double stranded DNA genome flanked by inverted terminal repeats (ITR). Transcription and replication of the AdV genome takes places in the nucleoplasm. Transcription can be divided into early (E), intermediate and late (L) units according to the time of gene expression relative to DNA replication (**Figure 3**). Each transcriptional unit contains its own promoter regulating the expression of primary transcripts. Except for polypeptide IX mRNA, the primary transcripts undergo extensive alternative RNA splicing, yielding 50 distinct mRNAs and ultimately 38 viral proteins that are AdV type dependent [89].

EARLY TRANSCRIPTION UNITS

The six early (E) transcription units (E1A, E1B, E2A, E2B, E3 and E4) function to initiate the synthesis of viral DNA replication proteins, induce the host cell to enter the S-phase cell cycle and dampen the cellular antiviral responses.

E1 genes

The E1A transcriptional unit encodes oncoproteins 243R and 298R that act as transactivators which dictate the transcription of E1B, E2A, E2B, E3 and E4 viral genes and cellular genes [90]. Since the availability of many cellular proteins may depend on the nature of the infected cell and its metabolic state, E1A proteins have evolved the capacity to interfere with cell division in different ways involving direct and indirect interaction with various cellular proteins [90]. For example, they can interfere by binding to cyclin-dependent kinase (CDK) complexes that regulate cell DNA synthesis [91] and CDK inhibitors that stimulate cell division and growth [92]. E1 proteins regulate nuclear transactivators such as NF-ĸB, that is responsible for activation of NF-ĸB-response genes. Among NF-ĸB-response genes, E3 genes [93] or p53 tumor suppressors regulate the transcription of genes involved in cell cycle arrest and apoptosis [90]. Cellular transcription patterns are modified in favor of AdV gene transcription by directly binding to TATA-box binding proteins [94]. As well as these examples, E1A can interfere with cell division on its own, or in cooperation with other viral proteins (e.g. E4 gene products) using numerous other mechanisms.

The two proteins encoded in the E1B transcriptional unit, 55Kd and 19Kd, block cellular apoptosis by acting on both the p53-dependent and independent pathways, thereby prolonging cell survival to enable efficient virus proliferation [90, 95, 96]. The 19K gene product acts as an analogue to a host cell protein and blocks cellular apoptosis by interacting with, and inhibiting, p53-inducible cell-death Bax protein [97]. The E1B 55K protein ensures AdV proliferation by cooperating with the viral E4orf6 gene product (34K). A E1B-55K-E4orf6 complex mediates p53 degradation, ensures selective transport and translation of late viral mRNA, while blocking the same process for host-cell mRNA and orchestrating shutoff of host-cell protein synthesis [98-102].

E2 genes and DNA replication

Transcriptional unit E2A encodes the DNA-binding protein (DBP) and E2B encodes TP and DNA polymerase (Pol) [103]. DBP, TP and Pol proteins and two cellular transcription factors, NFI and Oct-1 are all required for AdV DNA replication [104]. Replication of AdV occurs in the nucleus in newly formed replication centers (RC) [105, 106]. There are two kinetically distinct phases in RC that are also associated with a switch in RC morphology. Early RC produce low-levels of transcriptionally active AdV genomes associated with cellular histones for gene expression, while late RC produce high-levels of silenced AdV genomes for virion packaging [107, 108]. AdV genome replication is initiated at the two identical origins of replication located within the first 50bp of the ITR. The protein-primed DNA synthesis starts by the covalent addition of a dCMP to the TP precursor protein which uses nucleotide 4-6 (GTA) in the ITR as a template for the formation of the pTP-CAT complex. The intermediate pTP-CAT complex jumps back three bases to the left to pair with the 1-3bp nucleotide residues, allowing full AdV DNA genome synthesis by AdV Pol [109, 110]. DBP and the two cellular transcription factors NFI and Oct-1, work in syncrony to promote efficient DNA synthesis. The DBP modulates and protects the DNA progeny against nucleases, while the cellular transcription factors enhance DNA replication [111-113]. Finally, the precursor TP is cleaved by a viral protease resulting in progeny DNA linked to a mature TP protein that can subsequently be packaged into virions.

E3 GENES

E3 genes are unique to *Mastadenovirus* genus and dispensable for AdV replication in tissue culture. The E3 transcriptional unit encodes 12.5Kd, 6.7kD gp19Kd, adenovirus death protein (ADP), receptor internalization and degradation α and β (RIDα/β) (10.4kD and 14.5kD) and 14.7kD proteins, which all act to dampen the host innate and cellular immune response to the infected cell [114, 115]. AdV infection sensitizes the cell to tumor necrosis factor (TNF)-induced apoptosis, most probably by E1A proteins binding to p300 (transcription regulator) and viral DNA synthesis [116]. E3-14.7kD functions to inhibit TNF-induced apoptosis [116, 117], thereby prolonging cell viability and virus proliferation. Similarly, RID α&β-complex proteins protect the infected cells from apoptosis induced by the immune system, by ensuring the destruction of death receptors such as Fas and TNF-

ligand receptors [118, 119]. E3-gp19kD protein reduces the display of viral peptides to avoid detection of infected cells by specific cytotoxic T-cells, by inhibiting the transport of major histocompatibility complex class I (MHCI) complex [120]. In contrast, ADP facilitates late cytolysis of the infected cell to ensure efficient AdV progeny release [121].

E4 GENES

Proteins termed open reading frames 1-6/7 (orf1-6/7) encoded in the E4 transcriptional unit have various functions such as facilitating virus mRNA metabolism, orchestrating the shutdown of host protein synthesis, and promoting virus DNA replication [90, 122, 123]. AdV with deletion of the entire E4 region are growth-impaired, whereas deletions in individual orfs have minimal effects on virus growth [122]. For instance, E4orf3 is functionally redundant in the presence of E4orf6 during growth [122]. Similarly, even though E4orf4 product is believed to regulate viral DNA synthesis, it is not essential for virus growth [122]. E4orf6 can provide sufficient functions for viral replication in the absence of all other E4 proteins and is highly conserved across different AdV types [123]. E4orf6 forms a complex with E1B-55K protein which is involved in viral DNA replication, RNA processing, cytoplasmic transport of late viral mRNA, and host-cell protein synthesis shut down [122, 124]. Some of the E4 proteins, together with E1B, function to counteract the transforming activity of the E1A oncoproteins, mainly by stabilizing host-cell p53 levels [125], thereby ensuring AdV progeny release from host cells.

VA RNA

HAdV encodes two highly structured non-coding virus-associated RNA (VA RNA); VA RNAI and VA RNAII (~150-200 nt) [126, 127] that function to ensure efficient virus production. During AdV infection, VA RNAs can accumulate to exceptionally high levels ($\sim 10^8$ copies/cell) in both nucleus and cytoplasm [128]. VA RNAs are essential for efficient virus replication, and deletion of both VA RNAI and II results in a 60 fold decrease in viral titer [129].

The key role of VA RNAI is to inhibit the innate immune protein dsRNA-activated kinase. This activity allows the AdV to escape cellular responses to dsRNA—a potent pathogen-associated molecular pattern (PAMP)—thus ensuring AdV resistance to interferon [128]. In addition to dsRNA-activated kinase, VA RNAI interacts with RIG-I and OAS1 dsRNA-detecting innate immune system proteins [130] and host RNA interference (RNAi) machinery through cellular endoribonucleases, such as Dicer, within the RNA-induced silencing complex [131, 132]. A comprehensive understanding of the effects of VA RNA during AdV infection may promote our understanding of host-pathogen recognition as well as further advancement of AdV-based technologies.

INTERMEDIATE GENES, LATE GENES AND VIRAL ASSEMBLY

The gene products of the two intermediate (IVa2 and protein IX) and late (L) (major late transcription units, MLTU) units encode structural and regulatory proteins necessary for viral capsid assembly and cell release.

Intermediate genes

At the beginning of viral DNA synthesis, transcription of the intermediate units IVa2 and IX is initiated [133]. The replication-dependent activation of IVa2 protein is a result of titration of the cellular IVa2 promoter repressor as the number of AdV progeny DNA increases [134, 135]. Timed accumulation of IVa2 protein in the cell is essential for efficient AdV proliferation due to its multifunctional properties. IVa2 protein supports capsid assembly [136] and viral genome encapsidation [137, 138]. Importantly, IVa2 acts as a transcriptional enhancer of the major late promoter (MLP) activating the transcription of the MLTU [139, 140].

Protein IX gene is embedded between the E1B orf and the E1B polyadenylation signal. Active transcription of the E1B gene during the early transcription phase prevents activation of the protein IX promoter. As a result, protein IX gene transcription can only be achieved using the progeny DNA as a template [141]. Protein IX plays multiple roles in the AdV viral cycle. Besides its capsid-stabilizing protein properties as a minor capsid protein, it acts as a transcriptional activator of TATA-containing viral and cellular promoters [142]. Furthermore, protein IX acts to dampen the host-cell anti-viral responses by relocating the promyelocytic leukaemia (PML) protein—which among other things regulates cellular antiviral responses—within clear amorphous inclusion bodies during the late phase of infection, thereby contributing to optimal viral proliferation [143].

Late genes and viral assembly

Onset of viral DNA replication activates the transcription of the MLTU in the late phase of AdV infection. The MLP promoter drives the transcription of a primary transcript. By way of RNA splicing and polyadenylation using one of the five different sites located at the far-right end of the AdV genome, the primary transcript gives rise to late L1-L5 mRNA families [144] (**Figure 3**). Each major late mRNA contains a ~200bp tripartite leader sequence that ensure that the AdV late mRNA is efficiently translated by means of a cap-independent form of translation [145, 146]. This proves particularly important considering that the L4 encoded viral protein 100K orchestrates a shutdown of host cell translation by blocking the function of cap-initiation complexes [147, 148].

MLTU transcription is elevated considerably to ensure the production of adequate amounts of structural proteins (e.g. L3 hexon and L5 fiber) for the progeny virion assembly which takes place in the nucleus [149, 150]. AdV capsid assembly proceeds in a sequential order: (i) assembly of empty capsids; (ii) specific recognition of the viral genome by packaging proteins (e.g. IVa2 and 22K); (iii) insertion of the AdV genome into
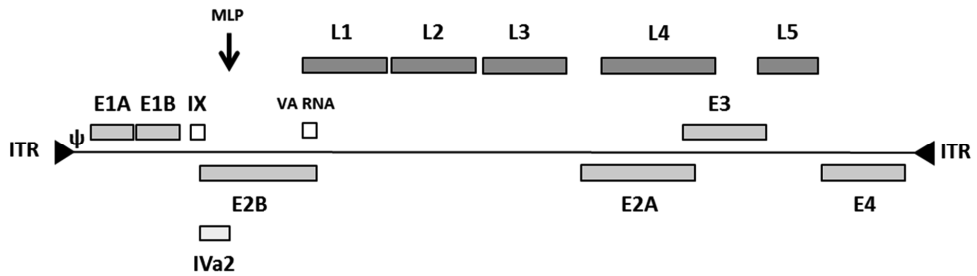
Figure 3. Adenoviral genome transcriptional units. Schematic illustration of a human adenoviral genome and the position of transcriptional units. The transcriptional units denoted with an 'E' are expressed early in the viral replication cycle (light grey). The E1 region is divided into E1A and E1B. E1A encodes three proteins; 9S, 12S and 13S. The E1B region encodes two genes; 55K and 21K. The E2A region encodes the single-stranded DNA-binding protein and E2B encodes two proteins; adenoviral polymerase and terminal protein. The E3 region encodes six proteins in total; 12.1K, 16K, gp19K, ADP, RIDαβ and 14.7K. The E4 region encodes Orfs 1-6/7. Protein IX which contains its own promoter, and protein Iva2, are expressed in an 'intermediate' phase in the replication cycle right after the early genes. The transcriptional units denoted with an 'L', are expressed late in the viral replication cycle (dark grey). The transcription of the late proteins is directed by the major late promoter (MLP) indicated by a vertical arrow. The L1 region encodes two proteins; IIIa and 52.55K. The L2 region encodes three proteins; III (penton), VII and V. The L3 region encodes three proteins; VI, II (hexon) and Pr. The L4 region encodes three proteins; 100Kd, 33Kd and VIII. The L5 region encodes protein IV (fiber). The VA RNAs I and 2 are transcribed in the intermediate/late phase of the viral life cycle. Adenoviral genome is flanked by the two left and right inverted terminal repeats (ITR). The packaging signal (ψ) is located just after the left ITR. Transcriptional units located above the schematic genome line are encoded on the positive DNA strand and transcribed from left to right. The lower transcriptional units are encoded in on the minus DNA strand and transcribed from right to left.

empty capsids; and cleavage of the immature proteins by the encapsulated AVP, resulting in mature infectious viral particles [150-153]. Cell lysis and progeny virion release (~10^4 per cell) occurs approximately 30 hours post-infection and is orchestrated by the ADP (encoded in the E3 region) which accumulates late in the infection [154, 155].

ADENOVIRUS VECTORS

AdVs thus have a small DNA genome that is well characterized and easy to engineer, allowing effective generation of gene delivery vectors [156, 157]. AdVs were initially vectored as gene therapy vehicles to replace damaged or abnormal genes in the host. The world's first commercial gene therapy is an AdV – p53 vector against head and neck squamous cell carcinoma, and is sold under the brand name *Gendicine* in China [158]. As vaccine vectors, AdVs have many intrinsic characteristics that promote their wide application. These include: their mild disease profile in immunocompetent human adults; broad cell tropism; ability to infect different dividing-, and non-dividing cells; generation of high amounts of progeny in cell culture; and genetic and particle stability [159, 160].

There are three types of gene delivery AdV vectors; replication-competent [161, 162], replicating single-cycle [163-165], and replication-incompetent vectors [159, 166] (**Figure 4**). Replication-competent AdV vectors are generated with more or less intact E1 genes (e.g. CR2 deletion) and deletions in E3 (and sometimes E4) genes [161, 162, 167] to accommodate insertions of therapeutic genes. They have been applied widely in cancer therapy as 'oncolytic vectors' often encoding therapeutic transgenes, with noteworthy successes in clinical trials [168, 169].
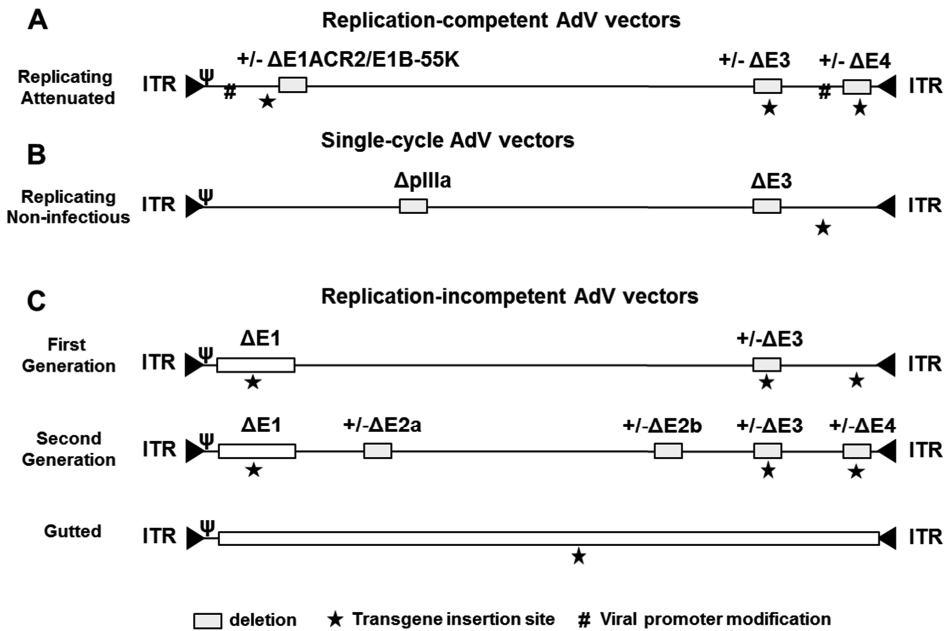
Figure 4. Different types of AdV vectors. Schematic genome structures of replication-competent (attenuated), replicating single-cycle, replication-incompetent first, second and third (gutted) generation adenoviral vectors (AdV). The inverted terminal repeats (ITRs) are indicated at each genome end with arrows. The packaging symbol ψ is denoted at its genome location near the left ITR. (A) Replication-competent AdV vectors can be generated by a small deletion (Δ) in the E1A gene termed "conserved region 2" (CR2) also referred to as Δ24 (deletion of 24 amino acids) or deletion of the E1-55K (ΔE1-55K) gene with additional deletions in parts of E3 and/or E4 -to generate space for transgene insertions. Modifications to the native promoters of E1A and/or E4 region (#) can also be introduced in replication-competent AdV vectors for selective and targeted transgene expression in host cells. (B) Replicating single-cycle AdV are generated by deletion of the minor capsid protein IIIa (ΔpIIIa) which results in vectors capable of replicating their DNA but unable to produce functional progeny viruses in the host cell. Additionally, single-cycle AdV vectors are deleted for most of the E3 region to generate space in the genome for transgene insertions. (C) Replication-incompetent AdV are deleted for the complete E1 region (ΔE1). Additional deletions are sometimes made to create space for foreign sequences in E3 (first generation) and E2A, E2B, E3 and E4 (second generation). In the gutless vector (third generation), all of the viral genes are deleted, and only the ITRs and the packaging symbol (ψ) are retained. The open box denotes the deletions and the stars possible transgene insertion sites.

Replicating single-cycle vectors are the most recent addition to the AdV vector repertoire. These vectors can replicate their own DNA but lack the gene (i.e. minor capsid protein IIIa) involved in the production of functional progeny [163, 164]. These vectors have the advantage of enhanced transgene expression due to DNA replication in the host cell but avoid the risks of causing an AdV infection. In pre-clinical settings they have been shown to induce potent immune responses against diseases such as influenza [163, 170].

As vaccine vectors, replication-competent AdVs and replicating single-cycle AdVs have several limitations. For instance, production of replicating single-cycle AdVs can be difficult given that the expression of capsid protein *in trans* may not be sufficient to produce good AdV vector yields. In addition, the lack of animal models supporting HAdV replication makes assessment of immunogenicity in pre-clinical settings particularly challenging. Nevertheless, the live oral replication competent HAdV4 and HAdV7 vaccine used successfully to prevent respiratory infections in military recruits [47, 48], demonstrates the acceptable safety of replicating AdV vaccines, and their potency to induce mucosal immunity. Replication-competent AdV vaccines against infectious diseases such as influenza [171-173] and HIV [161, 167] are slowly moving into clinical trials.

Replication-incompetent AdV vectors have a rich history as gene delivery vehicles in gene therapy and vaccine development [159, 174]. Their replication deficiency is generated by the deletion of essential E1 genes [157, 160, 175]. Additionally, AdV genes other than E1 can be deleted to create more space in the AdV genome for the therapeutic genes. A major challenge for all gene therapy vectors is the immunogenicity against the vector itself due to *de novo* 'leaky' viral gene expression (e.g. hexon and E2 genes) in vivo [176-178] which often results in elimination of transduced cells [166, 179]. Deletion of additional AdV genes reduces the immunogenicity against the vector.

Among first generation AdV E1-deleted vectors the E3 region is also sometimes deleted to provide space for up to 8 kb of therapeutic genes. Since the E1 genes are essential for AdV replication, they must be provided *in trans* by the 'producer' cell lines, such as HEK293 [180], 911 [181] and PER.C6® cells [182]. In contrast, E3 genes that encode proteins with immunoregulatory functions [114] are not essential for viral replication, and therefore do not need to be complemented.

In second generation AdV vectors, either the E2 and/or E4 regions are deleted, allowing ~14kbp of insertions for therapeutic genes [157, 160, 175, 183]. E2 and E4 genes are essential for viral growth. To complement the E2 and E4 deficiency, cells lines expressing E2 and E4 genes have been developed [184, 185].

Finally, third generation or 'gutted' AdV vectors have all viral genes deleted and contain only the ITRs and the packaging signal, allowing insertion of 35 kbp of transgenic sequence [160, 175, 186, 187]. Devoid of all viral genes, gutted vectors must be grown using a helper virus that provides all the necessary viral proteins *in trans* [185]. However, the application of 'gutted' vectors is limited due to the low titer yields in producer cells, as well as difficulties in purifying the vector without helper virus contamination [187, 188].

ADENOVIRUS VECTOR IMMUNOGENICITY

AdV vaccines induce potent immune responses by activating both innate and adaptive immunity against the vector itself and the genetically encoded antigen [189, 190].

Vaccination with AdVs triggers an innate immune response against PAMPs such as AdV capsid components and viral DNA, leading to the production of interferons and inflammatory cytokines by cellular effectors such as dendritic cells [191]. The recognition of AdV PAMPs by antigen presenting cells is mediated both by Toll-like receptor (e.g. TLR9) dependent and independent mechanisms (e.g. viral DNA) [191, 192]. In mice and monkeys, vaccination with AdV vectors activates antigen-presenting cells (vector dose-dependent) and causes rapid release of type I interferon , IL-6, IL-12, and TNF-α [191, 193-198]. The induced innate immune responses differ greatly by HAdV type, as exemplified by comparative studies between HAdV35, HAdV26, HAdV48 and HAdV5 vectors in non-human primates [199]. These differences may be due to the use of CD46 primary receptor versus CAR receptor for cell entry and the consequential variation in intracellular trafficking [199]. AdV intracellular trafficking and particularly endosome escape is important in triggering innate responses [200, 201]. Following cell entry, the HAdV5 vector moves rapidly to the nucleus, whereas HAdV35 and HAdV26 accumulate in late endosomes. This results in different innate immune responses as a consequence of (longer) exposure to toll-like receptor sensors [201]. The rapid trafficking to nucleus—where the transgene product can be expressed—may perhaps explain the higher transgene-specific B- and T-cell immune responses induced by low doses of HAdV5 vaccine vectors compared to, for example, HAdV35 [159, 202].

T-cell immunity is stimulated when professional antigen-presenting cells present antigen to CD4+ and CD8+ T-cells. Interaction of AdV with B-cells (with or without help from CD4+ T cells) leads to the production of anti-AdV specific or transgene-specific neutralizing antibodies (nAbs) [203]. Specific nAbs induced upon AdV vaccination (or natural infection) are primarily directed against the highly variable surface exposed hexon HVR loops, but also against penton base and fiber [204-208]. Consequently, antibody immune responses are AdV type-specific with limited cross-neutralization between AdV types [202, 209, 210]. In contrast, AdV-specific T-cells (e.g. CD4+ and CD8+) directed against the conserved regions of AdV structural proteins detected in humans as well as animal models, are highly cross-reactive between different AdV types [211-213].

ADENOVIRUS VACCINES

Replication-incompetent AdV vectors are a potent vaccine platform currently in development targeting numerous infectious diseases and cancers. AdV vaccines were initially based on the HAdV5 vector and typically engineered to genetically encode a single antigen in the E1 region. The effectiveness of HAdV5 to induce potent immune responses was demonstrated in animal models and in humans against a considerable number of different antigens including HIV, rabies, dengue, ebola and many more [159, 166].

However, the findings of a Phase IIb STEP trial that tested an HAdV5 HIV vaccine candidate (mixture of three HAdV5 encoding different HIV antigens) yielded conflicting results [214-216]. Pre-exposure to HAdV5 (through either natural infection or vaccination) induces HAdV5-specific NAbs which may reduce vaccine efficacy in humans [217]. Consequently, there has been a growing interest in generating vaccines from alternative AdV types.

AdV vaccine vectors derived from non-human primate AdV types and less-prevalent human Adv types may be less affected by pre-existing immunity in human populations. Several AdV from genera other than the *Mastadenovirus* genus have also been investigated as potential vaccine vehicles, including bovine AdV type 3, canine AdV type 2, ovine AdV type 7, porcine AdV type 3 and 5 and several fowl AdV types, mostly against infectious diseases in animals [218]. Some of these vectors have been explored as vaccines against human infectious diseases, but their further development remains challenging due to their overall poor efficiency in transducing human cells [218, 219]. Vectors derived from non-human primate AdV types such as chimpanzee or gorilla AdV share a close homology to the human AdV types [43, 220]. They are able to transduce human cells and can be efficiently produced on cells originally made for complementation of HAdV5 (e.g. HEK293 cells) [221, 222]. These characteristics enable their use as vaccine vectors against human diseases. In pre-clinical settings, non-human primate AdV vectors derived from ChAdV3, 7, 9, 32, 33, 63, and 68 [222, 223] have been tested against a wide range of pathogens including malaria [224-226], HIV [212, 227], influenza virus [228], ebola [229], SARS [230], hepatitis C [231, 232], rabies virus [233] and Rift Valley fever [234]. Other vectors derived from rhesus monkeys (against HIV) [235, 236], and gorillas (against malaria) [237] have also been tested in animal models. Some of these vectors such as chimpanzee PanAdV3 against RSV [238] and ChAdV3 against ebola and hepatitis C [232, 239-242] have proven potent at inducing antigen-specific immune responses in humans [222]. However, certain non-human primate AdV vectors may not be suitable as vaccine vectors in sub-Saharan Africa, Brazil and China due to high pre-existing immunity against such vectors in those countries [230, 243-245].

Alternative HAdV types vectors such as group B HAdV11 [246, 247] and HAdV35 [248-253], group D HAdV26 [252-256], HAdV48 [254] and HAdV64 (previously termed HAdV19a) [257-261], are highly immunogenic in animal models and in humans against infectious diseases such as RSV [255], human papilloma virus (HPV) [262] and others. These vectors are less prevalent in human populations and are therefore usually associated with low base-line anti-AdV titers [254]. HAdV26 and HAdV35 can bypass HAdV5 immunity [210] and elicit enhanced memory T-cell immunity compared to HAdV5 vectors [263, 264]. As vaccine vectors, HAdV26, HAdV48 and HAdV35 induce higher levels of innate cytokine responses compared to HAdV5, resulting in markedly different and potent antigen-specific adaptive immune responses in monkeys [199]. In humans, vaccination with HAdV35 against tuberculosis [265] and malaria [266, 267] and HAdV26 against ebola [268] and HIV [269-273], was well tolerated and induced strong antigen-specific immune responses.

THESIS SCOPE

This thesis focuses on advancing AdV engineering with the aim to utilize the full potential of AdV vectors as vaccines against infectious disaeses. For this purpose, low-seroprevalent HAdV26 and HAdV35 vectors were generated (i) displaying antigens on the outer capsid surface via protein IX (i.e. AdV capsid antigen-display vectors) to induce B-cell immune responses (**Chapter 2-5**) and (ii) genetically encoding multiple antigens to induce multivalent B- and T-cell immune responses using a single vector.

In **Chapter 2** the progress of the AdV capsid antigen-display vectors in vaccine development is discussed. AdV capsid antigen-display vectors are designed to harness the direct encounter of AdV particles with the immune system, and function much like protein or virus-like particle (VLP) vaccines. AdV capsid proteins such as hexon, fiber, penton and protein IX are exposed on the virus surface and are a target for host immune responses. This anti-AdV immunity is in most instances' undesirable, because it can neutralize the AdV vector and interfere with the vector's potency. By genetically inserting antigens in AdV capsid proteins, immune responses (particularly B-cell immune responses) are mounted against the displayed antigen rather than the AdV capsid components themselves. The many advantages and possible limitations of AdV antigen-display are outlined in detail in **Chapter 2**. The important distinguishing features of AdV antigen-display vectors compared to protein or VLP vaccines, are that they induce potent immune responses without an adjuvant and that they can be engineered to genetically encode specific proteins, thus combining the benefits of AdV-based genetic and protein vaccination within one vaccine vehicle.

In **Chapter 3** A HAdV35 vector was engineered to display a *Plasmodium falciparum* circumsporozoite (CS) protein B-cell epitope via pIX (CS$_{short}$) with or without a genetically encoded CS transgene in E1. To increase the antigen accessibility on the outer HAdV35 capsid surface, CS$_{short}$ antigen was fused to pIX with or without a flexible glycine-linker and/or a 45Å-spacer. To achieve high antigen-specific immunogenicity, antigen-capsid display via pIX was necessary. The pIX-display vectors induced strong CS-specific B-cell immune responses-which increased with the addition of the spacer molecule-exceeding the immune responses induced by the genetic HAdV35 CS vector expressing only the transgene and a recombinant CS protein control. The pIX-display vectors with a genetically encoded CS protein elicited high antigen-specific T-cell immune responses comparable to the genetic HAdV35 CS vector in mice, showing the feasibility of inducing potent T- and B-cell immune responses using a single AdV particle.

The benefit of pIX-display vectors over, for instance, the hexon-display is that larger peptides (e.g. green fluorescent protein, 32 kDa) can be fused to its C-terminus. This trait increases the repertoire of possible antigens which can be fused to the AdV capsid. The limits of AdV pIX-display in terms of antigen diversity (e.g. size) are yet to be determined in detail. In **Chapter 4,** HPV L2 protein linear epitopes were used as the basis for many different antigen designs fused to pIX. The antigen-pIX fusions were evaluated

to obtain the optimal pIX-L2 display. L2 antigens fused to pIX consisted of linear epitopes from different HPV types fused together in increasing order of size from 93 to 418 amino acids in HAdV35 and/or HAdV26 vectors. Based on capsid incorporation as determined by Western blot analysis, the optimal L2 repeats were 93 or 123 amino acids in size. The successful display of multiple L2 epitopes in HAdV35 and HAdV26 vectors enabled generation of a multivalent AdV pIX-display vector based prophylactic HPV vaccine.

A 9-valent prophylactic HPV vaccine based on the AdV35 pIX-L2 display vectors is described in **Chapter 5**. Carcinogenic viral HPV infections are responsible for ~5% of cancers worldwide with substantially higher numbers in developing countries. Highly efficacious L1-based prophylactic HPV vaccines are currently available, although they provide limited cross-protection against many disease-causing HPV types. Second-generation HPV vaccines are focused on the L2 protein containing the highly conserved N-terminus cross-protective linear neutralizing antibody epitopes. The repetitive L2 display—believed to notably enhance the L2-specific immune responses—was achieved by generating two heterologous HAdV35 vectors, each displaying four different HPV L2 epitopes via pIX. Each HAdV35 pIX-L2 vector elicited broad anti-L2 specific B-cell immune responses without an adjuvant in mice. Mixing of the two heterologous HAdV35 pIX-L2 display vectors significantly enhanced the anti-L2-specific humoral immune response. These findings strongly suggest that the pIX-L2 display vector strategy has the potential to offer a potent, multivalent prophylactic HPV vaccine.

To achieve a multivalent immune response using the current genetic AdV vector platform, multiple monovalent AdV vectors can be mixed. Alternatively, AdV vector can be modified to encode multiple antigens and many different approaches to achieve this are described in the literature. However, it is unclear whether these strategies equally preserve all the beneficial properties of the monovalent AdV vector. To assess this, thorough evaluation of different bivalent AdV vector designs was performed and compared to the respective monovalent vector and is described in **Chapter 6**. The bivalent HAdV35 and HAdV26 vectors were extensively tested for critical parameters such as potency to induce antigen-specific immune responses and genetic stability in producer cell lines. HAdV35 and HAdV26 vectors expressing two antigens from a bidirectional expression cassette in E1-region remained genetically stable in the producer cell line, and importantly, induced strong antigen-specific immune responses compared to the other tested bivalent vectors. This evaluation demonstrates the importance of head-to-head comparisons to select the optimal AdV vector-antigen combination for producible and potent AdV-based vaccine.

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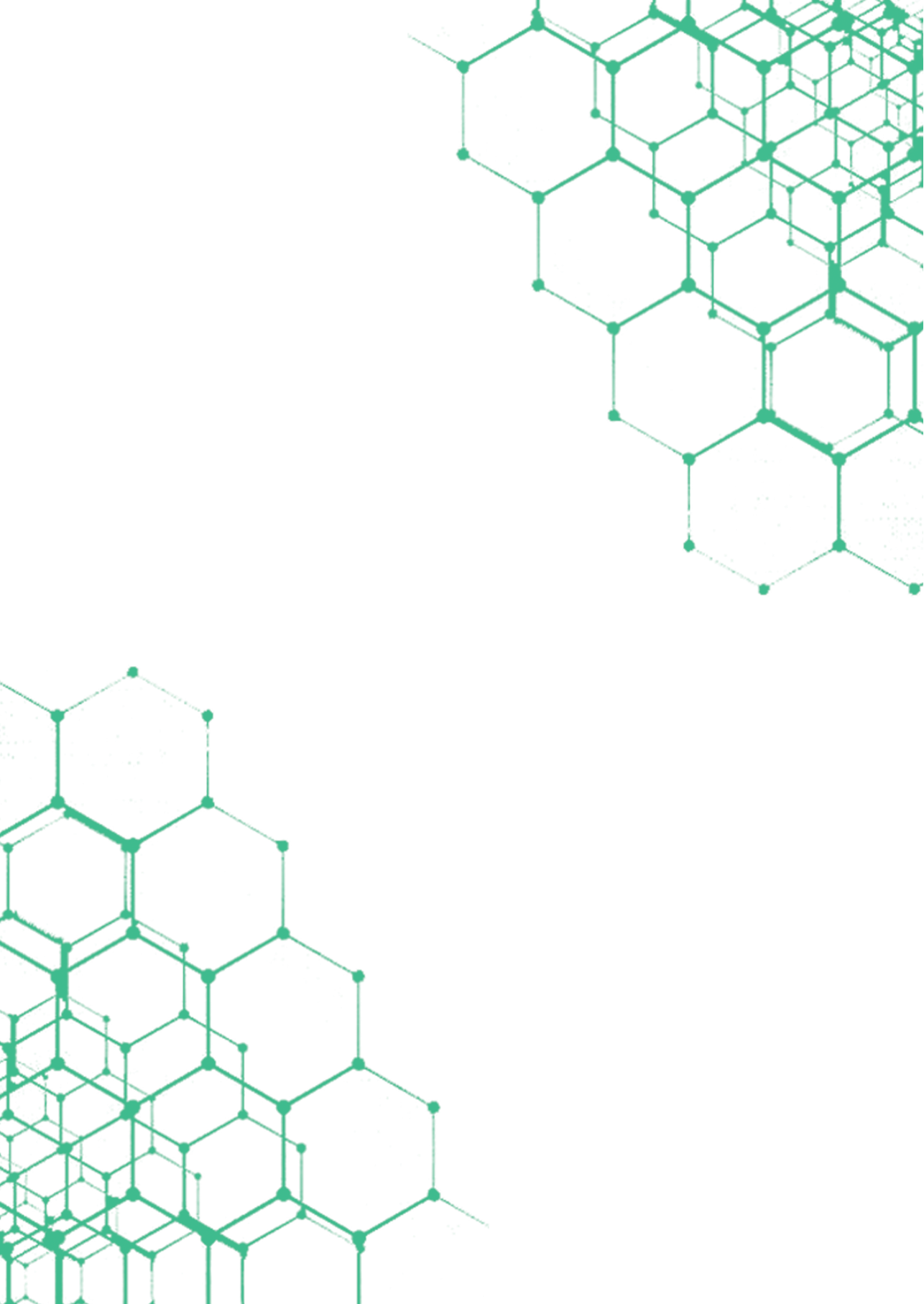
PROGRESS IN ADENOVIRAL
CAPSID-DISPLAY VACCINES

2

ABSTRACT

Adenoviral vectored vaccines against infectious diseases are currently in clinical trials due to their capacity to induce potent antigen-specific B- and T-cell immune responses. Heterologous prime-boost vaccination with adenoviral vector and, for example, adjuvanted protein-based vaccines can further enhance antigen-specific immune responses. Although leading to potent immune responses, these heterologous prime-boost regimens may be complex and impact manufacturing costs limiting efficient implementation. Typically, adenoviral vectors are engineered to genetically encode a transgene in the E1 region and utilize the host cell machinery to express the encoded antigen and thereby induce immune responses. Similarly, adenoviral vectors can be engineered to display foreign immunogenic peptides on the capsid-surface by insertion of antigens in capsid proteins hexon, fiber and protein IX. The ability to use adenoviral vectors as antigen-display particles, with or without using the genetic vaccine function, greatly increases the versatility of the adenoviral vector for vaccine development. This review describes the application of adenoviral capsid antigen-display vaccine vectors by focusing on their distinct advantages and possible limitations in vaccine development.

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INTRODUCTION

The hallmark of vaccine-mediated protection against infectious diseases is the induction of B- and T-cell immune responses [1]. Recombinant replication-incompetent adenoviral human (AdV) vaccine vectors are generally engineered to genetically encode a transgene in the E1 region and utilize the host cell machinery to express the transgene and to induce antigen-specific T-cell and B-cell responses [2–6]. AdVs broad application as vaccine vectors is greatly facilitated due to several valuable attributes: (i) They contain a well characterized ~36 kbp in size genome which can easily be engineered; (ii) they can be grown to high titers in a number of available complementing cell lines such as HEK293 [7], 911 [8] or PER.C6 cells [9,10], and iii) they have a broad cell tropism which enables them to efficiently transduce a wide range of dividing and non-dividing cells [11]. Importantly, in the clinical evaluations, AdV vaccines have been shown to induce potent T-cell and B-cell responses against pathogens, for instance HIV and Ebola, with a good safety profile [12–15]. By contrast, protein-based vaccines such as virus-like particles (VLP), typically in combination with an adjuvant, are usually highly effective at inducing mainly B-cell responses [16–20]. For some complex pathogens, effective vaccine-mediated protection requires potent multifaceted B- and T-cell immune responses [21,22]. This can be achieved using a prime-boost vaccination approach with, for example, an AdV prime and adjuvanted recombinant protein boost, containing the same antigens [23–26].

While the AdV prime-protein boost offers the advantages of well-known vaccine platforms, it is limited by increased cost of goods and potentially complex vaccine regimens. Combining the benefits of AdV and protein vaccination in a single vaccine formulation could be an attractive alternative.

AdV vector capsid proteins can be engineered to display antigens on the capsid surface—referred to as capsid-antigen display vectors herein—thus offering the potential to combine genetic and protein-based vaccination in a single AdV vector. The AdV capsid consists of seven structural proteins; three major capsid proteins hexon, fiber and penton; and four minor 'cement' proteins protein IIIa (pIIIa), VI, VIII and protein IX (pIX) [27–31] (Figure 1), some of which have been modified to display heterologous peptide sequences for different purposes.

Hexon, as a major capsid component, is a target for host immune responses against AdV [32], resulting in anti-vector immunity which may hamper with AdV vector efficacy [33–36]. To circumvent anti-vector immunity, hexon surface-exposed hypervariable regions (HVRs) can be replaced by alternative hexon sequences [32,37–40]. A human AdV5 (HAdV5) vector containing HAdV48 HVRs has been tested in phase I as an HIV vaccine [41]. Fiber and penton base are important for viral cell entry by binding to cellular receptor(s) (e.g., coxsackievirus and adenovirus *receptor (CAR))* [42,43], and can be modified to alter AdV vector tropism to a specific cell type by replacing the native receptor binding motif by an alternative receptor motif sequence for gene therapy purposes [44–46]. Protein IX functions as a 'cement' protein and contributes to overall capsid stability [47,48].

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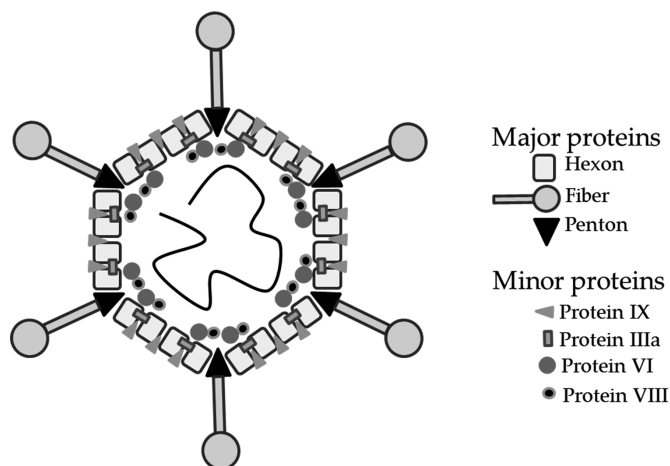
Figure 1. Adenoviral capsid. Schematic representation of an icosahedral AdV capsid organization with double-stranded DNA genome (black spiraling line). The outer capsid shell consists of three major capsid proteins hexon, fiber and penton base, and four minor capsid elements pIX, pIIIa and protein VI and VIII.

Its surface-exposed C-terminus can accommodate fusion of peptides and proteins, for instance to alter the tropism or by generating viruses that express reporter proteins on the outer capsid surface for use in AdV trafficking studies [49–52]. Even though pIIIa allows small heterologous peptide/epitope fusions to its N-terminus, its tolerance for modification seems to be very low ([53,54], and M. Vujadinavic et al. [55]. In this review, we focus on studies describing capsid-antigen display AdV vectors with antigen peptide insertions in AdV hexon, fiber, penton and pIX (Figure 2 and Table 1), with the aim to establish their value as vaccine vectors by highlighting their distinct advantages and possible limitations. Such knowledge can contribute to the successful future application of AdV vectors as an antigen display platform. The availability of multiple modification sites in the AdV capsid allows the selection of the optimal AdV antigen-display strategy. These AdV capsid-display vectors induce antigen-specific immune responses without an adjuvant in animal models. Next to the VLP-like antigen-display, AdV capsid-display vectors can be engineered to genetically encode antigens allowing the benefits of AdV-based genetic and protein vaccination to be combined within one vaccine vehicle.

HEXON ANTIGEN DISPLAY

Hexon is an attractive choice for the generation of AdV antigen-display vectors because of the high number of surface-exposed antigen insertion sites (Figure 2B and Table 1). There are 720 hexon monomer proteins per capsid, which consist of a relatively conserved base and a top section containing HVR 1–7 [28,29,55,56]. The size of each HVR loop differs per AdV type e.g., human adenovirus 5 (HAdV5) HVR1 44 amino acids (aa) [57].
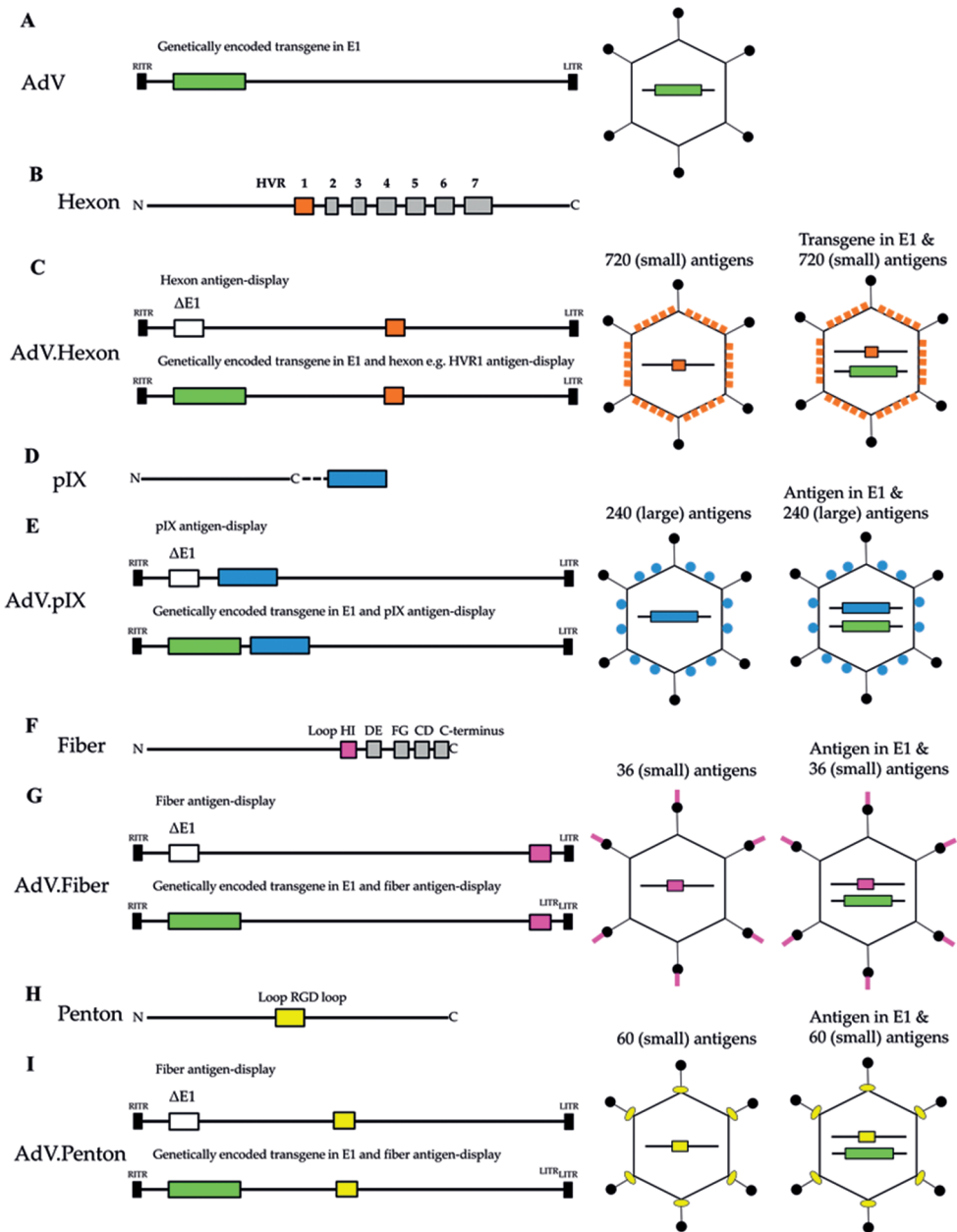
Figure 2. Schematic overview of capsid-display AdV vaccine vectors. (A) Replication-incompetent (ΔE1) AdV vectors encoding a transgene (green) in E1 (AdV.E1). (B) Hexon protein contains seven hyper variable regions (HVR1-7). The size in amino acids (aa) can vary per AdV type e.g., HAdV5 HVR1 44aa (137–181), HVR2 6aa (187–193), HVR3 7aa (211–218), HVR4 13aa (247–260), HVR5 15aa (267–282), HVR6 11aa (304–315) and HVR7 28aa (421–449). Single epitope can be inserted in or substitute the native HVR sequence resulting in 720 copies per capsid. AdV. Hexon display vectors are generated (e.g., HVR1 orange) with or without a genetically encoded transgene in the E1 region (green). (C) pIX can display 240 copies of linear peptides or globular proteins (blue) ▸

▸ by fusion of the antigen (blue) to the C-terminus (with or without a spacer, dotted line). AdV.pIX display vectors are generated with or without a genetically encoded transgene in E1 (green). (D) Epitopes can be inserted in fiber protein HI-, DE-, FG-, CD-, loops and C-terminus. For example, HI-loop (purple) AdV.Fiber display vectors contain 36 copies per capsid and can be generated with or without a genetically encoded transgene in E1 (green). (E) Penton Arg-Gly-Asp (RGD) loop (yellow) can be modified to present linear epitopes, resulting in 60 copies of epitopes per AdV capsid (AdV. Penton) with or without a without a genetically encoded transgene in E1 (green). Right and left Inverted terminal repeat (RITR-LITR).

HVRs are exposed on the outer capsid surface, however, their relative surface exposure can vary [57]. For instance, in HAdV5 the largest HVR1, HVR5 and HVR7 are located on top, while HVR2, HVR3, HVR4 and HVR6 are positioned lower towards the base [57]. Nonetheless, all HVRs contain AdV-specific epitopes and are at target for host antibody responses [32,57]. Consequently, pre-existing specific anti-AdV antibodies can neutralize the AdV vector and reduce vaccine immunogenicity [33–36]. To circumvent anti-vector immunity for gene delivery purposes, single (e.g., HVR1), or all (i.e., HVR1–7) HVRs, or the complete hexon have been replaced with their counterparts from alternative less-prevalent AdV [32,37–40,58]. Similar modifications have been made for the generation of antigen-display vectors by replacing some or all HVR sequences, or by inserting a target-specific epitope in the hexon HVR loop to display mostly linear epitopes on the outer capsid surface.

Successful epitope-display and antigen-specific immune responses in mice have been demonstrated for HAdV5 expressing the target epitope on HVR1 [59–64] (*Aotus nancymaae* [61]), HVR5 [64–66] and HVR2 [65]. In particular, antigen-display via HVR1 is highly versatile. HVR1 was shown to accommodate linear epitope from different pathogens (e.g., HIV gp120) in a size range of 8 to 30 amino acids (aa) resulting in potent antigen-specific immune responses [59–64]. However, the display of a 10aa- HIV gp120-epitope alone in HVR1 [62] or HVR5 [67] was unsuccessful. Surrounding this epitope with 'spacer'-sequences (~15aa) resulted in antigen display, suggesting that additional spacing or peptide engineering may rescue epitope presentation. The latter observations demonstrate the importance of epitope design (e.g., size of the epitope and presence of additional spacers) in combination with the appropriate HVR for successful epitope display to induce potent immune responses. HVR1 and HVR5 appear to display similar sized epitopes (~30 aa) inducing comparable antigen-specific immune responses [64]. HVR5 can display linear epitopes from different pathogens (e.g., Anthrax) [64–66] in the size range of 12 to 66 aa with potent antigen-specific immune responses in mice. In contrast, HVR2 has not been extensively utilized for epitope display. Similar sized epitope display via HVR2 or HVR5 demonstrate HVR2 to be less permissive for larger peptide insertions, with the induction of lower antigen-specific immune responses compared to HVR5 [65].

To generate AdV display vectors presenting more than one antigen per capsid or more copies of the same epitope from a single AdV vector, multiple hexon HVRs can

Table 1. AdV-display vectors.

AdV Location	Vector	Disease and Antigen/Epitope (Size aa)	Other Modifications		Reference
			Transgene in E1	Capsid	
Hexon HVR1	HAdV5	Polio type 3 (8 aa)	No	No	[59]
		Malaria Circumsporozoite protein (CSP) (20, 24 aa) and (12 aa)	No	No	[60], [61], [62]
		HIVgp120 (10, 21, 24, 26 aa) and HIV gp41 and His6 (7, 6 aa)	Yes	Yes	[128], [68]
		Chagas disease *Trypanosoma cruzi* gp83 (24 aa)	No	No	[63]
		Human papillomavirus (HPV) HPV16 L2 protein (29 aa)	No	Yes	[64]
	HAdV3	Enterovirus VP1 SP70 (15 aa) and Enterovirus VP1 SP70 and SP55 (15 aa)	Yes	Yes	[77], [78]
	SAdV25 (AdC68)	Influenza A M2e and NP antigen (13 aa)	Yes	Yes	[79]
		Coxsackievirus and Enterovirus VP1 (6, 15 aa)	Yes	Yes	[80]
Hexon HVR2	HAdV5	HIV gp41 and Gag protein (39 aa)	Yes	No	[71]
Hexon HVR5	HAdV5	Anthrax *Bacillus anthracis* Protective antigen (PA) (16, 36, 66,143, 245 aa (eGFP))	No	No	[66]
		Model antigen RGD motif + His- linker (45, 55, 65, 75, 85, 95 aa)	Yes	Yes	[65]
	HAdV5 and HAdV48	*Pseudomonas aeruginosa* outer membrane protein F (OprF) (14 aa)	Yes	Yes	[70] , [69]
pIX C-terminus	HAdV5	Plague *Yersinia pestis* (15, 37 aa)	Yes	No	[94]
		Ovalbumin (Ova) (43 aa)	No	Yes	[95]
		Chagas-disease vaccine ASP2 and gp83 (26, 24 aa)	Yes	No	[100]
	HAdV5	HIV gp120 (67 aa)	No	Yes	[128]
		Friend Murine Leukemia Virus gp70, Gag and Ova (22, 43, 60, 70, 92) HAdV5/ HAdV5.F35	Yes	Yes	[96]
	HAdV35	Malaria *P. falciparum Circumsprozoite protein (CSP)* (17, 20, 40 aa)	Yes	No	[98]
		Human papillomavirus (HPV) HPV6, 11, 16, 18, 31, 45, 52/58 L2 protein (93 aa)	No	No	[99]
Fiber Knob-loop	HAdV5	Influenza A HA (9 aa)	Yes	Yes	[126]
		Ovalbumin (20, 22 aa)	Yes	Yes	[127]
		Pseudomonas aeruginosa (14 aa)	Yes	Yes	[120]

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be modified. Antigen-specific immune responses were detected with display via HVR1 (HIV, 7aa) combined with His-epitope in HVR5, but not HVR2 [68], supporting previous results of poor immunogenicity against epitopes displayed via HVR2 [65]. The suboptimal antigen display via HVR2 may be due to poor accessibility of the epitope, since HVR2 is located away from the top relative to HVR1 and HVR5 [57]. Comparable immune responses against both antigens displayed via HVR1 and HVR5 [68] illustrate the potential of displaying different epitopes from a single AdV vector to generate a multivalent vaccine.

To combine the benefits of genetic and protein-based vaccination, hexon-display vectors can be engineered to genetically encode an antigen resulting in a (multivalent) display-expression. HAdV5 hexon display-expression vectors encoding and displaying antigens from pathogens such as *Pseudomonas aeruginosa* outer membrane protein F (OprF) epitope (HVR5, 14aa) [69–72], elicited potent (protective) immune responses against the displayed epitope and the encoded transgene in mice. Epitope display via HVRs may be a means to reduce or interfere with anti-vector immunity in the host and enhance transgene-specific immune responses with display-expression vectors [67,71,72]. For example, HAdV5 HVR1 malaria circumsporozoite (CS) display vectors were largely unaffected by anti-HAdV5 neutralizing antibodies in vitro, supporting the notion that the majority of anti-HAdV5 antibodies after immunization are directed against HVR1 [67,72].

To fully evade anti-vector immunity, replacement of all HVRs is necessary [32]. As a result, use of HAdV5-based hexon-antigen display vectors remains restricted [73,74]. Vectors derived from rare AdV types such as HAdV26 [75] and chimpanzee AdV type 3 (CAdV3) [76] are less likely to be limited by pre-existing immunity in the human population. Due to variations in the HVR loop aa sizes and possibly surface-exposure among AdV types (e.g., HAdV5 HVR1 has 30aa vs. HAdV48 HVR1 which has 8aa), it is important to determine whether alternative AdV types can be successfully used to display antigens via different HVRs. Epitope display via hexon using alternative AdV types was demonstrated with HAdV3 [77,78], and simian adenovirus 25 (SAdV25) [79,80] vectors, with both vectors inducing potent immune responses in mice. For the HAdV3 vector, a 15aa enterovirus epitope was successfully displayed via HVR1, HVR2 and HVR7, whereas display via HVR4 and HVR5 was unsuccessful [77,78]. Overall, these results suggest that alternative AdV types can be successfully used to display antigens via different HVRs to a similar extent as HAdV5 vectors.

PIX-DISPLAY VECTORS

The most important advantage of pIX-display vectors is that unlike hexon, pIX tolerates fusion of larger peptides to its C-terminus (Figure 2C and Table 1). Protein IX minor capsid protein acts as a capsid stabilizing element in the AdV capsid [47,81]. It is believed to have additional functions as a transcriptional activator [82] and suppressor of the host-cell anti-viral response, thereby contributing to optimal virus production [83]. AdV vectors lacking pIX can be generated without deleterious effects on viral replication

[84], albeit at a cost of reduced thermostability [85] and impaired packaging of full-length genomes [86]. There are 240 protein IX copies per capsid, arranged to protrude from the hexon cavities to expose the C-terminus tail to the outer surface [87,88] (Figure 1). Protein IX has proven suitable for the display of linear and globular functional proteins, for example, a polylysine motif or an eGFP protein, on the outer capsid surface [49–51]. This trait has been exploited for cell-specific AdV vector re-targeting and purification by fusion of specific ligands [51,89–91], or as a tool for AdV tracking in vitro and in vivo by fusion of functional reporter proteins [49,50,92]. Even though the pIX C-terminus is accessible on the outer capsid surface [93] it is embedded within the hexon cavity [87]. To increase the accessibility of the fusion peptide on the outer capsid surface, an alpha helical spacer can be added to lift the heterologous peptide fused to the pIX C-terminus out of the hexon grove [89].

In a HAdV5 vector, pIX was shown to display different antigens in a size range of 15 kDa to ~70 kDa (e.g., *Yersinia pestis* antigens) [94–96], inducing potent antigen-specific immune responses. Display of *Yersinia pestis* antigens via pIX elicited antigen-specific humoral responses and provided protection against lethal challenge in mice [94]. In addition, expression of pIX and the fused antigen in vivo is observed [97], which may contribute to potent immune responses against the pIX-fused antigen [95,96].

The potential of alternative AdV types for the generation of pIX-display vectors was confirmed by HAdV35 [98,99] and HAdV48 vectors [100], which induced potent antigen-specific immune responses in mice. HAdV35 displaying a ~15 kDa malaria CS antigen and encoding a CS transgene in E1 elicited higher immune responses than the transgene-only vector or CS protein alone. This effect could not be achieved by mixing the CS protein with the vector, suggesting that the antigen needs to be displayed on the outer capsid surface for induction of enhanced antigen-specific humoral responses [98]. HAdV35 vectors displaying HPV protein L2 epitope-repeats via pIX (e.g., HPV type 6, 31, 33, 16 (93aa)) elicited L2-specific immune responses against the HVP types included and not included in the vaccine, demonstrating the potential of using pIX to display multiple linear epitopes in a repetitive confirmation from a single AdV vector to generate a multivalent AdV display vaccine [99]. In some instances, alternative AdV vectors were shown be more potent at inducing antigen-specific immune responses. For example, HAdV48 displaying 16aa and 24aa *Trypanosoma cruzi* epitopes outperformed the HAdV5 display vector by inducing higher B- and T-cell responses in mice [100]. This difference may be explained by a difference in pIX-antigen capsid incorporation efficiency between HAdV5 and HAdV48, resulting in the observed variation in potency. Previous studies in non-human primates also indicated that HAdV48 and HAdV5 induce differential innate responses which might result in differences in adaptive immune responses to the vector and the antigen [5].

FIBER- AND PENTON-DISPLAY VECTORS

Fiber and penton proteins are appealing targets for epitope insertion because fiber trimers and penton pentamers are highly exposed on the 12 AdV icosahedral capsid vertices

[101] (Figure 2D,E and Table 1). Fiber and penton are pivotal for cell entry [101,102]. Fiber initiates the interaction with the primary cellular receptor followed by secondary receptor interaction by penton, resulting in cell entry [42,103]. Modifications to these proteins may impact their binding efficiency to cellular receptors and consequently cell entry, which can be detrimental to vector potency and/or production in producer cell lines. Fiber consists of three distinct domains: (i) a C-terminal knob domain which binds to the primary receptor, (ii) the central shaft region consisting of flexible β-sheets, (iii) and a highly conserved N-terminal tail which interacts with the penton base [101,104]. Penton binding with secondary integrin receptors is mediated by the highly conserved Arg-Gly-Asp (RGD) motif which is embedded in the most variable region of the otherwise highly conserved penton base [101,105–107].

Successful full fiber substitutions, knob and shaft and shaft-only substitutions with counterpart alternative AdV fiber domains have been demonstrated, mainly for AdV vector retargeting in gene therapy [108–112], although the potential of AdV vector retargeting for vaccination purposes has been demonstrated. Chimera HAdV5 containing a HAdV35 species B fiber showed efficient transduction of antigen-presenting dendritic cells, suggesting potential for targeting specific cells in vivo [113,114]. In AdV-based HIV vaccine development, HAdV fiber chimera vectors induced potent (protective) immune responses in mice and monkeys [115–117]. Less invasive fiber modifications for AdV vector retargeting have been demonstrated. Several different receptor ligand peptides up to 55aa in length were inserted into the surface-exposed fiber knob HI-, DE-, FG-, CD-loops and the C-terminus [118–123]. Similarly, to facilitate AdV binding to an alternative receptor penton base, modifications were achieved with replacement of the RGD motif for a different receptor binding motif [124,125].

Few studies demonstrate the potential of epitope-display via fiber [120,126–128] and only one via penton [126] (Figure 2D,E and Table 1). HAdV5 vectors displaying an influenza epitope via either the hexon HVR5, fiber HI-loop, penton RGD loop, or pIX, all induced antigen-specific immune responses in mice [126]. However, when mice were immunized with either the same AdV particle number or the same epitope copy number, influenza-specific humoral and cellular responses were highest with the fiber-display vector [126]. This observation is interesting considering that the fiber is the least abundant protein (36 copies vs. 60 of penton, 720 of hexon, and 240 of pIX) in the AdV capsid, suggesting that epitope accessibility and presentation is more important than the number of epitopes displayed per AdV capsid. HAdV5 vectors displaying a 14aa *P. aeruginosa* OprF epitope via fiber HI-loop, FG-loop, CD-loop, DE-loop, C-terminus and hexon HVR5 induced potent immune responses in mice [120]. In contrast to immunization with HAdV5 OprF transgene vector, the fiber FG-loop display vector induced protection against *P. aeruginosa* even in the presence of high anti-HAdV5 pre-existing immunity [120], clearly demonstrating the benefits of AdV fiber-antigen display vectors.

CONCLUDING REMARKS

AdV capsid-display vaccine vectors can be generated by inserting antigens or epitopes into hexon, fiber, penton and pIX. The tolerance for epitope insertion varies per capsid protein and seems to depend on the specific region that is targeted (e.g., hexon HVR1 vs. HVR2) and antigen properties (e.g., epitope size, conformation and biophysical properties). Hexon, fiber and penton proteins typically allow the insertion of small and linear T-cell or B-cell epitopes, and such limits to antigen size and confirmation might limit their utility for vaccine development. In contrast, by allowing fusion of both linear and larger globular antigens to its C-terminus, pIX has the potential to induce broader or stronger antigen-specific immune responses. The studies described in this review indicate a wide range of successful pIX-antigen fusions in terms of peptide size (i.e., 15 to ~70 kDa). However, there might be some peptide characteristic other than size, such as charge, which can influence successful pIX-display (e.g., pIX-HA) [129,130]. The pIX-antigen display as well as the expression of pIX-antigen contributes to the potency of these vectors, a characteristic which distinguishes the pIX-display platform from conventional VLP platforms. Since AdV vectors can be generated without pIX, suboptimal pIX-antigen capsid incorporation (e.g., 240 copies vs. 100 copies per capsid) may pose as a risk for consistent AdV manufacturing and/or vector quality.

VLP particle-based epitope display has been explored in vaccine development using, for instance, adeno-associated virus (AAV) [131] or various other viruses [132–135]. AdV display-vectors have the potential to carry multiple antigens for instance in hexon (e.g., HVR1 and HVR5) or fiber (e.g., HI-loop and FG-loop), or epitope insertion in different capsid proteins (e.g., hexon and pIX). Insertion of multiple epitopes can be exploited to generate single AdV vectors displaying multiple epitopes, or greater numbers of the same epitope per capsid. In addition, AdV display vectors can be engineered to genetically encode an antigen to enhance antigen-specific humoral and cellular responses, combining the benefits of protein and AdV-based vaccines.

Several strategies exist to overcome the impact of pre-existing immunity to highly prevalent vectors (e.g., HAdV5) on immunogenicity. For example, alternative AdV vectors (e.g., HAdV26 or ChAdV3) [75,76] with lower prevalence in human populations are available for genetic vaccination and/or capsid-display. Secondly, epitope insertions in the AdV capsid may reduce anti-AdV vector immunity [67,71,72]. Due to the spectrum of anti-AdV immune responses (e.g., anti-fiber and anti-hexon) in humans [136,137], it remains to be determined whether epitope display may indeed be sufficient to evade anti-AdV immunity in the clinic.

One possible limitation of antigen-display on the AdV capsid may be that the neutralizing antibodies raised against the displayed epitope (e.g., HVR1-CS display) can neutralize the AdV capsid-display vector encoding a transgene, resulting in reduced (T-cell) immune responses against the encoded transgene after second administration [72]. The AdV capsid-display studies so far focus on homologous prime-boost regimens; to test the full

potential of the platform it might be interesting to explore heterologous prime-boost regimens as well. However, in most homologous prime-boost regimens, AdV antigen-display vectors show potent (protective) antigen-specific immune responses against many different pathogens. The repetitive epitopes displayed on the AdV capsid enhances the induction of humoral immunity due to B-cell receptor crosslinking [138,139], which might be an advantage over genetic AdV vectors for the generation of antigen-specific nAb responses. Furthermore, the difference in responses of capsid-modified and genetic vectors might be attributed to differential processing of the antigen in hosts cell.

To date, available publications offer little insight into the possible effects of the modification on the capsid protein's additional biological function (e.g., AdV particle hexon-mediated transport to nucleus [140]), or critical AdV capsid-display particle characteristics such as viral titers yields, genetic stability in producer cell lines, particle stability, or appropriate formulation buffers. Yet, successful advancement of AdV capsid-display vectors depends highly on a potent vector generated at high yields in producer cell lines. Nonetheless, pre-clinical evaluations of the AdV capsid-display vectors describe potent (protective) antigen-specific immune responses against a range of complex infectious diseases, mostly without the addition of an adjuvant. Considering that the AdV capsid-display vectors are based on a well-established recombinant AdV vector platform, easily engineered and producible at high yields in producer cell lines, their suitability for commercial application is encouraging [10,141].

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CONFLICTS OF INTERES

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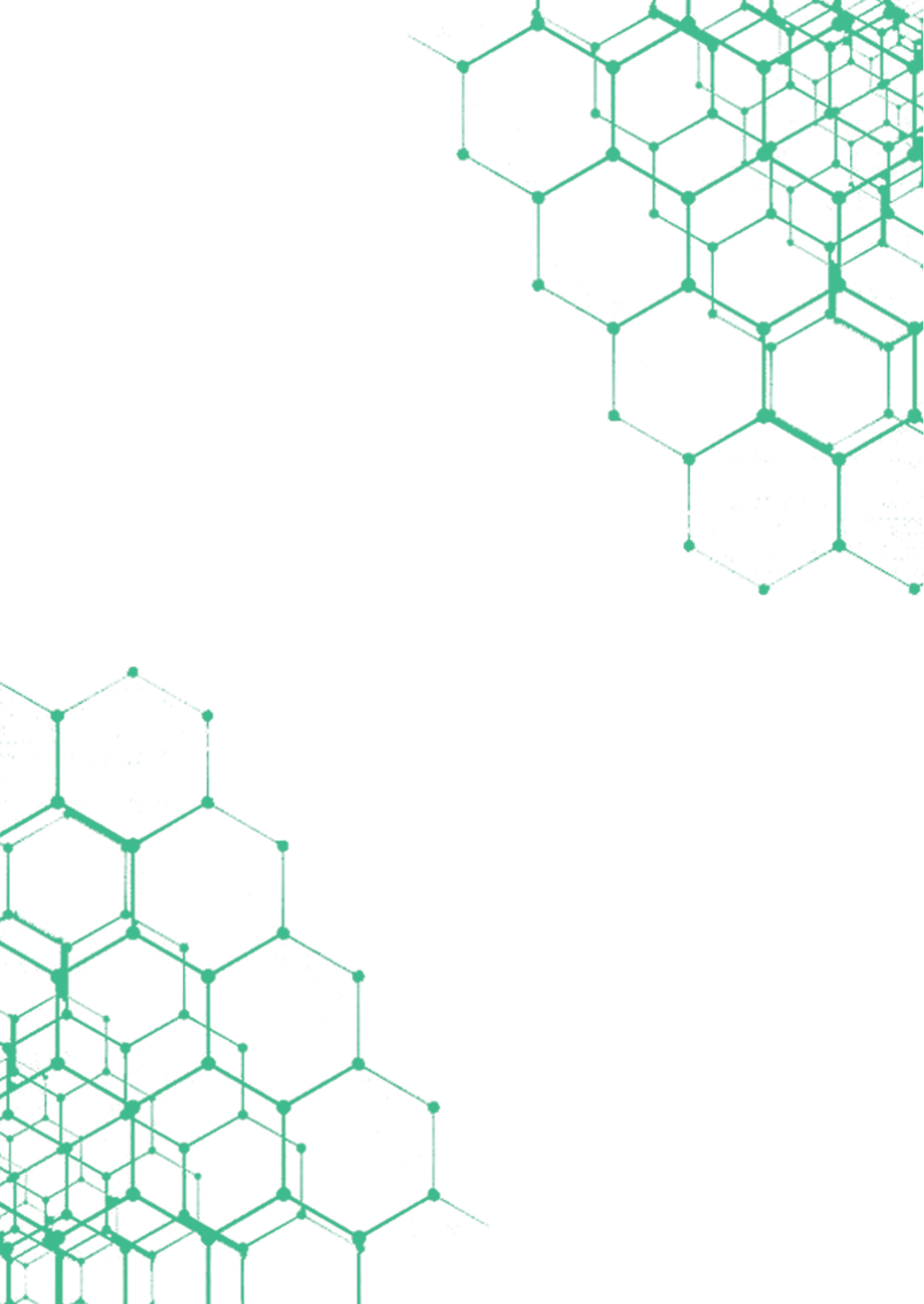
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ANTIGEN CAPSID-DISPLAY ON HUMAN ADENOVIRUS 35 VIA PIX FUSION IS A POTENT VACCINE PLATFORM

3

ABSTRACT

Durable protection against complex pathogens is likely to require immunity that comprises both humoral and cellular responses. While heterologous prime-boost regimens based on recombinant, replication-incompetent Adenoviral vectors (AdV) and adjuvanted protein have been able to induce high levels of concomitant humoral and cellular responses, complex manufacturing and handling in the field may limit their success. To combine the benefits of genetic and protein-based vaccination within one vaccine construct and to facilitate their use, we generated Human Adenovirus 35 (HAdV35) vectors genetically encoding a model antigen based on the *Plasmodium falciparum* (*P. falciparum*) circumsporozoite (CS) protein and displaying a truncated version of the same antigen (CS$_{short}$) via protein IX on the capsid, with or without a flexible glycine-linker and/or a 45Å-spacer. The four tested pIX-antigen display variants were efficiently incorporated and presented on the HAdV35 capsid irrespective of whether a transgene was encoded or not. Transgene-expression and producibility of the display-/expression vectors were not impeded by the pIX-display. In mice, the pIX-modified vectors induced strong humoral antigen-specific immunity that increased with the inclusion of the linker-/spacer molecules, exceeded the responses induced by the genetic, transgene-expressing HAdV35 vector, and surpassed recombinant protein in potency. In addition, the pIX- display/expression vectors elicited high antigen-specific cellular immune responses that matched those of the genetic HAdV35 vector expressing CS. pIX-modified display-/expression HAdV vectors may therefore be a valuable technology for the development of vaccines against complex pathogens, especially in resource-limited settings.

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INTRODUCTION

There is growing interest in recombinant, replication-incompetent Adenoviral (AdV) vectors for genetic vaccination due to their ability to induce strong cellular immune responses against the encoded transgenes [1-3]. While vectors based on human Adenovirus 5 (HAdV5) are most frequently used, their immunogenicity is impaired by high levels of pre-existing immunity [4, 5]. Alternative Adenovirus serotypes such as HAdV35, HAdV26 and ChAdV3 are highly immunogenic while less seroprevalent and unaffected by pre-existing immunity to HAdV5, making them suitable as vaccine vectors [6-8]. AdV vectors characteristically induce strong cellular responses against their encoded transgenes [9-11], compared to recombinant, adjuvanted protein that induces strong antibody responses [12]. Those antibody responses alone may, however not suffice to provide protection against complex pathogens where a synergistic T cell response has proven to be beneficial [13-15].

Heterologous prime-boost regimens are one means by which concomitant cellular and humoral immune responses can be induced. In preclinical studies, priming with HAdV35 or -26 vectors and boosting with adjuvanted, recombinant protein, or vice versa [10, 16], induced increased levels of both humoral and cellular antigen-specific immune responses compared to the individual vaccine components alone.[16] Despite this advantage, complex clinical handling may limit the success of heterologous prime-boost regimens in the field. Display of disease-relevant epitopes on the AdV surface by modifying the capsid proteins could serve as a platform to combine the benefits of AdV-based genetic- and protein-based vaccination within one vaccine construct [17]. While all three major capsid proteins - hexon, penton, and fiber - allow insertion of small heterologous peptides into their highly variable protein regions [18], the 240 copies of the minor capsid protein IX (pIX) [19, 20] tolerate fusion of relatively large, functional proteins to its surface-exposed C-terminus without drastically decreasing its function [21-23]. This so called 'pIX-display technology' has been used to influence the cell tropism of the vector [23, 24] or to display antigen on the vector surface [18, 25, 26]. The feasibility of pIX-antigen display on genetic HAdV5 vectors expressing a vaccine transgene has been successfully demonstrated in preclinical vaccination studies for different antigens [25, 26], indicating that AdV-based pIX-display-/expression vectors may be a potent approach to induce humoral and cellular immune responses with a single vaccine vector.

Based on previous observations with a HAdV35 vector that genetically encodes the *Plasmodium falciparum* circumsporozoite (CS) transgene in E1 [27] we generated display-/expression vectors presenting the central, B-cell epitope-rich four-amino acid (NANP) repeat region of the CS protein (CS_{short}) as a model antigen on the capsid via pIX. Optimal antigen display was explored by fusing the protein directly to pIX, or via a flexible glycine-linker and/or a 45Å-spacer [24]. We show that all pIX-display variants were efficiently incorporated and presented on HAdV35 vectors with and without the genetically encoded transgene and that transgene-expression or vector

yields in the E1-complementing producer cell line remained unaffected. In the mouse model, the unadjuvanted pIX-display vectors induced strong humoral antigen-specific immune responses that were increased by the presence of the linker-/spacer molecules and exceeded those induced by the parental genetic HAdV35 vector only expressing the transgene. Vector potency was higher than that of a recombinant protein control. In addition, the pIX-display/expression vectors elicited high antigen-specific cellular immune responses that matched those of the genetic HAdV35 vector expressing the transgene and that were not achieved by mixing protein with AdV vectors.

MATERIALS AND METHODS

Ethics statement

Animal work was performed according to the Dutch Animal Experimentation Act and Guidelines on the Protection of Experimental Animals by the Council of the European Committee (EU Dir. 86/609) after approval by the Dier Experimenten Commissie of Janssen Vaccines under permit numbers CRH0211 and CRH0233.

Vector design, generation and purification

Recombinant, replication-incompetent, genetically modified Advac® HAdV35 pIX-display-vectors with a mammalian-codon optimized *P. falciparum* CS protein gene in E1, a Luciferase reporter gene (Luc), or without a transgene in E1, were generated. The CS protein gene is a *P. falciparum* 3D7 protein based on the EMBL CAH04007 sequence of which the last 14 amino acids of the C-terminus are truncated (minus the GPI anchor) [27]. The CS_{short} antigen is a fragment of the CS protein consisting of 27 consecutive NANP repeats. Different variants of the pIX-display-vectors, with and without a transgene, were generated by fusing CS_{short} directly to pIX (HAdV35.CS.pIX-CS_{short}, HAdV35.empty. pIX-CS_{short}), via a 3-amino acid glycine-linker (HAdV35.CS.pIX-Gly-CS_{short}, HAdV35.empty. pIX-Gly-CS_{short}), via a 45Å-spacer [24] (HAdV35.CS.pIX-45-CS_{short}, HAdV35.empty.pIX-45-CS_{short}) or a combination of glycine-linker and 45Å-spacer (HAdV35.CS.pIX-Gly45-CS_{short}, HAdV35.empty.pIX-Gly45-CS_{short}). HAdV35-based vectors with the full-length CS sequence fused to pIX were also generated (HAdV35CS.pIX-CS, HAdV35.empty.pIX-CS). The control vectors included the pIX-unmodified vectors with (HAdV35.CS, and HAdV35. Luc) or without a transgene (HAdV35.empty, HAdV26.empty and HAdV5.empty), a HAdV35-based luciferase-expressing vector without the pIX protein (HAdV35.Luc.ΔpIX), and a luciferase-vector carrying a pIX-modification (HAdV35.Luc.pIX-Gly-CS_{short}).

HAdV35 Advac® vectors were generated by homologous recombination of three plasmids in which the right end of the genome ((pIX-deleted pWE.Ad35.ΔpIX.EcoRV, digested with NotI and EcoRV), pBr.Ad35 PRdE3 5E4orf6/7 (digested with PacI and NotI) and the left end of the genome,[28] (pAdapt35.Bsu.pIX-mod, digested with PacI) were transfected into PER.C6® cells. The vectors (S1 Table 1) were plaque-purified and up-scaled on PER.C6® cells at 37°C/10% CO_2 in Dulbecco's modified Eagle's medium supplemented

with 10% fetal bovine serum (FBS; Life Technologies Inc.) and 10 mM $MgCl_2$. Vectors were purified by two-step CsCl-gradient centrifugation, dialyzed in formulation buffer and tested for viral titers. The physical viral particle concentration (VP/ml) was determined by optical density (OD) in the presence of SDS [29] and the infectious units (IU/ml) were titrated by $TCID_{50}$ (tissue culture infectious dose 50%), followed by calculation of the viral particles to infectious units (VP/IU) ratios and the productivity expressed in viral particles per cm^2 of production flasks (VP/cm^2).The purified vector batches were tested for Bioburden and Endotoxin levels (Milipore).

Capsid-incorporation of modified pIX-CS$_{short}$ and transgene-expression

Surface-display of different pIX-modifications was determined by Western Blot of purified HAdV35 particles. For CS transgene-expression, A549 cells were transduced with 5000 VP/cell and incubated for 48 hours at 37°C/10% CO_2 The cells were harvested and lysed using a lysis buffer (Janssen) supplemented with complete protease inhibitor (Roche) and Benzonase® (EMD Milipore). For pIX capsid-incorporation and CS transgene-expression, the pIX-display-vectors were compared to the control vectors HAdV35.empty and HAdV35. CS. Purified viral particles (VP/well) or A549 cell lysates were denatured and reduced in protein loading buffer with reducing agent (Invitrogen) at 70°C, then separated on pre-cast 4-12% Bis/Tris Nu-PAGE gel (Invitrogen) in MOPS buffer (Invitrogen) at 175 Volt, 500 milli-Ampere. The protein was transferred to a nitrocellulose membrane according to manufacturer's recommendations using iBlot® Transfer stacks (iBlot system; Invitrogen). Protein staining was performed for 1 hour with antibodies specific for CS (2A10) or pIX (6740, monoclonal antibody generated in-house), or loading control antibodies specific for the Adenovirus fiber protein (AdV5 4D2, Abcam) for viral particles, or βActin (AC-15, Abcam) for cell lysate, in 5% non-fat dry milk (BioRad)/Tris buffered Saline Tween 20 (Invitrogen). The protein was visualized by staining with fluorescently labeled IRDye800CW/680RD® 1:10000 goat anti-mouse/rabbit and recorded on an Odyssey® Infrared Imaging System (Li-Cor).

pIX-CS$_{short}$ surface-display by Electron Microscopy

Capsid-display of the pIX-modifications was confirmed with Electron Microscopy (EM). Purified pIX modified vectors were coated with the primary anti-CS antibody (clone 2A10) on a copper grid with a carbo-coated Formvar film and incubated for 60 minutes at room temperature (1:1). After washing, the grids were stained with gold labeled anti-mouse Protein A Gold 10 nm (PAG10) 1:200 in **1 mM PBS** containing **2% bovine serum albumin (BSA)** and **0.1% Tween 20** buffer for 60 minutes at room temperature and fixed using 1.5% glutaraldehyde in cacodylate buffer. Samples were subsequently negatively stained with 2% Silicotungstic acid (STA) and visualized with a transmission electron microscope (FEI Tecnai 12 BioTwin).

Determination of capsid stability

Capsid stability was determined by a Heat Stability luciferase-based assay [23, 30]. Luciferase-encoding vectors containing a pIX-CS$_{short}$ modification were incubated in parallel with HAdV35.Luc as a positive reference, and HAdV35.Luc.(Δ)pIX at 45°C for up to 20 minutes. At 2-minute intervals, samples were taken and transferred to A549 cell cultures at 500 VP/cell. After 48 hours, cells were cryolysed in lysis buffer with 1mM dithiothreitol prior to the addition of luciferase substrate (Promega). The luciferase activity was measured on a Luminoskan microplate luminometer (Thermo Scientific).

Production and characterization of the *P. falciparum* CS protein

CS protein of the same sequence as in the HAdV-vectors was produced in the methylotropic yeast strain *Hansenula polymorpha* RB11 clone by ARTES Biotechnology GmbH (Germany). A C-terminal His tag sequence was introduced into the construct to facilitate Ni-column purification of the CS protein. The protein was characterized as previously described [10].

Mice and immunizations

Six- to eight-week old, specific pathogen-free female BALB/c (H-2D) mice were purchased from Charles River (L–Arbresle-Cedex, France) and kept at the AAALAC-certified institutional animal facility under specified pathogen-free conditions. Animals were co-housed per group in IVC cages using compressed sawdust as bedding, under controlled conditions of temperature, humidity and light (12-hour light, 12-hour dark cycles). Standard rodent diet and water were provided ad libitum and mice were provided with nesting material and enrichment. Animal wellbeing was checked daily and pre-set humane endpoints were used to define study-unrelated sacrifice criteria by a veterinarian. All measures were taken to minimize pain, distress and suffering and all procedures were performed by trained personnel. Mice were vaccinated intramuscularly in the quadriceps of both hind legs (50 µl/leg) with the indicated vector particle- or protein-doses in formulation buffer, under isoflurane anesthesia. Serum was obtained by submandibular bleeding throughout the study or by heart puncture under isoflurane anesthesia at the end time points, during which spleens were aseptically removed after cervical dislocation.

Determination of CS-specific antibodies by IgG-ELISA

P. falciparum CS protein-specific total IgG, or subclass-specific IgG1 and IgG2a in serum were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described [27]. Relative serum titers of total IgG (ELISA units/ml) were calculated in comparison to a *P. falciparum* CS-specific reference serum using a 4-parameter curve fit model. The IgG1- and IgG2a-specific measurements were used directly to calculate the ratio between IgG2a and IgG1, by dividing the reciprocal dilution at which the OD$_{492}$ reached three times that of the background measurement in a naïve control sample.

Determination of CS- and HAdV35 hexon-specific cellular immunity by IFNγ-ELISPOT

Antigen-specific cellular immunity in vaccinated mice was assessed using an interferon-gamma (IFNγ) enzyme-linked immune-spot assay (ELISPOT) as previously described [27]. Freshly isolated splenocytes were incubated either with a pool of 15-mer peptides overlapping by 11 amino acids, spanning the entire sequence of the *P. falciparum* CS protein, or with the described H-2Kd-MHC class I-restricted, immunodominant HAdV35 hexon epitope KYTPSNVTL. The peptide pool and single peptide were used at a final concentration of 1 ug/ml for each individual peptide. The numbers of spot-forming units (SFU) per 10^6 cells were calculated.

Indirect immunofluorescence assay

The binding of vaccination-induced antibodies to the native CS protein on the surface of *P. falciparum* sporozoites coated on glass slides (~5000 plasmodia/ well; Radboud University Medical Center, Nijmegen, The Netherlands) was evaluated using an indirect immunofluorescence assay (IFA) as previously described [27]. Sera obtained from animals immunized with HAdV35.empty were used as negative control for IFA specificity, the CS-specific monoclonal antibody clone 2A10 as a positive control.

Statistical analysis

ELISA Units/ml and $SFU/10^6$ cells were log-transformed and group comparisons performed using ANOVA models. For repeated measurements over time, a random intercept was added to the ANOVA model to account for correlated observations. Comparisons between groups containing values below the lower limit of quantification were analyzed using censored regression models. Correction for multiple testing was applied using the Dunnett method, since there was a fixed reference group in each analysis. Differences with a p≤0.05 were considered significant. All statistical analyses were performed using SAS® software, version 9.2 (SAS Institute Inc., 2011).

RESULTS

Generation of HAdV35 pIX-display-vectors

To evaluate pIX-display technology as a vaccine platform, we generated a panel of recombinant, replication-incompetent HAdV35 vectors, displaying a model antigen consisting of a truncated version of the *P. falciparum* CS protein (CS~short~) on pIX. To optimize the display design for B-cell responses, the panel included empty vectors in which CS~short~ was either directly fused to the C-terminus of pIX, or indirectly using an alpha-helical 45Å-spacer,[24] a glycine-linker for additional flexibility, or both combined ("pIX-display-vectors"). All four pIX-CS~short~ constructs were also implemented on HAdV35 vectors encoding the CS protein as a transgene in the E1 region ("pIX-display-/expression vectors"). To assess potential size-limitations of the pIX-fusion, we generated HAdV35

display- and display-/expression variants in which full-length CS protein was fused to pIX. Vector designs are shown in Fig 1.

All pIX-CS~short~ variants are incorporated into the capsid of pIX-display- and display-/expression vectors

To evaluate capsid-incorporation, Western blot analyses were performed on purified viral particles of all pIX-display and display-/expression vectors. As evident by anti-CS-specific antibody detection, all pIX-CS$_{short}$ display variants were successfully incorporated into the capsid of display- and display-/expression vectors and detected at similar levels (~50
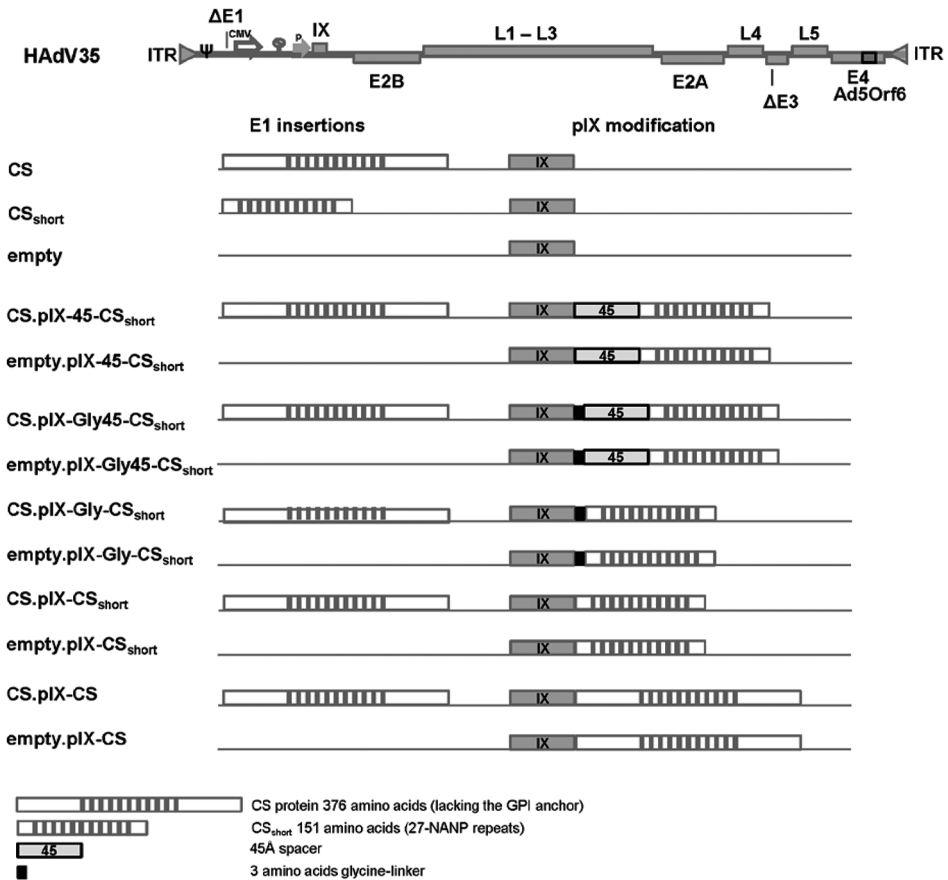
Figure 1. Design of HAdV35 pIX-display-vectors. Schematic representation of pIX-display HAdV35 Advac® vectors containing a human CMV promoter and SV40 poly-A expression cassette in E1 and the native pIX promoter (P). The vectors either express a 376 amino acid CS protein (lacking the GPI anchor) or no transgene (empty) in the E1 region. The pIX-display-vectors, with and without the CS-transgene in E1, are genetically modified to display the 151 amino acid CSshort with or without a 3 amino acid glycine-linker (Gly) and/or 45Å-spacer

kDa Fig 2A). In contrast, the pIX-CS fusion variant was not incorporated into the capsid of the HAdV35 display- or display-/expression vectors (data not shown) and excluded from further analysis. The genetically encoded CS protein was detected as an additional band at ~55kDa in purified viral particle preparations, suggesting co-purification or association of transgene product expressed during vector production in the producer cells. The highest level of this additional ~55kDa band was detected in the 45Å-spacer-containing vectors, whereas the lowest levels were observed for the pIX-Gly-CS$_{short}$ display-vector (Fig 2A). In the purified vectors containing the 45Å-spacer, a third band migrating at ~100 kDa was present both in the anti-CS as well as anti-pIX-specific staining, indicating the presence of a pIX-45-CS$_{short}$ dimer (Fig 2A). Anti-pIX staining showed that vector particles contained comparable levels of the pIX-45-CS$_{short}$, pIX-Gly45-CS$_{short}$ and non-modified pIX (S1 Fig). EM analysis with CS-specific staining confirmed capsid-display of the CS$_{short}$ model antigen on all HAdV35 display- and display-/expression vector particles (Fig 2B and S2 Table).

pIX-CS$_{short}$ modification does not affect in vitro expression of CS protein from the HAdV35 display-/expression vectors

Using Western blot, no differences in the expression level of CS were observed between the pIX-unmodified HAdV35.CS control vector and the four pIX-CS$_{short}$ display-/expression vectors in A549 cells (Fig 2C). Additional bands migrating at a size corresponding to the pIX-CS$_{short}$ variants (~50 kDa) in both the pIX-modified display- and display-/expression vectors indicate some expression of pIX-CS$_{short}$ modifications under non-replicating conditions, similar to previously reported observations [31].

Fusion of CS$_{short}$ to pIX reduces intrinsic capsid stability but has no effect on viral titers

To evaluate the impact of the pIX-CS$_{short}$ modification on capsid stability of the vector particles, a luciferase-based heat stability assay was used [23]. In contrast to the non-modified control vector, both the vectors lacking pIX (HAdV35.Luc.ΔpIX) and the pIX-CS$_{short}$ vectors showed rapid decline in luciferase expression, indicating capsid instability (Fig 2D, shown for representative vector HAdV35.Luc.pIX-Gly-CS$_{short}$). The physical viral particles (range 2.5×10^{11}-2.5×10^{12} VP/ml) and infectious units (range 1.5×10^{10}-3.5×10^{11} IU/ml) and the corresponding VP/IU ratios (range 3-19 VP/IU) were comparable for the eight HAdV35 pIX-modified vector preparations and the non-modified pIX control vectors HAdV35.empty and HAdV35.CS (S2 Table). The productivity (VP/cm^2) was equally unaffected compared to the non-modified pIX control vectors (S2 Table). These observations suggest that despite reduced capsid stability, the fusion of CS$_{short}$ to pIX did not affect vector producibility.
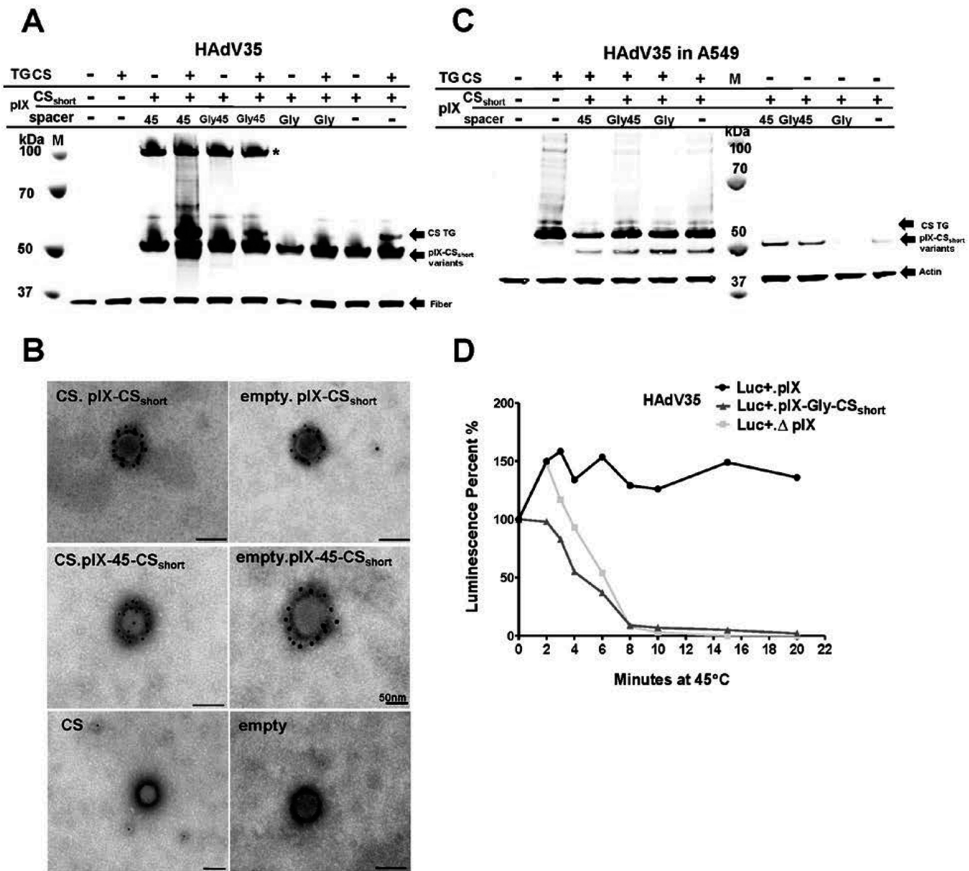
Figure 2. Characterization of the pIX-CS$_{short}$ display- and display-/ expression vectors. (A) pIX-CS$_{short}$ capsid incorporation in purified HAdV35 vector preparations. To confirm capsid incorporation of the pIX-CS$_{short}$ (~50 kDa variants) 5 x10^9 VP/well of each purified HAdV35 vector preparation was analyzed by Western blot using anti-CS antibody. To ensure equal loading, the blots were stained with anti-fiber 4D2 antibody (~35 kDa). The pIX-fusion proteins migrate higher than their predicted size in kDa due to the NANP-repeat in the CS protein. Marker (M) is indicated with the corresponding kDa band size. An additional band (~100 kDa) is indicated with an asterisk (*). (B) pIX-CS$_{short}$ capsid display by electron microscopy (EM) anti-CS staining and gold-label staining. Representative EM images of HAdV35.empty. pIX-CS$_{short}$, HAdV35.CS.pIX-CS$_{short}$ (upper row), HAdV35.empty. pIX-45-CS$_{short}$, HAdV35.CS.pIX-45-CS$_{short}$ (middle row), HAdV35empty, and HAdV35 (bottom row) vectors. The bars represent 100 nm unless stated otherwise. (C) *In vitro* expression of CS transgene under non-replicating conditions in A549 cells. Western Blot analysis of cell lysates from A549 cells infected with 5000 VP/cell, using an anti-CS antibody. From left to right, the two controls HAdV35.empty and HAdV35.CS followed by the pIX-display vector variants with a CS transgene in E1 and without the transgene. Marker (M) is indicated with the corresponding kDa band size. (D) Heat Stability Assay showing percent (%) variation in luminescence in lysate of A549 cells infected with HAdV35. luc vector preparations subjected to 45ºC temperature stress for 0 to 20 minutes. The percent variation was determined relative to the respective baseline time point (0 minutes).

pIX-CS$_{short}$ display induces strong CS-specific humoral immune responses that are increased by the presence of a spacer

Immunogenicity of the four HAdV35 pIX-CS$_{short}$ display-vectors was determined in Balb/C mice at a dose range of 1×10^7 to 1×10^{10} VP/mouse. At 1×10^7 VP/mouse, none of the tested constructs mounted detectable CS-specific IgG responses (Fig 3A). At 1×10^8 all four constructs displaying a pIX-CS$_{short}$ construct induced responses by week 4 post-immunization that were maintained until week 8. HAdV35.CS showed comparatively lower potency, with the lowest dose to induce CS-specific IgG responses being 1×10^9 VP. Titers elicited by the four pIX-CS$_{short}$ modified display-vectors were higher than those induced by the HAdV35.CS expression-vector (significant for titers 1×10^8 - 1×10^{10} VP. See S3 Table for p-values). CS$_{short}$ display on any of the three linker/spacer constructs further increased immunogenicity, resulting in an earlier onset of responses compared to the direct pIX-CS$_{short}$ fusion construct at 1×10^8 VP (S3 Table), and titers that were significantly higher than those induced by HAdV35.CS. Although the three different linker/spacer constructs induced CS-specific IgG titers of comparable magnitude, HAdV35.empty.pIX-45-CS$_{short}$
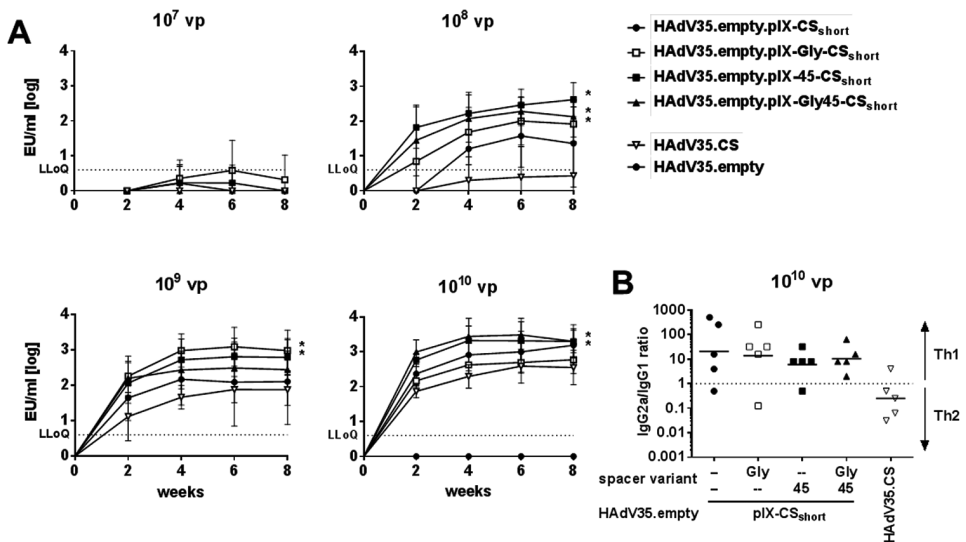
Figure 3. pIX-CS$_{short}$ display induces strong CS-specific humoral immune responses that are increased by the presence of a spacer. (A) Total CS-specific IgG titers in the serum of Balb/C mice over 8 weeks post-immunization with 1×10^7, 1×10^8, 1×10^9, or 1×10^{10} VP/animal of the HAdV35 vectors indicated in the legend. Symbols depict means (n = 5 animals per group and time point), error bars indicate standard deviation, EU indicates ELISA units. Statistical significance was determined using a mixed-model analysis with Dunnett correction for multiple comparisons on log-transformed data per dose across time points. Asterisks indicate statistical significance (p ≤ 0.05) (B) Ratio of CS-specific serum IgG2a to IgG1 levels, 8 weeks after immunization with 1×10^{10} VP of the HAdV35 vectors indicated in the legend. Horizontal bars depict mean ratios.

was the only vector to consistently reach responses that significantly exceeded HAdV35. CS at 1×10^8-1×10^{10} VP (S3 Table).

We analyzed the subtypes of the CS-specific IgGs to understand whether pIX-display of CS$_{short}$ and the usage of the linker/spacer constructs would introduce a Th1 or Th2 bias, with IgG1 indicative of a Th2- and IgG2a of a Th1-type response in this mouse model. Using sera from animals immunized with 1×10^{10} VP of all vectors, we saw that HAdV35. CS immunization induced an overall balanced response, while all pIX-CS$_{short}$ modified vectors induced a stronger IgG2a bias (Th1), independently of whether a linker or spacer was present (Fig 3B).

Taken together, pIX-display of CS$_{short}$ on empty HAdV35 vector particles proved to be highly immunogenic, with the presence of a spacer construct further increasing vector potency.

CS-specific antibodies induced by pIX-CS$_{short}$ displayed on HAdV35 vectors recognize native CS on *P. falciparum* sporozoites

Pooled sera from animals immunized with 1×10^{10} VP of each of the four pIX-CS$_{short}$ modified vectors or of HAdV35.CS as positive reference were equally capable of binding unfixed sporozoites, as determined by IFA with *P. falciparum* sporozoite-coated slides (S2 Fig).

pIX-CS$_{short}$ display on CS-transgene expression- vectors induces strong humoral and cellular immune responses

We assessed whether implementation of pIX-display of the B-cell epitope-carrying CS$_{short}$ antigen on HAdV35 encoding the CS protein sequence (including the H2kD-restricted CD8+ T-cell epitope NYDNAGTNL) would maintain the induction of strong humoral immune responses, concomitantly with strong T-cell responses against the encoded transgene. Animals were immunized with 1×10^8 to 1×10^{10} VP/mouse of HAdV35.CS that either did or did not carry the pIX-45-CS$_{short}$ construct (HAdV35.CS.pIX-45-CS$_{short}$ and HAdV35.CS), or with HAdV35.empty.pIX-45-CS$_{short}$ vector as a reference for the humoral immune response. To assess whether any potential interference between pIX-fusion technology and transgene-expression would be immune-mediated, animals were immunized with a mix of HAdV35.empty.pIX-45-CS$_{short}$ and HAdV35.CS (1×10^8 to 1×10^{10} VP/ mouse of each of the two vectors; final vector dose of 2×10^8 – 2×10^{10} VP/mouse). In all remaining groups, the total VP number was adjusted to the total vector dose of 2×10^8 – 2×10^{10} VP/mouse using HAdV35.empty. In contrast to the expression-vector HAdV35.CS, the display-vector HAdV35.empty pIX-45-CS$_{short}$, and the display-/expression vector HAdV35.CS.pIX-45-CS$_{short}$ induced measurable CS-specific antibody responses at the lowest dose (1×10^8) 2 weeks post-immunization, indicating higher potency of the vectors compared to the pIX-unmodified expression-vector HAdV35.CS (Fig 4A).
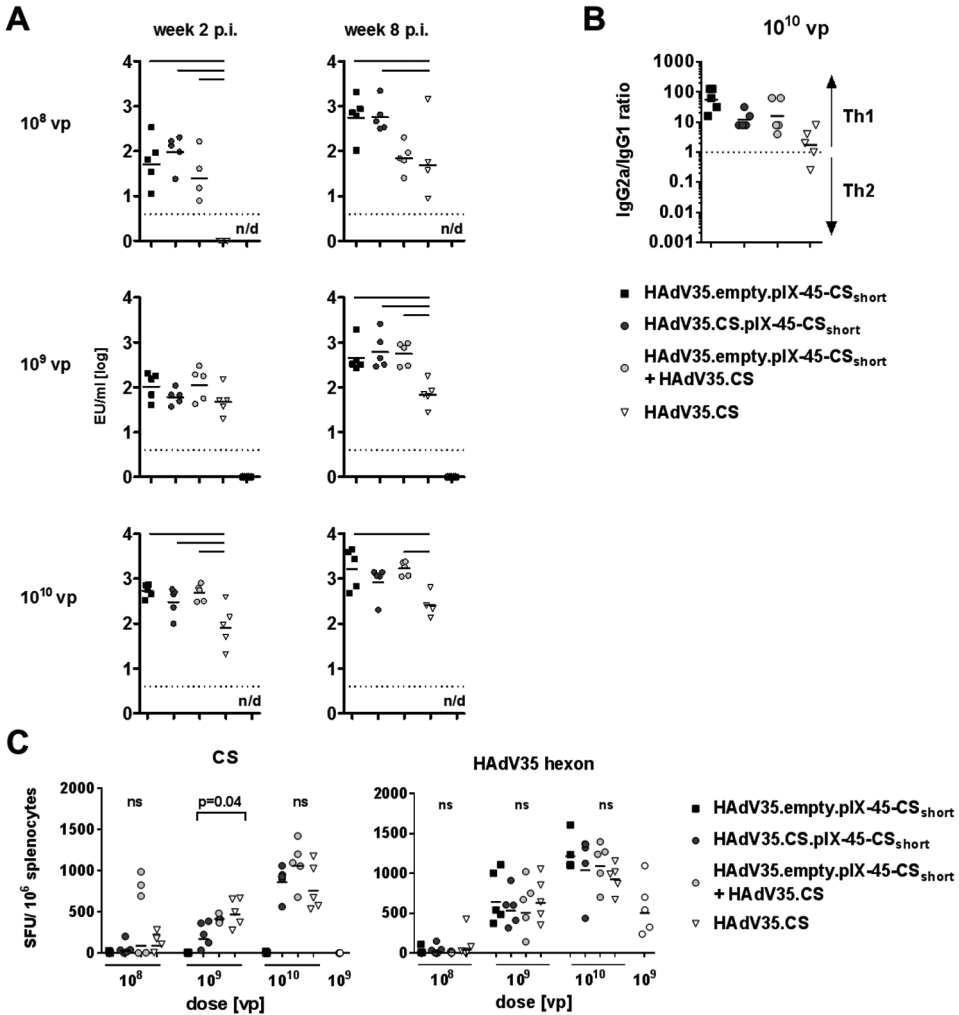
Figure 4. pIX-CS$_{short}$ display on CS-transgene expression-vectors induces strong humoral and cellular responses (A) Total CS-specific IgG titers in the serum of Balb/C mice 2 and 8 weeks post-immunization with 1×10^8, 1×10^9, or 1×10^{10} VP/animal of HAdV35.empty. pIX-45-CS$_{short}$, HAdV35. CS.pIX-45-CS$_{short}$, a mix of HAdV35.empty.pIX-45-CS$_{short}$ and HAdV35.CS, HAdV35.CS alone, or HAdV35.empty (10^9 VP/ animal only). n = 5 per group. Statistical significance was determined using a two-way ANOVA test with Dunnett correction for multiple comparisons on log-transformed data by time point and dose. Black horizontal bars indicate statistical significance (p ≤ 0.05). (B) Ratio of CS-specific serum IgG2a to IgG1 levels, 4 weeks after immunization with 1×10^{10} VP of the HAdV35 vectors indicated in the legend. Horizontal bars depict mean ratios (n = 5 animals/ group). (C) IFNγ ELISPOT responses to stimulation of splenocytes of Balb/C mice 8 weeks post-immunization with *P. falciparum* CS peptide pool (left panel; "CS") or the H-2Kd restricted immunodominant HAdV35 hexon epitope KYTPSNVTL (right panel; "HAdV35 hexon"). n = 5 animals/ group. Statistical significance was determined using a two-way ANOVA test with Dunnett correction for multiple comparisons on log-transformed data. ns = not significant.

In addition, the titers mounted by 1×10^{10} VP of HAdV35.CS.pIX-45-CS$_{short}$ and HAdV35.empty pIX-45-CS$_{short}$ at week 2 markedly exceeded those induced by HAdV35. CS (p ≤0.005 for all comparisons, Fig 4A). Although all titers increased in magnitude over time, those induced by the expression-vector HAdV35.CS remained significantly lower than those induced by the two pIX-45-CS$_{short}$ fusion constructs at week 8 post-immunization (p≤0.005 for all comparisons in the groups receiving 1×10^8 and 1×10^9 VP, Fig 4A). The mix of the display-vector HAdV35.empty.pIX-45-CS$_{short}$ with the expression-vector HAdV35.CS also resulted in significantly increased CS-specific titers at all doses and time points, except at the lowest dose, 1×10^8. This indicates that immune responses against the transgene do not interfere with the induction of humoral responses against the pIX-CS$_{short}$ fusion construct when provided to the immune system on separate vectors. Calculation of the IgG2a/IgG1 ratio showed that the Th1 bias induced by pIX-CS$_{short}$ on HAdV35.empty vectors was maintained on the HAdV35.CS pIX-45-CS$_{short}$ display-/expression vector (Fig 4B).

Next, CS-specific cellular immune responses against all four constructs were assessed by IFNγ ELISPOT at week 8 post-priming (Fig 4C). As expected, due to the absence of the immunodominant T-cell epitope NYDNAGTNL in the CS$_{short}$ sequence, HAdV35. empty pIX-45-CS$_{short}$ did not induce a response. All other immunization regimens induced responses that followed a dose-response relationship. IFNγ levels induced by the display-/expression vector HAdV35.CS.pIX-45-CS$_{short}$ did not significantly differ from those induced by the expression-vector HAdV35.CS at 1×10^8 and 1×10^{10} VP, but were slightly reduced at the intermediate dose of 1×10^9 VP (p=0.04). IFNγ levels induced by the mix of HAdV35. empty.pIX-45-CS$_{short}$ and HAdV35.CS did not differ from those induced by HAdV35.CS at any of the given doses. To exclude that differences in CS-specific immune responses were caused by slight variations in vaccine dosing, we measured IFNγ-producing T-cells targeting the immunodominant H-2Kd restricted epitope KYTPSNVTL in HAdV35 hexon, which, as expected, did not differ between groups immunized with the same dose (Fig 4C).

These results indicate that CS$_{short}$ display on pIX can be implemented on CS-transgene-expressing HAdV35 vectors, significantly increasing humoral immune responses while maintaining strong cellular immune responses.

Immunogenicity of pIX-CS$_{short}$ display is higher than that of CS protein and comparable levels cannot be achieved by mixing AdV vectors and protein.

Due to its high potency, recombinant CS protein [10] can be considered a benchmark for the induction of humoral immunity. To compare the immunogenicity of pIX-CS$_{short}$ HAdV35 display-vectors to that of CS protein, we immunized Balb/C mice with 1×10^{10} VP of either the display-vector HAdV35.empty.pIX-CS$_{short}$, the display-/expression vector HAdV35.CS.pIX-CS$_{short}$, or with 5µg of unadjuvanted CS protein (Fig 5A). Animals

immunized with 1×10^{10} VP of HAdV35.empty served as a negative control. 5µg CS protein per mouse was chosen since titrations of this CS protein preparation have shown that CS-specific IgG titers plateaued at a dose of 5µg/mouse in an adjuvanted [10] or unadjuvanted setting (data not shown). This dose contains the calculated equivalent of 1.88µg CS_{short} (the CS_{short} sequence consists of the central 27 NANP-repeats and accounts for 37.6% of the CS full-length protein sequence), approximately 30 times more than the theoretical amount of 0.06µg CS_{short} contained in the 1×10^{10} VP of HAdV35.empty. pIX-CS_{short} preparation, assuming full decoration of all 240 pIX- molecules per capsid with the 15.1 kDa CS_{short} modification. At week 4 post-immunization, both pIX-CS_{short}-modified vectors induced CS-specific total IgG titers that greatly exceeded those induced by CS protein ($p \leq 0.0001$), indicating that presentation of the CS_{short} antigen on viral particles is a potent platform, even compared to the highly immunogenic CS protein.

3
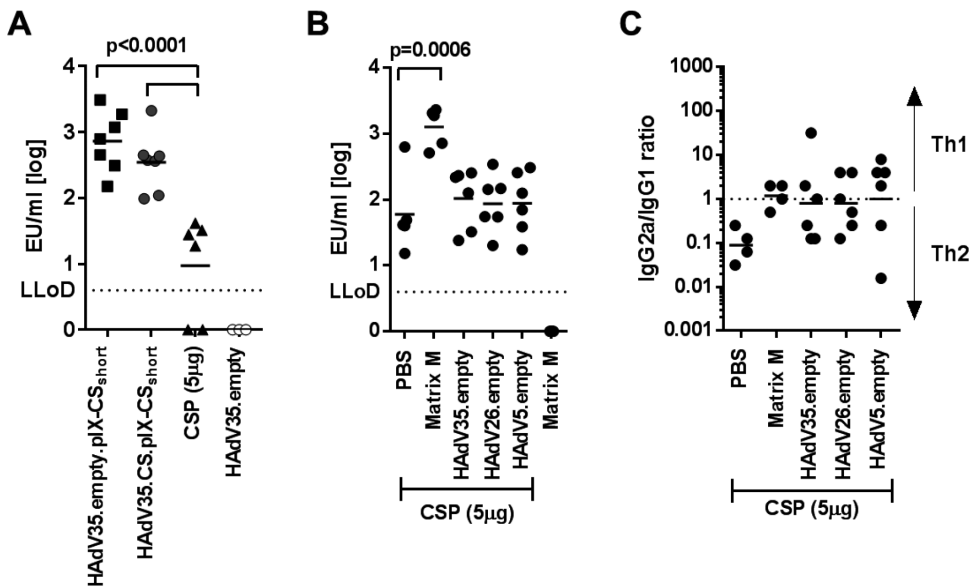
Figure 5. Immunogenicity of pIX-CS_{short} display is higher than that of CS protein and comparable levels cannot be achieved by mixing AdV vectors and protein. (A) Total CS-specific IgG titers in the serum of Balb/C mice 4 weeks post-immunization with 1×10^{10} VP/animal of HAdV35.empty. pIX-CS_{short}, HAdV35.CS.pIX-CS_{short}, with 5µg of CS protein in PBS, or with 1×10^{10} VP/animal HAdV35. empty. n = 7 per group. Statistical significance was determined using a two-way ANOVA test with Dunnett correction for multiple comparisons on log-transformed data. (B) Total CS-specific IgG titers in the serum of Balb/C mice 4 weeks after immunization with 5µg CS protein in PBS, or mixed with 1×10^{10} VP/animal of empty AdV-vectors (HAdV35, HAdV26, or HAdV5), animals injected with Matrix-M only serve as control. n = 6 per group. Statistical significance was determined using a two-way ANOVA test with Dunnett correction for multiple comparisons on log-transformed data. Ratio of CS-specific serum IgG2a to IgG1 levels, 4 weeks after immunization with 5µg CS protein in PBS only, or mixed with Matrix-M, or 1×10^{10} VP/animal of empty Ad-vectors (HAdV35, HAdV26, or HAdV5). Horizontal bars depict mean ratios (n = 5 animals/ group).

To determine whether the comparatively high immunogenicity of the pIX-CS$_{short}$ modified vector particles was mediated by an inherent adjuvant-effect of the AdV backbone [32], we immunized Balb/C mice with 5µg CS protein alone or mixed with $1x10^{10}$ VP of HAdV35.empty and determined CS-specific total IgG levels 4 weeks later (Fig 5B). As a reference point, we chose 5µg CS protein adjuvanted with Matrix-M, and Matrix-M alone as negative control. CS protein adjuvanted with Matrix-M induced CS-specific IgG titers that significantly exceeded those induced by CS protein alone (p = 0.0006) and reached levels similar to those observed in animals immunized with $1x10^{10}$ VP of pIX-CS$_{short}$ modified HAdV35 vectors (Fig 5A). By contrast, the mixture of CS protein and HAdV35.empty induced only slightly increased titers compared to CS protein alone (p=0.08). Similarly, ad-mixing $1x10^{10}$ VP of HAdV26.empty or HAdV5.empty, induced slightly, but not significantly increased IgG titers, excluding a serotype-specific difference in the adjuvant capability of AdV particles in this setting. Mixing of CS protein with Matrix-M or any of the AdV serotypes did, however, produce a more pronounced shift towards a Th1-type response compared to CS protein alone (Fig 5C), which was similar to that observed with pIX-CS$_{short}$ modified vectors (Figs 3B and 5B). Inherent AdV-mediated adjuvation is therefore not likely to be the decisive mediator of the strong immunogenicity of pIX-CS$_{short}$ modified vectors, confirming that display of the antigen on the surface of the vector particles is an essential aspect that mediates the high immunogenicity of the pIX-modified vector platform.

DISCUSSION

We describe the development and immunological evaluation of replication-incompetent HAdV35 vectors displaying a truncated version of the *P. falciparum* CS (CS$_{short}$) as a model antigen via the small capsid protein IX. Multiple pIX-antigen display variants were efficiently incorporated into, and presented on the HAdV35 viral capsid without affecting vector yields. The pIX-display vectors induced strong humoral antigen-specific immunity that increased with the inclusion of linker-/spacer molecules and exceeded the responses induced by a genetic HAdV35 vector expressing a transgene. The potency of the display-/ expression vectors surpassed that of recombinant protein control. pIX- display could successfully be implemented on transgene expressing HAdV35, resulting in significantly increased antigen-specific humoral responses comcomitantly with strong cellular immune responses that were not achieved by mixing the protein with Adenoviral vectors.

As reported, pIX can tolerate relatively large fusions of functional proteins (30-120 kDa) to its C-terminus [21, 22, 25, 26]. The finding that the ~41 kDa (376 a.a.) CS protein cannot be incorporated into the capsid was therefore unexpected. This suggests that factors other than size may influence the extent of successful capsid-incorporation, and that empirical selection of protein antigens for pIX antigen fusion might be necessary. Similarly, a vector encoding CS$_{short}$ as transgene failed to express the CS$_{short}$ protein (RNA synthesis confirmed but protein not detected, data not shown), suggesting that in some instances the fusion to pIX could be beneficial in stabilizing the (artificial) antigen.

Some CS antigen-specific observations were made, which may not necessarily be translated to other antigen combinations using the pIX- display/ expression technology. The detection of an additional 55 kDa CS-staining band in all four preparations of HAdV35 vectors encoding CS as a transgene suggest that co-purified CS-transgene expressed during vector production may physically associate with the pIX-display vectors. Proteins containing NANP-repeats demonstrate a high nonspecific association [33], which may also explain the presence of the additional band migrating at approximately 100kDa in the four vectors containing the 45Å-spacer. There, the spacer might increase accessibility of the NANP-repeat, thus enhancing the propensity for dimerization inherent to the CS protein [34], which is also supported by the observation that under non-reducing conditions, the 50 kDa band decreases and the 100 kDa band increases in intensity (data not shown). This, however, remains speculative since no obvious differences in decoration intensity between the vectors containing the 45Å-spacer or the direct pIX-CS$_{short}$ fusion were observed in the EM analysis.

The detection of pIX-display variant expression under non-replicating conditions in A549 cells is consistent with previous observations [31] and might be due to the activity of the pIX promoter itself or possible enhancing effects of the CMV promoter directly upstream of the pIX region. However, the consequences of possible pIX-antigen fusion *in vivo* expression, in addition to capsid-display, on the induction of antibody responses remain to be determined.

In agreement with previous observations in HAdV5 vectors, our pIX-modified HAdV35 vector preparations exhibited the same heat instability as vectors lacking pIX (ΔpIX) [23]. Despite this instability, all pIX-display modified HAdV35 vectors were comparable to the pIX-unmodified vectors in terms of physical viral particle yields, infectious virus titer, vector infectivity and productivity. Overall we conclude that the fusion of the CS$_{short}$ model antigen by itself or in combination with the linker and/or spacer to pIX did not interfere with pIX capsid-incorporation, the transgene-expression in A549 cells or productivity in the producer cell line.

Immunological characterization of the vectors demonstrated that surface-display of CS$_{short}$ on HAdV35 vector particles significantly increased the potency of the model antigen to induce CS-specific antibody titers over those induced by the CS-transgene expressed from HAdV35.CS, or by soluble CS protein. Compared to immunization with soluble protein, fusion of the CS$_{short}$ antigen to pIX may ensure presentation of the antigen in its native conformation and in a highly repetitive nature inherent to viral capsids, both of which were shown to deliver strong activation signals to the recognizing B-cells [35, 36]. Accessibility of the antigen on the surface of the AdV vector particle may be of additional importance, as demonstrated by the increased humoral responses induced by the pIX-CS$_{short}$ fusion constructs including the glycine-linker and/or 45Å-spacer in comparison to the direct fusion construct. The glycine-linker may be acting as a molecular hinge, making the rigid, rod-shaped NANP-repeat domain of the CS protein more flexible in its interaction with the two antigen-binding domains of the B-cell receptor. By lifting

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the antigen up towards the surface of the hexon molecules, the 45Å-spacer molecule may ensure display of the antigen over its full length at an optimal distance from the surface of the AdV capsid. However, this effect may be dependent on length and conformation of the linker and/or the antigen [25].

We further showed that mixing soluble CS protein with unmodified, empty AdV vector particles failed to match responses reached by pIX-modified vectors or adjuvanted CS protein. AdV particles have previously been reported to mediate a strong adjuvant-effect on co-administered lipopeptide antigens that is independent of their vector function and likely mediated by capsid components [32]. AdV vectors induce the release of pro-inflammatory cytokines [37, 38], including type-I interferons, which enhances immune responses to peptides [39] or vector-encoded transgenes [40]. Although HAdV26- and HAdV35-based vectors induce higher levels of innate cytokine responses than HAdV5-based vectors [41], in our study all HAdV vectors (HAdV26, HAdV35, HAdV5) mixed with CS protein equally failed to increase CS-specific antibody responses, indicating that potential adjuvant-effects of AdV vector particles on protein-based vaccines are likely independent of innate cytokine responses but depend on the biophysical properties of the mixed protein; for example, its propensity to adhere to the surface of the viral particle. Therefore, our findings indicate that the high immunogenicity of pIX-modified display-vectors is related to a physical association of the CS_{short} antigen with the viral particles.

Our findings of increased humoral immune responses in addition to strong cellular responses induced by AdV vectors corroborate those reported by Bayer et al. [25], who demonstrated immunogenicity and efficacy of simultaneous pIX-display and expression of the gp70 envelope protein of Friend Murine Leukemia virus. AdV vectors are known to induce high levels of antigen-specific IFNγ-producing CD8^{+} T-cells [42] and combining them in a prime-boost regimen with other vaccine types has proven efficient in eliciting strong T-cell as well as humoral responses [43-46]. We previously demonstrated that HAdV35-based vectors encoding the CS antigen are highly effective in improving the T-cell responses induced by a CS protein prime in a heterologous prime-boost schedule [10]. Here we demonstrate that implementing pIX-display technology on CS-transgene expressing HAdV35 vectors induces strong antibody and IFNγ-producing CD8+ T-cell responses after a single immunization. It remains to be determined whether homologous prime-boost with the pIX-modified display-/expression vectors would further increase the magnitude of the immune responses, and whether this could serve as a potent, simplified regimen compared to heterologous prime-boost regimens.

In conclusion, we demonstrated that pIX-display technology is highly immunogenic and offers the opportunity to induce both T- and B-cell responses using a simplified vaccination regimen. Moreover, the pIX-CS_{short} display-vectors can be efficiently produced at high viral particle yields that are in the same range as the conventional transgene-expression vectors. Considering that many of the pathogens which require a complex immune response for protection are endemic to resource-limited settings, simplification

of vaccine regimen and high yield makes pIX-display technology particularly valuable to develop vaccines for underserved communities where they may have the highest impact.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION
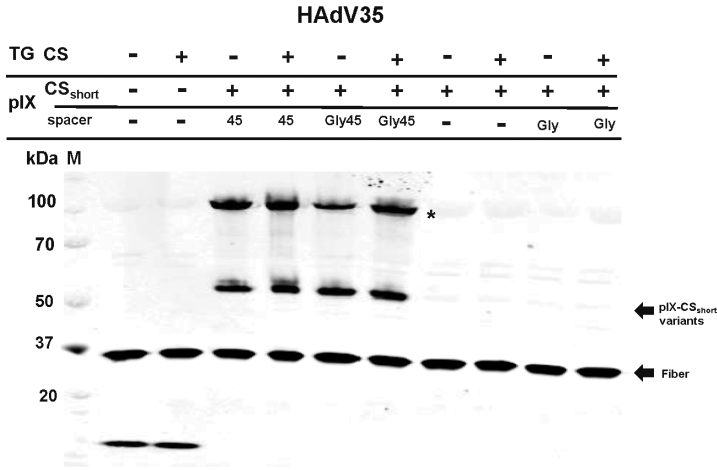
Figure S1. pIX-CS$_{short}$ capsid incorporation in purified HAdV35 vector preparations using the anti-pIX antibody. To confirm capsid incorporation of the pIX-CS$_{short}$ variants (~50 kDa), purified HAdV35 vector preparations (1.5 x10^{10} VP/well) were analyzed by Western blot using the monoclonal anti-pIX antibody. To ensure equal loading the blots were also stained with anti-fiber antibody (~35 kDa). Marker (M) is indicated with the corresponding kDa band size. An additional band (~100 kDa) is indicated with an asterisk (*). The pIX-fusion proteins migrate higher than their predicted size in kDa due to the NANP-repeat in the CS protein, a feature probably also affecting the detection of the pIX-CS$_{short}$ and pIX-Gly-CS$_{short}$ variants due to anti-pIX epitope masking (single epitope).
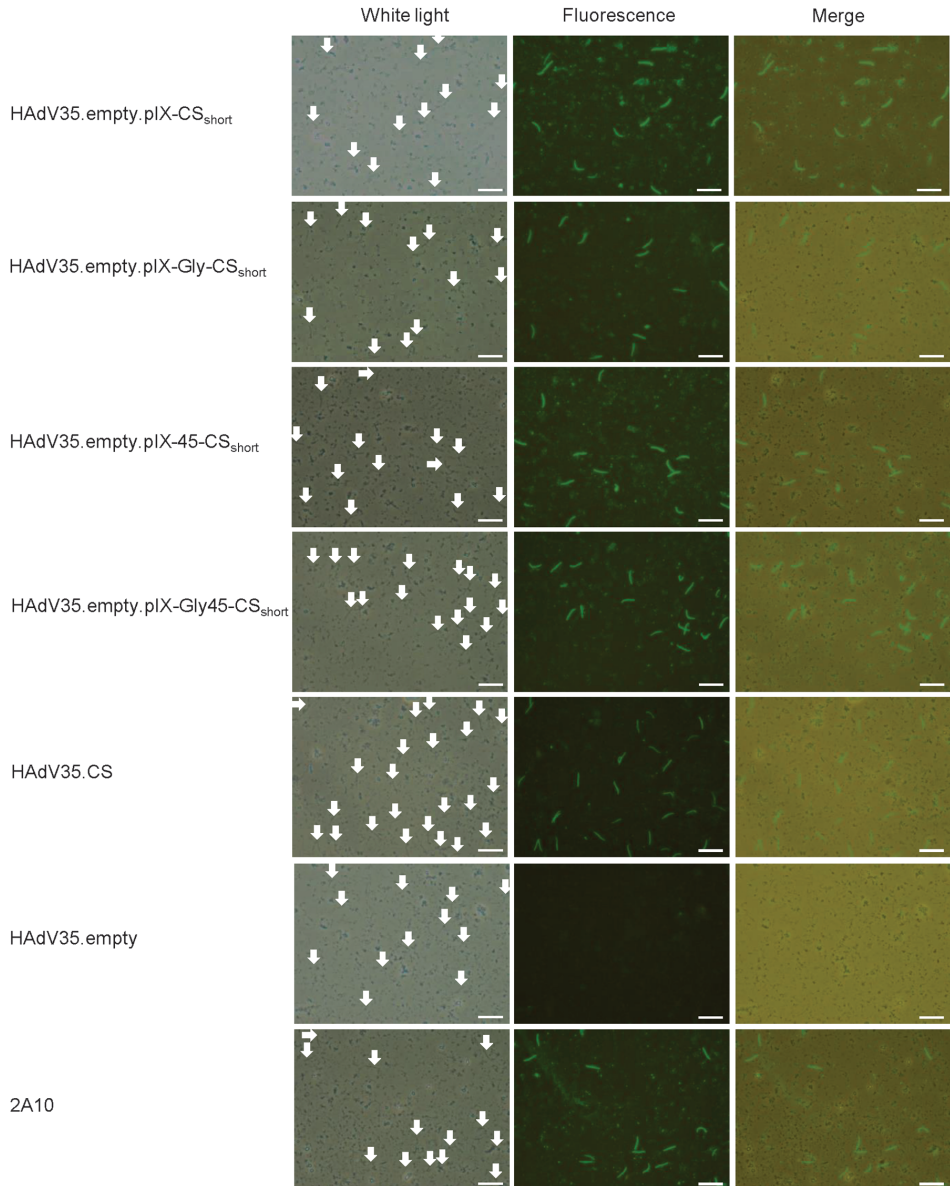
Figure S2. CS-specific antibodies induced by pIX-CS~short~ displayed on HAdV35 vectors recognize native CS on *P. falciparum slides.* Binding of CS-specific IgG in pooled sera of Balb/C mice 6 weeks after immunization with 1×10^{10} VP of the indicated vectors. Images taken at 40-fold magnification. Left panels show the white light (white arrows indicate sporozoite location), middle panels the fluorescence and the right panels show the merged images. White bars correspond to 20μm length.

Table S1. HAdV35 pIX-display vectors: overview of pIX capsid incorporation and CS transgene expression in A549 cells

| Vector | TG | pIX-modification | pIX-CS$_{short}$ capsid incorporation | | | pIX-CS$_{short}$ expression in producer cell line | | CS transgene expression |
| | | | WB | | EM | | | |
			α-pIX	α-CS	α-CS	α-pIX	α-CS	α-CS
HAdV35	CS	n/a	+	n/a	-	-	+	+
	Empty	n/a	+	n/a	-	+	-	n/a
	CS$_{short}$	n/a	n/d	n/d	n/d	+	-	-
	Empty	45-CS$_{short}$	+	+	+	+	-	n/a
#	CS	45-CS$_{short}$	+	+	+	+	+	+
	Empty	Gly45-CS$_{short}$	+	+	+	+	-	n/a
	CS	Gly45-CS$_{short}$	+	+	n/d	+	+	+
	Empty	GlyCS$_{short}$	-	+	+	-	-	n/a
	CS	GlyCS$_{short}$	-	+	n/d	-	+	+
	Luc	GlyCS$_{short}$	n/d	+	n/d	-	-	n/a
	Empty	CS$_{short}$	-	+	+	-	-	n/a
	CS	CS$_{short}$	-	+	+	-	+	+
	Empty	CS	-	-	n/d	+	-	n/a
	CS	CS	-	-	n/d	+	+	+

n/a: not applicable, (-) not detected, n/d: not determined, # no batch-to-batch differences observed, WB: Western blot, EM: electron microscopy, CS: circumsporozoite protein, TG: transgene in E1

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Table S2. Overview of HAdV35 pIX-CS$_{short}$ display vectors: viral titers and producibility as determined by optical density

Vector	TG	pIX modification	VP/ml	IU/ml	VP/IU ratio	VP/cm$_2$
HAdV35	CS		1.9×10^{12}	5×10^{11}	3	n/d
	Empty		1.0×10^{12}	3.3×10^{11}	3	6.1×10^8
	Empty	45-CS$_{short}$	1.4×10^{12}	1.8×10^{11}	8	2.5×10^8
#	CS	45-CS$_{short}$	2.8×10^{11}	1.7×10^{10}	17	1.3×10^8
	Empty	Gly45-CS$_{short}$	1.1×10^{12}	6.0×10^{10}	19	6.3×10^8
	CS	Gly45-CS$_{short}$	1.9×10^{12}	1.8×10^{11}	11	1.8×10^9
	Empty	GlyCS$_{short}$	3.3×10^{12}	2.9×10^{11}	11	n/d
	CS	GlyCS$_{short}$	2.8×10^{12}	2.0×10^{11}	13	n/d
	Luc	GlyCS$_{short}$	4.8×10^{11}	4.9×10^{10}	10	n/d
	Empty	CS$_{short}$	2.6×10^{12}	4.2×10^{11}	6	n/d
	CS	CS$_{short}$	1.5×10^{12}	2.0×10^{11}	8	8.8×10^8

CS: circumsporozoite protein
TG: transgene in E1
n/d: not determined, # second batch comparable titers
VP: physical viral particles
IU: infectious units
VP/IU: viral particle to infectious units ratio
VP/cm²: viral particles generated per cm² production- flask
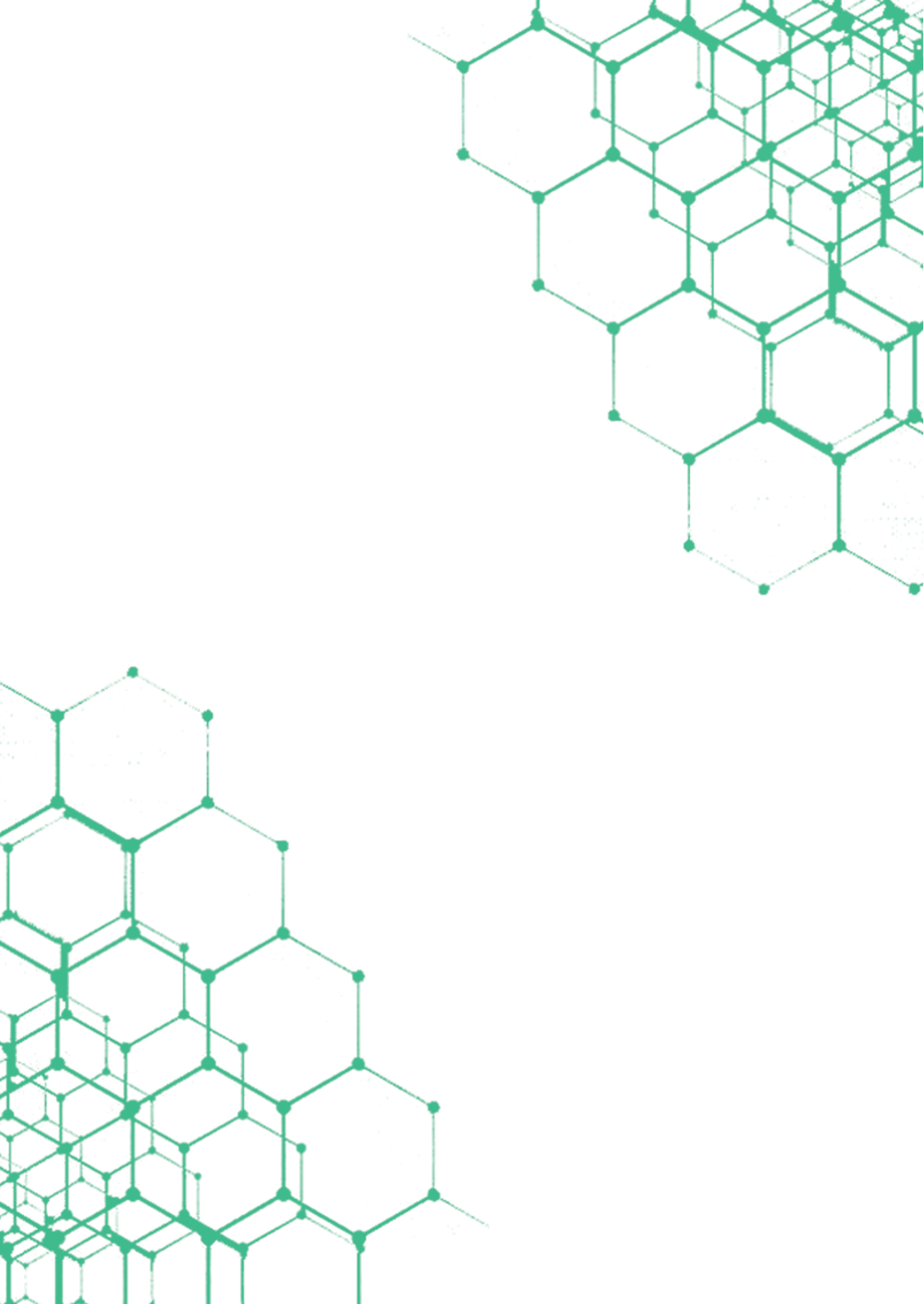
Table S3. Statistical significance in differences in CS-specific serum IgG titers elicited by the three pIX-CSshort modified HAdV35.empty vectors compared to HAdV35.CS (corresponding to figure 3A).

	p-value*			
	10^7 VP/mouse	10^8 VP/mouse	10^9 VP/mouse	10^{10} VP/mouse
HAdV35.epmty.pIX-CS$_{short}$	n/d	0.1169	0.6661	0.0658
HAdV35.epmty.pIX-45-CS$_{short}$	n/d	**0.000**	**0.0366**	**0.0029**
HAdV35.epmty.pIX-Gly-CS$_{short}$	n/d	**0.003**	**0.0082**	0.6378
HAdV35.epmty.pIX-Gly45-CS$_{short}$	n/d	**0.0002**	0.227	**0.0007**

n/d: not done (indicated dose excluded from statistical analysis since no titers above lower limit of quantification for the CS-IgG ELISA assay were elicited).

* Adjusted p-values for the titers elicited over time by the indicated vectors compared to HAdV35.CS given at the same dose (fixed reference group). P-values $\leq$ 0.05 were considered significant and are indicated in bold.

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DESIGN AND SELECTION OF THE OPTIMAL HPV L2-ANTIGEN FOR PROTEIN IX DISPLAY ON HUMAN ADENOVIRUS 35 AND 26 VECTOR PARTICLES

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ABSTRACT

Replication-incompetent adenoviral vector (AdV) vaccines are attractive as a platform for particle display of antigens via the hexon and protein IX (pIX) capsid components. Since pIX can be used to display larger heterologous peptides on the outer particle surface, this technology was used to generate a multivalent human papillomavirus (HPV) prophylactic vaccine based on the HPV L2 protein. The L2 protein N-terminus contains several conserved linear neutralizing antibody (nAb) epitopes. It has been shown that for a potent L2-based HPV vaccine, inclusion of linear nAb epitopes from different HPV genotypes displayed via particle-like structures may be beneficial. We designed a large panel of N-terminus L2 epitope repeats (i.e. concatemers) from different HPV genotypes such as HPV16, 18 and 45 (Sx3), or HPV16, 18, 45 and 31 (Sx4), that were fused to pIX in low-seroprevalent non-replicating human AdV35 and AdV26 vectors. In contrast to larger (≥153kDa) L2 concatemers, designs containing three heterologous (Sx3), and four heterologous (Sx4) L2 concatemers, were successfully incorporated into the human AdV35 or AdV26 capsid. This evaluation provides insight in the pIX-antigen designs that allow efficient capsid incorporation.

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INTRODUCTION

Replication-incompetent adenoviral vector (AdV) vaccines have shown promise as genetic vaccine vehicles in human trials [1-3]. AdV vectors are potentially attractive as a platform to display antigens on the vector particles via their hexon and protein IX (pIX) capsid components [4]. In preclinical settings, antigen display vaccines induce antigen-specific immune responses against infectious diseases such as *Yersinia pestis* and malaria [4-7]. Immune responses induced by an unadjuvanted malaria antigen pIX-display vector exceeded those induced by a genetic vector and recombinant malaria protein [7].

An AdV capsid contains 240 copies of the minor capsid pIX which functions to stabilize the capsid [8, 9]. AdV particles can be generated without pIX, but such particles demonstrate reduced capsid stability [10]. In addition to antigens, the pIX C-terminus can be engineered to contain heterologous proteins for vector retargeting [11, 12] and virus tracking [13, 14]. In these studies, the heterologous proteins fused to pIX were large (up to ~70kDa), globular and functional in terms of their receptor binding capacity or fluorescent/enzymatic activity.

We hypothesized that pIX-display technology can be used to generate a multivalent human papillomavirus (HPV) prophylactic vaccine based on the HPV L2 protein. Efficacious multivalent HPV vaccines containing L1 protein virus-like particles (VLPs) are currently available [15-17]. However, due to the limited cross-protection conferred by the L1 protein and the variety of cancerogenic high-risk HVP viruses—HPV16, HPV18, HPV45, HPV31, HPV33, HPV52, HPV58, HPV35, HPV56, HPV51, HPV39, HPV68, HPV73 and HPV82 (in descending order of frequency) [18-20]—wide HPV coverage could only be achieved by the inclusion of L1 protein of each HPV genotype [21]. This would notably increase the vaccine complexity. In contrast to L1, the HPV L2 protein contains highly conserved, cross-protective and linear neutralizing antibody epitopes (nAbs) on its N-terminus. The L2 N-terminus is a suitable candidate to develop a single- or multi-peptide antigen vaccine to provide broad protection against many circulating HPV types [22-24]. To maximize coverage, inclusion of linear nAbs epitopes from several different HPV genotypes may be beneficial [25, 26]. For example, HPV16- and HPV18-derived peptides were not sufficient to induce neutralizing antibodies against the divergent HPV31 [25, 27, 28]. However, immunization with adjuvanted 'multitype' L2 N-terminus peptide repeats mix was sufficient to induce cross-protective antibody responses in animal models [25]. Of note, repetitive L2 epitope-display via particle-like scaffolds notably enhanced the L2 specific immune response [29-36].

In this study, low-seroprevalent human AdV35 and AdV26 (HAdV35, HAdV26) [37-40] vectors were engineered to display different HPV L2 epitope repeats (i.e. concatemers) via pIX. Multiple pIX-display vectors containing multitype HPV L2 concatemers (size range 93-418 amino acids (a.a.)) were generated in the producer cell line and tested for pIX-L2 capsid incorporation. This evaluation study resulted in successful display of certain HPV L2 epitopes on the outer AdV capsid surface while failure to efficiently incorporate other L2 concatemers demonstrated the limitations of pIX antigen fusion designs.

MATERIAL AND METHODS

Vector generation and cell culture

The recombinant E1-deficient HAdV35 and HAdV26 vectors displaying human HPV L2 protein concatemers (repeats) via the minor capsid protein IX (pIX), and the control vectors, were generated in E1-complementing PER.C6© cells essentially as previously described [7]. In short, the L2-concatemers fused to pIX were synthesized and cloned in the pAdapt35 or pShuttle26 plasmids by GeneArt (Thermo Fisher Scientific). HAdV35 pIX-L2 modified vectors were rescued in PER.C6© cells by transfecting three genome plasmids using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. The HAdV35 and HAdV26 genomes contain all the native genes, including native pIX and its promoter (P), packaging signal (ψ) and the left and right inverted terminal repeats (ITR). These were further modified to contain an 'Empty' E1-cassette ΔE1 (i.e., with cytomegalovirus promoter and poly A signal), ΔE3 and HAdV5 E4orf6 (E45orf6). The genome plasmids containing overlaps for the homologous recombination, included the right end of the genome divided over two plasmids pBr.Ad35 PRdE3 5E4 orf6/7 (digested with PacI and NotI) and pWE.Ad35.ΔpIX.EcoRV (digested with NotI and EcoRV), and the left end of the genome (pAdapt35.Bsu.pIX-mod, digested with PacI). The HAdV26 vectors were generated by either allowing homologous recombination between the left (pShuttle26. pIX-mod, digested with PmeI and SbfI) and the right-end cosmid (pWE.Ad26.dE3.5orf6, digested with PacI) in PER.C6© cells, or BJ5183 *E.coli* cells to generate the whole HAdV26 genome plasmid as previously described [41]. All HAdV35 and HAdV26 vectors were plaque purified (expect for the whole genome HAdV26 rescues) and further propagated on adherent PER.C6® cells in DMEM (Thermo Fisher Scientific) supplemented with 10% of fetal bovine serum (Life Technologies Inc.) and 10 mM $MgCl_2$.

The vectors were purified in a two-step CsCl-gradient and dialyzed in formulation buffer (10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 75 mM NaCl, 5% sucrose, 0.02% PS-80, 0.1 mM EDTA, 10 mM Histidine, 0.5% ETOH). Viral particles (VP) per ml titer were determined by optical density (OD) in the presence of SDS [42]. The infectious units (IU) per ml were determined by a $TCID_{50}$ assay. The corresponding VP/IU ratios and the productivity (VP/cm^2) were calculated. Vector identity and integrity (e.g. pIX region) were determined by a PCR and confirmed by sequencing (Baseclear B.V.). The Bioburden and the Endotoxin (Microsafe, Milipore) levels were determined on each purified vector.

pIX capsid incorporation by Western blot

To determine pIX-L2 fusion proteins capsid incorporation, Western blot analysis was performed by loading viral particles on gel in different concentrations (e.g. 1.5, 1, and 0.5 x10^{10} VP/well). Viral particles were resolved on pre-cast 12% Bis/Tris Nu-PAGE gel (Invitrogen) in MOPS buffer (Invitrogen) at 175 V, 500 mA. After separation by size, total protein was transferred to nitrocellulose membrane using iBlot® Transfer stacks (iBlot system; Invitrogen) according to the recommendations provided by the manufacturer.

The capsid incorporated pIX-L2 variants were stained with anti-L2 specific (pooled HPV 16 mouse serum, Janssen) or anti-pIX monoclonal antibody (6740, Janssen) [7] and anti-fiber (HAdV5 4D2, Abcam) as loading control for 1 hour in 5% non-fat dry milk (BioRad)/Tris buffered Saline Tween 20 (Invitrogen). The proteins were visualized by staining with a fluorescently labeled secondary antibody IRDye800CW® 1:10 000 goat anti-mouse and recorded on the Odyssey® (Li-Cor).

HAdV proteome analysis by RP-UPLC and MS/MS

Reversed-Phase-Ultra-Performance-Liquid-Chromatography (RP-UPLC) on an ACQUITY BEH C4 Column, 300Å, 1.7µm, 2.1mmX150mm with ACN+0.17% TFA gradient was performed as described previously [43]. Protein sequences of pIX-L2 were determined by Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis as previously described [43].

RESULTS

L2-concatemer design and capsid incorporation: the trivalent (Sx3) HPV L2-concatemer is consistently displayed on the HAdV35 capsid via pIX

HAdV35 vectors displaying conserved, linear and cross-protective HPV L2 [22-24] concatemers via pIX were engineered and tested for capsid incorporation using Western blot.

The L2-concatemers consisted of: (i) a fragment spanning amino acids (aa) 10-40 containing a highly conserved aa 17-36 neutralizing B-cell epitope [28], termed the (S)-fragment, and (ii) a fragment spanning aa 11-89 containing the aa 17-36 [28] epitope plus the aa 68-85 neutralizing linear B-cell epitope [23], termed the (L)-fragment (Figure 1A). Concatemers of a single HPV type peptide or multiple HPV types have been shown to induce cross-neutralizing antibodies [29-36]. To enhance the repetitive presentation of the L2 epitopes, (S)- and (L)-fragments of the most prevalent HPV types 16, 18 and 45 were combined into multitype L2 concatemers - resulting in four HAdV35 pIX-L2 vectors: (Sx3), (Lx3), (SSSx3) and (SSLx3) (Figure 1B). All HAdV35 pIX-L2 vectors were successfully generated in the E1-complementing producer cell line and purified (data not shown).

To determine pIX-L2 capsid incorporation, purified VPs were separated on gel under reducing conditions and detected with anti-L2 serum (Figure 1C). The results showed major differences in pIX-L2 detected in the purified vaccine batches. pIX-L2(SSL) was not detected in the HAdV35 capsid, and only minor amounts of pIX-L2(SSSx3) and pIX-L2(Lx3) were detected. In contrast, pIX-L2(Sx3) was readily detectable at abundant levels in the HAdV35 capsid. An additional band (~20 kDa) directly below the pIX-L2(Sx3) band (25 kDa) was also detected. MS/MS analysis indicated that this minor band lacked the HPV16 (S)-fragment thus consisting of HPV45 and HVP18 (S)-fragments alone.

To assess the consistency of pIX-L2(Sx3) capsid incorporation, three different batches were produced and L2-concatemer incorporation was evaluated by Western blot
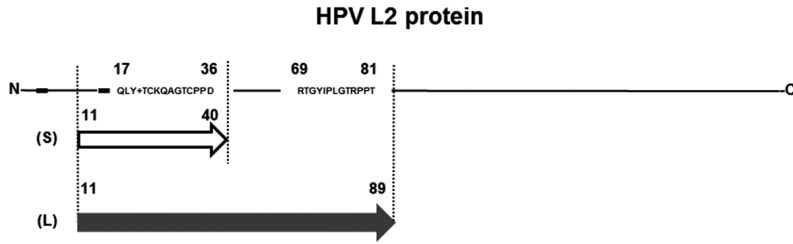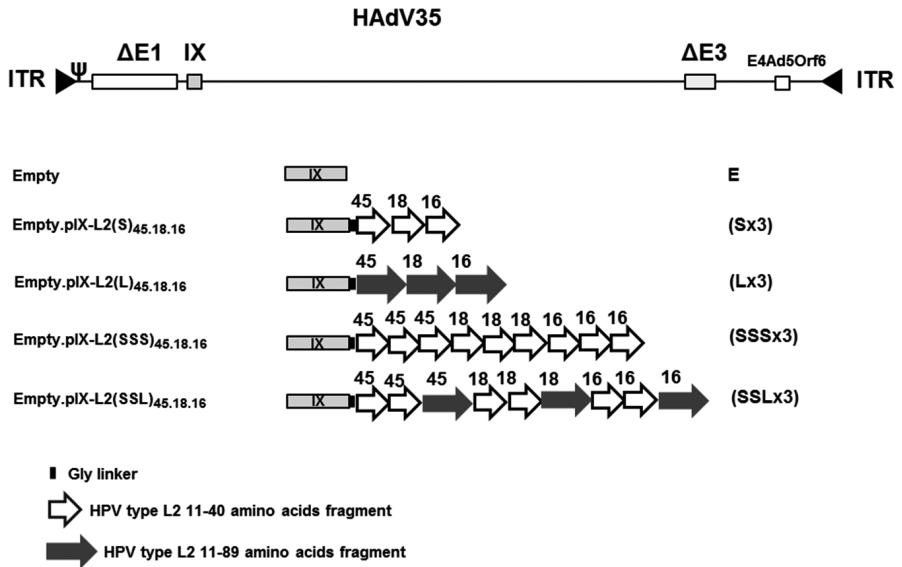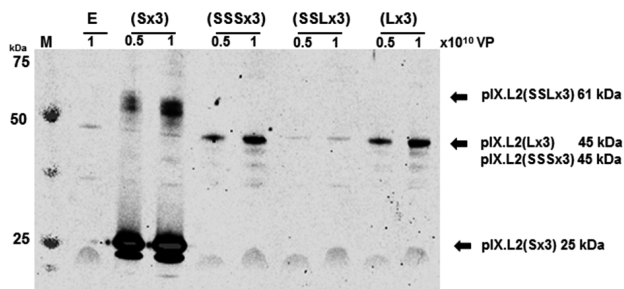
Figure 1. HPV L2-concatemer display on HAdV35 vector. **(A)** Schematic drawing of HPV L2 protein, epitope amino acid (aa) sequence and the fragments used to generate the L2-concatemers displayed on HAdV-vectors via pIX. Two fragments were selected for the generation of a L2-concatemer; the (S)-design is an aa 10-40 L2 protein fragment containing the aa 17-36 (QLY+TCKQAGTCPPD) epitope, and the (L)-design is an aa 11-89 fragment containing the aa 17-36 (QLY+TCKQAGTCPPD) and aa 69-81 (RTGYIPLGTRPPT) epitopes. **(B)** Schematic drawing of replication-incompetent HAdV35 Advac© vectors (see material and methods for details) genetically encoding and displaying the L2 protein epitope repeats (i.e., concatemers) on the capsid via the minor capsid protein IX. ▶

▶ HAdV35.Empty (E) is a control vector and contains native pIX. HAdV35.Empty.pIX-L2(Sx3)$_{45.18.16}$ (Sx3) and HAdV35.Empty.pIX-L2(SSS)$_{45.18.16}$ (SSSx3) vectors contain pIX and HPV45, 18 and 16 L2 protein (aa 10-40 (S)) concatemer fusion-protein. HAdV35.Empty.pIX-L2(Lx3)$_{45.18.16}$ vector (Lx3) contains pIX and HPV45, 18 and 16 L2 protein (aa 11-89 (L)) concatemer fusion-protein. In the HAdV35.Empty. pIX-L2(SSLx3)$_{45.18.16}$ vector, the pIX and L2 protein fusion protein contains both the aa 10-40 (S) and aa 11-89 amino acids fragments of HPV45, 18 and 16. All the fusion-proteins are linked with a flexible aa 3 Gly-linker. **(C)** Capsid incorporation pIX-fusion proteins (Sx3) (25 kDa), (Lx3) (45 kDa), (SSSx3) (45 kDa) and (SSLx3) (61 kDa) by Western blot (overexposed) in purified 1 and 0.5 x10^{10} VP/well of the purified HAdV35 (vector stained with anti-L2 (HPV 16 positive mouse serum). HAdV35. Empty (E) negative control.

(Table 1) and confirmed by RP-UPLC [43]. The results showed comparable capsid incorporated pIX-L2(Sx3) amongst the three HAdV35 pIX-L2 batches.

Expanding the L2-concatemer design: consistent display of a quadrivalent (Sx4) HPV L2-concatemer on the HAdV26 capsid via pIX

An HAdV35 vector containing a trivalent L2 protein aa 10-40 fragment of HPV types 45, 18 and 16 was successfully generated, and was the best amongst the tested variants in terms of (consistent) pIX-L2 incorporation. However, to ensure protection against multiple HPV types, inclusion of other (distant) HPV types such as HPV31 may be necessary. For this purpose, additional L2 protein aa 10-40 fragments of different HPV types were engineered and displayed via pIX in HAdV26 vector. The rational for using the HAdV26 was mainly to determine whether the L2 (Sx3) display (successful in HAdV35) was interchangeable amongst different HAdV vectors.

HAdV26 vectors displaying up to six different clinically relevant HPV types 16, 18, 31, 33, 45 and 52/58 (HPV 52 and 58 are identical in amino acid sequence in the L2 protein aa 10-40 region) in various combinations were generated and characterized; HAdV26. Empty.pIX-L2(Sx3), two different variants of pIX-L2(Sx4), two different variants of pIX-L2(Sx5) and one pIX-L2(Sx6) (Figure 2A). All pIX-L2(S) variants were incorporated into the HAdV26 capsid (Table 1). Consistent with the observations in HAdV35, the pIX-L2(Sx3) was efficiently incorporated in the HAdV26 capsid. The two pIX-L2(Sx4) variants were comparable to the pIX-L2(Sx3). The pIX-L2(Sx5) were less abundant than pIX-L2(Sx3)/(Sx4) in HAdV26 vector. The pIX-L2(Sx6) was the least abundant in the HAdV26 capsid (Table 1). Comparison of two batches showed that the pIX-L2(Sx4) variants consistently incorporate in the HAdV26 capsid (Figure 2B and Table 1).

DISCUSSION

Prophylactic HPV vaccines based on the L2 protein linear nAb epitopes are considered an alternative to currently available L1-based vaccines [22-24]. Inclusion of conserved and cross-reactive linear L2 epitopes in the vaccine provides broader protection across multiple clinically relevant HPV types [22-26]. Several L2-based vaccine strategies have

Table 1. Capsid incorporation pIX-L2 variants.

	HPV type	Concatemer	Size pIX fusion a.a.	Capsid incorporation (WB)
HAdV35 Advac©				
Batch 1	45.18.16	(Sx3)	93	++++ (4)
Batch 2	45.18.16	(Sx3)	93	++++ (2)
Batch 3	45.18.16	(Sx3)	93	++++ (2)
Batch 1	45.18.16	(SSSx3)	273	+/- (2)
Batch 1	45.18.16	(Lx3)	238	+/- (2)
Batch 1	45.18.16	(SSLx3)	418	-
HAdV26 Advac©				
Batch 1	52.33.31.45.18.16	(Sx6)	183	++ (3)
Batch 1	33.31.45.18.16	(Sx5)	153	+++ (3)
Batch 2	33.31.45.18.16	(Sx5)	153	+++ (3)
Batch 1	52.31.45.18.16	(Sx5)	153	+++ (3)
Batch 2	52.31.45.18.16	(Sx5)	153	+++ (3)
Batch 1	33.45.18.16	(Sx4)	123	++++ (3)
Batch 2	33.45.18.16	(Sx4)	123	++++ (3)
Batch 1	31.45.18.16	(Sx4)	123	++++ (3)
Batch 2	31.45.18.16	(Sx4)	123	++++ (3)
Batch 1	45.18.16	(Sx3)	93	++++ (1)
Batch 2	45.18.16	(Sx3)	93	++++ (1)

++++: very good, +++: good, ++: poor, +: barely detected, -: not detected, (n)= number of experiments, a.a.: amino acids

been described: (i) concatemer peptides consisting of L2 epitopes of different HPV types (multitype concatemers) [25, 44, 45], (ii) adjuvanted peptides [46, 47] and (iii) repetitive surface display of L2 epitopes via virus particles and/or scaffold proteins [29-36, 48-52].

Considering that repetitive L2 epitope-display via particle-like structures using L2 epitopes—possibly from multiple HPV types—may increase the L2 specific immune response, we set out to develop an HAdV pIX-L2 display vector using the low-seroprevalent HAdV35 and HAdV26 vectors. The L2 N-terminus is highly conserved amongst the different HPV types (e.g. aa 17-36 epitope sequence homology is ~80%). However, minor sequence variations can result in reduced protection against certain (distant) HPV types [25]. For broad coverage, inclusion of the most prevalent HPV types covering the full variation in L2 sequences in the vaccine may be necessary. Therefore, the first step was to design a multitype L2-concatemer which can be displayed on the outer surface of the HAdV particle.

The L2-concatemers were initially designed to contain different N-terminal fragments of the most prevalent HPV types 16, 18 and 45. The fragment includes the L2 protein N-terminal aa 10-40 ((S)-fragment), and aa 11-89 ((L)-fragment), thus incorporating
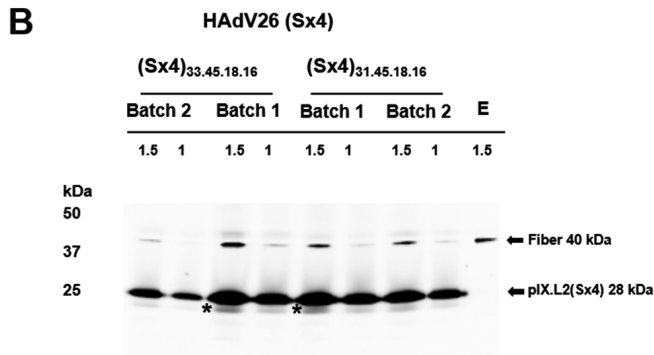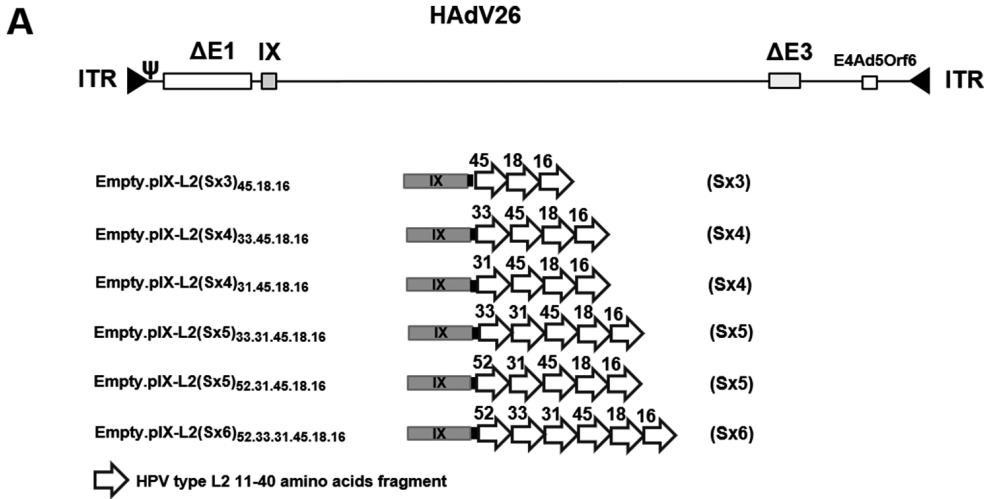
Figure 2. Extension of the L2-concatemer in HAdV26. **(A)** Schematic drawing of HAdV26 vectors encoding and displaying different L2(S) concatemers via protein IX. The vectors contain the aa 10-40 or (S)-design concatemers in a range from Sx3 up to Sx6 fused to pIX via an aa 3 Gly-linker. Included in the concatemers are different combinations of HPV types 16, 18, 31, 33, 45 and 52/58 (identical in the 10-40 aa). See material and methods for details on the HAdV26 vector genome. **(B)** Capsid incorporation by Western blot of the pIX-L2(S) modified HAdV26 vectors. Shown are two different small vector preparations (batch 1 and 2) of (Sx4) variants HAdV26 pIX-L2(Sx4) (28 kDa) containing HPV types 33, 45, 18, 16, and HAdV26 pIX-L2(Sx4) containing HPV types 31, 45, 18, 16 at two concentrations 1.5 and 1 x10^{10} VP/well detected with anti-L2 (HPV 16 positive mouse serum) and the loading control anti-fiber (40 kDa fiber) (HAdV5 fiber knob, 4D2). A minor band (indicated by an (*) asterisk) detected in all the batches although at different intensities. HAdV26.Empty (E) negative control.

the highly conserved linear nAb epitopes at aa 17-36, and aa 69-81 [23]. Efficient incorporation was only observed with the pIX-L2(Sx3) concatemer containing the L2 protein aa 17-36 linear nAb epitope. To achieve broader HPV protection, we sought to identify the maximum number of aa 10-40 ((S)-fragments) of different HPV types that could be included in one L2-concatemer, while retaining efficient capsid incorporation.

In an HAdV26 vector, systematic addition of aa 10-40 fragment from one HPV type at a time to the successful pIX-L2(Sx3) was evaluated. In the HAdV26 vector, pIX-L2(Sx3) was incorporated into the capsid, suggesting that the L2(Sx3) antigen is easily transferred from HAdV35 to other AdV types. In addition, efficient and consistent capsid incorporation was observed with the two heterologous pIX-L2(Sx4) vectors (two batches each) (Figure 2B), suggesting some sequence independence. The least efficient capsid incorporation was observed with pIX-L2(Sx6), followed by pIX-L2(Sx5) (Table 1). We attempted to semi-quantify pIX-L2 compared to the non-modified pIX using Western blot or RP-UPLC. However, due to varying results in anti-pIX detection in Western blot and the A280 absorption in RP-UPLC between modified and non-modified pIX, such comparisons did not yield reliable results.

The difference in capsid incorporation between the pIX-L2 variants may be explained by the fact that the L2 aa 17-36 epitope contains two highly conserved (across all HPV types) cysteine residues (C22 and C28) which form intra-molecular disulfide hairpin loops [53, 54]. The pIX-L2 fusion proteins are produced in the cytosol and transported back to the nucleus where the virion assembly takes place [14, 55], reducing environments typically not conducive for stable disulfide formation. However, there are various examples of cytosolic proteins undergoing disulfide bond formation in oxidant-stressed and non-stressed cells [56]. Therefore, we speculate that due to the stress in the cell induced by virion production, the abundance of cysteine residues in the pIX-L2(Sx6) and pIX-L2(Sx5) can form covalent bonds, yielding intra- and inter-molecular disulfide bridges and protein aggregates which may interfere with capsid incorporation.

The additional 'minor' band was observed in all batches and was consistent with the size in kDa representative for the loss of HPV16. MS/MS analysis of the additional band observed in the Western blot (~20 kDa) directly below the pIX-L2(Sx3) band (25 kDa), suggests that the HPV16 (S)-fragment was 'cleaved' off. We speculate that the incomplete furin cleavage site (e.g. HPV16: RTKR/ASA) [57] in the aa 10-40 fragment results in partial digestion of the pIX-L2(Sx3).

This study assessed the design and selection of the optimal HPV L2-antigen and showed that AdV vectors can be successfully loaded with multitype HPV peptides via fusion to the pIX C-terminus. Future manufacturability and immunogenicity assessments are necessary to determine whether this AdV pIX-display-based technology can be used to generate a viable and broadly protective prophylactic HPV vaccine.

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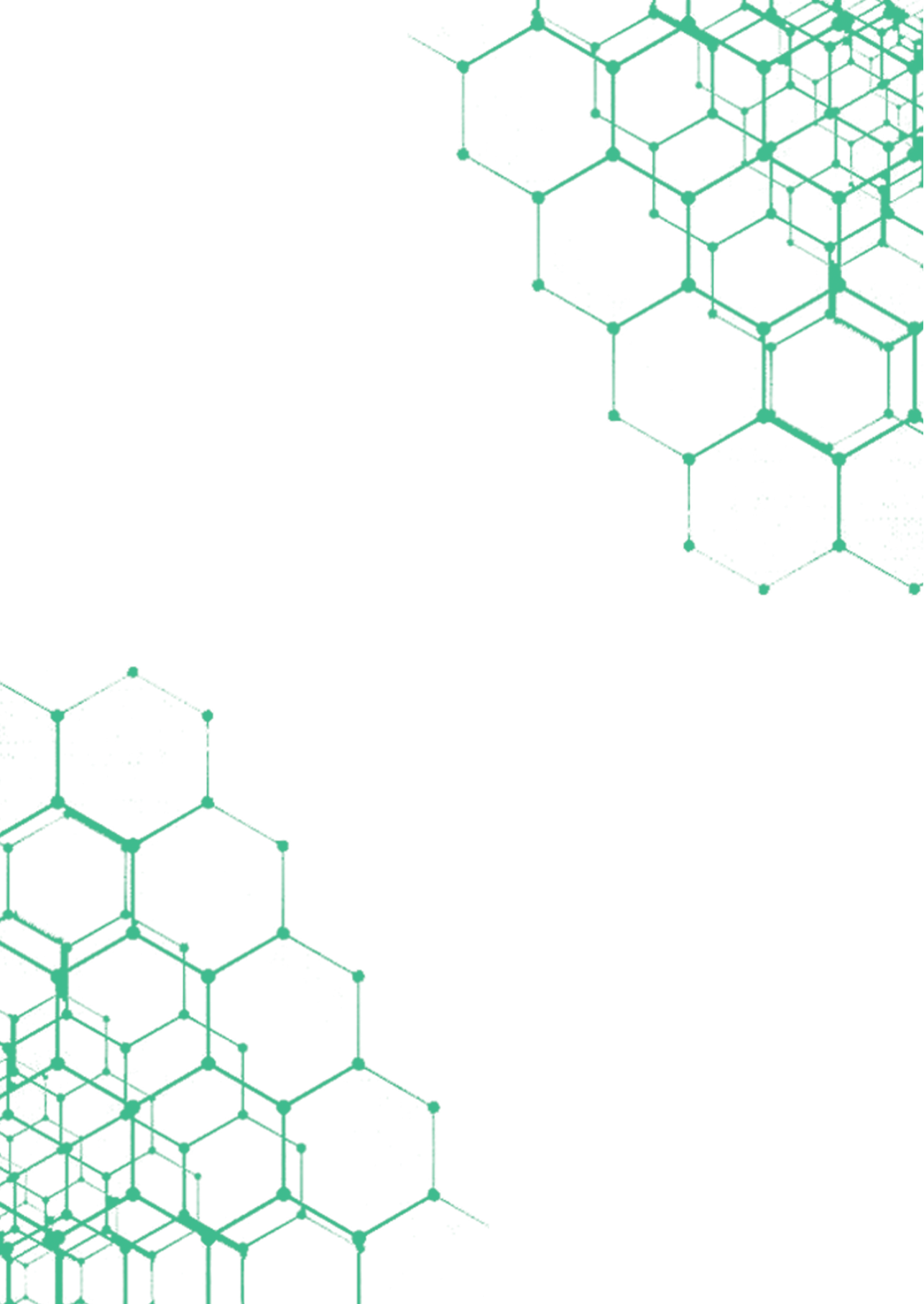
ADENOVIRUS BASED HPV L2 VACCINE INDUCES BROAD CROSS-REACTIVE HUMORAL IMMUNE RESPONSES

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ABSTRACT

Oncogenic high-risk human papillomavirus (HPV) infections cause a substantial number of genital and non-genital cancers worldwide. Approximately 70% of all cervical cancers are caused by the high-risk HPV16 and 18 types. The remaining 30% can be attributed to twelve other high-risk HPV-types. Highly efficacious 2-valent, 4-valent and 9-valent L1 protein based prophylactic HPV vaccines are available however with limited cross-protection. To further increase the coverage, development of a multivalent cross-protective HPV vaccine is currently focused on the conserved N-terminus of HPV's L2 protein. We have developed a vaccine candidate based on the rare human adenovirus type 35 (HAdV35) vector that displays a concatemer of L2 protein epitopes from four different HPV-types via protein IX (pIX). A mix of two heterologous HAdV35 pIX-L2 display vectors present highly conserved linear epitopes of nine HPV-types. Each HAdV35 pIX-L2 display vector exhibits a good manufacturability profile. HAdV35 pIX-L2 display vaccine vectors were immunogenic and induced neutralizing antibodies against HPV-types included in the vaccine and cross-neutralizing antibodies against distant a HPV-type not included in the vaccine in mice. The HAdV35 pIX-L2 display vectors offer an opportunity for a multivalent HAdV-based prophylactic HPV vaccine.

ABBREVIATIONS

a.a. – amino acid; AdV – adenoviral; HAdV35 - human adenovirus 35; HPV - human papillomavirus; MSD - Meso Scale Discovery; nAb - neutralizing antibody; pIX - protein IX; VLP - virus-like particle; VNA - virus neutralization assay; VP – viral particles; VPN - viral passage number

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INTRODUCTION

Oncogenic high-risk human papillomavirus (HPV) infections are responsible for almost 5% of all cancers worldwide, with substantially higher rates in developing countries [1, 2]. Twelve high-risk HPV genotypes are implicated as the causative agents of ano-genital and oropharyngeal cancers in woman and men [1, 3]. Approximately 70% of the cervical cancer cases worldwide are caused by the high-risk HPV16 and 18 genotypes. The remaining 30% of cervical cancer cases are caused by other high-risk types: HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59 and probably HPV68 [2, 4]. Low-risk HPV genotypes such as HPV6 and 11 can cause benign but debilitating genital and cutaneous warts [1, 2]. Three L1 virus-like particles (VLP) based prophylactic HPV vaccines are currently available: 2-valent (HPV16 & 18) *Cervarix* (GSK), 4-valent *Gardasil* (HPV6, 11, 16 and 18) and 9-valent *Gardasil*9 (HPV6, 11, 16, 18, 31, 33, 45, 52 and 58) (Merck)[5-7].

Although L1 VLP vaccines have proven to be highly efficacious, due to limited cross-protection wide HPV coverage can only be achieved by addition of L1 component of each HPV genotype [8] increasing the vaccine complexity. Therefore, there is a need for an alternative less complex prophylactic HPV vaccine with broad HPV coverage [9, 10], and efforts have been invested in generating an HPV vaccine based on the other HPV capsid component, the L2 protein [9, 10]. L2 protein-based vaccine might have potential for broad HPV coverage with a single vaccine due to the highly conserved cross-protective linear neutralizing antibody (nAb) epitopes in the L2 protein N-terminus [10, 11]. Immunization with adjuvanted N-terminus L2 peptide concatemers (i.e. repeats) induced cross-protective antibody responses in different animal models [12-16]. Surface exposed repetitive presentation of the L2 epitope is believed to improve the L2-specific cross-protection as demonstrated by some antigen-display platforms [17]. For instance, the adjuvanted scaffold L2 epitope presentation induced L2-specific antibodies in mice [18]. Similarly, several different experimental adjuvanted VLP-based L2-display vaccine designs induced L2-specific broad and cross-reactive antibodies in animal models: HPV L1-based VLP [19, 20], bacteriophage VLP [21-23], adeno-associated virus particles capsid display [24] and human adenovirus 5 (HAdV5) L2 hexon-display [25].

Replication-incompetent AdV vectors are attractive as vaccine vectors due to their clinically acceptable safety profile, potent antigen-specific immune responses and good manufacturability [26]. Vaccination with AdV vectors against infectious diseases such as HIV and Ebola has proven promising in clinical trials without an adjuvant [27-30]. Additionally, AdV vectors can be modified to display antigens via capsid proteins such as hexon and pIX [31]. AdV pIX-display vectors induce potent immune responses against *Yersinia pestis* and malaria in animal models [32-34]. We hypothesized that with the adenoviral (AdV) protein IX (pIX)-display vaccine platform we could generate a multivalent L2 protein display prophylactic HPV vaccine.

To evaluate the feasibility of a multivalent replication-incompetent AdV pIX-display based prophylactic HPV vaccine, HAdV35 vectors displaying L2-epitope concatemers

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fused to the pIX C-terminus (pIX-L2) were generated and extensively characterized. Unlike HAdV5 vectors [35, 36], vectors derived from rare HAdV35 and HAdV26 types [36-39] are less likely to be hampered by high levels of pre-existing immunity. HAd35 pIX-L2 vectors showed good manufacturability that was comparable to the non-modified control vector, with consistent batch-to-batch, viral titer yields, genetic stability and stability after 1 year incubation at cold-chain relevant 2-8°C temperatures [40, 41]. Each single HAdV35 pIX-L2 vector displaying different HPV L2 concatemers elicited humoral immune responses in mice, against the HPV types included and not included in the vaccine, which were further enhanced by mixing two heterologous HAdV35 pIX-L2 vectors in one vaccine formulation. These findings indicate that the pIX-L2 display vector offers an opportunity for an AdV-based multivalent prophylactic HPV vaccine.

METHODS

Vector generation

The replication-incompetent E1/E3-deleted HAdV35 vectors displaying HPV L2 protein concatemers (synthesized and codon-optimized [*Homo sapiens*] by GeneArt] ThermoFisher Scientific]) via pIX were generated in E1-complementing PER.C6® cells, CsCL-purified and characterized as previously described [34].

pIX capsid incorporation

Capsid incorporation of pIX-L2 proteins was determined by western blot with reduced/denatured CsCL-purified viral particles (VP) as previously described [34]. ELISA was performed by immobilizing CsCL-purified intact VP (10^{10} VP/well) as previously described [42].

HAdV proteome analysis by RP-UPLC and LC-MS/MS

Reversed-Phase-Ultra-Performance-Liquid-Chromatography (RP-UPLC) on an ACQUITY BEH C4 Column, 300Å, 1.7μm, 2.1mmX150mm with ACN+0.17% TFA gradient was performed to evaluate the protein content of the purified HAdV35 vectors (280nm absorption). pIX/pIX-L2 peak area abundance (%) was determined relative to the peak area sum of all the viral proteins.

Protein sequences of pIX-L2 were determined by Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis. pIX/pIX-L2 bands were isolated from purified HAdV35 vector, separated on a 4-12% Bis-Tris gel SDS-PAGE gel (Thermo Fisher Scientific), stained with SilverQuest™ and treated with Trypsin-V5111 (Promega) according to manufacturer's recommendations. The digested proteins were separated on a 150x2.1mm reversed-phase C18 BEH300 UPLC column with 2-50 % ACN + 0.1% FA gradient connected to mass spectrometer Waters Synapt G2 ESI-Q-TOF. Peptide sequence analysis was performed using BiopharmaLynx version 1.3.2 (Waters).

Viral growth kinetics and genetic stability in producer cell line

HAdV35 vectors growth kinetics in the suspension PER.C6® producer cell line were evaluated by analyzing the VP/mL titers with CMV-promoter-specific quantitative PCR (QPCR) (0-4 days post infection). The cells were lysed with 1% Triton™ X-100 (Sigma-Aldrich) and treated with DNaseI (Roche). TaqMan Gene expression master mix (Life Technologies), 1000x diluted sample and 25 mM $MgCl_2$ with 10 pmol of each primer were used. In the reaction, DNA was denatured at 95°C for 8 minutes, followed by 10 seconds at 95°C and 30 seconds at 60°C for 35 cycles in the QPCR machine (Applied Biosystems Viia7). Genetic stability of the pIX-L2 modification in the HAdV genome during vector production in PER.C6® cells was evaluated at the fourth viral passage number (VPN) beyond the commercial process stage as described by Vogels et al.[43].

Incubation at 2-8°C and the infectivity assessment by QPA

HAdV35 vectors were incubated for up to 1 year at 2-8°C under controlled conditions. HAdV vectors (n=3) were diluted in formulation buffer (10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 75 mM NaCl, 5% sucrose, 0.02% PS-80, 0.1 mM EDTA, 10 mM Histidine, 0.5% ETOH) to $1x10^{11}$ VP/mL in a glass 1mL vial. Infectivity (IU/mL) testing by Quantitative-PCR-based Potency Assay (QPA) was performed as described using CMV primers/probe [44]. Change from baseline values (Δlog_{10} values) were calculated by subtracting the log10 value at time point 0 from the log10-transformed values at later time points.

Mice immunization and ethical statement

Six-to-eight-week-old female CB6F1 mice (Charles River) were vaccinated intramuscularly two times at an 8-week interval with HAdV-vectors in the quadriceps of both hind legs with $1x10^8$ or $1x10^{10}$ VP/mouse of each HAdV35 vector type (e.g. total $2x10^8$ or $2x10^{10}$ VP). As a positive control for the L2 pseudovirions virus neutralization assay (VNA), mice were vaccinated three times at 4-week interval with 1/10 of a full-human dose of 4-Gardasil (Merck). Animal handling was performed according to the Dutch Animal Experimentation Act and Directive 86/609 of the Council of the European Committee after approval by Janssen Dier Experimenten Commissie (permit number 21300).

L2-specific-antibody in mice by MSD-ELISA

L2 HPV-type-specific serum IgG antibody responses were determined by using the Meso Scale Discovery (MSD) multivalent immunoassay. Each plate contained ten spots per well covered with one of HPV L2 (10-38 amino acids [a.a.]) antigen peptides of HPV6, 11, 16, 18, 31, 33, 45, 52(58), 59 or BSA protein control. Mouse serum samples were analyzed in the assay according to manufacturer's recommendations and read on a MSD SectorS-600-reader. Antibody titers-log_{10} were calculated using R3.1.1 software (R Core Team 2013).

HPV pseudovirions and VNA

The HPV-pseudovirions were essentially produced as described previously [45]. In summary, HEK293FT cells (DMEM [Thermo Fisher] with 10% Fetal Bovine Serum [FBS]) were transfected with HPV L1-L2 capsid-encoding pcDNA2004Neo(-).HPVx.L1S.IRES. L2.WPRE plasmids and the pCLucF plasmid encoding firefly luciferase and eGFP proteins (a kind gift from Dr. John Schiller, NIH).

HPV-type-specific L2 nAb were determined using a previously described *in vitro* HPV VNA [46], with the exception that firefly luciferase expression was measured and recombinant furin (R&D Systems) was used instead of supernatant from furin producing cells. Luciferase *bioluminescence (*neolite™ PerkinElmer) was measured according to manufacturer's recommendation using the Synergy Neo2 Multi-Mode Reader (BioTek).

Statistical analysis

One-year stability of the vector was tested using the slope over time based on a linear mixed-effects model that accounted for correlated measurements per lot over time. Per HPV-type and week, humoral responses (ELISA and VNA titers) between treatment groups were compared across doses using a Cochran–Mantel–Haenszel test with rank scores based on the titer values, with dose as the stratification factor. Wilcoxon Rank Sum tests were used for secondary analysis on the 10^{10} VP/animal dose, and for group comparisons per HPV type and week at the 10^{10} dose. P-values were adjusted using a 3-fold Bonferroni correction with a 5% significance level. Statistical analyses were performed in SAS 9.4 (SAS Institute, Cary NC). A quantitative comparison between the ELISA and VNA titers was assess using Spearman correlation analysis in GraphPad Prism 7.0 (GraphPad Software Inc.).

RESULTS

Multivalent prophylactic HPV vaccine design: 3- 4- and 5-valent HPV L2-concatemer display in HAdV35 vectors

To develop a multivalent HAdV capsid-display prophylactic HPV vaccine based on the L2 protein, HAdV35 vectors displaying multiple HPV-type L2-fragments via pIX were generated. L2 protein fragment (S), containing the highly conserved 17-36 a.a. nAb binding site [10] (Supplemental Figure 1), was selected for HAdV35 pIX-display. The (S)-fragments of nine clinically relevant HPV-types 6, 11, 16, 18, 31, 33, 45, 52 and 58 were fused to pIX in different combinations. This resulted in concatenated multiple HPV-type L2(S)-fragment display per HAdV35 capsid. Three vectors were generated: 3-valent HAdV35 pIX-L2(Sx3) vector displaying HPV45.18.16, 4-valent HAdV35 pIX-L2(Sx4)16 vector displaying HPV6.31.33.16 and 5-valent HAdV35 pIX-L2(Sx4)18 vector displaying HPV11.52.58.45.18 (Figure 1). All three HAdV35 pIX-L2 display vectors were successfully rescued, produced at small-scale in producer cells, CsCl-purified and extensively characterized to determine the production profile and immunogenicity *in vivo*.
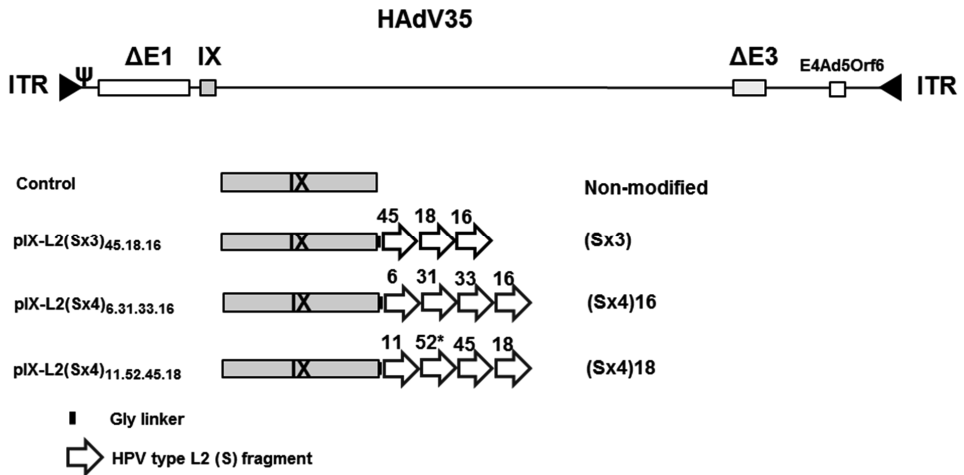
Figure 1. HAdV35 pIX-L2 concatemer vectors. Schematic drawing of replication-incompetent HAdV35 vectors genetically encoding and displaying the L2 protein epitope concatemers on the capsid via pIX. The HAdV35 vector genome contains all the native HAdV35-genes including the native pIX and its promoter (P), packaging signal (ψ) and the left and right inverted terminal repeats (ITR). The vector is further modified to contain an 'Empty' E1-cassette ΔE1 (i.e. with CMV promoter and SV40 poly A signal), ΔE3 and HAdV5 E4orf6 (E45orf6). L2 protein concatemers displayed on the HAdV35 capsid via pIX and a 3 a.a. Gly-linker for added flexibility are encoded in the HAdV35 genome. The concatemers include the L2 protein (S) fragment of in total nine different clinically relevant HPV-types 6, 11, 16, 18, 31, 33, 45, 52 and 58. Resulting in, HAdV35.pIX-L2(Sx3)$_{45.18.16}$ (pIX-L2(Sx3)), HAdV35.pIX-L2(Sx4)$_{6.31.33.16}$ (pIX-L2(Sx4)16) and HAdV35.pIX-L2(Sx4)$_{11.52/58.45.18}$ (pIX-L2(Sx4)18). HPV52 and 58 are identical in the 11-40 a.a. L2 protein region (*). HAdV35 is a control vector and contains the non-modified pIX.

pIX capsid display of multiple HPV-type L2-concatemers in HAdV35 vectors

The quality of HAdV35 pIX-L2 small-scale vector preparation was determined with pIX-L2 capsid incorporation by western blot, pIX-L2 surface display by ELISA and capsid incorporation consistency by RP-UPLC analysis. Consistent capsid incorporation in at least two small-scale vector preparations shows the batch-to-batch variation which is considered an indicator of a reliable manufacturing process [41]. pIX-L2(Sx3), pIX-L2(Sx4)16 and pIX-L2(Sx4)18 were incorporated into the HAdV35 capsid and exposed to the capsid surface (Figure 2A and B). RP-UPLC analysis confirmed pIX-L2 capsid incorporation and indicated consistent batch-to-batch pIX-L2 capsid incorporation (Supplemental Table 1). The capability of pIX to consistently display concatenated L2 epitopes on the AdV capsid allows the generation of a multivalent L2 based AdV pIX-display vaccine vector.

HPV L2-display HAdV35 vectors grow at high titer yields and are genetically stable in producer cells

To determine the effect of capsid incorporated pIX-L2 on HAdV35 vector producibility, the vector yields, replication kinetics and genetic stability were evaluated in the producer
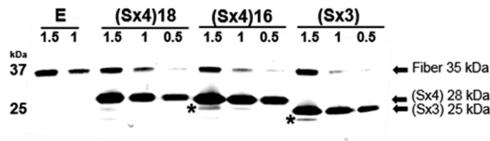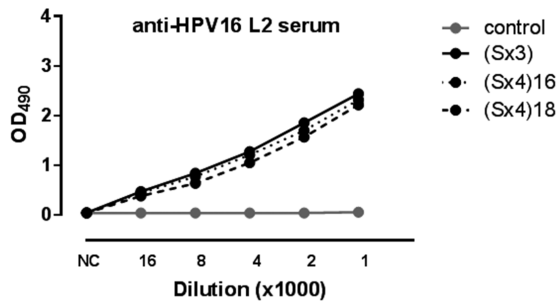
Figure 2. HPV L2 (Sx3) and (Sx4) capsid incorporation and display. **(A)** pIX-L2 capsid incorporation by western blot of purified HAdV35 pIX-(Sx3), pIX-L2(Sx4)16 and HAdV35 pIX-L2(Sx4)18 vectors. Three different concentrations 1.5, 1 and 0.5 x10¹⁰ VP/well of the pIX-L2 vectors were analyzed by western blot and stained with anti-L2 (HPV16 positive mouse serum) and the loading control anti-fiber antibody (HAdV5 fiber knob, 4D2). pIX-L2(Sx4) (28 kDa), pIX-L2(Sx3) (25 kDa) and the loading control fiber (35 kDa) are indicated with an arrow. The minor protein fragment is marked with an asterisk (*) indicates. **(B)** pIX-L2 capsid surface display by ELISA. Plates were coated with 1x10¹⁰ VP/well of purified HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16 and pIX-L2(Sx4)18 vectors. HAdV35.Empty vector was used as a negative control (1x10¹⁰ VP/well). Diluted HPV16 L2 positive mouse serum (two wells per dilution) was added followed by an anti-mouse horse radish peroxidase (HRP) labeled secondary antibody. After the addition of substrate, the OD$_{490}$ levels were measured as indicated in the material and methods. A representative of two experiments is shown.

cells [40]. HAdV35 pIX-L2 display vectors yields were assessed by comparing VP/mL of the small-scale vector preparations, infectious units (IU/mL) and VP/IU ratio to the non-modified control vector. HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16 and pIX-L2(Sx4)18 vector preparations were comparable to the non-modified control vector (Supplemental Table 1). These observations were confirmed by the HAdV35 pIX-L2 display viral replication analysis in suspension producer cells (Figure 3A and B).

The HAdV35 pIX-display vectors encode a highly repetitive L2-concatemer with an average homology of ~70% (data not shown), which might lead to genetic instability of the pIX-L2 concatemer in producer cells [47, 48]. To assess this risk, multiple clones and/or small-scale vector preparations were propagated extensively in producer cells, analyzed by pIX-specific PCR and confirmed by sequencing as previously described [39]. All HAdV35 pIX-L2 vectors remained genetically stable in the producer cells for four additional VPNs beyond the manufacturing-scale passage (Figure 3C, 3D and Supplemental Table 1).

In summary, HAdV35 pIX-L2 display vectors showed comparable producibility to the non-modified control vector and remained genetically stable in the producer cell line.

HAdV35 pIX-L2 display vectors remain infectious after 1 year incubation at 2-8°C

L2 concatemers surface displays may affect the physical properties of the HAdV35 capsid, therefore long-term stability was assessed [49]. Long-term stability was determined by incubating purified HAd35 pIX-L2 vector preparation at 2-8°C for up to 1 year under controlled conditions and measuring the IU/mL by QPA. QPA analysis showed that the HAdV35 pIX-L2 infectivity decreased after 1 year incubation at 2-8°C (approximately
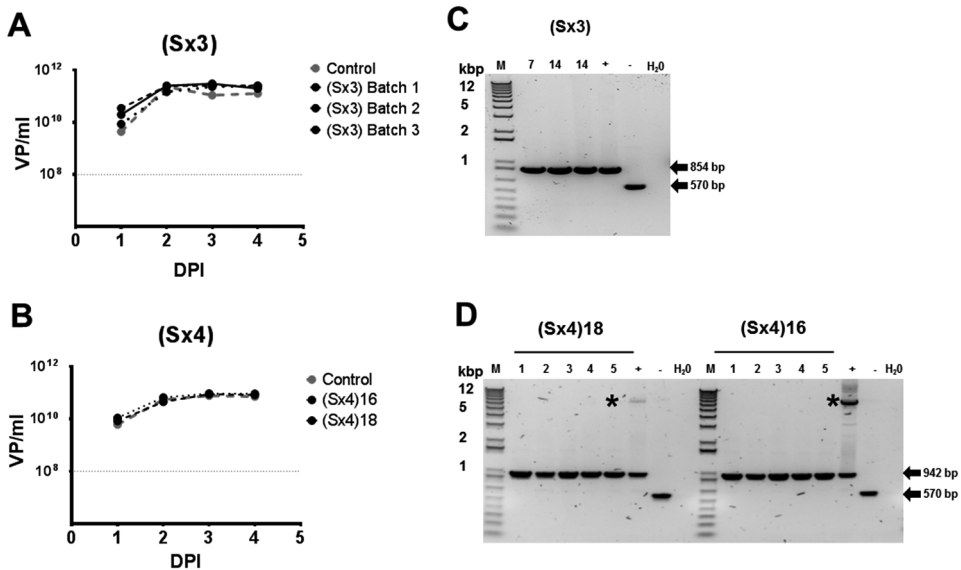
Figure 3. HAdV35 pIX-L2 viral replication kinetics and genetic stability in the producer cell line. **(A)** HAdV35 pIX-L2(Sx3) vector replication in suspension producer cell line. 10 mL of 1x10^10 viable cells/mL were infected with three small-scale vector preparations (batch 1-3) of the (Sx3) vectors or non-modified HAdV35 control vector (control) at 70 VP/cell and incubated in a shaker-flask for up to 5 days. Samples were taken at 0-4 days post infection (DPI) and evaluated for VP titer (log VP/mL) increase by QPCR (n=3, assay replicates) as described in the material and methods. Representative results of at least two experiments is shown. **(B)** Replication kinetics of the HAdV35 pIX-L2(Sx4)18 and pIX-L2(Sx4)16 modified vectors in suspension producer cells as described in 3A. **(C)** Genetic stability of HAdV35 pIX-L2(Sx3) batch 1 after extended passaging in the producer cell line (n=2). Shown are pIX-PCR results of VPN 7 (i.e. purified small vector preparation) and 14 (four passages beyond the envisioned commercial scale) (854 bp). **(D)** Genetic stability of five clones (i.e. plaques) of HAdV35 pIX-L2(Sx4)18 and HAdV35 pIX-L2(Sx4)16 after extended passaging in the producer cell line. Shown are VPN 14 pIX-PCR results of five (1-5) viral clones (942 bp). (+) indicates the positive plasmid control, (–) is the non-modified pIX plasmid control (570 bp) and H_2O the PCR water control. M stands for the molecular weight markers. Non-specific bands are marked by asterisk (*).

0.7 Log IU/mL) in AdV-formulation buffer, which was comparable (no significant differences) to the non-modified control vector (Figure 4).

HAdV35 pIX-L2 display vectors induce broadly cross-reactive L2-specific binding antibodies

Cross-reactive L2-specific antibody responses was determined by vaccinating mice (n= 8/ group) with 10^8 or 10^{10} VP HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 or a (1:1) mix of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 vectors (9-valent) in a 0-8 week homologous prime-boost regimen. HPV L2-specific IgG antibodies were measured in serum at weeks 8 and 12 post-prime immunization against HPV-types included in the vaccine (HPV6, 11, 16, 18, 31, 33, 45 and 52/58) and one distant HPV-type (HPV59) using MSD-ELISA. All vectors induced binding antibodies against all HPV-types (Figure 5, Supplemental Figure 2 and Table 3). The mixture of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 vectors induced significantly higher HPV18-L2-specific antibody titers compared to pIX-L2(Sx3) after prime immunization, higher HPV18-L2-specific antibody titers compared to pIX-L2(Sx4)16 after both prime and boost immunization, and higher HPV18- HPV45- and HPV59-L2-specific antibodies than pIX-L2(Sx4)16 (Figure 5). There were no other significant differences in other HPV-type-specific antibody responses after immunization with single vectors or the AdV vector mix at 10^{10} VP doses. However, both the 10^8 and 10^{10} VP doses showed enhanced type-specific binding antibody responses induced by the 9-valent AdV display vector mixture compared to the single vectors (Supplemental Table 2, Supplemental Figure 2 and Figure 5).

HAdV35 pIX-L2 display vectors induce HPV neutralizing L2 antibodies

HPV neutralizing L2 antibodies measured using the *in vitro* VNA are considered a surrogate for *in vivo* protection [46]. To assess L2-specific cross-neutralization induced
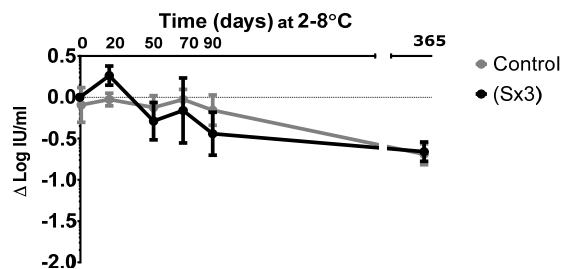
Figure 4. HAdV35 pIX-L2 particle aggregation and 1 year stability at 2-8°C. HAd35 pIX-L2 vector 1 year stability at 2-8°C measured by QPA (IU/mL). Viral titers (IU/mL) were determined as indicated in the material and methods at 0, 20, 50, 70, 90 and 365 days. Depicted in the graph is the Δlog/ IU decrease relative to time point 0. Data were analyzed by a linear mixed-effects regression model and no significant differences were observed related to the interaction terms between vector type and day.

Figure 5. Humoral responses induced by 10^{10} VP HAdV35 pIX-L2(Sx4)16, pIX-L2 (Sx4)18 and pIX-L2 (Sx3) vectors in mice. Binding antibodies measured in serum by MSD ELISA for HPVHPV6 **(A)**, HPV11 **(B)**, HPV16 **(C)**, HPV18 **(D)**, HPV31 **(E)**, HPV33 **(F)**, HPV45 **(G)**, HPV52/58 **(H)** and HPV59 **(I)**. Endpoint titers-$\log_{10}$ were calculated values were calculated for each sample (as described in the material and method section). Induction of antibodies were measured prime immunization at week 8 (P) and after boost at week 12 (B); are shown for 1×10^{10} VP/mouse HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 and mixture of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 per HPV-type. Horizontal lines indicate the group mean. HAdV35.empty (E) is a negative control. The dotted lines indicate the lowest titer $\log_{10}(20) = 1.30$ and the highest titer $\log_{10}(14580) = 4.16$ measured in the assay. P-values are based on a Wilcoxon Rank Sum test and were adjusted using a 3-fold Bonferroni correction. Tests were performed at the 5% significance level (<0.05).

A. VNA HPV16
B. VNA HPV18
C. VNA HPV31
D. VNA HPV59

by the pIX-L2 display vectors, four clinically-relevant HPV types (HPV16, 18, 31 and 59) were selected based on their L2 sequence diversity [50] (Supplemental Figure 1), and used in the *in vitro* VNA assay (Figure 6). Neutralization of HPV16 and HPV18 was induced in the majority of animals immunized with the HAdV35 pIX-L2 display vectors with titers comparable to that induced by 4-valent *Gardasil* (HPV6, 11, 16 and 18) (Figure 6A and 6B). In addition, the single HAdV35 pIX-L2 display vectors and the mixture induced HPV31 and 59 nAb titers in most animals (Figure 6C and 6D). The quality of the humoral immune responses is supported by a moderate correlation between binding Ab titers and nAb titers for HPV16 (r =-0.33) and strong correlation for HPV18 (r =-0.67), HPV31 (r=-0.61) and HPV59 (r=-0.72) across groups (Supplemental Figure 3). Similar to the HPV-specific binding antibody responses, statistical analysis across both the 10^8 and 10^{10} VP doses showed higher type-specific nAb responses induced by the 9-valent AdV display vector mixture compared to that induced by the single vectors (Supplemental Table 3 and Figure 6), for all HPV types except for HPV16. This suggests an overall added value for including multiple L2 epitopes displayed in one vaccine formulation.

DISCUSSION

Second generation prophylactic HPV vaccines, focusing on the highly conserved and cross-protective L2 protein linear nAb epitopes are considered a promising approach to ensure a broad protection against multiple HPV-types within one vaccine [9-11].

Antigen display via pIX has proven highly potent at inducing antigen-specific B-cell immune responses, superior to genetic AdV particle expressing a transgene, protein or non-modified AdV particles mixed with protein [32-34, 51]. Therefore, we generated an immunogenic multivalent prophylactic L2-protein vaccine based on the HAdV pIX-display vector. The vaccine consists of two heterologous HAdV35 vectors displaying either HPV6, 31, 33, 16 or HPV11, 52/58, 45, 18 L2 protein epitopes in a repetitive conformation fused to the pIX C-terminus.

To select a L2 protein fragment which can efficiently be fused to pIX and incorporated into the HAdV capsid, different L2 concatemers designs were screened (data not shown). Consistent capsid incorporation was achieved with the (S)-fragment (Supplemental Figure 1) resulting in display of L2(Sx3) and L2(Sx4) repeats on the HAdV capsid. Characterization

◂ **Figure 6.** *In vitro* neutralization of HPV16, 18, 31 and 59. *In vitro* pseudovirions VNA shown in Luminescence Log_{10} expression for HPV16 **(A)**, HPV18 **(B)**, HPV31 **(C)** and HPV59 **(E)**. Serum of mice immunized with $1x10^{10}$ VP/mouse HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 and mix of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 was analyzed in an *in vitro* pseudovirions VNA. Serum from 4-valent Gardasil immunized mice served as a positive control for HPV16 and 18 neutralization assay. HAdV35.empty (E) is a negative control. Horizontal lines indicate the group mean. The dotted lines in each graph depict the average of luminescence detected in samples with the HPV pseudovirions without furin as a background control. P-values were adjusted using a 3-fold Bonferroni correction and tests were performed at the 5% significance level (<0.05).

of these vectors indicated that a fraction of the capsids incorporated with pIX-L2(Sx3) or pIX-L2(Sx4)16 proteins lacked the exteriorly located HPV16 fragment (Figure 2A and MS-analysis data not shown). pIX-L2(Sx3) and pIX-L2(Sx4)16 fragments contain a partial furin cleavage site (e.g. HPV16: RTKR/ASA)[52] which might explain the fragment cleavage, providing an opportunity to further improve L2 concatemer designs.

HAdV35 pIX-L2 vector yields and batch-to-batch variation was comparable to the non-modified control vector suggesting efficient large-scale vaccine production [40, 41]. A well characterized trait of pIX-display vectors or vectors without pIX (ΔpIX) is the capsid instability profile which influences the vectors' infectivity [53]. The HAdV35 pIX-L2 vector infectivity (IU/mL) after 1 year incubation at 2-8°C in AdV-formulation buffer was not different from the non-modified vector control. In contrast to the purified HAdV35 pIX-L2 small-scale vector preparation, increased aggregation was observed for HAdV35 pIX-L2 incubated 1 year at 2-8°C in AdV-formulation buffer (data not shown), which might be prevented by an improved AdV-formulation buffer [54].

Immunizing with HAdV35 pIX-L2(Sx3), HAdV35 pIX-L2(Sx4)16, HAdV35 pIX-L2(Sx4)18 alone or pIX-L2(Sx4)16 & HAdV35 pIX-L2(Sx4)18 mixture induced cross-reactive HPV L2-specific binding and nAbs. Across groups a moderate (HVP16) and strong (HPV18, HPV31 and HPV59) binding antibody and nAb titers correlation was observed, indicating good quality humoral immune response. Compared to the single vectors, the mixture induced higher binding and nAb responses across 10^8 and 10^{10} VP doses. Interestingly, 5-valent HAdV35 pIX-L2(Sx4)18 induced more potent immune responses than the 4-valent HAdV35 pIX-L2(Sx4)16 and 3-valent HAdV35 pIX-L2(Sx3) across both doses. At 10^{10} VP dose the immune responses induced by the 5-valent HAdV35 pIX-L2(Sx4)18 were comparable to the 9-valent mixture. One possible explanation could be the reduced amount of HPV16 in the 4-valent HAdV35 pIX-L2(Sx4)16 and 3-valent HAdV35 pIX-L2(Sx3) vectors due to pIX-L2 furin cleavage. Nonetheless, it has been reported that to achieve broad HPV protection, it is not necessary to include L2 N-terminus epitopes derived from each HPV type [55]. HPV11, 18, 45 and 52/58 included in the 5-valent HAdV35 pIX-L2(Sx4)18 vector possibly offer an optimal L2 N-terminus sequence diversity coverage resulting in potent cross-reactive HPV immune responses and preventing the need for a 9-valent HPV vaccine. Our observations support the notion that the inclusion of multiple L2 epitopes within one vaccine results in an enhanced antibody immune response [13, 56].

The levels of induced HPV L2-specific antibody in serum are generally lower than HPV L1 protein-specific antibodies [55, 57, 58]. However, in clinical trials, even low levels of serum L2-specific antibody induced by HPV16 L2 peptides or L2 fusion protein were sufficient to neutralize distant HPV types such as HPV52 [59] and HPV18 [60]. Even though *in vivo* cross-protection after a vaginal HPV challenge remains to be determined in future studies for the 9-valent or 4-valent/5-valent HAdV35 pIX-L2 vectors, it is noteworthy that in contrast to the majority of currently described L2-based vaccines [25, 57, 58, 61, 62] the potent cross-reactive humoral immune responses induced by the HAdV35 pIX-L2 vectors were achieved without the addition of an adjuvant.

The HAdV35 pIX-L2 vaccine showed good manufacturability potential and induces broadly cross-reactive humoral immune responses without the addition of an adjuvant. Based on the well-established AdV platform, HAdV35 pIX-L2 holds promise as a next generation, broad coverage, prophylactic HPV vaccine.

DECLARATION OF INTEREST

All authors are employees of Janssen Vaccines and Prevention, Janssen Pharmaceutical Companies of Johnson & Johnson. Marija Vujadinovic, Taco G. Uil, Koen Oosterhuis, Jort Vellinga and Jerome Custers are authors on a HPV L2 vaccine patent application (WO/2018/011196).

AUTHORS CONTRIBUTION

Conceptualization: MV SK JV JC RZ. Data curation: MV JK AV. Formal analysis: MV JS JK AV. Investigation: MV SK JC JV. Methodology: MV SK KW TU JK AV JS SD SB RZ JV. Supervision: JV GS JC HS. Visualization: MV. Writing – original draft: MV SK AV JK JS. Writing – review & editing: MV SK KO TU KW SD SB AV JK JS RZ GS HS JC JV.

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17-36 a.a. nAb epitope

→ HPV16 (AAD33258) L2 11-40 a.a.   KRASAT QLYKTCKQAGTCPPD I IPKVEGKT 30 →   (Sx3)   (Sx4)16
→ HPV18 (AGG40790) L2 10-39 a.a.   . . . . V . D . . . . . . S . . . . . . VV . . . . . T . 30 →   (Sx3)   (Sx4)18
→ HPV31 (ALT54656) L2 11-40 a.a.   . . . . Q . . . A . . . . . S . V . . . I . HT . 30 →   (Sx4)16
   HPV33 (ACL12332) L2 10-39 a.a.   . . . . Q . . . AT . . . . . . V . . . . . . S . 30 →   (Sx4)16
   HPV35 (AEP23091) L2 11-40 a.a.   . . . . R . . . A . . . . N . 30
   HPV39 (AEP23093) L2 10-39 a.a.   . . . . . AD . . R . . . . S . . . . . . VVN . . . . T . 30
   HPV45 (X74481) L2 10-39 a.a.   . . . . . . D . . R . . . . S . . . . . V . N . . . . T . 30 →   (Sx3)   (Sx4)18
   HPV51 (GQ487712) L2 10-39 a.a.   . . . . V . . . . S . . . A . . . . . . V . N . . . . T . 30
   HPV52 (ACX32361) L2 10-39 a.a.   . . . . Q . . . AS . . . . . . V . . . . . T . 30 →   (Sx4)18
   HPV56 (EF177181) L2 10-39 a.a.   . . . . LS . . . . E . VVN . I . Q . . 30
   HPV58 (ACL12324) L2 10-39 a.a.   . . . . Q . . . AS . . . . . . V . . . . . T . 30 →   (Sx4)18
→ HPV59 (ACL12340) L2 10-39 a.a.   . . . . . . D . . . . S . V . N . . . . T . 30
   HPV68 (ACL12357) L2 10-39 a.a.   . . . . . . E . . . . S . . . . . . V . D . . . . T . 30
   LR HPV6 (AF092932) L2 10-39 a.a.   . . . . Q . . . LT . . . . . . V . . . . . HN . 30 →   (Sx4)16
   LR HPV11 (HE962024) L2 9-38 a.a.   . . . . Q . . . AT . . . . . . V . . . . . HT . 30 →   (Sx4)18

Figure S1. HPV L2 (S)-fragment. Sequence alignment of HPV6, 11, 16, 18, 31, 33, 45, 52 and 58 L2 protein (S)-fragments 30 a.a. used to generate pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 concatemers. Remaining high-risk HPV35, 39, 51, 56, 59 and 68 are included in the alignment. The HPV L2 sequence accession numbers and the exact (S)-fragments a.a. per type are shown. The 17-36 a.a. QLY+TCKQAGTCPPD linear nAbs epitope is indicated (black box). Identical a.a. in the sequence fragments are indicated with dots and non-identical a.a. are highlighted in grey. The arrows show the HVP type using the *in vitro* VNA assay.

Figure S2. Binding antibody responses induced by 10^8 VP HAdV35 pIX-L2(Sx4)16, pIX-L2 (Sx4)18 and pIX-L2 (Sx3) vectors in mice. Binding antibodies measured in serum by MSD ELISA for HPV6 (A), HPV11 (B), HPV16 (C), HPV18 (D), HPV31 (E), HPV33 (F), HPV45 (G), HPV52/58 (H) and HPV59 (I). Endpoint titers-log_{10} values were calculated for each sample. Antibodies measured after prime at week 8 (P) and after boost at week 12 (B) are shown for $1x10^8$ VP/mouse HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 and mix of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 per HPV-type. Horizontal lines indicate the group average. HAdV35.empty (E) is a negative control. The dotted lines indicate the lowest titer $log_{10}(20) = 1.30$ and the highest titer $log_{10}(14580) = 4.16$ measured in the assay. P-values are based on a Wilcoxon Rank Sum test and were adjusted using a 3-fold Bonferroni correction. Tests were performed at the 5% significance level (<0.05).
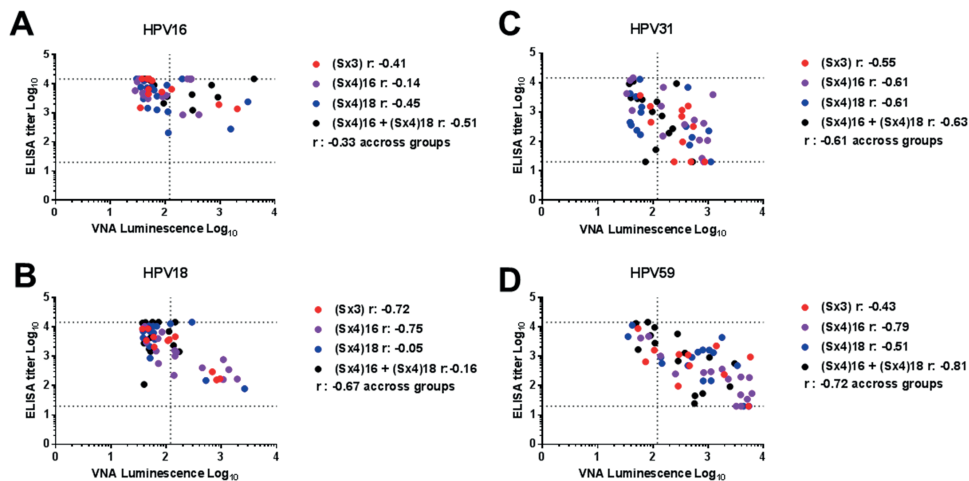
Figure S3. The relationship between HPV16, 18, 31 and 59 antibody titers measured by ELISA and VNA across doses 10^8 and 10^{10}. Antibodies titers (log_{10}) measured in serum by MSD ELISA and *in vitro* VNA for HPV16 **(A)**, HPV18 **(B)**, HPV31 **(C)** and HPV59 **(D)** after the boost at week 12 with HAdV35 pIX-L2(Sx3), pIX-L2(Sx4)16, pIX-L2(Sx4)18 and mix of pIX-L2(Sx4)16 & pIX-L2(Sx4)18 VP/animal doses 10^8 and 10^{10} were compared using Spearman correlation analysis. A negative correlation is observed since a high binding Ab titer corresponds with HPV neutralization, thus low a luminescence value. The dotted line indicates the lowest titer $log_{10}(20) = 1.30$ and the highest titer $log_{10}(14580) = 4.16$ measured in the ELISA assay and the average of luminescence detected in samples with the HPV pseudovirions without furin as a background control. The nonparametric Spearman correlation coefficient (r) is indicated in the figure.

Table **S1**. Consistent small vector preparations production in producer cell line.

	Capsid modification		Viral titers			Genetic stability		RP-UPLC (%)
	HPV types	Concatemer	VP/ml	IU/ml	VP/IU ratio	Batch	Extended passaging	
HAdV35 Advac©								
Empty	NA	NA	2.9×10^{12}	4.0×10^{11}	7	NA	NA	NA
Batch 1	45.18.16	(Sx3)	1.6×10^{12}	3.6×10^{11}	4	Stable	Stable	2.4
Batch 2	45.18.16	(Sx3)	2.1×10^{12}	3.3×10^{11}	6	Stable	ND	2.4
Batch 3	45.18.16	(Sx3)	2.3×10^{12}	ND	ND	Stable	ND	ND
Batch 1	6.31.33.16	(Sx4)16	1.8×10^{12}	3.3×10^{11}	5	Stable	Stable	1.8
Batch 2	6.31.33.16	(Sx4)16	5.6×10^{11}	4.4×10^{10}	13	Stable	Stable	1.8
Batch 1	11.52/58.45.18	(Sx4)18	1.8×10^{12}	2.7×10^{11}	7	Stable	Stable	2.5
Batch 2	11.52/58.45.18	(Sx4)18	7.3×10^{11}	5.3×10^{10}	14	Stable	Stable	2.3

ND: not determined, NA: not applicable
% Area: percentage of each peak compared to total peak area (total protein on the column) at comparable loading (peak-area penton)

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Table S2. Statistical analysis humoral responses across 10^8 and 10^{10} VP/mouse dose immunization.

Comparator	Reference	HPV type	Week	ELISA MSD P value* (< 0.05)
(Sx3)	(Sx4)16 + (Sx4)18	HPV6	12	0.0029
			8	0.0184
		HPV11	12	0.0353
			8	0.0088
		HPV16	12	ns
			8	ns
		HPV18	12	ns
			8	0.0109*
		HPV31	12	ns
			8	0.0168
		HPV33	12	0.011
			8	0.0066
		HPV45	12	ns
			8	0.0418
		HPV52/58	12	0.0042
			8	0.0018
		HPV59	12	ns
			8	ns
(Sx4)16		HPV6	12	ns
			8	ns
		HPV11	12	ns
			8	ns
		HPV16	12	ns
			8	ns
		HPV18	12	0.0019
			8	0.0009
		HPV31	12	ns
			8	ns
		HPV33	12	ns
			8	ns
		HPV45	12	0.0049
			8	0.0014
		HPV52/58	12	ns
			8	ns
		HPV59	12	ns
			8	0.0037
(Sx4)18		HPV6	12	ns
			8	ns
		HPV11	12	ns
			8	ns
		HPV16	12	0.0495

Comparator	Reference	HPV type	Week	ELISA MSD P value* (< 0.05)
			8	ns
		HPV18	12	ns
			8	ns
		HPV31	12	ns
			8	ns
		HPV33	12	0.0428
			8	ns
		HPV45	12	ns
			8	ns
		HPV52/58	12	ns
			8	ns
		HPV59	12	ns
			8	ns

*Cochran–Mantel–Haenszel test

Table S3. Statistical analysis VNA titers across 108 and 1010 VP/mouse dose immunization

Comparator	Reference	HPV type	Week	VNA P value* (<0.05)
(Sx3)	(Sx4)16 + (Sx4)18	HPV16	12	ns
		HPV18	12	ns
		HPV31	12	.015
		HPV59	12	ns
(Sx4)16		HPV16	12	ns
		HPV18	12	0.0246
		HPV31	12	0.0246
		HPV59	12	0.0195
(Sx4)18		HPV16	12	ns
		HPV18	12	ns
		HPV31	12	ns
		HPV59	12	ns

*Cochran–Mantel–Haenszel test

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ADENOVIRAL TYPE 35 AND 26 VECTORS WITH A BIDIRECTIONAL EXPRESSION CASSETTE IN THE E1 REGION SHOW AN IMPROVED GENETIC STABILITY PROFILE AND POTENT TRANSGENE-SPECIFIC IMMUNE RESPONSE

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ABSTRACT

Genetic vaccines based on replication-incompetent adenoviral (AdV) vectors are currently in clinical development. Monovalent AdV vectors express one antigen from an expression cassette placed in most cases in the E1 region. For many vaccines, inclusion of several antigens is necessary in order to raise protective immunity and/or target more than one pathogen or pathogen strain. Based on the current technology, a mix of several monovalent vectors can be employed. However, a mix of the standard monovalent AdV may not be optimal with respect to manufacturing costs and the final dose per vector in humans. Alternatively, a variety of bivalent recombinant AdV vector approaches is described in the literature. It remains unclear whether all strategies are equally suitable for clinical development while preserving all the beneficial properties of the monovalent AdV (e.g. immunogenic potency). Therefore, a thorough assessment of different bivalent AdV strategies was performed in a head-to-head fashion compared to the monovalent benchmark. The vectors were tested for rescue efficiency, genetic stability, transgene expression, and potency to induce transgene-specific immune responses. We report that the vector expressing multiple antigens from a bidirectional expression cassette in E1 shows a better genetic stability profile and a potent transgene-specific immune response compared to the other tested bivalent vectors.

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INTRODUCTION

Recombinant replication-incompetent adenoviral (AdV) vectors have proven to be particularly useful for genetic vaccination due to their capacity to induce strong immune responses against the encoded antigens.[1, 2] The majority of replication-incompetent vectors currently in clinical development contain an expression cassette in the E1 region, from which a single protein or fusion protein is expressed.[3-7] The complex nature of infectious diseases frequently requires expression of more than one antigen from a genetic vaccine.[8-10] For example, in the case of *Filovirus* hemorrhagic fever, immunity against one Ebola strain is not expected to provide cross-protection against other Ebola and Marburg viruses.[11] Since recent documented outbreaks were caused by different filovirus species[12, 13] an effective vaccine should offer protection against all of the relevant species. This could be achieved by mixing together several monovalent vectors that each express one antigen.[14-16] While the mixing-approach offers the advantages of a well-established and potent vaccine platform, it is limited by increased cost of goods and a decreased dose per vector in the final vaccine formulation. Vectors expressing more than one antigen are attractive alternatives. In order to provide a viable alternative to the monovalent vector platform for clinical development, the new bivalent vectors should be comparable to their monovalent counterparts with respect to production properties, immunogenicity and safety. These properties can be assessed by testing rescue efficiency, genetic stability, protein expression and induction of an immune response against the encoded antigens.

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Interestingly, a multitude of different approaches for expression of more than one protein from a recombinant AdV vector is described (Supplemental Table 1), suggesting that generation of a potent bivalent vaccine vector can be easily achieved. In a first approach, two transgene expression cassettes can be placed at different insertion sites in the AdV genome, using the E1 region, E3 region[17-24] and the right end of the genome between the E4 region and the right inverted terminal repeat.[19, 25-28] In a second approach, two transgene expression cassettes are placed in the E1 region in tandem. [15, 29-33] Alternatively, one insertion site can be exploited by direct fusion of the transgenes without or with regulatory sequences; encompassing an internal ribosomal entry site (IRES), a so called "self-cleaving" peptide sequence (2A) or splicing sites separating the transgenes.[18, 34-39] Yet another approach to express two transgenes from the E1 region is by means of a bidirectional promoter.[31, 40] While the described literature suggests that bivalent AdV can be readily generated, two more recent reports[24, 41] show that bivalent AdV require careful design to achieve genetically stable vectors that express both antigens at levels able to induce potent immune responses.

In this study we identified an optimal method for bivalent AdV vector production by testing different bivalent AdV designs in a head-to-head comparison using the respective monovalent AdV as a benchmark. Rather than the typically used human adenovirus 5 (HAdV5) derived vector, we used the low-seroprevalent vectors human adenovirus

35 and 26 (HAdV35 and HAdV26). HAdV5 has the limitation that high levels of pre-existing immunity in the human population can negatively affect immunogenicity.[42, 43] Low-seroprevalent vectors such as HAdV35 and HAdV26 are not faced with this drawback. [44-46] HAdV35 and HAdV26 vaccine vectors have shown promising results in clinical trials of malaria, HIV and Ebola virus vaccines.[47-49] Induction of T cell, B cell responses, as well as favorable innate cytokine responses[44] by these low-seroprevalent vectors supports their advancement in vaccine development.

For bivalent vectors, rescue efficiency, the genetic stability profile, transgene protein expression, and immunogenicity were used as selection criteria. Major differences were found between the different bivalent designs. However, the use of a bidirectional mouse cytomegalovirus (CMV) promoter cassette in the E1 region performed better than the other tested strategies by combining good rescue efficiency, high genetic stability and induction of a potent immune response, thereby providing an attractive new vaccine vector design.

MATERIALS AND METHODS
Vector generation and cell culture
HAdV26 and HAdV35 vectors were generated as previously described in Vogels et al. 2007. [17] In short, the monovalent E1, bivalent E1-E3 (inverted), E1-E1 tandem, E1-2A and the E1 bidirectional promoter containing vectors were generated by inserting the expression cassettes in either the E1, and if applicable, the E3 position. The expression cassettes in E1-E3 contain identical human CMV immediate early (hCMV) promoters however differ in the polyadenylation-signal sequences derived either from SV40 or BGH,[17] respectively. The vectors containing the expression cassettes in tandem in the E1 region were designed as such to reduce the sequence homology[29, 30] and contain heterologous promoters; hCAG[50] promoter modified to contain a mouse CMV enhancer (mCAG), and hCMV, and SV40 and BGH-derived polyadenylation signals, separated by a stop codon. In the E1-2A vectors a foot-and-mouth disease virus-derived 2A self-cleavage site (NFDLLKLAGDVESNPGP)[51, 52] was placed between two transgenes expressed under the control of an hCMV promoter and SV40 derived polyadenylation-signal sequences. In the E1- bidirectional promoter vectors two genes were placed up- and downstream of the bidirectional mCMV promoter containing heterologous polyadenylation signal sequences derived from SV40 and BGH. The inserted genes Filovirus Marburg Angola (MARV), Ebola Zaire (EBOV), Ebola Cote d'Ivoire (CIEBOV)/Taï Forest virus (TAFV) or Ebola Sudan Gulu (SEBOV) glycoprotein (GPs) were codon-optimized for human expression, and for EBOV and SEBOV, to reduce the homologous sequence stretches where necessary by GeneArt, Thermo Fisher Scientific. The reporter genes Firefly Luciferase and enhanced Green Fluorescent Protein (eGFP) were also codon-optimized for human expression by GeneArt, Thermo Fisher Scientific. The transgenes were cloned into pAdapt35 or pAdapt26 plasmids.[17] A Kozak sequence (5' GCCACC 3') was included directly in front

of the ATG start codon, and two stop codons (5' TGA TAA 3') were added at the end of the coding sequences.

The HAdV35 and HAdV26 vectors were generated by a two plasmid system by transfection into PER.C6® cells using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. The homologous sequences in the HAdV genome plasmids allowed for homologue recombination in PER.C6®cells, giving rise to full length HAdV vectors. The vectors were subsequently plaque purified and further propagated on adherent PER.C6® cells at 37°C/10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% of fetal bovine serum (Life Technologies Inc.) and 10 mM $MgCl_2$. Virus was purified by standard two-step CsCl-gradient and dialyzed in formulation buffer 10 mM Tris (pH 7.4), 1 mM MgCl2, 75 mM NaCl, 5% sucrose, 0.02% PS-80, 0.1 mM EDTA, 10 mM Histidine, 0.5% ETOH). Final viral particle (VP) concentration was determined by optical density (OD) in the presence of SDS and the viral infectious units (IU) titers by $TCID_{50}$ assay. The corresponding VP/IU ratio, as well as the productivity (VP/cm^2) was calculated. Transgene expression and vector identity were tested for all purified vectors, followed by sequencing of the transgene expression cassette plus flanking regions (Baseclear B.V.). Prior to the testing *in vivo* Bioburden (MicroSafe, Milipore) and Endotoxin (Microsafe, Milipore) were determined for all purified vectors.

Genetic stability

The genetic stability of the expression cassettes was determined by passaging five different plaques serially up to, and beyond passage 13 (p13) (representative of 3 viral passages beyond the bulk drug substance), on adherent PER.C6® cells in T25 flasks ($3.5x10^6$ cells/flask). After infection, predetermined amounts of virus were added that give rise to full cytopathic effect (CPE) two days post-infection. Based on previous observations, for optimal batch quality the recombinant vectors were harvested either two (HAdV35) or one day (HAdV26) post full CPE. Viral DNA was isolated at viral passage number (VPN) 2, 5, 10 and 13 or 15. PCR analysis was performed to test for presence of the expression cassette both in E1 and E3 with primers flanking these regions. At VPN 13 the expression cassettes PCR fragments were confirmed by sequencing. E3 and E4 region PCRs were performed for all vectors as an additional readout for general genome identity. For HAdV35 E1 Fw (ID 2271) 5'GGAGGTTCGATTACCGT3', Rv(ID2272)5'CCTCGATCTCGATATCATCA3', E3 Fw (ID 1985) 5'GCTGCTTTGCCCGGGAACTTATTG3', Rv (ID 1986) 5'CAAGTTCGTAAGAGAGGCGATGG 3' , E4 Fw (ID 478) 5'GGGTAGAGTCATAATCGTGCATCA 3' and Rv (ID 479) 5'CATGACACTACGACCAACACGATCTCG 3' and HAdV26 E1 Fw (ID 2741) 5' TGGCGCGAAAACTGAATGAG '3 , Rv (ID 2742) 5' GCAGGCGGGTTGTCAAATAAG '3 , E3 Fw (ID 669) 5' GAGTCTCACCTGGTCAGGTTC '3, Rv (ID 670) 5' GCTGAACAACTACACCAGAGAC '3, E4 Fw (ID 671) 5' TTACACCAGCACGGGTAGTCAG '3 and Rv (ID 672) 5' CGGAAGTTGAGTCACGAAATCG '3 primer sequences were used.

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Transgene expression

Transgene expression of Ebola and Marburg GP was analyzed both by Western Blot (WB) and FACS in transduced A549 cells. For WB analysis cells were transduced with predefined VP/cell ratios, namely for HAdV35 1000, 2500 and 5000, or for HAdV26 vectors 10 000, 20 000 and 50 000. Cells were harvested 48-72 hours post-transduction and lysed. The protein in cell lysates was separated on pre-cast 4-12% Bis/Tris Nu-PAGE gels (Invitrogen) in MOPS buffer (Invitrogen) at 175 V, 500 mA. Protein was subsequently transferred to a nitrocellulose membrane according to manufacturer's recommendations using iBlot® Transfer stacks (iBlot system; Invitrogen). The immune-staining was performed for 1 hour with Filovirus GP specific antibodies (non-commercial monoclonal and polyclonal antibodies) in 5% Non-fat dry milk (BioHAdV)/Tris buffered Saline Tween 20 (Invitrogen). Visualization of the protein of interest was performed by staining with fluorescently labeled secondary antibody IRDye800CW® 1:10000 goat anti-mouse/rabbit and recorded on Odyssey® (Li-Cor). The surface display of different GPs was determined by transducing A549 with increasing amount of virus. Surface staining of the GPs was completed 48h post-transduction as recommended by the manufacturer, with mouse-serum raised against the respective GPs and anti-mouse APC-coupled secondary antibody to facilitate detection of the positive cells (%) by FACS (BD FACSCantoTM II). The geometric mean of positive cell fraction was determined plotted relative to the single monovalent benchmark (ratio (candidate/benchmark).

Animal experiments

The animal experiments were approved by the Institutional Review Board and the National Ethical Committee for animal experiments. BALB/c mice were immunized intramuscularly (IM) with a total of $2x10^9$ VP or $1x10^9$ VP per HAdV35 vector. The IM immunization with the bidirectional promoter containing HAdV26 vector was performed with two different concentrations; $2x10^9$ VP, $1x10^9$ VP per vector and $2x10^{10}$ VP, $1x10^{10}$ VP per vector. The HAdV double insert vectors were supplemented with the HAdV.E1 Empty vector to compensate for the HAdV monovalent control mix of $1x10^9$ VP per vector. Either two or eight weeks (with biweekly bleedings) post-immunization, mice were sacrificed, after which spleen or sera were isolated and further analyzed. The spleens were prepared as previously described.[53] Filovirus specific T cell responses were detected using gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay.[54] In brief, the relative number of GP protein-specific IFNγ-secreting T cells in the spleen, was determined by stimulating the cells with three different 15-mer peptide pools per Filovirus antigen to cover the whole Filovirus GP. As a result, the numbers of spot-forming units (SFU) per 10^6 cells were determined and calculated. The serum B cell response in mice was determined by measuring the GP-specific antibody titers in an Ebola and Marburg GP-specific ELISA (EU/ml) as previously described.[14] Briefly, Lectin coated ELISA Maxisorp plates (Nunc) were blocked for two hours prior to coating with the PBS diluted Filovirus GP containing

HEK293 supernatant. After washing, the diluted reference standard serum and the test serum were added (in duplicate) to the plates with sample buffer and incubated at room temperature for one hour. Naïve mouse serum was used as a negative control. After secondary antibody treatment with horseradish peroxidase labeled IgG, the plates were developed using the o-phenylenediamine dihydrochloride (Sigma) according to manufacturer's recommendations. After stopping the enzymatic reaction with 1M H_2SO_4 the OD was measured at 492 nm using an ELISA plate reader. All the analyses were performed using the Gen5 software.

Intra-cellular cytokine staining was performed by flow cytometry using the BD Biosciences FACSCanto™. Cells were stained with antibodies against CD4, CD8 and INFy, TNFα and IL-2 prepared in BD CompBead (BD Biosciences) preparation according to the manufacturer's recommendations. In FACSCanto™, data collection was stopped after 1.2×10^5 events in the lymphocyte gate and the final analysis were performed using FlowJo software.

Statistical analysis

The T cell responses were compared between the vectors using analysis-of-variance with $\log_{10}$ SFU/million splenocytes as the response variable. P-values were adjusted for multiple testing using a Dunnett correction.

The B cell responses were compared between the vectors using analysis-of-variance for potentially censored values (Tobit model) using $\log_{10}$ ELISA Units (EU) per ml as the response variable. P-values were adjusted for multiple testing using a Bonferroni correction. In both analyses, the monovalent group was compared to the double insert groups. Adjusted p-values, less than 0.05 were considered as statistically significant. All analyses were performed using the SAS 9.2 software.

RESULTS

Design and selection criteria for bivalent vectors

In order to identify the most favorable bivalent AdV design among those currently described in the literature, we selected a panel of bivalent designs and generated the respective HAdV35 vectors encoding two heterologous transgenes. First, vectors encoding transgenes in E1 and E3 (E1-E3) were designed and generated. Then, three different strategies based on the E1 insertion site were tested: E1-based bivalent vectors with two expression cassettes in tandem (E1-E1); the transgenes separated by the self-cleavable 2A sequence (E1-2A) and a bidirectional promoter containing cassette (E1-bidirectional) (Figure 1A).

The main selection criteria for identification of the optimal bivalent vector design were producibility and induction of potent immune responses. These criteria were evaluated for the bivalent vectors using: (i) rescue efficiency in the producer cell line, (ii) genetic stability by extended passaging of several viral clones (plaques) followed by PCR amplification
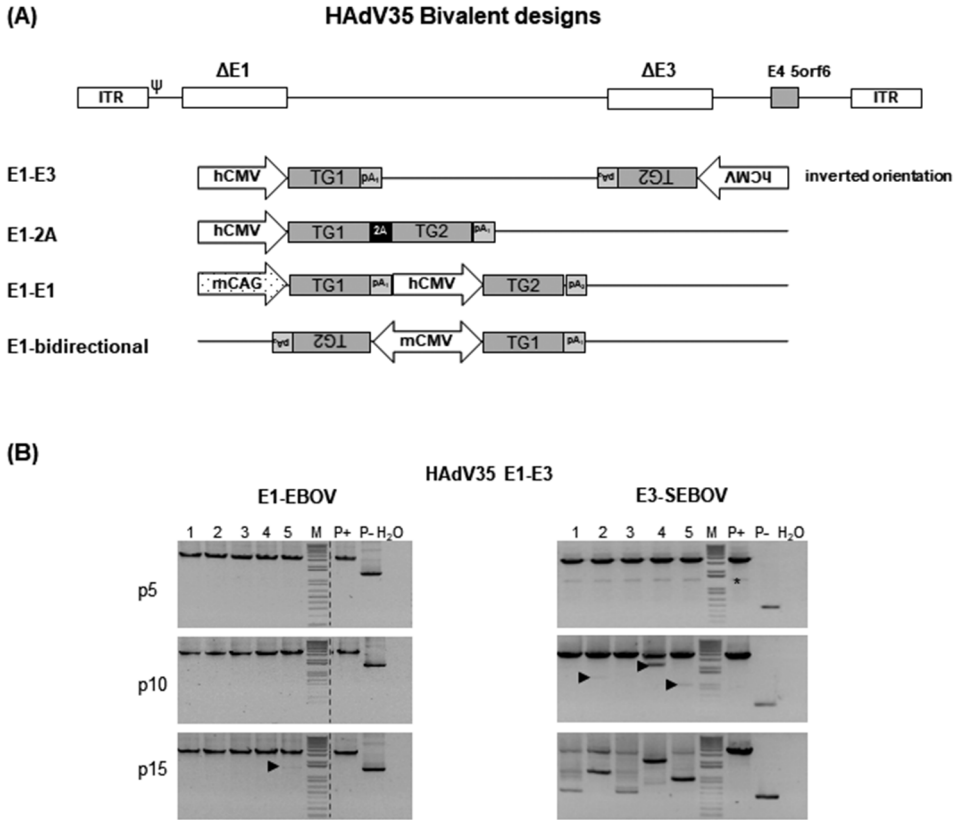
6

Figure 1. Bivalent vector designs and poor genetic stability profile of E1-E3 vectors. (A) Schematic representation of the replication-incompetent bivalent vector genome designs showing the deleted (Δ) E1 and E3 genome regions including the left and right inverted terminal repeats (ITR), the genome packaging signal (ψ) and the E4 Orf6 replaced by the HAdV5 Orf6 (E4 5orf6) to complement the growth in the HAdV5.E1-complementing producer cell line. Together the E1 and E3 regions are used to generate the E1-E3 inverted orientation bivalent vectors encoding two different transgenes (TG1 and TG2) under direct control of the human CMV (hCMV) promoter and heterologous poly A signals (pA$_1$ and pA$_2$). In the E1-2A bivalent vector, two transgenes are separated by the self-cleavable 2A peptide with hCMV driving the expression. The E1-E1 vector contains two heterologous expression cassettes with the mCAG and hCMV promoter and heterologous poly A signals. E1-bidirectional vector encodes an expression cassette containing the mouse CMV bidirectional promoter to drive the expression of two transgenes placed on either side with heterologous poly A signals. (B) Genetic stability testing by extended passaging in the producer cell line and E1 - E3 expression cassette. PCR shows multiple deletion bands at passage 10 (p10) and passage 15 (p15), predominantly in the E3 region. 1-5 plaques, P+ positive control plasmid DNA, P- negative control plasmid DNA, H$_2$0 control and M for 1 kb plus DNA marker. * indicates a background band detected in the positive control and plaques at passage 5 (p5).

and sequencing[17] (Supplemental Figure 1A), (iii) successful generation of a small scale vector preparation, (iv) transgene protein production in a non-complementing cell line (i.e. A549), and (v) transgene specific humoral and cellular immune responses in the mouse model. All assays were performed using the respective monovalent vector as a benchmark control.

In order to establish the benchmark, individual test criteria were evaluated for three HAdV35 and one HAdV26 monovalent vectors encoding different filovirus GP proteins in the E1 region. All four monovalent vectors were successfully rescued and expanded. Using a genetic stability by extended passaging assay, all the tested monovalent vectors were found to be genetically stable up to viral passage 13 (p13) (Supplemental Figure 1B). After upscaling and small scale vector preparations, the monovalent vectors were also found genetically stable (data not shown). The physical titer (range: $2\text{-}3.5\text{x}10^{12}$ VP/ml), infectious titer (range: $1\text{-}9\text{x}10^{11}$ IU/ml), VP/IU ratio (range 4-19) and the productivity (range: $1\text{-}5\text{x}10^{9}$ VP/cm^2) were not impaired, regardless of the type of vector and/or the encoded transgene.

A large panel of bivalent E1-E3 vectors is genetically unstable

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Since E1-E3 bivalent vectors were previously described for HAdV35 vectors[17] , we chose E1-E3 bivalent vectors as a first strategy to evaluate. The transgene expression cassettes were placed in the E3 region in an inverted orientation compared to Vogels et al.[17]. In order to test the E1-E3 strategy, an HAdV35 vector encoding two different Ebola strain GPs E1.EBOV and E3.SEBOV was generated in the producer cell line. The vector was rescued successfully and five viral populations (plaques) were subsequently evaluated for genetic stability in the described assay (Supplemental Figure 1A). While the E1.EBOV expression cassette remained stable up to a high passage number in most clones (faint deletion band for 1 out of 5 viral clones at p15) for this transgene combination, the transgene expression cassette in E3 showed major deletions in 5 out of 5 tested plaques (Figure 1B) up to a level where almost no correct transgene expression cassette was left in E3. Two possible contributing factors for the observed genetic instability were addressed by generating a panel of additional vectors: Different transgenes were combined in one vector to address problems caused by the particular combination of the two transgene sequences. Combinations of one filovirus GP and a reporter gene were tested to rule out the combination of two filovirus genes in one vector as a cause for genetic instability (Supplemental Table 2).

All four HAdV35.E1-E3 vectors encoding two filovirus GP proteins, two vectors encoding one filovirus GP protein, and one reporter gene were successfully rescued and expanded. All six HAdV35.E1-E3 vectors showed deletions in the transgene expression cassettes, mainly in the E3 region (Supplemental Table 2). The two HAdV26.E1-E3 vectors were rescued, yet proved to be difficult to expand and were genetically unstable. In light of the poor genetic stability of transgene expression cassettes placed in the E3 region, this

bivalent strategy was not pursued any further. In the tested setup, the combination of E1 and E3 insertion sites is detrimental for genetic stability of the bivalent vectors. Based on these genetic stability results we hypothesized that bivalent vector designs in which both transgenes are expressed from the E1 region may have a better genetic stability profile. Therefore, further efforts focused on the E1-based bivalent designs.

Expression of a fusion gene separated by the 2A sequence from the E1 region of HAdV35 vectors results in an acceptable genetic stability profile, but poor transgene expression

E1-2A vectors were generated as the first of three different E1-based bivalent vector designs. In these vectors, two antigens are expressed from one expression cassette, separated by a 2A site which allows separation of the two proteins by ribosome skipping (Figure 1A).[51, 52] The three vectors HAdV35.E1.eGFP-2A-Luc, HAdV35.E1.Luc-2A-eGFP and HAdV35.E1.MARV-2A-SEBOV were efficiently rescued and propagated. The genetic stability testing by extended passaging of several viral populations (plaques) in the producer cell line confirmed that this strategy yielded genetically stable vectors. However, minor deletion bands were observed for HAdV35.E1.MARV-2A-SEBOV at p13 (Figure 2A). From a genetically stable clone the HAdV35.E1.MARV-2A-SEBOV vector was further up-scaled, purified and the small scale preparation was found to be genetically stable. In this vector, the tested viral titers (VP and IU/ml), the VP/IU ratio and the productivity (VP/cm2) were not impaired compared to the benchmark vectors (data not shown). Although the two antigens were efficiently separated by the presence of the 2A sequence, the transgene expression by HAdV35.MARV-2A-SEBOV was reduced compared to the respective monovalent benchmark vectors HAdV35.MARV and HAdV35. SEBOV, as shown by WB (Figure 2B) and by FACS assay (Figure 2C).

HAdV35 vector with two expression cassettes in tandem in E1 region yields vectors with a improved genetic stability profile and potent transgene expression

As a second E1 insertion site based bivalent strategy, we tested vectors with two expression cassettes in tandem under control of heterologous regulatory sequences (Figure 1A): mCAG and human CMV promoters. Individual testing of the two promotors showed that they have similar transgene expression potency (data not shown). Two vectors HAdV35. mCAG.Luc-hCMV.eGFP and HAdV35.mCAG.MARV-hCMV.SEBOV were rescued and propagated successfully. In the genetic stability testing by extended passaging of five viral populations per vector, 1 out of 5 tested plaques of HAdV35.mCAG.Luc-hCMV.eGFP showed a deletion, whereas HAdV35.mCAG.MARV-hCMV.SEBOV showed no deletions in 5 out of 5 tested plaques (Figure 3A). After upscaling and preparation of small scale batches, the HAdV35.mCAG.MARV-hCMV.SEBOV vector was found to be genetically stable (data not shown). The viral titers (VP and IU/ml), VP/IU ratio and productivity (VP/cm^2)
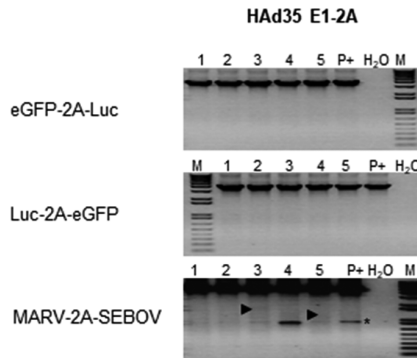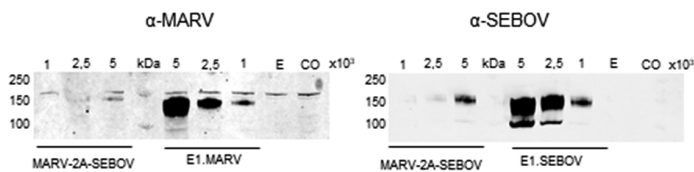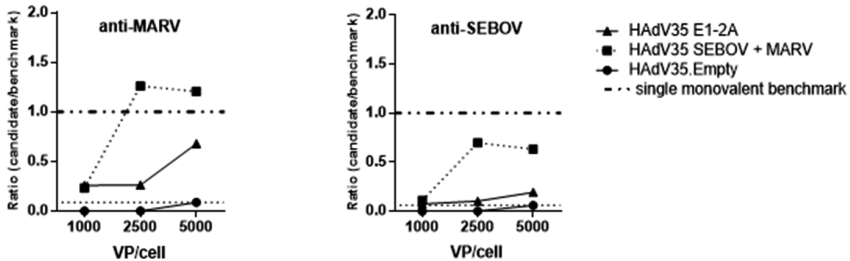
Figure 2. Genetic stability assessment and transgene expression of the bivalent E1-2A vector. *In vitro* characterization of the bivalent HAdV35.E1-2A vector. (A) Genetic stability assessment after extended passaging in the producer cell line. No deletion bands were detected by the E1 PCR at p13 of HAdV35.E1.eGFP-2A-Luc and HAdV35.E1.E1.Luc-2A-eGFP vectors. By contrast, E1 PCR of HAdV35.E1.MARV-2A-SEBOV shows, in two out of 5 plaques, additional (faint) bands at p13 (arrows). 1-5 plaques, P+ positive control plasmid DNA, P- negative control plasmid DNA, H_2O control and M for 1 kb plus DNA marker. (B) Total Filovirus glycoprotein (GP) expression in A549 cells of the HAdV35.E1.MARV-2A-SEBOV, directly compared to the respective monovalent controls, stained by anti-MARV and anti-SEBOV specific antibodies. The data presented is obtained in one experiment for all bivalent vector designs together with data presented in figures 3C, 4C. A549 cells were infected with 1000, 2500 and 5000 VP/cell HAdV35.E1.MARV-2A-SEBOV and the mixed monovalent MARV and SEBOV controls (HAdV35 SEBOV + MARV). (C) Surface exposed Filovirus GP was detected by staining A549 cells transduced with 1000, 2500 and 5000 VP/cell with HAdV35.E1.MARV-2A-SEBOV vector in parallel with the monovalent controls. The cells were stained with anti-MARV and anti-SEBOV 48 hours after infection and plotted relative to the HAdV35.SEBOV or HAdV35.MARV (ratio (candidate/benchmark)). The dotted line indicates the background staining in cells transduced with HAdV35.Empty vector.

were comparable to the monovalent benchmark (data not shown). Transgene expression testing showed MARV GP expression to be comparable to the monovalent benchmark, and SEBOV GP expression that was lower than that of the monovalent benchmark by WB (Figure 3B). In FACS analysis MARV GP expression was higher than the monovalent vectors, whereas SEBOV GP expression was slightly lower than the monovalent vectors (Figure 3C).

HAdV35 vectors with a mCMV bidirectional promoter expression cassette in E1 show a good genetic stability profile and transgene expression in the range of the monovalent benchmark vectors

Vectors with a bidirectional mouse CMV promoter expression cassette (Figure 1A) were tested as the third E1 insertion site-based bivalent strategy. Three vectors, HAdV35.Luc-mCMV-eGFP, HAdV35.eGFP-mCMV-Luc and HAdV35.MARV-mCMV-SEBOV, were rescued and propagated successfully. Five out of 5 viral populations of the three vectors were genetically stable after genetic stability testing by extended passaging (Figure 4A). The vector preparation of HAdV35.MARV-mCMV-SEBOV was successfully generated and genetically stable (data not shown). The viral titers (VP and IU/ml), the VP/IU ratio and the productivity of the HAdV35.MARV-mCMV-SEBOV vector were not impaired when compared to the monovalent benchmark (data not shown). In WB, transgene expression testing showed potent MARV and SEBOV GP expression in A549 cells transduced with the purified HAdV35.MARV-mCMV-SEBOV, comparable to the HAdV35.MARV and HAdV35.SEBOV monovalent vectors (Figure 4B). The FACS analysis of A549 cells transduced with the purified HAdV35.MARV-mCMV-SEBOV vector showed higher MARV and SEBOV GP expression than the monovalent controls (Figure 4C). Five additional HAdV35 vectors expressing two different Filovirus GP proteins from a mCMV bidirectional expression cassette were successfully rescued, propagated and were shown to be genetically stable at p10 (Supplemental Table 3). At p13 however, 1 out of 5 plaques of the HAdV35.CIEBOV.mCMV.MARV vector showed a faint deletion band (data not shown). Cumulatively these data support the robustness of this bivalent vector design.

Immune responses induced by the HAdV35 vector containing the bidirectional promoter cassette in E1 is comparable to the monovalent benchmark and performs better than other E1 based strategies

The E1-E3 bivalent strategy was deselected for immune response evaluation based on a poor genetic stability profile. The E1 insertion site-based bivalent vectors showed similar genetic stability profiles, but differed in their transgene protein expression. Potent transgene expression is considered a prerequisite (although not predictive) for inducing potent immune responses. Since immunogenicity is considered a key outcome, all three bivalent vectors, HAdV35.MARV-2A.SEBOV, HAdV35.mCAG.MARV.hCMV.SEBOV and
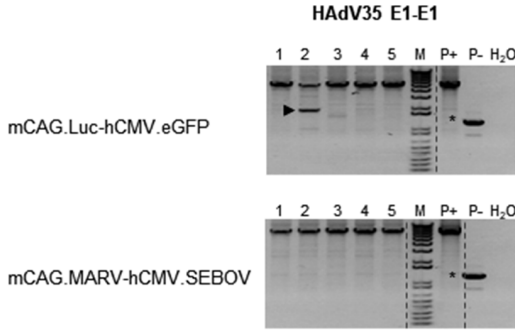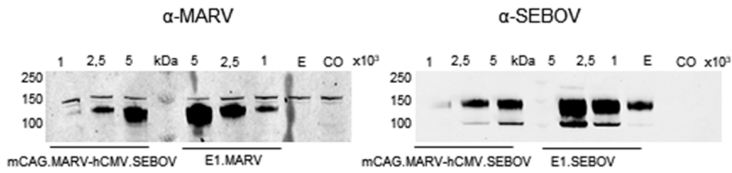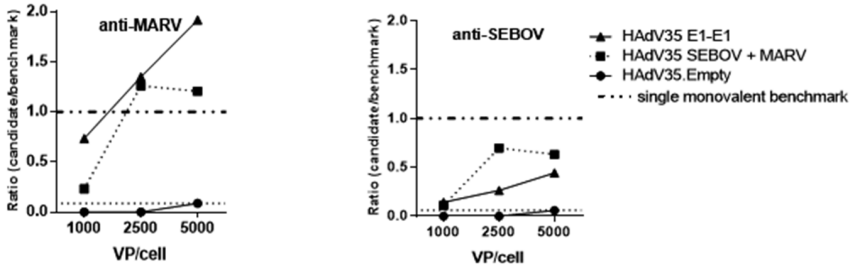
Figure 3. Genetic stability assessment and transgene expression of the bivalent E1-E1 vector. *In vitro* characterization of the bivalent HAdV35.E1-E1 vector. (A) Extended passaging of five plaques of HAdV35.mCAG.MARV.-hCMV.SEBOV shows no additional bands. The vector containing the mCAG. Luc.-hCMV.eGFP expression cassette shows one clone (out of five) with an additional smaller band detected by the E1 PCR. 1-5 plaques, P+ positive control plasmid DNA, P- negative control plasmid DNA, H_2O control and M for 1 kb plus DNA marker. (B) Total Filovirus glycoprotein (GP) expression in A549 cells of the HAdV35.mCAG.MARV.-hCMV.SEBOV. Stained by anti-MARV and anti-SEBOV specific antibodies showing the E1-E1 vector and the mixed monovalent controls (HAdV35 SEBOV + MARV) transduced with 1000, 2500 and 5000 VP/cell. (C) Surface exposed Filovirus GP was detected by staining A549 cells transduced with 1000, 2500 and 5000 VP/cell with HAdV35.mCAG. MARV.-hCMV.SEBOV vector in parallel with the mixed monovalent controls. The data presented is obtained in one experiment for all bivalent vector designs together with data presented in figures 2C, 4C. The cells were stained with anti-MARV and anti-SEBOV 48 hours after infection and plotted relative to the HAdV35.SEBOV or HAdV35.MARV (ratio (candidate/benchmark)). The dotted line indicates the background staining in cells transduced with HAdV35.Empty vector.
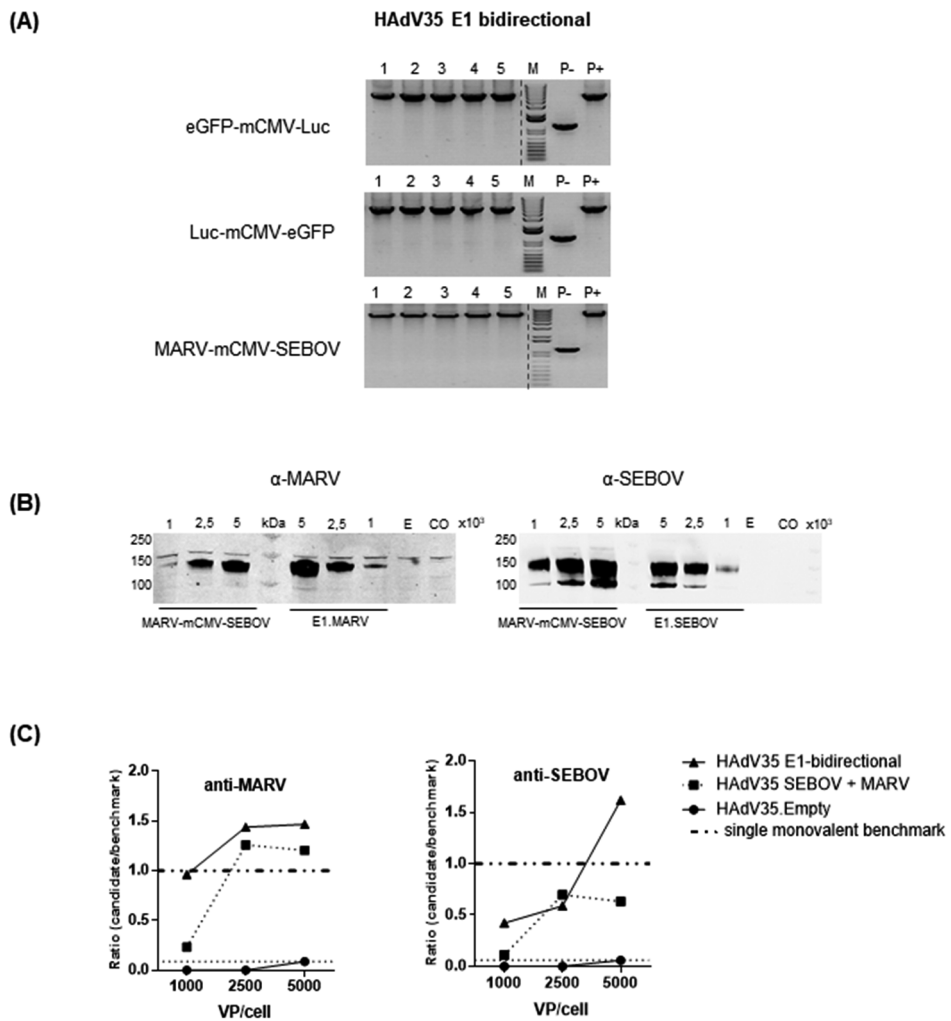
Figure 4. HAdV35 E1 bidirectional promoter containing vectors. *In vitro* characterization of the bivalent HAdV35.E1 bidirectional promoter containing vector. (A) Extended passaging of five plaques of HAdV35.eGFP.mCMV.Luc, HAdV35.Luc.mCMV.eGFP and HAdV35.MARV.mCMV. SEBOV shows no additional bands in the E1 PCR. 1-5 plaques, P+ positive control plasmid DNA, P- negative control plasmid DNA, H_2O control and M for 1 kb plus DNA marker. (B) Total Filovirus glycoprotein (GP) expression in A549 cells of the HAdV35.MARV.mCMV.SEBOV, directly compared to the respective mixed monovalent controls (HAdV35 SEBOV + MARV), stained by anti-MARV and anti-SEBOV specific antibodies. A549 cells were transduced with 1000, 2500 and 5000 VP/ cell HAdV35.E1.MARV-mCMV-SEBOV vector and the controls. (C) Surface exposed Filovirus GP was detected by staining A549 cells infected with 1000, 2500 and 5000 VP/cell with the HAdV35. E1.MARV-mCMV-SEBOV vector in parallel with the mixed monovalent controls. The data presented is obtained in one experiment for all bivalent vector designs together with data presented in figures 2C, 3C. The cells were stained with anti-MARV and anti-SEBOV 48 hours after infection and plotted relative to the HAdV35.SEBOV or HAdV35.MARV (ratio (candidate/benchmark)). The dotted line indicates the background staining in cells transduced with HAdV35.Empty vector.

HAdV35.MARV-mCMV-SEBOV, were compared for their ability to induce humoral and cellular immune responses against MARV and SEBOV GP in the mouse model. The mix of the monovalent vectors HAdV35.MARV and HAdV35.SEBOV was used as a benchmark indicative of potent immunogenicity. To this end, BALB/c mice were immunized IM with a total of $2x10^9$ VP ($1x10^9$ VP per vector) and the humoral and cellular immune responses were analyzed by ELISA and ELISPOT, respectively. The functionality of the induced CD4+ and CD8+ T cell fractions was determined by measuring cytokines in an intracellular cytokine staining (ICS) assay. The effector phase (two weeks post-immunization) as well as long-term immune responses (8 weeks post-immunization) were examined, but only data for the week 8 time point is presented here.

Compared to the monovalent mix, the HAdV35.MARV-2A.SEBOV vector induced low GP specific humoral (Figure 5A) and cellular (Figure 5B) immune responses by week 8. The humoral and cellular immune responses induced by the HAdV35.mCAG.MARV. hCMV.SEBOV were higher for the MARV and lower SEBOV GPs when compared to the monovalent vector mix (Figure 5). Unlike the other two E1-bivalent vectors, at week 8 the HAdV35.MARV-mCMV-SEBOV vector was comparable to the monovalent mix for both the MARV and SEBOV GP specific humoral and cellular response as measured by ELISA and ELISPOT (Figure 5).

At week 2, all of the vectors induced functional helper CD4+ (IL-2, INFγ and TNFα) T cells. However, only the HAdV35.MARV-mCMV-SEBOV vector induced INFγ secreting CD8+ T cells (data not shown). The INFγ positive CD8+ T cells induced by the HAdV35. MARV-mCMV-SEBOV vector at week 2 were also confirmed and comparable at week 8 post-immunization to the monovalent mix (data not shown). In that respect, by showing humoral, cellular and CD8+ immune responses that were comparable to the monovalent benchmark, the E1-bidirectional strategy performed better than the other E1-based designs in the mouse model.

HAdV26 vector containing an E1 mCMV bidirectional promoter cassette shows a good genetic stability profile and potent immunogenicity in mice

To assess whether the bidirectional approach for bivalent vectors can be applied in AdV vectors derived from different types, HAdV26 vectors encoding two transgenes in E1 driven by the bidirectional promoter were designed. The HAdV26.MARV-mCMV-SEBOV vector was generated, up-scaled, purified and compared to the respective monovalent HAdV26. MARV and HAdV26.SEBOV controls. Five out of 5 viral populations of the HAVd26.MARV-mCMV-SEBOV were shown to be genetically stable after extended passaging (Figure 6A). The purified small scale batch preparation of HAVd26.MARV-mCMV-SEBOV was shown to be genetically stable. The corresponding viral quality characteristics such as the viral titers (VP and IU/ml), VP/IU ratio and the productivity (VP/cm$_2$), were comparable to the monovalent vector preparations (data not shown). Transgene expression in A549 cells showed MARV GP expression was lower than the monovalent control, and SEBOV GP
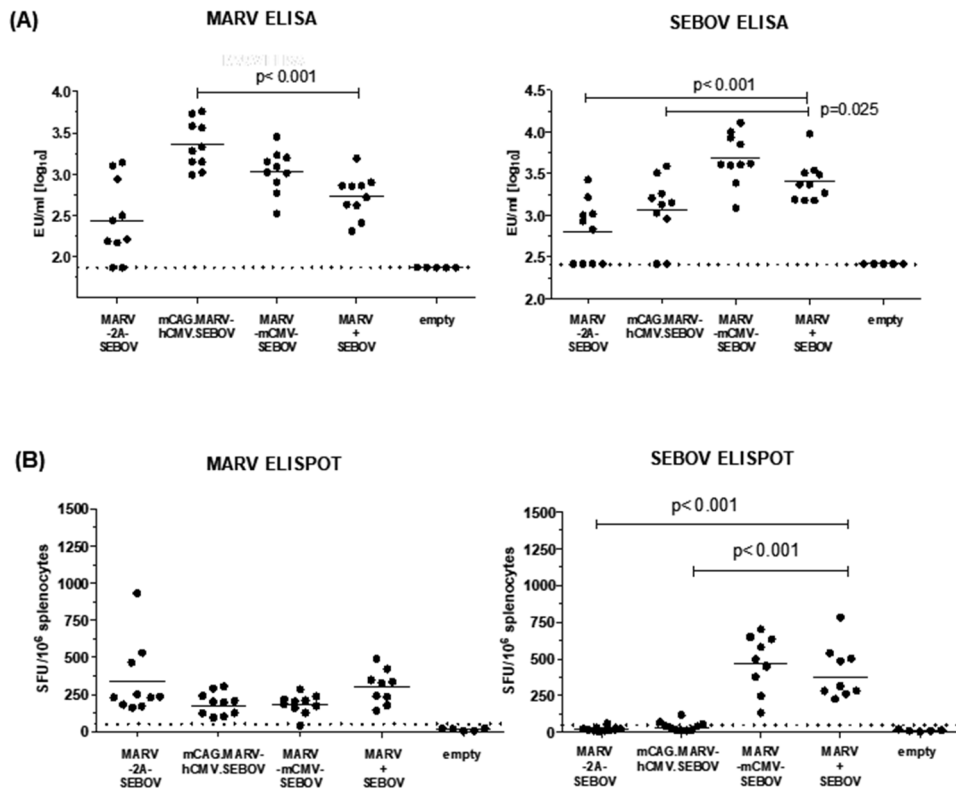
Figure 5. Filovirus specific B- and T cell responses in mice. Filovirus specific B- and T cell responses were measured in mice 8 weeks post immunization after confirming correct vaccination by vector (hexon) induced T cell responses. The dots represent individual mice. (A) ELISA specific for SEBOV GP and MARV GP were performed relative to a reference serum. An arbitrary value of ELISA unit (EU) per ml was assigned to the reference serum. The line in each group of mice represents the mean of the $\log_{10}$ transformed EU/ml per group and the dotted line indicates the limit of detection for each assay. (B) Splenocytes were stimulated with either MARV or SEBOV specific peptide pools in ELISPOT assay. The bar represents the geometric mean of the group and the dotted line the lower cut-off of the assay namely 50 SPU/10^6 splenocytes. SPU: spot forming units. Statistically significant differences between the groups are indicated in the graphs (p<0.001).

expression that was comparable to the monovalent control by WB (Figure 6B). The FACS analysis showed MARV and SEBOV GP expression that was comparable to the monovalent controls (Figure 6C).

To determine MARV- and SEBOV-specific immune responses, mice were immunized with HAdV26.MARV-mCMV-SEBOV 2×10^9 or 2×10^{10} VP. At week 8, MARV-specific humoral responses induced by the HAdV26.MARV-mCMV-SEBOV were significantly lower compared to HAdV26.MARV (p<0.01), for both doses. However, SEBOV GP-specific humoral response were comparable to the monovalent control (Figure 6D). Even

though cellular immune responses were significantly lower with the $2x10^9$ VP dose, the responses induced by the $2x10^{10}$ HAdV26.MARV-mCMV-SEBOV were not different from the monovalent controls for both antigens (Figure 6E).

In addition to the HAdV26.MARV-mCMV-SEBOV vector, four more HAdV26 mCMV-vectors with different transgene combinations were successfully rescued, propagated and found genetically stable (Supplemental Table 3). Together, the presented data generated with the E1-bidirectional cassette in HAdV26 indicate that the E1 bidirectional approach can be transferred to other AdV types, such as HAdV26.
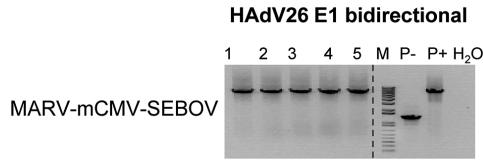
DISCUSSION

Current AdV technology allows induction of protective immunity against more than one antigen if several monovalent vectors are mixed. In order to establish an alternative to the monovalent vector mixing approach we thoroughly characterized available bivalent vector designs, and established their rescue efficiency, producibility, genetic stability profile[55] and immunogenicity compared to their monovalent counterparts.

We first focused on the E1-E3 bivalent vector design since this bivalent strategy is commonly used in literature and HAdV35 E1-E3 vectors were successfully generated in previous reports (Supplemental Table 1). The observed marked genetic instability of the E1-E3 vectors in this study, which was preceded by poor rescue efficiency of some of the vectors, was therefore surprising. Generally, events of AdV genetic instability are most probably caused by homologous-recombination as a consequence of sequence homology between the expression cassettes, transgenes, regulatory sequences and the orientation in the AdV genome.[17, 24, 29, 30, 56] To generating a successful E1-E3 vector a systematic assessment of the E3 function and the consequences of its deletions (e.g. effects on the fiber expression) might be necessary,[24, 57] which limits its further development.
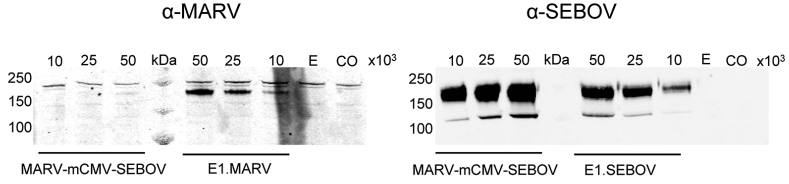
Unlike the E3 region, the E1 insertion region in the E1-E3 bivalent vectors remained genetically stable. We hypothesized that E1-based bivalent vectors would remain genetically stable and focused on generating designs which would allow placing two transgenes in the E1 region.

In these designs, the close proximity of homologous sequences in the E1-based bivalent vectors can pose a risk for deletion events during vector generation.[56] Nevertheless, all E1 based strategies showed a superior genetic stability profile after extended passaging and E1-PCR analysis compared to E1-E3 bivalent vectors and allowed selection of stable clones. In addition, batch viral genomes were further analyzed by E3/E4-PCR (data not shown) and multiple restriction enzyme digestions as shown for StuI enzyme in Supplemental figure 2. However, in the E1-2A vectors transgene expression levels and transgene-specific immunogenicity were poor. In the light of successful reports using the 2A sequence for transgene separation in literature[37, 39], the poor protein expression and immunogenicity observed in this study (*Thosea asigna* virus-2A gave similar results, data not shown) leaves room for improved design.

HAdV26 E1 bidirectional

1 2 3 4 5 M P- P+ H$_2$O

MARV-mCMV-SEBOV

α-MARV

10 25 50 kDa 50 25 10 E CO x10^3

250
150
100

MARV-mCMV-SEBOV   E1.MARV

α-SEBOV

10 25 50 kDa 50 25 10 E CO x10^3

250
150
100

MARV-mCMV-SEBOV   E1.SEBOV

anti-MARV

Ratio (candidate/benchmark)

2.0
1.5
1.0
0.5
0.0

10 000   25 000   50 000
VP/cell

anti-SEBOV

Ratio (candidate/benchmark)

2.0
1.5
1.0
0.5
0.0

10 000   25 000   50 000
VP/cell

- ▪ - HAdV26 SEBOV + MARV
- ▲ HAdV26 E1-bidirectional
- ● HAdV26.Empty
- ···· single monovalent benchmark

MARV ELISA

EU/ml [log$_{10}$]

4.5
4.0
3.5
3.0
2.5
2.0

p < 0.01

MARV   MARV   empty
+   -mCMV-
SEBOV   SEBOV

SEBOV ELISA

EU/ml [log$_{10}$]

5.0
4.5
4.0
3.5
3.0
2.5

ns

MARV   MARV   empty
+   -mCMV-
SEBOV   SEBOV

MARV ELISPOT

SFU per 10^6 splenocytes

800
600
400
200
0

ns

MARV   MARV   empty
+   -mCMV-
SEBOV   SEBOV

SEBOV ELISPOT

SFU per 10^6 splenocytes

800
600
400
200
0

ns

MARV   MARV   empty
+   -mCMV-
SEBOV   SEBOV
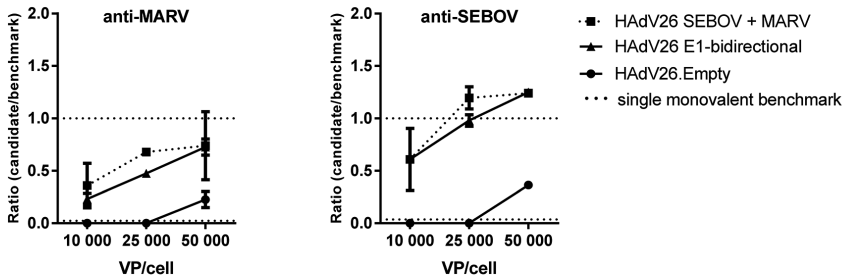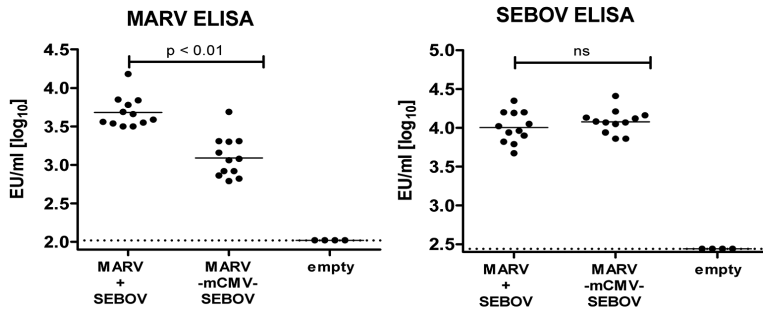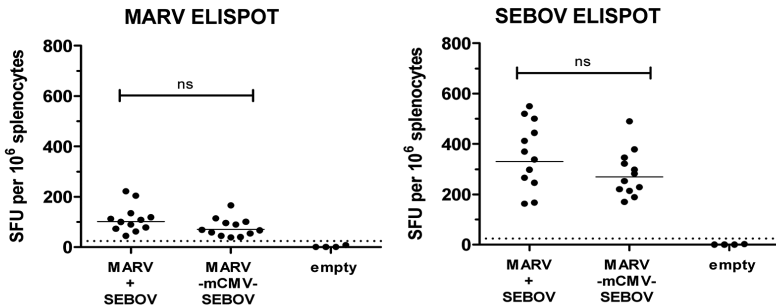
Analysis of the transgene expression levels and immunogenicity of the E1-E1 vectors using mCAG and hCMV promoters clearly showed higher levels of the gene inserted in the mCAG driven 5'-expression cassette as opposed to the hCMV driven transgene expression in the 3'-expression cassette. This observation was unexpected considering that the hCMV promoter drives robust transgene expression in monovalent benchmark vectors. Presumably the close proximity of the highly potent promoters influences their function, an effect previously termed promoter 'interference'.[58, 59] Possibly, the left ITR and the E1a enhancer might boost expression from the more proximal mCAG promoter.[60, 61] Nevertheless, the E1-E1 strategy in which different regulatory sequences are used to prevent genetic instability can be regarded as a viable bivalent vector design for further development. If balanced expression is desired with this strategy, insertion of (small) insulator sequences between the cassettes may be worthy of further investigation.[62, 63]

In this study, the E1-bidirectional design HAdV35 vectors showed a favorable genetic stability profile, potent antigen expression and immunogenicity, comparable to the tested mix of monovalent vectors. Therefore the E1-bidirectional design using a mCMV bidirectional promoter was chosen as the best tested design and feasibility to transfer the design to another human AdV serotype was tested. Transfer of the E1-bidirectional design to HAd26 vectors was successful yielding genetically stable vectors with potent transgene expression and immunogenicity. However, interestingly transgene expression of the two filovirus GPs showed to be more imbalanced than observed in the context of HAdV35 vectors.

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Figure 6. Bivalent E1-bidirectional design in HAdV26 vector. A HAdV26 vector containing an E1-bidirectional cassette expressing Filovirus transgenes MARV and SEBOV (A) was tested for genetic stability in the producer cell line by extended passaging of five plaques in parallel. Shown here are five plaques analyzed by E1 PCR at passage 13. 1-5 plaques, P- plasmid negative control, P+ plasmid positive control, H_2O control and M for 1 kb plus DNA marker. Total protein and surface expression of the Filovirus transgenes in A549 were analyzed by Western blot (B) and FACS (C) in parallel with the respective monovalent controls 48 hours after transduction. Analysis of total protein was performed with 10 000, 25 000 and 50 000 VP/cell transduced A549 cells. Staining of surface exposed MARV and SEBOV GPs was analyzed after infection of A549 cells with a 10 000, 25 000 and 50 000 VP/cell (in duplo). Both analyses were performed with anti-MARV and anti-SEBOV specific antibodies (B and C). The B- and T cell Filovirus specific responses were assessed in mice in parallel with the respective monovalent controls and an E1-Empty vector (D and E). ELISA specific for SEBOV GP and MARV GP were performed relative to a reference serum. An arbitrary value of ELISA unit (EU) per ml was assigned to the reference serum. The line in each group of mice represents the mean of the $\log_{10}$ transformed EU/ml per group and the dotted line the limit of detection for each assay. (E) Splenocytes were stimulated with either MARV or SEBOV specific peptide pools in ELISPOT assay. The geometric mean of each group is indicated with a bar and the dotted line the lower cut-off of the assay, 50 SPU/10^6 splenocytes. SPU: spot forming units. Statistically significant differences between the groups are indicated in the graphs (p<0.001 and non-significant, ns).

This imbalance seems to be AdV type-specific, suggesting some (unknown) effect of the AdV backbone on the 5'-reverse oriented transgenes, such as the described adenovirus group and/or cell line specific ITR influences on the proximal promoters.[64-66] Rethinking the surrounding sequences for instance by placing insulators,[62] using an alternative insertion site,[67] or changing the bidirectional promoter, may tailor the bidirectional vector design for other AdV serotypes. It should be noted that in all instances the humoral immune responses of the HAdV35 bivalent vectors, closely followed the GP surface expression in A549 and may have been predictive for the HAdV26 bidirectional vector.

While the E1-bidirectional strategy showed to be better than other tested designs in this study, bivalent vectors in which one expression cassette each is placed at the left end of the AdV genome (E1) and at the right end of the AdV genome (between E4 and the right ITR) might offer a viable alternative and remain to be explored in HAdV35 and HAdV26 vectors (Supplemental Table 1).

Our data highlight the importance of meticulous screening and systematic assessment of different vector characteristics prior to their employment in further clinical development to ensure selection of the optimal (bivalent) vector design. As a result of this study E1-bidirectional vectors using a mCMV bidirectional promoter are proposed as alternatives to the mix of monovalent vectors for further development.

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AUTHOR DISCLOSURE STATEMENT

The authors are employees of Johnson and Johnson.

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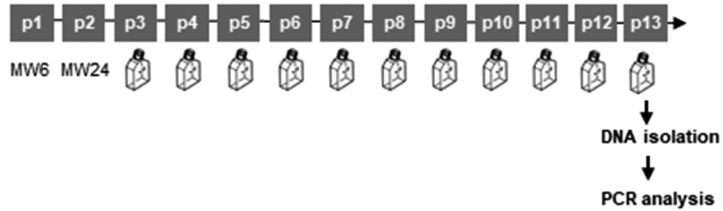
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Figure S1. Genetic stability assessment by extended passaging in the producer cell line. (A) Schematic representation of the genetic stability assessment by extended passaging in producer cell line, showing the amplification from plaque purification (MW6) up to passage 13 (p13) in T25 format, followed by DNA isolation and E1 PCR analysis. (B) Shown here are E1 PCR analysis of five individual plaques of the monovalent benchmark vectors HAdV35.EBOV, HAdV35.SEBOV, HAdV35.MARV and HAdV26.EBOV. 1-5 plaques, P+ positive control plasmid DNA, P- negative control plasmid DNA and H_2O control.

HAdV35 E1-bivalent vectors
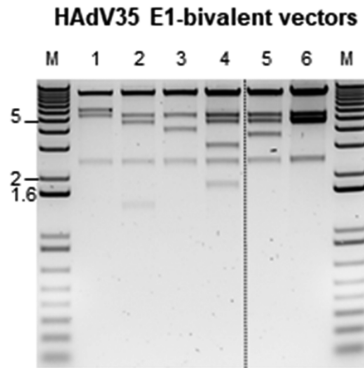
Figure S2. HAdV genome genetic stability assessment by restriction enzyme analysis. Purified HAdV35.E1 bivalent vectors genome digestion with StuI. Lane 1 HAdV35.MARV (2404, 5275, 5781, 9243, 9566 bp), Lane 2 HAdV35.SEBOV (1320, 2404, 4596, 5275, 9243, 9566 bp), Lane 3 HAdV35.emtpy (2404, 3950, 5275, 9243, 9566 bp), Lane 4 HAdV35.MARV.mCMV.SEBOV (1783, 2404, 2999, 4596, 5275, 9243, 9566 bp), Lane 5 HAdV35.MARVA-F2A.SEBOV (2404, 3550, 4596, 5275, 9243, 9566 bp), Lane 6 HAdV35.mCAG.MARVA-cCMV.SEBOV (2404, 4593, 4596, 5275, 9243, 9566 bp). M stands for 1 kb plus DNA marker.

Table S1.

Strategy / position	Reference	Characterization
Two complete expression cassettes		
E1-E3	Vogels et al., 2007	rAd35 , E3 right orientation , stability shown by PCR for five rAd clones at p8
	Bramson et al., 1996	Double rAd5 expresing murine IL-12 from E1 and E3 (forward and reverse orientation) driven by homologous promoters and poly A
	Danthinne & Werth, 2000	Renila (E3) and firefly luciferase (E1) in rAd5
	Ghosh-Choudhury et al., 1986	Ad5 E1 and E3 deletion mutant generation
	Putzer et al., 1997	Two Il-12 subunits encoded in E1 seperated by IRES (tandem) and in E3 B7-1 both cassettes driven by mCMV
	Pham et al., 2009	sCEA, betahCG or hNIS were inserted in E1 and E3 or E4. The levels of transgene expression can be inferred from the marker expression encoded by second transgene inserted elsewhere in the vector genome
	Berenjian & Akusjarvi, 2006	Rabies glycoprotein (gp60) in E1 and Tet-On or OFF in E3 (inverted and right orientation) (rAd5) 3x LacO regulator sites inserted behind gp60 in E1
	Small et al 2013	Heterologous promoter and poly A expression cassettes in E1 and E3 (both orientations) Expressing gag and NP (AdC7) Poor rescuability for forward E3 oriented cassette Genomic stability p1 and p10 shown with restriction enzyme analysis
E1-E4_rITR	Holman et al., 2009	CAdVax-RVF (Ad5) reverse orientation inserted between right ITR and E4
	Pham et al., 2009	See above
	Norris et al., 2006	FasL and small molecule for cancer treatment
E1-tandem	Diaz et al. 2013	Heterologous promoter and pA E1-E1 cassette
	Harro et al., 2009	MRKAd5 and MRKAd6 HIV 1 nef, gag-pol safety and immunogenicity (and fusion gag-pol)
	Belousova et al., 2006, Hemminki et al. 2001	Homologous promoters and poly A;CMV promoter driven tandem expression cassettes for cancer chemotherapy and imagine , found genetically unstable (rAd5) Genetic stability tested by three additional Ad amplification rounds and testing cassette by PCR

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Table S1. (continued)

| Strategy / position	Reference	Characterization
	Gonzalez-Nicolini & Fussenegger, 2005	rAd5 E1 inducible expression of reporter genes and cell arrest regulator ,number of promoters tested including bidirectional for expression
	Lee et al., 2005	CMV driven sodium/iodide symporter (NIS) and eGFP tandem in rAd5 E1
	WO2011/110864	Promoter sequences for DNA and viral vectors
Direct fusion of the transgenes	Catanzaro et al., 2006	Phase I Ad5 HIV fusion protein (GenVec)
	Huang et al., 2001	Immunogenicity Gag-pol HIV-1 in mice
	Bennekov et al., 2006	Immunogenicity TB fusion protein
	Havenga et al., 2006	TB antigens fused directly, resulting in a single poly protein expressed from a E1 cassette in rAd35
	Masaki-Shoji et al, 2012	HIV Gag and Env in E1 either directly fused, separated by IRES or F2A sequence. Different promoters were tested for optimal expression. F2A variants most immunogenic (rAd5.F35)
Internal ribosomal entry site (IRES)	Putzer et al., 1997	See above
	Tan et al., 2010	rAd5 E1 expression cassette containing encoding multiple Rabies GP and reporter gene separated by IRES and/or F2A sequence . Southern blot analysis showed no deletions of RVG copies after ' normal' rAd5 replication
	Masaki-Shoji et al, 2012	See above
	Tan et al., 2010	See above
Self-cleavable peptides	Masaki-Shoji et al, 2012	See above
Splicing	Cheng et al., 1992	Encoding of simian immunodeficiency virus Env and Rev proteins by splicing from E3 region (of rAd5).
Bidirectional promoter architecture		
E1-bidirectional	Na et al., 2010:	Synthetic bidirectional promoters in rAd5.F35 E1 region to coordinately express a marker gene and a beta-globin therapeutic gene in haematopoetic stem cells.
	Gonzalez-Nicolini & Fussenegger, 2005	rAd5 based E1 bidirectional auto-regulated promoter expressing reporter genes and cell arrest regulator

Table S2. Bivalent HAdVs with one antigen encoded in E1 and one in E3 are genetically unstable and difficult to expand. The table shows HAdV35 and HAdV26 vectors using the genetic setup described in rescue attempts, rescue success and genome stability of these vectors in number of plaques with detected deletion bands in E3.

Vector	# Rescue	Result	Genome stability	# Plaques
HAdV35 Advac™				
E1.EBOV-E3.SEBOV	1	successful	Deletion bands detected	5/5[1]
E1.SEBOV-E3.EBOV	1	successful	Deletion bands detected	2/2[1]
E1.MARV-E3.EBOV	1	successful	Deletion bands detected	5/5*
E1.MARV-E3.SEBOV	1	successful	Deletion bands detected	5/5*
E1.eGFP-E3.EBOV	1	successful	Deletion bands detected	5/5*
E1.eGFP-E3.SEBOV	1	successful	Deletion bands detected	5/5*
HAdV26 Advac™				
E1.EBOV-E3.SEBOV	2	Difficult to expand	Deletion bands detected	1/1#
E1.eGFP-E3.SEBOV	2	Difficult to expand	Deletion bands detected	1/1#

[1] p15, * p10 and # p14

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Table S3. Genetic stability testing of vectors harboring the mCMV bidirectional expression cassette.

Vector	# rescue	Result	genome stability p10
HAdV35 Advac©			
Luc-mCMV-eGFP	1	Successful	stable
eGFP-mCMV-Luc	1	Successful	stable
MARV-mCMV-SEBOV	1	Successful	stable
SEBOV-mCMV-MARV	1	Successful	stable
MARV-mCMV-CIEBOV	1	Successful	stable
CIEBOV-mCMV-MARV	1	Successful	stable
EBOV-mCMV-SEBOV	1	Successful	stable
SEBOV-mCMV-EBOV	1	Successful	stable
HAdV26 Advac©			
Luc-mCMV-eGFP	1	Successful	stable
MARV-mCMV-SEBOV	1	Successful	stable
EBOV-mCMV-SEBOV	1	Successful	stable
SEBOV-mCMV-EBOV	1	Not successful	-
MARV-mCMV-CIEBOV	1	Successful	stable
CIEBOV-mCMV-MARV	1	Successful	stable
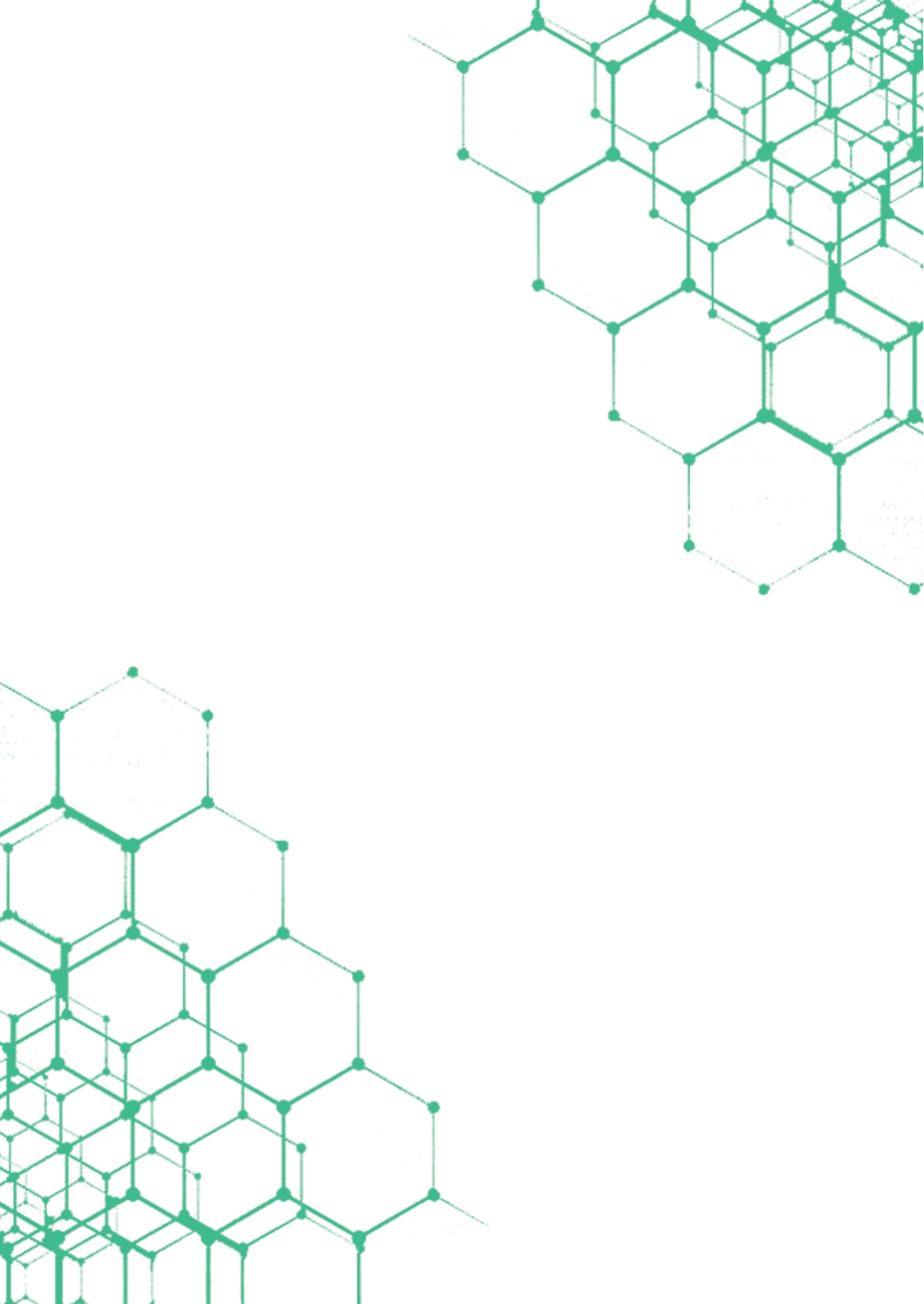
SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVE

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SUMMARIZING DISCUSSION

Replication-incompetent adenoviral (AdV) vectors are being developed as platform for vaccines against infectious diseases because of their ability to elicit potent antigen-specific immune responses, their acceptable safety profile and relative ease of manufacturing [1]. In human studies AdV-based viral vectored vaccines have shown promising immunogenicity results that potentially can translate in protection against infectious diseases such as HIV and Ebola [2-5]. Much effort has gone into the development of the highly prevalent HAdV5 vector which has the limitation of highly prevalent and high titer pre-existing immunity in the human population which was demonstrated to affect its potency [6-9]. Vectors derived from low-seroprevalent human AdVs such as HAdV35 and HAdV26 [9-12] are less likely to be hampered by pre-existing immunity due to low prevalence and generally low anti-vector antibody in the population [9, 13, 14].

For effective vaccine-mediated protection against infectious diseases, potent B- and T-cell immune responses are believed to be required [15, 16]. There are several ways this can be achieved using currently available technologies; a heterologous vaccination regimen with first single or multiple doses of an AdV based vaccine followed by a single or multiple doses of an alternative vector (e.g. AdV or MVA) [17-19], mixing several monovalent AdV vectors [19-21], or first an AdV based immunization followed by a (adjuvanted) protein vaccination [17, 19, 22-24]. While these approaches induce potent immune responses with a favorable safety profile, they may be complex for general implementaion.

To address this limitation alternative AdV vaccine designs are available. The AdV capsid can be engineered to display an antigen on the outer capsid surface, thus harnessing the host immune response mounted against the vector itself. These so-called AdV antigen-display vectors are particularly designed to induce B-cell antigen-specific immune responses. In this respect, they serve a similar purpose to protein/VLP but without the need for an adjuvant. Importantly, unlike their VLP counterparts, AdV antigen-display vectors can be engineered to genetically encode a specific antigen, thereby combining genetic and protein-based vaccines within one vector. Most of the genetic AdV vaccines are monovalent, designed to encode a single gene in the E1 region. However, the AdV genome can be further engineered to encode multiple antigens simultaneously, resulting in the so-called multivalent vector. This thesis focused on generating AdV antigen-display and multivalent AdV vaccines against infectious diseases using the low-seroprevalent HAdV35 and HAdV26 vectors.

AdV antigen-display vectors can be generated by modifying capsid proteins hexon, fiber, penton and protein IX (pIX). Hexon is the major capsid component counting 720 monomers per capsid. Each hexon monomer consists of a highly conserved base and hyper variable loops 1-7 (HVR1-7) [25-28] which are located on the outer capsid surface. HVRs contain AdV-specific epitopes and are at target for hosts antibody responses against the AdV [29, 30]. These antibody responses may neutralize the AdV vector, reducing its potency [31-34]. HVRs can be modified to contain heterologous peptides such as

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counterpart sequences from alternative less-prevalent AdV, to circumvent the anti-vector immunity [29, 35-39]. Alternatively, disease-relevant epitopes can be inserted to generate a vaccine. For vaccine generation, mainly HVR1 and HVR5 have been modified using ~30 aa linear epitopes from various pathogens antigen-specific immune responses in mice [40]. There are two main advantages of hexon-display; (i) single epitope insertions in, for instance, HVR1 result in a vector displaying 720 surface exposed epitopes per capsid in a highly structured fashion and (ii) multiple HVRs can be modified at the same time, allowing for insertion of multiple different epitopes or more of the same.

The second most utilized approach to present antigens on the outer capsid surfaces is by using pIX. Minor capsid pIX is located between the hexon groves in the AdV capsid exposing its C-terminus tail to the outer capsid surface [41, 42]. pIX can accommodate linear and globular proteins [43-45]. It has been modified for different purposes such as cell-specific AdV vector re-targeting and purification by specific ligand fusion [43, 46-48], or as a tool for AdV tracking *in vitro,* and *in vivo* of functional reporter proteins fusion [44, 45, 49]. In vaccine development, large antigens in the range of 15 kDa to ~70 kDa have been successfully fused to the pIX C-terminus and displayed on the AdV capsid surface resulting in potent antigen-specific immune responses in mice [40, 50-52]. A major advantage of pIX-display vectors is that pIX tolerates fusion of larger and possibly correctly folded proteins to its C-terminus [50-52]. This would allow a selection of more complex multi-epitope antigens for generation of a pIX-display vaccine.

The major capsid proteins fiber and penton are imperative for cell entry [53, 54] and are typically modified for vector retargeting [55-59]. However, there are some examples of using these proteins (mainly fiber) to display epitopes [60-63]. Antigens displayed via fiber are highly accessible on the AdV capsid which might explain their potency to induce antigen specific immune responses in mice, despite their relatively low abundance (36 copies) per capsid [40, 60-63].

The majority of AdV capsid antigen-display vectors have been generated using the HAdV5 vector, however there are some examples where alternative AdV types such as HAdV48 and simian AdV type 25 have been successfully employed for capsid antigen-display [40].

AdV pIX-display vectors with or without a transgene in E1, have proven highly potent at inducing antigen-specific B-cell immune responses, and in some instances are superior to genetic AdV vector or protein [50-52, 64]. In **Chapter 3** HAdV35 vectors were generated to encode a *P. falciparum* CS transgene and display a truncated version of the CS protein (CS_{short}) via pIX C-terminus [64]. To increase the CS_{short} accessibility on the outer capsid surface, pIX-display vectors were generated with or without a glycine linker (for flexibility) and/or a 45Å-spacer [46].

The pIX-CS_{short} display vectors induced strong humoral antigen-specific immunity in mice which increased with the addition of a spacer molecule, exceeding the immune responses induced by the HAdV35 CS genetic vector or CS recombinant protein control.

Co-administration of AdV particles with peptide antigens has been shown to mediate a strong adjuvant-effect [65] most likely due to the induction of pro-inflammatory cytokines [66, 67] such as type-I interferons that enhance immune responses to peptides [68]. However, mixing of the CS protein with HAdV particles (HAdV26, HAdV35, HAdV5) failed to increase CS-specific antibody responses, implying that the potential adjuvant-effects of AdV vector particles are likely independent of innate cytokine responses. This observation suggests that for potent antigen-specific immune responses, physical association and display of the antigen on the AdV capsid may be necessary. Potential explanations are that firstly, pIX-antigen fusion would ensure their expression within the same cell (e.g. antigen presenting cell) enhanced by the transgene promoter located upstream of the pIX-region. Supporting this hypothesis is the observation that immune responses against expressed pIX were previously noted as a cause for AdV-induced immune responses [69]. Secondly, antigen presentation in a dense, repetitive manner via the particle may enable cross-linking of B-cell receptors, ensuring a qualitatively superior antibody response [70]. In addition, the pIX-display vectors with a genetically encoded CS transgene elicited high antigen-specific T-cell immune responses comparable to the genetic HAdV35 CS genetic vector, demonstrating the immune-directing potential of combining the antigen pIX-display and a genetically encoded transgene [64].

Using the pIX-display vaccine platform, a potent second-generation prophylactic HPV vaccine based on highly conserved minor L2 proteins was generated (**Chapter 4 and 5**). Although L1 protein-based HPV vaccines are shown to be highly efficacious, due to limited cross-protection wide HPV coverage can only be achieved by addition of L1 component of each HPV genotype [71] increasing the vaccine complexity. To provide broader protection across multiple clinically relevant HPV types, prophylactic HPV vaccines based on the conserved L2 protein are considered an alternative to L1 protein-based vaccines [72-74]. The L2 protein contains a highly conserved N-terminus with many well-defined linear cross-reactive neutralizing antibody (nAb) epitopes (e.g. aa 17-36 epitope sequence homology is ~80%) [72-74]. However, minor sequence variations for instance in the aa 17-36 epitope can result in reduced protection against certain (distant) HPV types (e.g. HPV16 vs. HPV31) [75]. Therefore, to achieve coverage of the most prevalent HPV types worldwide inclusion of multitype (covering the sequence variation) linear nAb epitopes in the vaccine may still be necessary.

Even tough pIX can tolerate fusion of larger polypetides to its C-terminus [50-52]. To successfully generate AdV pIX-display vectors, assessment of the optimal antigen design (e.g. size or spacers) is necessary [40, 64]. Different L2 epitope repeats (concatemers) fused to pIX were generated and tested in HAdV35 and HAdV26 vectors (**Chapter 4 and 5**). L2 epitope repeats in a size range of 93-418 amino acids (aa) combining different HPV types were fused to pIX and tested for capsid incorporation [76] (**Chapter 4**). In contrast to pIX-L2 repeats which contained epitopes from three (93 aa) or four (123 aa) HPV types, the larger repeats (153-418 aa) were not efficiently incorporated into the HAdV35

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or HAdV26 capsid. These observations indicate the importance of antigen design and suggest that polypeptide characteristics other than size (or a combination of factors) may play a role in efficient capsid incorporation [77].

A 9-valent AdV pIX-display prophylactic HPV vaccine consisting of two heterologous 4-valent and 5-valent HAdV35 vectors displaying via pIX either HPV6, 31, 33, 16 or HPV11, 52/58, 45, 18 (HPV52 and HPV58 identical epitope sequence) L2 protein epitopes in a repetitive conformation (**Chapter 5**) was generated and extensively tested. Each HAdV35 pIX-L2 display vector exhibited a good manufacturability profile, showing high titer yields in producer cell line, genetic stability, and stability comparable to the non-modified AdV vector at cold-chain relevant temperature 2-8°C for 1 year [78]. Single 4-valent and 5-valent HAdV35 pIX-L2 display vaccine vectors were immunogenic in mice, inducing nAb responses against HPV-types included in the vaccine and cross-reactive nAb against distant HPV-types not included in the vaccine [78]. Immunization with a mix of 4-valent and 5-valent HAdV35 pIX-L2 display vectors induced higher binding and nAb responses in mice, supporting the notion that the inclusion of multiple L2 epitopes within one vaccine results in an enhanced antibody immune response [75, 79]. The degree of *in vivo* HPV cross-protection remains to be determined for the 9-valent or 4-valent/5-valent HAdV35 pIX-L2 vectors. However, it should be noted that in contrast to the currently described L2-based vaccines [80-84], the HAdV35 pIX-L2 vectors induced potent cross-reactive nAb responses without an adjuvant. These findings strongly suggest that the pIX-L2 display platform may offer the opportunity for an improved multivalent prophylactic HPV vaccine.

The majority of replication-incompetent AdV vectors currently in clinical development are monovalent encoding a single antigen in the E1-region [33, 85-88]. For potent immune responses against infectious diseases more than one antigen per AdV vector may be required [89-91]. For this reason, the work presented in **Chapter 6** focused on modifying the AdV genome to encode two antigens (i.e. bivalent AdV vectors) [92]. Many bivalent AdV vector approaches are described in the literature, such as two antigens in the E1 region using different genetic regulatory elements, E1- combined with E3-region or E1- combined with the E4-region insertions [92]. An evaluation of different bivalent AdV vector designs using HAdV35 vector encoding different Ebola antigens was performed and compared to the respective monovalent AdV vectors [92]. In contrary to the published results, HAdV35 vectors encoding transgenes in E1 and E3 region, were genetically unstable in the producer cell line, most probably as a consequence of homologous-recombination between regions with sequence homology between the insertions [93-97]. Amongst the tested E1-based bivalent HAdV35 vectors, the E1-bidirectional promoter design showed a favorable genetic stability profile, potent antigen expression and immunogenicity in mice, comparable to the tested mix of monovalent AdV vectors. In HAdV26 the E1-bidirectional design was shown to be more unbalanced in terms of transgene expression than observed with the HAdV35 vectors. This observation suggests an AdV type-specific genome effect on

the transgene expression (e.g. AdV ITR influences on the proximal promoters [98-100]). Redesigning the surrounding sequences, for instance by placing insulators [101], or using an alternative insertion site in the AdV genome [102], may tailor the bidirectional promoter design for other AdV types. This study highlights the importance of systematic assessment of different AdV vector characteristics prior to clinical development to ensure the selection of an optimal bivalent AdV vector design. Bivalent vectors containing a bidirectional promoter can be an interesting alternative to the mix of monovalent AdV vectors for further development [92].

FUTURE PERSPECTIVE

Antigen-specific B- and T-cell immune responses can be elicited by genetically engineering antigens in the AdV capsid and/or modifying the AdV genome to encode multiple transgenes. In preclinical settings, AdV antigen-display vectors and bivalent AdV genetic vaccines have been shown to induce potent immune responses. While such vectors may be a viable alternative for the AdV prime/(adjuvanted) protein boost or mixing of several monovalent vectors, additional efforts are necessary to ensure their further advancement into human trials. For instance, characteristic such as viral titer yields, genetic stability in producer cell lines, particle stability, purification during large scale production, formulation buffers, toxicity, pre-existing immunity in humans and potency in humans should be addressed for both AdV display and bivalent vectors.

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The success of AdV display and bivalent vectors as vaccines is determined by the design choice. It is imperative to choose the right epitope, as well as the optimal capsid insertion site, the appropriate transgene expression regulatory elements, and a suitable producer cell line. Testing of several designs is vital, but such evaluations are often laborious and time-consuming. These activities could be significantly reduced by an advanced understanding of the AdV-genome, -biology, -capsid, the selected antigen and using the latest technological developments. A great conceptual design can make all the difference between a failure and a success.

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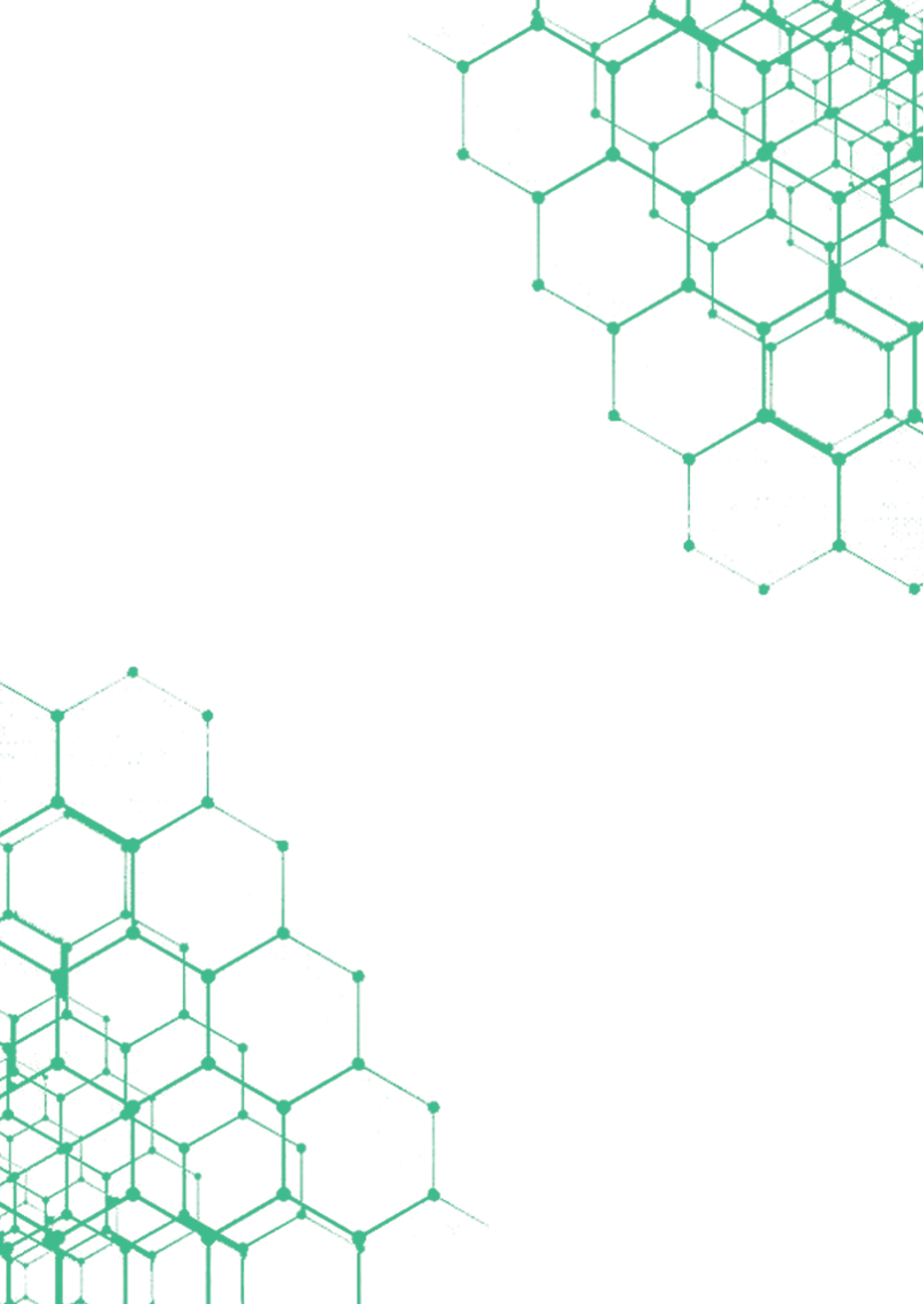
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SUMMARY

Replication-incompetent AdV vaccine vectors have a demonstrated ability to induce potent antigen-specific immune responses and an acceptable safety profile in humans. Several immunization schedules have currently been tested in clinical trials; single or multiple doses of an AdV based vaccine followed by a single or multiple doses of an alternative vector (e.g. AdV or MVA), a mixture of monovalent AdV vectors, or an AdV prime combined with (adjuvanted) protein/virus-like particle (VLP) boost. While these schedules have resulted in potent immunity with an favorable safety profile, they may be complex for general implementation after licensure.

Advances in the design of AdV vector vaccines offer several alternatives: (i) AdV vectors engineered to display an antigen on the outer capsid surface, functioning much like a VLP vaccine to induce B-cell antigen-specific immune responses (**Chapter 2-5**). Unlike a typical VLP platform, AdV antigen-display vectors induce potent immune responses without the need for an adjuvant. Importantly, AdV antigen-display vectors can be designed to genetically encode specific antigens. (ii) The AdV genome can further be engineered to genetically encode more than one antigen resulting in a 'multivalent' vector (**Chapter 6**). This thesis focused on generating AdV antigen-display and multivalent AdV vaccines against infectious diseases using the low-seroprevalent HAdV35 and HAdV26 vectors.

To generate AdV antigen-display vectors, capsid proteins hexon, fiber, penton and pIX can be engineered to present antigens/epitopes on the outer capsid surface (**Chapter 2**). Hexon protein, which makes up the majority of the AdV capsid, contains a conserved base and highly variable top with 7 hypervariable loops (HVR1-7). HVRs can be engineered to display heterologous peptides for various purposes such as anti-AdV vector immunity evasion and/or epitope display in vaccine development. Mainly HVR1 and HVR5 have been used to display ~30 aa linear epitopes from various pathogens inducing potent antigen-specific immune responses in mice. Insertion of antigens into hexon results in many surface exposed epitopes per capsid. In addition, multiple HVRs can be engineered simultaneously resulting in an even greater number of a single antigens or the option to insert multiple different pathogen epitopes per capsid. Major proteins fiber and penton are imperative for cell entry and are typically engineered for vector retargeting.

In the AdV capsid, the minor capsid protein IX is nested in the hexon cavities exposing its C-terminus tail to the outer capsid surface. Heterologous peptides can be fused to pIX to create vectors for AdV vector re-targeting or antigen-display. AdV pIX-antigen display vectors containing large antigens in the size range of 15 kDa to ~70 kDa have been generated and shown to be able to induce potent antigen-specific immune responses in mice. Since pIX allows fusion of larger antigens to its C-terminus, it offers the opportunity to select more complex (multi-epitope) antigens for display on the AdV capsid.

Combining the benefits of AdV genetic and pIX-display vaccination to induce potent B- and T-cell immune responses, in **Chapter 3** HAdV35 vectors were generated to encode a *P. falciparum* CS transgene and display a truncated version of the CS protein (CS_{short}) via

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pIX C-terminus. The accessibility of the CS_{short} on the outer capsid surface was increased by pIX fusions with or without a glycine linker (for flexibility) and/or a 45Å-spacer. B-cell immune responses induced by the pIX-CS_{short} display vectors increased with the addition of the spacer, and exceeded the immune responses induced by the HAdV35 CS genetic vector without transgene or a CS recombinant protein control. Mixing of the CS protein with (HAdV26, HAdV35, HAdV5) particles did not increase CS-specific antibody responses, suggesting no adjuvant-effect of the AdV vector particles. For potent humoral immune responses, it seems that association of the antigen to the AdV particle is necessary. AdV pIX-display vectors genetically encoding a CS transgene elicited high antigen-specific T-cell immune responses which were comparable to the genetic HAdV35 CS genetic vector, demonstrating the immune-directing potential of combining the antigen pIX-display and a genetically encoded transgene in a single vector.

pIX-display was used to generate a potent second-generation prophylactic HPV vaccine based on the highly conserved minor L2 protein (**Chapter 4 and 5**). The currently available prophylactic HPV vaccines based on the L1 VLP are highly efficacious, however due to limited cross-protection, wide HPV coverage can only be achieved by addition of L1 components, thus increasing the vaccine complexity. The highly conserved L2 protein N-terminus contains many well-defined linear cross-reactive nAb epitopes with minor sequence variation between the HPV genotypes (e.g. HPV16 vs. HPV31). To ensure broad protection across multiple clinically relevant HPV types, inclusion of linear epitopes from several clinically relevant HPV types may be necessary.

To successfully generate AdV pIX-display vectors, assessment of the optimal antigen design (e.g. size or spacers) was performed. Different L2 epitope concatemers (repeats) in a size range of 93-418 amino acids (aa) were fused to pIX and tested for capsid incorporation using HAdV35 and HAdV26 vectors (**Chapter 4**). The pIX-L2 concatemers containing epitopes from three (93 aa) or four (123 aa) HPV types were successfully incorporated into the HAdV35 or HAdV26 capsid, whereas concatemers larger than 153 aa were not. Since pIX was shown to tolerate fusion of larger polypeptides to its C-terminus, it seems that characteristics other than size (e.g. charge) may play a role in efficient capsid incorporation.

A 9-valent AdV pIX-display prophylactic HPV vaccine containing two heterologous 4-valent and 5-valent HAdV35 vectors displaying via pIX either HPV6, 31, 33, 16 or HPV11, 52/58, 45, 18 the sequences of HPV52 and HPV58 are identical in the region that was included in the vaccine L2 protein epitopes (**Chapter 5**) mixed in a single formulation was assessed. Each HAdV35 pIX-L2 display vector exhibited a good manufacturability profile, showing high titer yields in producer cell line, genetic stability and stability comparable to the non-modified AdV vector at cold-chain relevant temperatures (e.g. 2-8°C) for 1 year. Single 4-valent and 5-valent HAdV35 pIX-L2 display vaccine vectors were immunogenic in mice, inducing nAb responses against HPV-types included in the vaccine and cross-reactive nAb against distant non-vaccine HPV-types. Immunization of mice with a mix

of 4-valent and 5-valent HAdV35 pIX-L2 display vectors induced higher binding and nAb responses than when administered alone, suggesting that inclusion of multiple L2 epitopes within one vaccine may result in enhanced immune responses. In contrast to the currently described L2-based vaccines, the HAdV35 pIX-L2 vectors induced potent cross-reactive nAb responses without an adjuvant. This offers the opportunity for an improved multivalent prophylactic HPV vaccine.

Generation of a 'multivalent' AdV vector genetically encoding multiple antigens can be achieved using many different strategies such as genetic regulatory elements, E1-combined with E3-region, or E1- combined (E1-E3) with the E4-region insertions (E1-E4). A head-to-head comparison of different multivalent designs (E1-E3 and two antigens in E1 region using different gene expression regulatory elements) in HAdV35 and HAdV26 vectors was performed (**Chapter 6**). HAdV35 E1-E3 vectors were genetically instable in the producer cell line and therefore not tested further. Amongst the tested E1-based bivalent HAdV35 vectors, the E1-bidirectional promoter design was genetically stable, showed good transgene expression and potent antigen-specific immunogenicity in mice. The E1-bidirectional design was successfully transferred to HAdV26 demonstrating the use of this technology in different vectors derived from alternative AdV types. Overall, bivalent AdV vectors containing a bidirectional promoter may be used as an alternative to the mix of monovalent AdV vectors for further development.

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NEDERLANDSE SAMENVATTING

Van replicatiedeficiënte adenovirale (AdV) vaccinvectoren is bekend dat ze sterke antigeenspecifieke immuunresponsen kunnen opwekken en dat ze een acceptabel veiligheidsprofiel in mensen hebben. Voor het bereiken van optimale immuniteit zijn momenteel verschillende immunisatie schema's beschikbaar; een of meerdere doses van een AdV vaccin gevolgd door een of meerdere doses van een alternative vector (bijv. AdV of MVA), een mengsel van monovalente AdV-vectoren, en een AdV prime gecombineerd met een (geadjuveerd) eiwit/virusachtig deeltje (VLP (virus like particle)) boost. Hoewel klinische studies laten zien dat deze vaccinatieschema's uitstekende immuniteit geven zijn deze schema's mogelijk ingewikkeld voor algemene implementatie na registratie.

Door vooruitgang in het ontwerp van AdV-vectorvaccins zijn er verscheidene alternatieven: (i) AdV-vectoren kunnen genetisch worden gemodificeerd om een antigeen aan de oppervlakte van de capside te presenteren (AdV-presentatie) en daardoor – als een VLP-vaccin te functioneren en B-cel antigeenspecifieke immuunresponsen te induceren (**Hoofdstuk 2-5**). In tegenstelling tot gangbare VLP-platforms induceren AdV-presentatie vectoren sterke immuunresponsen zonder dat daarvoor toevoeging van een adjuvans nodig is. Eveneens van groot belang is het feit dat AdV-presentatie vectoren ontworpen kunnen worden die een (ander) antigeen genetisch coderen. (ii) Het AdV-genoom kan bovendien worden gemodificeerd om meerdere antigenen genetisch te coderen waardoor een 'multivalente' vector ontstaat (**Hoofdstuk 6**). Dit proefschrift richt zich op het genereren van capside oppervlakte presentatie AdV-vaccins en multivalente AdV-vaccins tegen infectieziekten door gebruik te maken van HAdV35 en HAdV26 vectoren die een lage prevalentie hebben.

Door middel van genetische modificatie van capside-eiwitten hexon, fiber, penton en pIX is het mogelijk om antigenen/epitopen op de buitenkant van de capside van AdV-vaccins te presenteren (**Hoofdstuk 2**). Het hexon eiwit, waaruit het grootste deel van de AdV-capside is opgebouwd, bestaat uit een geconserveerde basis en een zeer gevarieerde top met 7 hypervariabele lussen (HVR1-7). Deze HVR's kunnen worden gemodificeerd om heterologe peptiden voor verscheidene doeleinden te presenteren, zoals immuno-evasie van de anti-AdV-vector en/of de weergave van een epitoop bij de ontwikkeling van een vaccin. Voornamelijk HVR1 and HVR5 worden gebruikt voor de weergave van ~30 aminozuur-lange lineaire epitopen van verschillende ziektekiemen die sterke antigeenspecifieke immuunresponsen in muizen induceerden. Door de insertie van antigeen in hexon worden per capside vele epitopen aan de oppervlakte gepresenteerd. Daarnaast kunnen meerdere HVR's gelijktijdig worden gemodificeerd waardoor een nog groter aantal enkelvoudige antigenen wordt verkregen en bestaat de optie tot insertie van meerdere verschillende pathogene epitopen per capside. De grote fiber en penton eiwitten zijn een absolute vereiste voor het binnendringen van AdV in cellen en modificatie van deze eiwitten wordt meestal gebruikt voor vector retargeting.

In de groeven tussen de hexon eiwitten van de AdV-capside zit het kleine capside-eiwit IX (pIX) genesteld, waarvan de C-terminale staart aan de buitenkant van de capside

tevoorschijn komt. Heterologe peptiden kunnen worden gefuseerd met het pIX om vectoren te creëren voor AdV vector retargeting of capside oppervlakte presentatie AdV-vaccins. Er zijn pIX-antigeen capside oppervlakte presentatie AdV-vaccins gegenereerd met antigenen met een grootte van 15 kDa tot ~70 kDa en er is aangetoond dat zulke vectoren sterke antigeenspecifieke immuunresponsen kunnen oppwekken in muizen. Aangezien het mogelijk is om grotere antigenen te fuseren aan de C-terminus van pIX, biedt dit de mogelijkheid meer complexe (multi-epitope) antigenen te presenteren op de AdV-capside.

Om de voordelen van AdV-genetische- en pIX-antigeen capside oppervlakte presentatie AdV vaccinatie te koppelen en zodoende krachtige B-cel- en T-cel- immuunresponsen te induceren, zijn in **Hoofdstuk 3** HAdV35 vectoren gegenereed die een *Plasmodium falciparum* CS-transgen coderen en een ingekorte versie van het CS-eiwit (CS$_{short}$) presenteren wat aan de C-terminus van pIX tot expressie komt als een fusie-eiwit. Om de toegankelijkheid van CS$_{short}$ op de buitenkant van de capside te vergroten zijn pIX-fusies met of zonder glycine-linker (voor flexibiliteit) en/of een 45Å-spacer gegenereerd. Inderdaad namen de door de vectoren geïnduceerde B-cel immuunresponsen toe door toevoeging van de spacer, en waren deze sterker dan die geïnduceerd door de HAdV35 CS vector zonder transgen en CS-recombinant eiwit. Mengen van het CS-eiwit met (HAdV26, HAdV35, HAdV5) deeltjes deed de CS-specifieke antilichamen responsen niet toenemen, hetgeen suggereert dat er geen sprake is van een adjuvans-effect van de AdV-vector deeltjes. Om sterke humorale immuunresponsen te verkrijgen, lijkt verbinding van het antigeen met het AdV-deeltje noodzakelijk te zijn. pIX-antigeen capside oppervlakte presentatie AdV vectoren die een CS-transgen coderen, wekten sterke antigeen-specifieke T-cel immuunresponsen op, vergelijkbaar met de genetische HAdV35-CS vector, waarmee is aangetoond dat koppeling van de antigeen aan de capside oppervlakte en een genetisch geëncodeerd transgen in een enkelvoudige vector mogelijk is.

Vervolgens hebben we pIX-antigeen capside oppervlakte presentatie technologie gebruikt om een tweede-generatie profylactisch humaan papillomavirus (HPV) vaccin te genereren, gebaseerd op het zeer geconserveerde kleine L2-eiwit (**Hoofdstuk 4 en 5**). De huidig beschikbare HPV-vaccins gebaseerd op de L1 VLP zijn bijzonder doeltreffend, maar wegens beperkte kruisbescherming kan een brede HPV-dekking slechts bereikt worden door toevoeging van de L1-component van ieder afzonderlijke HPV-genotype, waarmee de complexiteit van het vaccin toeneemt. De zeer geconserveerde N-terminus van het L2 eiwit bevat vele goed gedefinieerde lineaire epitopen van kruisreactieve neutraliserende antilichamen (nAb), met geringe sequentievariatie tussen de HPV-genotypen (bijv. HPV16 vs. HPV31). Om zeker te zijn van een brede bescherming tegen meerdere klinisch relevante HPV-typen zou inclusie van lineaire epitopen van verscheidene klinisch relevante HPV-typen noodzakelijk kunnen zijn.

Om successvol pIX-antigeen capside oppervlakte presentatie AdV vectoren te genereren, hebben we een studie gedaan naar het optimale ontwerp van het antigeen (bijv. wat betreft grootte en het al of niet gebruiken van spacers). Verschillende L2-epitope

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concatameren (herhalingen), variërend in grootte van 93 tot 418 aminozuren (a.a.), zijn gefuseerd aan pIX en getest op capside-incorporatie in HAdV35- en HAdV26-vectoren (**Hoofdstuk 4**). De pIX-L2-concatameren met epitopen van drie (93 a.a.) of vier (123 a.a.) HPV-typen erin konden succesvol in de HAdV35- en HAdV26-capsides geïncorporeerd worden terwijl dit niet het geval was voor concatameren die groter waren dan 153 a.a. Aangezien eerder grotere popuppeptiden gefuseerd aan de C-terminus van pIX succesvol zijn geïncorporeerd, lijkt dit erop te duiden dat, naast grootte, ook andere eigenschappen (bijv. lading) een rol kunnen spelen bij een efficiente opname in de capside.

Door heterologe 4-valente en 5-valente HAdV35 vectoren die middels pIX-antigeen capside oppervlakte presentatie AdV de L2-epitopen van respectievelijk HPV6, 31, 33 en 16, en van HPV11, 52/58, 45, 18 (HPV52 en HPV58 hebben identieke sequenties in het gedeelte dat in het vaccin is opgenomen) presenteren met elkaar te mengen hebben we een profylactisch 9-valent pIX-L2 capside oppervlakte presentatie AdV HPV vaccin gemaakt (**Hoofdstuk 5**). Beide vectoren konden efficient geproduceerd worden met een hoge titer opbrengst in de producerende cellijn. Daarnaast konden genetische stabiliteit en een vergelijkbare stabiliteit met een niet-gemodificeerde AdV-vector worden vastgesteld bij een 'Cold Chain' temperatuur van 2-8°C gedurende 1 jaar. Enkelvoudige 4-valente en 5-valente pIX-L2 capside oppervlakte presentatie HAdV35 vaccin vectoren waren immunogeen in muizen waarbij nAb-responsen tegen in het vaccin opgenomen HPV-typen en kruisreactieve nAb-responsen tegen niet in het vaccin opgenomen HPV-typen werden geïnduceerd. Immunisatie met een combinatie van 4-valente en 5-valente pIX-L2 capside oppervlakte presentatie HAdV35 vectoren induceerde hogere titers van bindende en van neutraliserende antistoffen, hetgeen erop lijkt te duiden dat inclusie van meerdere L2-epitopen in één vaccin tot sterkere immuunresponsen kan leiden. In tegenstelling tot de tot op heden beschreven vaccins die gebaseerd zijn op L2 induceerden de HAdV35 vectoren met pIX-L2 capside op de oppervlakte sterke kruisreactieve nAb-responsen zonder toevoeging van een adjuvans. Deze technology biedt de mogelijkheid voor een verbeterd multivalent profylactisch HPV-vaccin.

Voor het genereren van een 'multivalente' AdV-vector die meerdere antigenen genetisch codeert, zijn er vele verschillende benaderingen mogelijk, zoals middels modificatie van genetische regulatoire elementen, E1- gecombineerd met E3-regio of E1-gecombineerd (E1-E3) met de E4-regio inserties (E1-E4). Er is een directe vergelijking gemaakt tussen verschillende multivalente ontwerpen (E1-E3 en twee antigenen in de E1-regio met verschillende genexpressie regelende elementen) - in HAdV35- en HAdV26-vectoren (**Hoofdstuk 6**). HAdV35 E1-E3-vectoren waren genetisch instabiel in de productie cellijn en zijn om deze reden niet verder getest. Onder de geteste op E1-gebaseerde bivalente HAdV35-vectoren bleek het E1-bidirectionele promoter design genetisch stabiel en vertoonde goede transgen expressie en krachtige antigeenspecifieke immunogeniciteit in muizen. Het E1-bidirectionele design is met succes toegepast in HAdV26, waarmee de mogelijkheid van het gebruik van deze technologie in verschillende van andere AdV-typen

afkomstige vectoren is aangetoond. Over het geheel genomen kunnen bivalente AdV-vectoren met een bidirectionele promoter verder ontwikkeld worden als een alternatief voor de combinatie van monovalente AdV-vectoren.

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"As I review the events of my past life, I realize how subtle are the influences that shape our destinies."

Никола Тесла (Nikola Tesla), **My Inventions**

CURRICULUM VITAE

Marija Vujadinovic was born in ex-Yugoslavia, Sarajevo on the 1st of January 1984. At the age of 10 she moved to the Netherlands where she completed her high school education in Amsterdam. She enrolled in a Bachelor of Sciences, Life Sciences Molecular Cell Biology at the Hogeschool Utrecht (HU) in 2002. Research towards her Bachelor thesis was conducted at the University of Utah (UofU) Department of Pathology, United States of America (USA) under the supervision of Dr. Sherwood R. Casjens. With Dr. Casjens, Marija studied the Horizontal Gene Transfer in Lyme Disease Agent *Borrelia burgdorferi,* which yielded two co-authored publications on the subject. Marija continued as a Technician in a diagnostics laboratory at the Free University of Amsterdam Medical Center, Medical Microbiology and Infection Control led by Prof. Dr. Paul Savelkoul. In September 2007 she moved back to the USA to work as a Technician in a research laboratory supervised by Prof. Dr. Wai Mun Huang at the UofU Department of Pathology. In 2009 Marija enrolled at the Free University of Amsterdam and successfully completed two Master degrees in Life Sciences, Biomolecular Sciences & Cell Biology and Management, Policy Analysis & Entrepreneurship (MPA). Her MPA thesis studied ovarian cancer survivors and their need for psychosocial care, and was conducted at the National Cancer Institute, Antonie van Leeuwenhoek (NKI-AVL), Amsterdam Department Psychosocial Research and Epidemiology under the supervision of Dr. Marieke van Leeuwen. The Biomolecular Sciences & Cell Biology MSc thesis was completed at Crucell Holland, Vaccine Generation (now Janssen, Vaccines and Prevention, pharmaceutical companies of Johnson & Johnson) and supervised by Dr. Jort Vellinga and Dr. Taco Uil. In June 2012 Marija accepted the offer to continue her graduate studies at Janssen, Vaccine and Prevention as a University of Amsterdam Ph.D Student, with Prof. Dr. Hanneke Schuitemaker as her promoter and Dr. Jort Vellinga as co-promoter. She has performed her studies in the Vaccine Generation group and produced the thesis 'Advances in the design of adenoviral vectored vaccines' which contains research focusing on the advancement of the replication-incompetent adenoviral vaccine vectors platform. A significant part of the work has resulted in two patents filings in the field of replication-incompetent adenoviral vaccines ("An improved Adenovirus based Malaria vaccine encoding and displaying a Malaria antigen" and "HPV vaccines"). During her Ph.D, Marija participated in the Industry-Academia Partnerships and Pathways, ADenovirus VECtor Technology (AD-VEC) Next Generation Systems for Medical Therapy collaboration in 2016. As a representative from Janssen she completed a three-month secondment at the Umea University, Sweden Department Clinical Microbiology – Virology Unit led by Dr. Niklas Arnberg. Together with Dr. Lijo John, Marija has focused on elucidating the receptor usage of a novel adenoviral vector candidate. Since 2017, Marija has been working as a Scientist at Janssen, Vaccines and Prevention - Viral Vaccines, Vaccine generation group where she studies the manufacturing processes for candidate adenoviral vaccines.

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PH.D. PORTFOLIO
AMC GRADUATE SCHOOL FOR MEDICAL SCIENCES

Summary of Ph.D. Training, Teaching and Publications

Ph.D. Candidate: Marija Vujadinovic

Period: June 2012 – December 2019

Ph.D. Supervisors: Prof. Dr. H. Schuitemaker and Dr. J. Vellinga

	Year	Hours	ECTS
Ph.D. Training			
Courses			
Advanced Immunology	2013	80	2.9
ADVance Training Event Academia meets Industry	2013	56	2
Laboratory Animals (Article 9)	2014	110	3.9
VacTrain The Future in Vaccinology	2014	40	1.4
Cell culture-based viral vaccines	2017	24	0.9
Seminars and Meetings			
Viral Vaccines meetings (Group, Scientific, Review Meetings etc.)	2012-18	60	2.1
Inspired by Synthesis of Evidence ZonMw/NWO Den Haag	2014	8.5	0.3
Conferences			
European Society of Gene and Cell Therapy (ESGCT) Versailles, France	2012	32	1.1
American Society of Gene and Cell Therapy (ASGCT) Salt Lake City, USA	2013	32	1.1
EMBO Modern DNA concepts and tools for safe gene transfer and modification Evry, France	2015	40	1.4
Bioprocesse International European Summing: Vaccine Manufacturing Amsterdam, The Netherlands	2018	24	0.9
Poster Presentations			
11th Adenovirus Meeting San Diego, USA (Conference)	2014	40	1.4
Power of Viral Vectors In gene therapy and basic science Primosten, Croatia (Conference)	2014	32	1.1
Janssen R&D day	2017	8	0.4
Oral Presentations			
Vaccine R&D Baltimore, USA (Conference)	2015	24	0.9
12th Adenovirus Meeting Hannover, Germany (Conference)	2016	40	1.4
13th Adenovirus Meeting Morelos, Mexico (Conference)	2018	40	1.4

Ph.D. Portfolio (continued)

	Year	Hours	ECTS
Teaching and Supervision			
Internship Master Student Drug Discovery and Safety Free University of Amsterdam, Esmee van Geffen (8 months)	2013-14	160	5.7
Thesis Master Student Drug Discovery and Safety Free University of Amsterdam, Esmee van Geffen (3 months)	2014	60	2.1
Traineeship MSc Hanna Visser (6 months)	2015	120	4.3
Internship Master Student Biomolecular Sciences Free University of Amsterdam, Sarah Jane Remak (11 months)	2015	220	7.6
Internship Medical Biotechnology Student Wageningen University, Marloes Grobben (7 months)	2016-17	140	5

List of Publications	Year
Progress in Adenoviral Capsid-Display vaccines. *M. Vujadinovic* and J. Vellinga *Published in Biomedicines*	2018
Adenovirus based HPV L2 vaccine induces broad cross-reactive humoral immune responses. *Marija Vujadinovic*, Selina Khan, Koen Oosterhuis, Taco G. Uil, Kerstin Wunderlich, Sarra Damman, Satish Boedhoe, Annemiek Verwilligen, Jonathan Knibbe, Jan Serroyen, Hanneke Schuitemaker, Roland Zahn, Gert Scheper, Jerome Custers, Jort Vellinga *Published in Vaccine* (*Patent filed*)	2018
Adenoviral type 35 and 26 vectors with a bidirectional expression cassette in the E1 region show an improved genetic stability profile and potent transgene-specific immune response. *M. Vujadinovic* & K. Wunderlich, B. Callendret, M. Koning, M. Vermeulen, BP. Sanders, E. van der Helm, A. Gecgel, D. Spek, K. de Boer, M. Stalknecht, J. Serroyen, M. Grazia Pau, H. Schuitemaker, R. Zahn, J. Custers, J. Vellinga *Published in HGT* (*Patent filed*)	2018
Antigen capsid-display on human adenovirus 35 via piX fusion is a potent vaccine platform. N.C. Salisch & *M. Vujadinovic*, E. van der Helm, D. Spek, L. Vorthoren, J. Serroyen, H. Kuipers, H. Schuitemaker, R. Zahn, J. Custers, J. Vellinga *Published in PloS ONE* (*Patent filed*)	2017
Plasmid diversity and phylogenetic consistency in the Lyme disease agent Borrelia burgdorferi. S. R. Casjens, E.B. Gilcrease, *M. Vujadinovic*, E.F. Mongodin, B.J. Luft, S.E. Schutzer, C.M. Fraser and W. Qiu	2017
Genome Stability of Lyme Disease Spirochetes: Comparative Genomics of Borrelia burgdorferi Plasmids. S. R. Casjens, E. F. Mongodin, W. Qiu, B.J. Luft, S.E. Schutzer, E.B. Gilcrease, W.Huang, *M. Vujadinovic*, J.K. Aron, L.C. Vargas, S. Freeman, D. Radune, J.F. Weidman, G. I. Dimitrov, H.M. Khouri, J.E. Sosa, R.A. Halpin, J.J. Dunn, C.M. Fraser	2012

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AUTHOR AFFILIATIONS

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