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Chapter 5

Viral Vectors for Vaccine Development

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Additional information is available at the end of the chapter

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1. Introduction

Recombinant vectors can be used to deliver antigens and to stimulate immune responses in humans. Viral vectors possess various intrinsic properties which may lend to advantages and disadvantages for usage for a given therapeutic application [reviewed by Larocca and Schlom] [1]. The safety and flexibility of recombinant viral vectors have lead to their usage in gene therapy, virotherapy, and vaccine applications. In this chapter, we will discuss the utility and importance of recombinant vectors as vaccine agents. This chapter will highlight some of the uses of recombinant viral vectors for therapeutic vaccines; and will mostly focus on the application of a range of recombinant viral vectors for prophylactic vaccines against infectious agents. More specifically, this chapter will focus in depth on the use of recombinant adenovirus (Ad) for vaccine development against infectious agents.

2. Gene therapy vectors and oncolytic vectors

Viruses can be used as gene delivery tools for a variety of diseases and conditions [1]. Most viruses are naturally immunogenic and can be engineered to express genes that modulate the immune system or express tumor antigen transgenes. Human Ad vectors have been widely used as vehicles for gene therapy [2]. Replication-defective Ads were the first vectors to be evaluated for *in vivo* gene transfer in a wide variety of preclinical models. For instance, Stratford-Perricaudet, and group reported efficient, long-term *in vivo* gene transfer throughout mouse skeletal and cardiac muscles after intravenous administration of a recombinant Ad vector [3]. This study focused on the transfer of the report gene, *β-galactosidase*; however, studies similar to this lead to the delivery of therapeutic genes by means of recombinant vectors. Routinely, viral vectors such as human Ad vectors have been engineered to express

[4–6] or display [7–9] herpes simplex virus type 1 thymidine kinase which kills proliferating tumor cells in the presence of the prodrug gancyclovir. This strategy is commonly referred to as cancer gene therapy. Concerning non-cancerous diseases, viral vectors have been utilized to deliver genes to a multitude of cells types ranging from dental cells [10], islets cells [11], and many other cell types. In these instances, viral vectors transduce cells to deliver genes which may lead to an overexpression or knock down of protein, leading to a corrective phenomenon or destruction of damaged cells.

Viruses can also be used as oncolytic agents. Oncolytic viruses which have been identified or engineered belong to several viral families. They include herpes simplex viruses, adenovirus, retroviruses, paramyxoviruses, and poxviruses [12]. These viruses can be categorized into four major groups on the basis of their oncolytic restriction: (1) mutation/deletion derived viruses, (2) transcriptionally targeted oncolytic viruses, (3) transductionally targeted oncolytic vectors, and (4) "naturally smart" viruses [13]. Oncolytic viruses for cancer exploit the difference of the molecular makeup between the tumor cells and their normal counterparts; they also utilize recombinant DNA technology to engineer viral vectors to selectively replicate in the tumor cells and destroy them. Conditionally replicative Ads (CRAds) [14,15], measles virus [16,17], herpes simplex virus [18], and vesicular stomatitis virus [reviewed in [1]], have been shown to preferentially infect and propagate in tumor cells. It has been demonstrated that these vectors not only have direct cytopathic effect on tumor cells but in addition, these oncolytic vectors are likely to enhance immune-mediated killing of tumor cells likely through the release of tumor antigens. Tumor antigens have been demonstrated to be more immunogenic when delivered as transgenes in a viral vector, compared with employing tumor antigens used as a peptide or protein vaccine [19,20].

However, some oncolytic vectors are unlikely to be used as cancer vaccines due to the short duration of transgene expression in infected cells given the onset of lysis; this might limit their ability to elicit a robust immune response against the transgene. Many types of recombinant vectors can infect antigen presenting cells (APC), specifically dendritic cells (DCs). Once engineered recombinant vectors infect APCs and then express antigens or transgenes which are then presented to the immune system [21–26]. However, some oncolytic vectors have a limited tropism for DCs. Ads or CRAds do not infect DCs well due to the fact that DCs possess limited expression of the primary Ad5 docking receptor, Cosxackie-Adenovirus Receptor (CAR). Naturally, DCs are virtually resistant to Ad5 infection, presenting a challenge for effective transduction of DCs by Ad [27]. Direct *in vivo* administration of untargeted Ad5 may result in cytopathic effects due to ectopic gene transfer to CAR expressing bystander cells rather than DCs. Moreover, additional antigen presentation by these transduced non-processional APCs may lead to suboptimal T cell activation, or even tolerance induction [28]. Despite these caveats, DCs are key orchestrators of the adaptive immune system. DCs have an exceptional ability to capture, process, and present antigens to activate naïve T cells. DCs have the ability to regulate the nature of the T cell response by providing appropriate co-stimulatory signals that dictate immunogenic or tolerogenic T cell stimulation. These unique features make targeted manipulation of DCs an attractive approach for modulating immune response against

cancer [29]. Therapeutic strategies related to DCs, cancer vaccination, and oncolytic vectors are summarized in the following reviews [17,29–31].

3. Viral vectors for vaccine development

Each viral vector has its own distinct advantage and disadvantage. The most extensively studied viral vectors are from the poxviridae family. They include derivatives of vaccinia virus from the orthopoxvirus genera, and members of the avipoxvirus genera, such as fowlpox and canarypox (ALVAC). Poxviruses have a long and successful track record related to vaccination. In particular, vaccinia virus was used to vaccinate over one billion people against smallpox, leading to the eradication of the disease in 1978. Poxviruses are double stranded DNA viruses with a linear genome. Poxviruses have the ability to accept large inserts of foreign DNA, and therefore can accommodate multiple genes. Vaccinia virus has a genome of ~190 kilobases (kb), which encodes for ~250 genes [32]. Fowlpox viruses have a ~260 kb to 309 kb genome with approximately 260 recognized genes. Attenuated canarypox strain ALVAC has an approximate 330 kb genome with ~320 putative genes [33].

Viral replication and transcription of the poxvirus genome is limited to the cytoplasm of the host cell. This extranuclear replication eliminates the risk of insertional mutagenesis, the random insertion of viral genetic sequences into host cell genomic DNA [34]. Vaccinia virus infects mammalian cells, and expresses recombinant genes for about 7 days before the infected cell are eliminated by the immune system [21]. Avipoxviruses infect mammalian cells and express their transgenes for 14 to 21 days [35,36]. Despite the attractive features of poxviruses, replication competent viruses like vaccinia should not be administered to severely immunocompromised patients. To circumvent this problem, an attenuated vaccinia virus called modified vaccinia virus Ankara (MVA) was developed for high-risk individuals. MVA was developed by over 500 serial passages of a smallpox vaccine from Ankara, Turkey, in chicken embryo fibroblasts (CEF). This technique resulted in over 15% loss of the vaccinia virus genome [37]. MVA can infect mammalian cells and express transgenes, but it cannot produce infectious viral particles. Similarly, fowlpox and canarypox, which are pathogenic in some avian species, are unable to productively infect humans because they cannot complete their life cycle and form infectious particles [38]. As a result, mammalian poxviruses generate a stronger immune response compared to avipoxviruses. Unfortunately, MVA and vaccinia virus vectors can only be given once or twice to vaccinia immune or vaccinia naïve patients due to the development of host neutralizing antibodies against these vectors [39]. Neutralizing antibodies (NAbs) are not developed against the avipoxvirus vectors, allowing them to be given several times to patients as booster vaccinations [40]. Similarly, alphaviruses, like avipox viruses, are also desirable vectors because infected hosts do not develop neutralizing antibodies to the vectors, allowing for multiple administrations.

In 2010, adenovirus-based vectors accounted for 23.9% of gene-therapy clinical trials [41]. The broad utility of these vectors is derived from several key characteristics: (a) the recombinant viral genome is readily manipulated; (b) replication-defective Ads can be propagated in

complementing cell lines; (c) Ads infect a broad range of target cells, [42,43] and (d) Ads can achieve high levels of *in viv*o gene transfer with concomitantly high levels of transgene expression [44]. Adenovirus is a non-enveloped double stranded DNA virus. The 36 kb genome can accommodate cDNA sequences of up to 7.5 kb. Replication of the adenovirus occurs in the nucleus but remains extrachromosomal, minimizing the risk associated with insertional mutagenesis. The majority of Ad vectors are replication- incompetent because of a deletion of the viral gene, E1. This limits the vectors' pathogenicity, while still allowing for humoral and cellular responses to the transgene. Most Ad vectors are E3-deleted [E1-, E3-], for the potential to have increased cloning capacity. However, retainment of the E3 gene-encoding regions within an [E1-, E3+] Ad vector would given an optimal effect related to vector characteristics. There has been some speculation that E3 gene promoters are dependent primarily upon the trans-activation capabilities of the E1 gene products. There have been various studies where the E3 region (or selected genes from E3) is re-introduced into the Ad vector under appropriate control of E1 independent promoters. These studies have shown some improvement in small animal models, including reduced humoral and CD8 T cell responses to the vector, and/or long-term transgene expression [45–47]. Oncolytic vectors have, in some cases, the E1 regions intact and, therefore, could potentially benefit from expression of these immune evasion proteins [48]. Most importantly, Ad vectors can be easily manipulated in the laboratory setting, which allows researchers to easily modify these vectors. This includes retargeting the viral tropism to infect DCs which are usually resistant to Ad infection. These properties have also led to Ads being used as molecular vaccine agents.

4. Adenoviral vectors as vaccine agents

Traditionally, Ad vaccination embodies the concept that the vector uses the host-cell machinery to express antigens that are encoded as transgenes within the viral vector, specifically within the E1 and/or the E3 regions. Cellular and humoral immune responses are generated against these antigens for a vaccine effect. Several preclinical successes have used this approach in animal model systems. In one example, an Ad serotype 5 (Ad5) vector encoding Ebola surface glycoprotein generated neutralizing antibodies and protected monkeys after a single administration of Ebola [49]. In murine models protection against malaria has also been observed using Ad vectors that express the circumsporozite antigen in *Plasmodium yoelii* [50–52] or *Plasmodium berghei [53].* Ad vectors are currently being used in clinical trials for vaccine development against tuberculosis [54], HIV [55–57], and malaria [58–60]. Ad5-based HIV and malaria vaccines were well tolerated and induced antigen-specific CD4+ T cell, CD8+ T cell, and antibody responses in volunteers [55,59,60].

However, in some instances, these conventional Ad-based vaccines have yielded suboptimal clinical results. These suboptimal results are attributed, in part, to preexisting Ad5 immunity. It is estimated that 50% to 90% of the adult population has preexisting immunity (PEI) to Ad serotype 2 (Ad2) or Ad5 [61–65]; and this Ad PEI can limit efficacy of Ad based vaccinations due to Ad clearance by the immune system. In this regard, innovative strategies have been developed to circumvent drawbacks associated with Ad5 PEI, some of these strategies include

the "antigen capsid-incorporation" strategy, vector chimeras, covalent modifications (i.e. such as polyethylene glycol, PEGylation) [66–68], and Gutless (helper-dependent) Ad vectors. Gutless vectors are devoid of the majority of viral genes. Therefore, they avoid cellular immunity to Ad viral genes and diminish liver toxicity, thus promoting long-term transgene expression [67,69,70].

5. Antigen capsid-incorporation strategies for vaccination schemes

The "antigen capsid-incorporation" or "capsid-display of antigen" strategies are currently being used to circumvent drawbacks associated with conventional transgene expression of antigens by viral vectors. Initially, the "capsid-display" strategy had been developed and utilized to present ligands [8,71,72], imaging motifs [7–9,73–75], and more recently immunomodulatory inhibitors and/or activators ligands [76–78]. More recently, the capsid-display of antigen strategy has been used to present antigens for vaccination applications. The antigen capsid-incorporation strategy embodies the incorporation of antigenic peptides within the capsid structure of viral vectors. The human rhinovirus has been utilized for HIV vaccination in the context of the antigen capsid-incorporation strategy. Researchers have constructed human rhinovirus: HIV chimeras to stimulate immunity against HIV-1 [79]. As well, researchers have designed combinatorial libraries of human rhinovirus capsid incorporated HIV-1 glycoprotein 41 (gp41) epitope, eliciting antibodies whose activity can mimic the NAb effect [80]. Commercial and clinical development of Ad-based HIV vaccines has progressed faster than the development of vector systems such as human rhinovirus because the tremendous flexibility of Ad generally exceeds that of current rhinovirus systems. For instance, since human rhinovirus is a relatively small RNA virus, the human rhinovirus platform can only display 60 copies of a single HIV-1 epitope [79]. In contrast, the Ad vector capsid platform could allow incorporation of HIV-1 epitopes into 4 structurally distinct domains including hexon [81], fiber, penton base, and protein IX (pIX), similar to the illustration depicted in Figure 1 [82,83].

Fiber, penton base, and pIX have been used for antigen capsid-incorporation strategies [84]. However, the major capsid protein hexon has been involved in the majority of antigen capsid-incorporation strategies. Hexon is the most plentiful of the capsid's structural proteins, accounting for 63% of the total protein mass [85,86]. Current hexon sequence analysis from different species revealed that, in addition to the conserved regions, there were 9 discrete hypervariable regions (HVRs). The HVRs of hexon contain serotype-specific epitopes [85,87]. The loops at the top of the HVRs are the most amenable to modification by genetic engineering. Some research groups have shown that short heterologous peptides can be incorporated within the HVRs of the hexon without affecting the virion's stability or function. Of note, a subset of these modifiable loops were exposed on the surface of the capsid [88,89]. HVRs1, 2, and 5 have been utilized respectively for peptide or antigen incorporation [84,88–94].

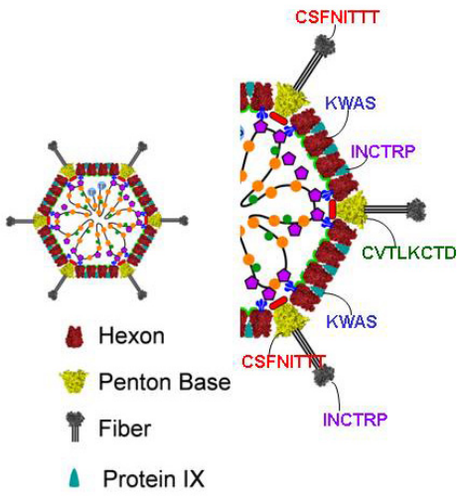

Figure 1. Antigen Capsid-Incorporation within Adenovirus Structural Proteins. Adenoviral capsid protein consists of: Hexon (II), Penton Base (III), Fiber (IV), and protein IX (pIX). Antigenic epitopes can be incorporated into these capsid structural proteins to induce antigen-specific immune responses. For example, this figure depicts the incorporation of HIV antigens from the variable region 2 (CSFNITTT), glycoprotein 41(KWAS) and glycoprotein 120 (INCTRP). This figure is adapted from Nemerow et al., 2009. Virology 384 (2009) 380–388, copyright Elsevier.

One drawback associated with conventional transgene expression of antigen is the inability of Ad-based vectors to produce a potent humoral immune response against certain antigens (as seen in the case of some malaria antigens) [95]. The antigen capsid-incorporation strategy may circumvent this drawback because this strategy embodies the incorporation of antigenic peptides within the capsid structure of viral vectors. By incorporating antigens directly into the capsid proteins, the capsid-incorporated antigen is processed through the exogenous pathway leading to strong humoral response, similar to the response generated by native Ad capsid proteins. Incorporating immunogenic peptides into the Ad capsid offers potential advantages. This strategy may allow vectors to circumvent Ad5 PEI allowing a more robust immune response to either the antigen presented on the vector capsid or the antigen that is expressed as a transgene. Additionally, because anti-Ad capsid responses are augmented by administering the vector repeatedly, immune responses against antigenic epitopes that are part of the Ad capsid should be increased by this approach as well, thus allowing boosting of the response [96–98]. This strategy may also allow for cross-priming [99,100] and activation of CD8+ T cells by means of incorporating T cell helper epitopes into the Ad capsid proteins [90]. Therefore, this antigen capsid-incorporation approach offers feasible opportunities to create Ad-based vaccine vector strategies that circumvent the major limitations associated with traditional Ad-based vaccine vectors.

Preclinically, incorporating antigens into viral capsid structures has been used as a vaccination approach for several diseases [84,90,91,93,94,101]. In 1994, Crompton and colleagues used this strategy for the first time in the context of Ad [101]. Crompton's group genetically incorporated an 8 amino acid sequence of the VP1 capsid protein of poliovirus type 3 into 2 regions of the adenovirus serotype 2 hexon. One of the chimeric vectors produced was able to grow well in tissue culture, and antiserum raised against the Ad with the polio antigen specifically recog-

nized the VP1 capsid of the polio virus [89]. More recently, similar studies have been performed by other research groups. For example, Worgall and colleagues used the antigen capsid-incorporation strategy to vaccinate against *Pseudomonas aeruginosa* (*pseudomonas*), a Gram-negative bacterium that causes respiratory tract infections in individuals who are immunocompromised or who have cystic fibrosis [102]. Because *pseudomonas* is an extracellular pathogen, anti-*pseudomonas* humoral immunity should be sufficient to provide protective immunity. Therefore, *pseudomonas* can be a candidate agent for vaccine development. Several immunogenic peptides have been identified in the outer membrane protein F (OprF) of *pseudomonas*, including the immunodominant 14-mer peptide Epi8. This study characterizes genetic incorporations of a neutralizing epitope from the *pseudomonas* Epi8 into Ad5 HVR5 (AdZ.Epi8) [90]. BALB/c mice immunized with the capsid-modified vectors showed an increase in antibody response consisting of both anti-*pseudomonas* IgG1 and IgG2a subtypes. In addition, mice immunized with the vector containing the OprF epitope were subjected to pulmonary challenge with *pseudomonas*, 60% to 80% of them survived. This group also performed additional studies where they attempted DC targeting in combination with the antigen capsid-incorporation strategy [103].

To expand on knowledge gained from previous antigen capsid-incorporation studies, our group set out to create novel vaccine vectors that would yield optimal vaccine efficacy by maximizing the size of antigens which could be incorporated within the capsid protein, hexon. Our 2008 manuscript evaluated the use of Ad5 HVR2 or HVR5 vectors containing identical antigenic epitopes in either region. To compare the capacities and flexibility of Ad5 HVR2 to those of HVR5, we genetically incorporated identical epitopes of increasing size within HVR2 or HVR5 of the Ad5 hexon. The epitopes ranged in size from 33-83 amino acids. Stable vectors were produced with incorporations of 33 amino acids plus a 12 amino acid linker at HVR2 or HVR5. In addition, stable vectors were produced with incorporations up to 53 amino acids plus a 12 amino acid linker in HVR5. With respect to the selected antigens, HVR5 was more permissive, allowing an epitope incorporation of 65 amino acids. Whole virus enzyme-linked immunosorbent assay (ELISA) analysis revealed that the model antigens were virion surface-exposed, and *in vivo* immunization with these vectors elicited antigen-specific immune responses [93].

In our most recent published study we evaluated the antigen capsid-incorporation strategy further by using novel vectors that were constructed to provide cellular and humoral HIV immunity [104]. Our study was the first of its kind to genetically incorporate an HIV antigen within the Ad5 hexon's HVR2, alone or in combination with the genomic/E1 incorporation of the HIV Gag gene (Ad5/HVR2-MPER-L15(Gag)). In this study, we incorporated a 24 amino acid epitope of HIV within Ad5 HVR2. The HIV region selected was the membrane proximal ectodomain region (MPER) derived from HIV gp41. When the MPER epitope was incorporated within HVR2 in combination with transgene incorporation, we observed growth kinetics and thermostability changes similar to those observed in other studies after using antigen capsid-incorporated vectors [7,105], indicating that incorporation of the MPER epitope within HVR2 was not substantially detrimental to vector characteristics [9,105]. In this study we demonstrated for the first time that a disease-specific antigen could be incorporated within HVR2 of

Ad5. Also, we demonstrated that the MPER epitope is surface exposed within HVR2. Most importantly, we observed a humoral anti-HIV response in mice immunized with the hexon-modified vector. Immunization with the MPER-modified vector allows boosting when compared to that of immunization with AdCMVGag vector, possibly because the Ad5/HVR2-MPER-L15(Gag) vector elicits less of an anti-Ad5 immune response. It is plausible that the MPER epitope which is incorporated within this vector reduces the immunogenicity of the Ad5 vector. This finding is notable because HVR2 has not been fully explored for its potential use in antigen capsid-incorporation strategies.

While many studies have examined the efficacy of targeting genetic vector based vaccines to DCs to enhance cellular immune responses, our group will examine a novel question. How does DC targeting affect the vector capsid antigenicity with respect to focusing humoral immune responses to finite amounts of capsid-incorporated HIV antigen? Specifically, we are interested in evaluating how DC targeting impacts the quality and potency of humoral responses generated from our capsid-incorporated antigen approach. As previously mentioned, in our 2010 manuscript, we illustrated that immunizations with Ad5/HVR2-MPER-L15(Gag) and Ad5/HVR2-MPER-L15ΔE1 yielded MPER-specific humoral responses in BALB/c mice [104]. However, we eventually plan to use the antigen-capsid incorporation system in combination with DCs activation. The C57BL/6 mouse model will allow us to better evaluate the antigen capsid-incorporation strategy in combination with DC targeting. Our initial data illustrates (data not shown) that there are substantially more DCs available for targeting in C57BL/6 mice as compared to BALB/c mice. Therefore, it was necessary to evaluate our antigen capsid-incorporation strategy in C57BL/6 mice. In brief, C57BL/6 mice were immunized with Ad5/HVR2-MPER-L15(G/L) (green fluorescent protein/ luciferase) and Ad5/HVR2-MPER-L15(Gag), respectively. 17 days later these mice were boosted in a similar manner with the same vectors. This data indicates that there is MPER-specific humoral response produced after immunizations with both vectors in C57BL/6 mice (Figure 2). In summary, we observed a similar outcome with our antigen capsid-incorporated vectors in C57BL/6 mice; therefore, we can continue with our DC targeted antigen capsid-incorporated studies. These experiments are likely to be very informative because DCs represent a unique junction for intervention by antigen-specific vaccination strategies.

With the vast diversity of many bacterial pathogens and viral pathogens, such as HIV, the need remains for vaccine vectors that yield a broad immune response. Successful HIV vaccination remains a tremendous challenge because HIV-1 vaccine strategies must contend with the enormous global sequence diversity of HIV-1. To attempt to overcome this obstacle, mosaic vectors and Ad vectors schemes that utilize "heterologous inserts" in prime-boost regimens have been developed in order to increase the breadth and depth of cellular immune responses in nonhuman primate models [106,107]. These vectors have shown promise; however, these constructs focused primarily on cellular immunity. It is likely that the most successful prophylactic HIV-1 vaccine will elicit a broad and robust cellular and humoral response. In order to create vectors that could provide a varied humoral response we generated multivalent proof-of-concept vectors. Our definition of a multivalent vector is, a vector that has the ability to vaccinate against several strains of an organism or vaccinate against

two or more distinct organisms. In this regard, our current unpublished data herein demonstrates for the first time ever that multiple antigens can be incorporated in combination at two sites within the major capsid protein, hexon (Figures 3, 4 and 5). In order to create a multivalent vaccine vector, we created vectors that display antigens within HVR1 and HVR2 or HVR1 and HVR5. Our unpublished findings focus on the generation of proof-of-concept vectors that can ultimately result in the development of multivalent vaccine vectors displaying dual antigens within the hexon of one Ad virion particle. These novel vectors utilize HVR1 as an incorporation site for a seven amino acid region (ELDKWAS) (called KWAS in this chapter) derived from HIV gp41; in combination with a six Histidine (His6) incorporation within HVR2 or HVR5. After these vectors were rescued they were designated as Ad5/H5-HVR1-KWAS-HVR2-His6 and Ad5/H5-HVR1-KWAS-HVR5-His6. In order to determine the quality of these vectors, we determined the viral particle (VP)/infectious particle (IP) ratios for the hexon-modified vectors. We compared these parameters to unmodified Ad5. Importantly, we observed similar VP/IP ratios for Ad5/H5-HVR1-KWAS-HVR2-His6 and Ad5/H5-HVR1-KWAS-HVR5-His6 as compared to Ad5 (Figure 3). These values are similar to what we observed in our previous 2008 study [93].

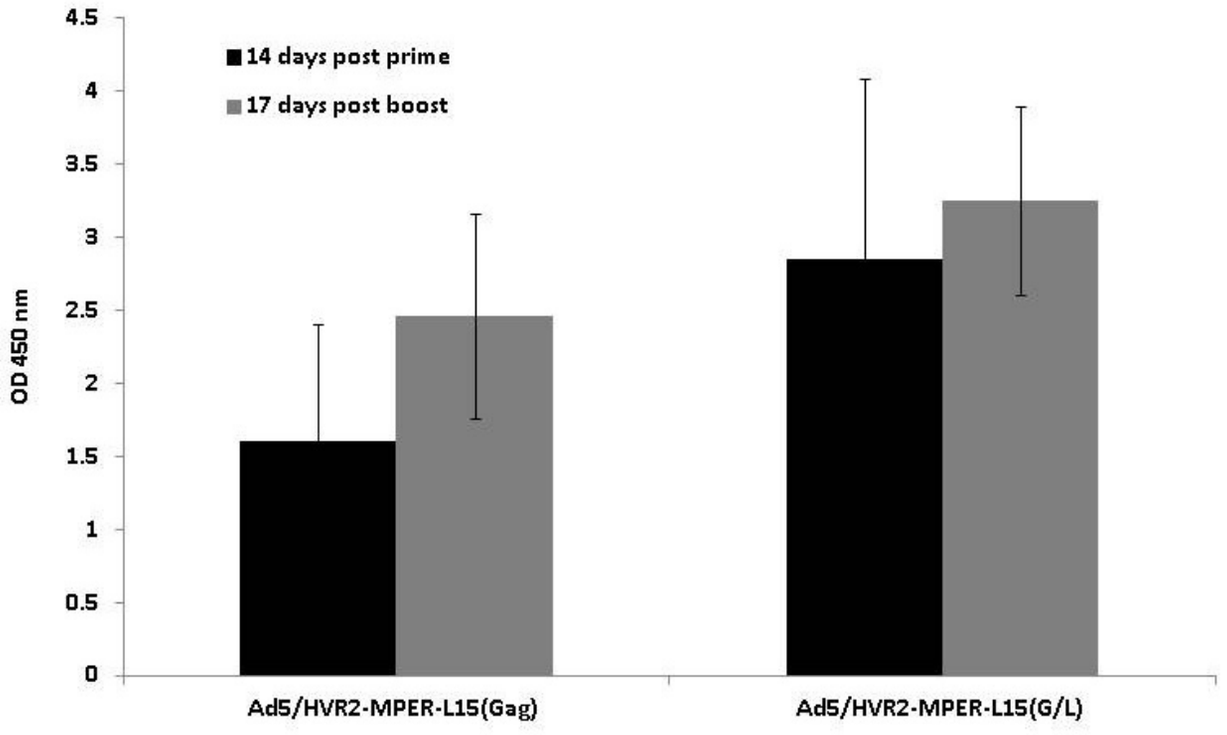

Figure 2. Adenovirus Presenting Capsid-Incorporated HIV Antigen Elicits an HIV Humoral Immune Response in C57BL/6 Mice. C57BL/6 mice (n=8) were primed and boosted with 1 x 10^{10} VP of Ad vectors. Post-prime and post-boost sera was collected at various time points for ELISA binding assays. 10 μM of purified MPER (EKNEKELLELDK-WASLWNWFDITN) antigenic peptide was bound to ELISA plates. Residual unbound peptide was washed from the plates. The plates were then incubated with immunized mice sera and the binding was detected with HRP conjugated secondary antibody. OD absorbance at 450 nm represents MPER antibody levels in sera.

Modified Vectors	Viral Particles (VP)	Infectious Particles (IP)	VP/IP
Ad5	3.1 x 10^{12} VP/ml	1.0 x10^{11} IP/ml	31
Ad5/H5-HVR1-KWAS-HVR2-His_6	2.0 X 10^{12} VP/ml	3.5 x 10^{10} IP/ml	57
Ad5/H5-HVR1-KWAS-HVR5-His_6	1.3 x 10^{11} VP/ml	1.4 x 10^{9} IP/ml	92

Figure 3. Virological Characterization of Multivalent Vaccine Vectors Displaying Dual Antigens.

After the successful rescue of the multivalent vectors we next sought to verify expression of genetic incorporations at the protein level by Western blot analysis. In order to determine if the dual hexon-modified vectors were presenting His6 tag within HVR2 or HVR5, the vectors were subjected to Western blot analysis. The His6 tag was detected as a 117 kilodalton (kDa) protein band associated with Ad5/H5-HVR1-KWAS-HVR2-His6 and Ad5/H5-HVR1-KWAS-HVR5-His6. Figure 4, lanes 2 and 3, respectively. The size of the 117 kDa band corresponds to the expected size of the Ad5 hexon protein with His6 peptide genetically incorporated into the HVR2 or HVR5 region. As expected, there was no His6 protein detected on Ad5 wild type particles (Figure 4, lane 1). Similar results were observed when these vectors were analyzed in order to verify expression of KWAS incorporations within the HVR1 locale of our dual hexon-modified vectors (Figure 5, lanes 2 and 3, respectively).

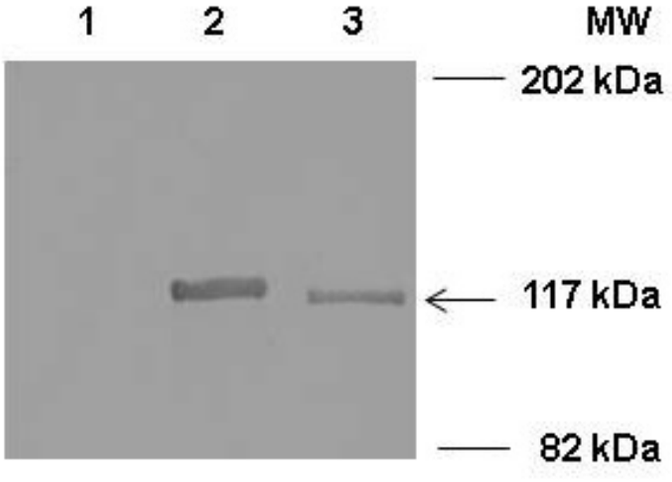

Figure 4. Western Blotting Confirms the Presence of His6 on Multivalent Vaccine Vectors Displaying Dual Antigens. Western blotting confirmed the presence of His6 incorporation within the dual modified vectors. In this assay, 1 x 10^{10} VP of Ad5 (lane 1), Ad5/H5-HVR1-KWAS-HVR2-His6 (lane 2), and Ad5/H5-HVR1-KWAS-HVR5-His6 (lane 3) were separated on 4 to 15% polyacrylamide gradient SDS-PAGE gel. The proteins were transferred to polyvinylidene fluoride membrane then blotted with anti-His antibody. The arrow indicates the His tag is genetically incorporated into the hexon protein.

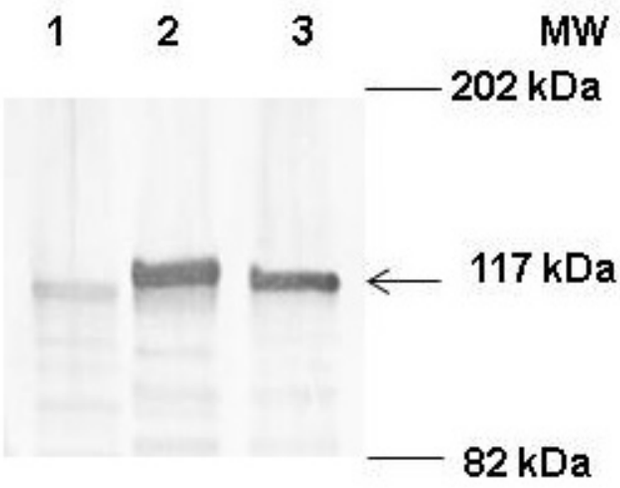

Figure 5. Western Blotting Confirms the Presence of KWAS on Multivalent Vaccine Vectors Displaying Dual Antigens. Western blotting confirmed the presence of KWAS incorporation within the dual modified vectors. In this assay, 1 x 10^{10} VP of Ad5 (lane 1), Ad5/H5-HVR1-KWAS-HVR2-His6 (lane 2), and Ad5/H5-HVR1-KWAS-HVR5-His6 (lane 3) were separated on 4 to 15% polyacrylamide gradient SDS-PAGE gel. The proteins were transferred to polyvinylidene fluoride membrane then blotted with anti-gp41 antibody (NIH AIDS Reagent Program). The arrow indicates KWAS/gp41 protein genetically incorporated into the hexon.

The size of the 117 kDa band corresponds to the expected size of the Ad5 hexon protein with KWAS peptide genetically incorporated into the HVR1 region. There was a slight KWAS protein detected on Ad5 wild type particles, we attribute this to a cross reactive sequence within the Ad vector (Figure 5, lane 1). Most importantly, Figures 4 and 5 illustrate that KWAS and His6 proteins were incorporated at comparable levels within HVR1 and HVR2 or HVR5.

We also performed a series of ELISA assays to verify that the KWAS and His6 motifs were surface accessible on the hexon double-modified virions. These results indicated that the His6 epitope was properly exposed on the virion surfaces when incorporated within HVR2 or HVR5 (data not shown). In addition, the HIV-specific ELISA also illustrated that that the HIV motif was accessible on the virion surface within the HVR1 region. Our results showed significant binding of the anti-HIV antibody to the Ad5/H5-HVR1-KWAS-HVR2-His6 and Ad5/H5-HVR1-KWAS-HVR5-His6, whereas no binding was seen in response to Ad5 control (data not shown). Currently, we are in the process of testing these vectors *in vivo*. Our initial findings lead us to believe that these vectors can have tremendous impact for multivalent vaccine development.

6. Chimeric serotype Ad vectors and rare serotype vectors for vaccine usage

In the near future, it is possible that viral vector-based vaccination will become a common clinical intervention; therefore, it has become increasingly necessary to design vectors that can overcome Ad5 pre-existing immunity [108,109]. In order to overcome Ad5 pre-existing immunity rare and non-human Ad serotypes have been used. Chimeric Ad vectors consist of

either a sub-portion of the Ad5 vector genome that is replaced with genomic portions of another alternative serotype, thus creating "chimeric" Ad vectors, or, in a more drastic approach, the entire Ad vector genome is composed of proteins solely derived from alternative Ad serotypes [27,109–114]. Ad hexon and fiber have been the proteins manipulated genetically in chimeric strategies, primarily because these proteins are known to be the target of vector neutralizing antibodies [115–118]. Several chimeric fiber and hexon strategies have been endeavored [109]. Specifically, NAbs generated against hexon HVRs account for 80-90% of the Anti-Ad NAb response. These antibodies appear to be most critical for vector clearance, therefore, diminishing therapeutic efficacy of the vaccine vector administered [119]. The importance of the seven HVRs as NAbs epitopes remains unclear as it relates to Ad5 and other serotypes [120]. Therefore, exact mapping of the NAb epitopes in these HVRs, maybe necessary to obtain improved chimeric Ad5-based vectors [121].

One of the first reports of Ad5-based chimeric vectors generated was performed in 1998. This was done by replacing Ad serotype 5 hexon gene with sequences from Ad2 [122]. This study was the launching point for other chimeric vectors, such as experiments performed in 2002 by Wu and group. They constructed a chimeric adenoviral vector, Ad5/H3, by replacing the Ad5 hexon gene with the hexon gene of serotype Ad3. The chimeric vector was successfully rescued in 293 cells. Ad5/H3 had a significantly lower growth profile as compared to Ad5/H5. Indicating that the Ad3 hexon could encapsidate the Ad5 genome, but with less efficiency than the Ad5 hexon. The gene transfer efficiency of Ad5H3 in HeLa cells was also lower than that of Ad5/H5. They also tested the host neutralization responses against the two vectors after immunizing C57BL/6 mice. The neutralizing antibodies against Ad5/H3 and Ad5/H5 generated by the immunized mice did not cross-neutralize each other in the context of *in vitro* infection of HeLa cells. Preimmunization of C57BL/6 mice with one of the two types of vectors also did not prevent subsequent infection of the other type. These data suggest that replacing the Ad5 hexon with the Ad3 hexon can circumvent the host neutralization response to Ad5 [117]. Along these same lines, another research group constructed a chimeric Ad vector, Ad3/H7. This construction was generated by replacing the Ad3 hexon gene (H3) with the hexon gene (H7) of Ad7. The chimeric vectors were successfully rescued in HEp-2 cells, and the Ad7 hexon was able to encapsidate the Ad3 genome, and functioned as efficiently as the Ad3 hexon. In addition, this group tested the host neutralization responses against the vectors using BALB/c mice. Up to 97% of the NAbs produced by mice that were infected with these vectors were specific for the hexon protein *in vitro*. Preimmunization of mice with one of Ad7 and Ad3/H7 significantly prevented subsequent intranasal infection of the other vector *in vivo*. In contrast, preimmunization of mice with either Ad3 or Ad3/H7 did not remarkably prevent subsequent infection of the other vector [123].

Roberts et. al, previously demonstrated that replacing seven of the HVRs in Ad5 with that of rare serotype, Ad48, resulted in a chimeric vector, Ad5HVR48 (1-7). Ad5HVR48 (1-7) was able to evade the majority of Ad5 pre-existing immunity in preclinical studies in mice and rhesus monkeys, [112] Ad5 chimeric vectors in which all seven HVRs were exchanged induced the same level of anti-antigen immune responses in mice with Ad5 PEI as in naïve mice. However, replacing only one HVR provided little improvement over non-chimeric Ad5 vectors. Since the role of the seven individual HVRs as NAb epitopes remains unclear, there are several

studies which are currently underway. Recent studies suggested that Ad5 responses maybe focused on one specific HVR, such as HVR1 or HVR5 [81,124]. Bradley and group attempted to answer some of these issues; in their study they characterized the contribution of the individual hexon HVRs as Ad5 NAb epitopes. They constructed chimeric Ad5 vectors in which subsets of Ad5 HVRs were exchanged for Ad48 HVRs. These partial HVR-chimeric vectors were evaluated by NAb assays and immunogenicity studies with and without baseline Ad5 immunity. Through a series of complex and thorough experiments they demonstrated that Ad5-specific NAbs are targeted against several of the HVRs. This data suggest that it is necessary to replace all HVRs to optimize evasion of Anti-Ad5 immunity [125]. Along those same lines, another group evaluated Ad5-based vectors where the hexon HVRs are replaced with that of the HVRs of rare serotypes, Ad43 and Ad34. Ad43 and 34 are group D and B viruses, each of these have low prevalence of neutralizing antibodies in humans. They demonstrated that these hexon-modified Ad vectors are not neutralized efficiently by Ad5 neutralizing antibodies *in vitro* using sera from mice, rabbits, and human volunteers. This research yielded significant findings related to malaria antigen expression, in combination with hexon-modified vectors. Their data also demonstrates that hexon-modified vectors can be highly immunogenic in the presence of Ad5 pre-existing immunity. The authors comment that these hexon-modified vectors may have useful applications in places such as sub-Saharan Africa where there is high prevalence of pre-existing Ad5 immunity [126].

Liver sequestration of Ad5-based vectors is another major drawback that hinders Ad5-based therapies. Previous studies illustrate that human coagulation factor X (FX) binds Ad5 hexon through an interaction between HVRs and the FX Gla domain leading to liver infection after systemic delivery [127,128]. The binding affinities for FX vary among vector serotypes, and may explain differences in heptaocyte transduction *in vivo* previously observed between serotypes. Although, some differences in binding affinities were noted in this report, overall, Ad2 and Ad5 bound factor X with the highest affinity, however, weak or no binding was detected with Ad9, Ad35, Ad48, and Ad51. This interaction has been observed in multiple human adenovirus serotypes and shows diversity and affinity. The domains and amino acid sequences in the HVRs are integral for high-affinity interaction with FX, however, several aspects of this binding and mechanism remain unclear [121]. In recent studies, Yu and colleagues evaluated the role of chimeric hexon HVRs with FX binding and affinity. In this study they constructed and expressed several chimeric HVR proteins and demonstrated that the native proteins were oligomers and had consistent structure and function with that of the virus. Their data demonstrated that HVR5 and HVR7 had only a fraction of hexon activity to NAbs compared to a group of HVRs, 1 through 7. In addition, they demonstrated a differential high-affinity interaction of the HVR proteins with FX and indicated that the HVRs had similar binding activity with corresponding Ad vector serotypes. This study highlighted some properties of chimeric HVR proteins and exposed the influences on the structure and function of hexon proteins and Ad vectors resulting from the incorporations of these HVRs [129].

The use of vectors derived completely from alternative human serotypes (including Ad26 and Ad35) have also shown great promise, in particular, in terms of ability to deliver transgenes [110,113,130,131]. The development of vectors based on Ads which normally infect nonhuman

species have also shown a great deal of promise. These nonhuman Ad vectors have been developed from multiple species, including, bovine, canine, chimpanzee and porcine [67]. Vectors derived from chimpanzee Ads C1 or C8 (AdC), have been recently developed, initially these vectors gained popularity since it was demonstrated that human sera does not significantly neutralize AdC vectors [132]. Importantly, unlike some other serotypes, the E1-deleted version of AdC7 is easily propagated [133]. An AdC7 vector expressing the SARS-coronavirus antigen elicited higher T-and B-cell responses than an Ad5 vector in mice with Ad5 PEI [134]. Importantly, a single injection of AdC7 encoding the Ebola glycoprotein provided protection from a lethal challenge, unlike the corresponding Ad5 vector [133].

It is essential to note that several Ad epitopes recognized by T cells are conserved among a broad range of human and nonhuman primate-derived Ads, making it possible that the T cells in patient with Ad5 PEI will also recognize vectors derived from these viruses [135–138]. Bovine Ads have been examined, since NAbs to bovine Ad3 (BAd3) have not been reported in humans. In a mouse model, a single immunization of BAd3 encoding the hemagglutination antigen of H5N1 influenza induced greater levels of cellular immunity than Ad5 vectors, and this was not diminished by Ad5 PEI [139]. It is important to note that, mice which had Ad5 PEI and received a prime-boost regimen of BAd3-Ad5 vectors encoding HA were fully protected from lethal influenza virus challenge. However, those receiving a homologous Ad5-Ad5 regimen were not. Therefore, Ad vectors that normally infect nonhuman species may induce responses and offer protection comparable or superior to Ad5, while maintaining protection in the presence of Ad5 PEI. The use of alternative serotype Ads allow for improved induction of immune responses to vector re-administration in host that have Ad5 PEI [110,113,140]. As a result of these earlier studies, alternative serotypes vectors have been tested in patient populations for HIV vaccine development [141]. In addition, human clinical trials utilizing Ad26 as a HIV vaccine agent have been initiated.

There are benefits to using alternative serotype vectors, however, the use of alternative serotypes vectors can also have limitations as well as potential side effects for human use. One limitation of alternative serotype usage is that, some alternative serotypes do not afford the same benefits of Ad5 because they are unable to induce high levels of transgene expression and are less amenable to large scale purification [108]. Humans have evolved with previous exposure to human Ad vectors, and have not been exposed to Ads derived from other species. Consequently, it may be predicted that the human innate immune system may react to the capsid proteins of alternative serotype Ads in a way that is different from that of human Ad vectors. It is also possible that the human immune system may have a response which is more robust when challenged with alternative serotypes as compared with human serotypes Ads. Recently, it has been demonstrated that the innate immune response to capsid proteins of alternative serotypes Ads have not only been shown to be significantly more robust as compared to Ad5, but in some cases toxic in animal models [110,142–144]. Alternative serotype vectors have different tropism than Ad5; therefore the biodistribution of these vectors could be quite different than that of Ad5-based vectors. Ad5 vectors have been proven to be safe in humans and animals over the last decade and the knowledge gained from this experimentation must be applied and tested as it relates to alternative serotype vectors.

7. Conclusion

Recombinant viral vectors have been utilized as therapeutic agents to prevent or cure disease because of their tremendous capacity to deliver antigens and to stimulate immune responses in humans. Viral vectors are generally more immunogenic than antigen administered adjuvant [19,145]. In addition, it is easier to generate recombinant vectors as compared to tumor cell or DC-based vaccines. Tumor cell or DC-based vaccines can be complex to acquire. They are often time consuming to produce and costly because they are customized treatments. Whereas, on the other hand, recombinant vectors are thought to be "off the shelf" treatments because they are relatively easy to produce, purify, and store. One major advantage of utilizing viral vectors for vaccination in multi-center clinical trials is the relatively low cost of vector production. However, the paramount factor to overcome when using viral vectors for gene therapy, virotherapy, and vaccine applications is the development of host-induced neutralizing antibodies to the vector itself which limits continued usage.

The past few years have bought forward exciting technical advances, along with critical structure/function analyses of viral vectors which have allowed for better understanding of the interaction of recombinant vector and host immune systems. It has become increasingly more obvious that there are many factors which must be evaluated to optimize each specific vaccine. In order to achieve optimal therapeutic outcomes when treating patients with vector PEI, careful consideration must be given to determine prime-boost schemes, epitope-capsid incorporation (monovalent versus polyvalent), transgene selection (homologous versus heterologous), vector dosing, and serotype selection.

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