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# Adenoviral Vector Vaccines Antigen Transgene

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## 1. Introduction

Vaccines save millions of human lives. They increase the lifespan of our pets and reduce the loss of livestock. Traditionally, vaccines were based on attenuated or inactivated pathogens, or in some cases bacterial toxins. Advances in molecular biology now allow for the generation of subunit vaccines that express one or a few of the pathogens' antigens that induce protective immune responses. Most viral and bacterial infections can be prevented by sufficiently high titers of neutralizing antibodies, which are elicited by viral surface antigens, such as the glycoprotein of rabies virus, the hemagglutinin of influenza virus, or the envelope protein of human immunodeficiency virus (HIV). For other pathogens such as those that are highly variable and thereby escape neutralization, cellular immune responses in form of CD8<sup>+</sup> T cells may need to be elicited to prevent clinically symptomatic infections. Although unlike neutralizing antibodies CD8<sup>+</sup> T cells fail to provide sterilizing immunity, they can rapidly clear infected cells and thereby block the spread of pathogens and ensuing disease.

CD8<sup>+</sup> T cells come in different varieties.<sup>1</sup> Upon activation, CD8<sup>+</sup> T cells expand and assume effector functions. Most effector cells, which in general produce the antiviral cytokine interferon (IFN)-gamma and lytic enzymes such as perforin and granzyme B that allow for lysis of infected cells, die once the infection is cleared. A fraction differentiates into effector or central memory cells. Effector memory cells, which are longer-lived than effector cells although their numbers gradually decline, circulate through the periphery. They can rapidly assume effector functions although their proliferative capacity is limited. Central memory cells reside in lymphatic tissues. They require reactivation and expansion before they commence effector functions. Unlike effector or effector memory cells, their proliferative capacity is high and they persist through cytokine-driven cell renewal for the lifespan of an individual. Central memory cells in general produce interleukin (IL)-2 but not IFN-gamma or lytic enzymes. Which type of CD8<sup>+</sup> T cell response is most suited to protect against an infection remains debated and most likely depends on the type of invading pathogen. For example, infections of mice with lymphocytic choriomeningitis virus can be prevented by central memory CD8<sup>+</sup> T cells,<sup>2</sup> whereas chronic infections of rhesus macaques with simian immunodeficiency virus (SIV) can be averted by effector memory CD8<sup>+</sup> T cells.<sup>3</sup> The nature of vaccine-induced immune responses is largely dictated by the type of vaccine carrier. Protein vaccines and most viral vector vaccines, with the exception of vectors based on cytomegaloviruses (CMV), can induce antibodies including

neutralizing antibodies to foreign antigens. Viral vectors that are rapidly cleared, such as those based on poxviruses or most ribonucleic acid (RNA) viruses, for example, recombinant influenza or rabies virus vectors, induce sustained central memory CD8<sup>+</sup> T cell responses. Vectors that persist, such as those derived from CMV or adenoviruses, maintain more activated effector or effector memory CD8<sup>+</sup> T cell responses.<sup>4,5</sup> Adenoviral vectors persist at low levels and therefore, unlike CMV vectors, induce both sustained effector-like and central memory T cell responses.

This chapter described some basic characteristics of adenoviruses and vectors based on adenoviruses and their performance as vaccine carriers. Vaccine carriers based on adenoviruses can be designed to retain the ability to replicate or they can be rendered replication-defective upon deletion of gene segments needed for viral reproduction. Numerous serotypes of adenoviruses have been isolated from humans, nonhuman primates, and other species, which allow for generation of vectors that can be used sequentially for prime-boosting or for immunizations against different pathogens. Preexisting neutralizing antibodies to adenoviruses, which are commonly found in humans to many of the human serotypes, can impair the immunogenicity of adenoviral vectors; this can be circumvented by using vaccines based on rare human serotypes or adenoviruses isolated from other species such as chimpanzees. Unless used at excessively high doses, adenoviral vectors are well tolerated. They induce exceptionally potent and sustained B and CD8<sup>+</sup> T cell responses to foreign antigens encoded by a transgene. Adenoviral vectors thus provide a highly suitable platform as vaccine carriers for numerous pathogens.

## 2. Characteristics of Adenoviruses

Adenoviruses are members of the Adenoviridae family. They are nonenveloped viruses with a double-stranded genome ranging from 25 to 48 kilobases (kb). Adenoviruses infect numerous species, including mammals, birds, and even frogs. They are subdivided into five genera depending on their target species.<sup>6</sup> Human adenoviruses, which belong to the genus *Mastadenovirus*, are divided into seven families (A–G) and then further into 57 distinct serotypes (HAdV-1–57). Chimpanzee adenoviruses, which have been vectored for gene transfer, are grouped within human adenoviruses.

The adenovirus genome encodes from both deoxyribonucleic acid (DNA) strands a number of early and late gene products. Products of three of the four early domains, that is, E1, E2, and E4, are essential for viral replication, whereas products of E3, which are antiapoptotic or allow the virus to escape immunosurveillance,<sup>7</sup> are non-essential. The late genes L1–L5 encode the icosahedral viral capsid composed of the major proteins hexon, penton, and fiber and minor proteins IIIa, VI, VIII, and IX. Hexon, the most abundant viral protein, has a conserved stalk and a head domain with several highly variable loops, which serve as targets of virus-neutralizing antibodies.<sup>8,9</sup> Different serotypes of adenoviruses of the same family mainly differ in the sequence of these variable loops.<sup>10,11</sup> Fiber binds the viral receptor, which for most adenoviruses is the Coxsackie adenovirus receptor (CAR<sup>12,13</sup>), whereas others bind to CD46.<sup>14</sup> Coxsackie adenovirus receptor, a type I membrane protein, is expressed on

endothelial and epithelial cells; it is not expressed on antigen-presenting cells such as dendritic cells or macrophages. CD46, an inhibitory complement receptor, is ubiquitously expressed. It also serves as receptor for measles virus<sup>15</sup> and human herpes virus-6.<sup>16</sup> Penton, which anchors fiber into the viral capsid, contains an arginyl-glycyl-aspartic acid (RGD) sequence, which binds to integrins and together with CAR facilitates the virus' entry into its target cells.<sup>17</sup>

Adenoviruses spread mainly by aerosols. Most cause upper respiratory diseases, conjunctivitis, tonsillitis, and ear infections. Adenovirus 40 and 41 infections are associated with gastroenteritis. In general, adenoviruses cause mild disease although severe and even fatal infections can occur in immunocompromised individuals, and on rare occasions in healthy individuals. After infection, humans shed adenoviruses in the feces for a few days whereas nonhuman primates such as chimpanzees shed viruses for very long periods of time. Adenoviruses persist at low levels in activated T cells; they can be recovered for years after infection from lymphatic tissues.<sup>18</sup>

A live attenuated vaccine is available for human adenoviruses 4 and 7; it is used by the United States (US) Army for immunization of recruits.<sup>19</sup>

### 3. Characteristics and Construction of Adenovirus Vectors

Adenoviruses have been constructed as replication-competent or replication-defective vectors. Replication-competent vectors used in a nontarget species are in fact replication-defective whereas E1-deleted replication-defective vectors can replicate to some degree in cells with transcription machineries that substitute for the deleted E1 gene products.<sup>20</sup> Foreign sequences of up to 1.8 kb can be incorporated into the adenovirus genome with no deletions. Incorporation of longer sequences interferes with packaging and viral rescue. The packaging size can be increased by deletion of the E3 domain. E3 only-deleted adenovirus vectors remain replication-competent and such vectors are used as vaccine carriers. Deletions of E1, E2, or E4 render the vector replication-defective and the deleted gene products have to be provided in trans during vector production. Most vaccine vectors used to date are deleted in E1, which allows for insertion of approximately 4 kb of foreign sequences. Some are also deleted in E3, which increases the packaging capacity to about 7.5 kb. Further deletions of E4, which encodes polypeptides that affect host cell proliferation and survival and provide for nuclear export of RNA, have been explored. Additional deletion of E4 reduces the synthesis of adenoviral proteins; this in turn may reduce the stimulation of T cell response to adenoviral antigens.<sup>21</sup> Originally, E1-deleted adenovirus vectors were constructed by homologous recombination in a packaging cell line that provides E1 in trans.<sup>22</sup> Currently, adenoviruses are constructed from viral molecular clones by ligating a transgene expression cassette directly into the deleted E1 domain.<sup>23</sup> The bacterial clone upon linearization is then transfected into the packaging cell line for viral rescue. Several packaging cell lines are available. HEK 293 cells carry the 5' gene region of HAdV-5 virus.<sup>24</sup> They are suited for rescue of E1-deleted HAdV-5 vectors as well as for some of the family

E simian adenoviruses (SAdV) that are being explored as vaccine carriers. Homologous recombination between the adenovirus sequences within HEK 293 cells and the E1-deleted vector can lead to outgrowth of replication-competent viruses<sup>25,26</sup>; this is not a problem for SAdV vectors grown on HEK 293 cells because sequence differences between the E1-flanking regions of HAdV-5 and SAdV viruses prevent homologous recombination. PER.C6 cells offer an alternative<sup>27</sup>; they only carry the E1 domain of HAdV-5, which reduces the chance of insertion of E1 into the genome of HAdV-5 vectors.<sup>28</sup> For other serotypes, packaging cells have to be constructed typically by stable E1 transfection of a cell line that is readily infected with adenoviruses.

Upon transfection of packaging cells with a recombinant molecular clone of adenovirus, viral plaques typically become visible after 7–10 days. Virus once expanded in packaging cells is then purified. For small-scale production, vectors are purified by CsCl gradient centrifugation, whereas for large-scale production chromatographic separation is more suited.<sup>29–31</sup> The virus particle (vp) content of purified vectors is determined by spectrophotometry. Content of infectious virus particles (IU) is measured by plaque assays by staining for hexon or by reverse transcription polymerase chain reaction methods. Although the level of transgene product expression depends on IU, adenovirus vectors are dosed according to vp, which determines the toxicity of a preparation. The vp to IU ratios vary and are typically 5:1–400:1. Vectors with higher vp to IU ratios tend to perform poorly.

Depending on the diluent, adenovirus vectors are stable for several days at room temperature and for several months if kept on ice. Highly concentrated adenovirus vectors can be lyophilized with minimal loss in titers. Adenovirus vectors become unstable at low pH, which may pose problems for shipment in dry ice because CO<sub>2</sub> seeping into the vials may lower the pH, causing loss of viral titers.<sup>32</sup>

Transgene product expression by adenoviral vectors is influenced by a number of parameters. In our hands, HAdV-5 vectors express higher levels of their transgene compared with HAdV-26 or family E chimpanzee-origin adenovirus vectors.<sup>23,33,34</sup> A chimeric vector based on a CD46 binding chimpanzee vector was shown to express lower levels of transgene products compared with HAdV-5 vectors or a CAR-binding chimpanzee origin adenovirus vector.<sup>35</sup> E1- versus E1- and E3-deleted HAdV-5 or chimpanzee-origin vectors had comparable expression whereas further deletions in E4 reduced expression. The length of the transgene product also affected levels of protein expression; longer transgenes resulted in reduced expression.<sup>35</sup> The promoter regulating transgene expression was shown to influence both the magnitude and the kinetics of protein expression.<sup>36,37</sup> The orientation of the transgene expression cassette within E1 or E3 significantly influences protein expression.<sup>37,38</sup> We attempted to produce dual-expression adenoviral vectors, which carried one cassette in the deleted E1 domain and the other in the deleted E3 domain. Although we were able to generate stable vectors that expressed both transgene products, vectors that shared regulatory sequences within both expression cassettes were unstable, presumably owing to excision of large fragments of the genome upon homologous recombination.<sup>38</sup>

## 4. Preexisting Immunity to Antigen of Adenoviruses

Infections with adenoviruses are common and most humans carry T cells and binding and neutralizing antibodies to adenoviruses. Neutralizing antibodies directed mainly against the adenovirus hexon are serotype-specific whereas other antibodies or T cells, which are directed to multiple antigens, including those that are highly conserved between adenovirus serotypes and even families, are highly cross-reactive.<sup>39</sup> Seroprevalence happens early in life. A study in Northern China showed that newborns compared with toddlers commonly have higher titers of antibodies to HAdV-2 and -5, which presumably reflects transmission of antibodies from their mothers. Infants aged 6–12 months tend to have low titers, which then steadily increase.<sup>40</sup> A study conducted in India showed that neutralizing antibodies to HAdV-5 virus are slightly higher in infants aged 1–6 months than aged 7–12 months. Titers then steadily increase until adulthood.<sup>41</sup> A study conducted in North America, South America, Sub-Saharan Africa, and Southeast Asia showed low seroprevalence rates in infants (up to age 6 months) to HAdV-26 and HAdV-35 virus. Children had high prevalence rates of antibodies to HAdV-5 and HAdV-26, although titers to HAdV-26 tended to be lower than those to HAdV-5.<sup>42</sup> Prevalence of neutralizing antibodies in human adults varies depending on the serotype and geographic region. For example, neutralizing antibodies to common serotypes such as HAdV-5 are found in about 40% of individuals residing in the US and in up to 90% of those living in Sub-Saharan Africa.<sup>43,44</sup> The prevalence of neutralizing antibodies to HAdV-26, which was initially described as a rare serotype, is low in the US and Europe but high in most African countries.<sup>36</sup> Seroprevalence rates to HAdV-48 were shown to be high only in Africa but low in the US and Asia.<sup>42</sup> HAdV-35 appeared to be a truly rare serotype; less than 20% of sera from children or adults independent of their geographic localization were able to neutralize this virus.<sup>42</sup> Because adenoviruses are species-specific, most humans lack antibodies to those that infect other species, although this depends on the serotype as well as potential contact with the infected species. For examples, we described antibodies to three different chimpanzee adenoviruses (SAdV-23, SAdV-24, and SAdV-25) that were virtually absent in sera from humans residing in the US but that could be detected in up to 20% of individuals from Sub-Saharan Africa.<sup>44,45</sup> We assumed that this reflected close contact between humans and chimpanzees in Sub-Saharan Africa, where hunting and eating monkeys is common. We subsequently conducted a study in Brazil and again found slightly enhanced prevalence rates of neutralizing antibodies to chimpanzee adenoviruses in humans residing in Brazil; this was especially pronounced in cohorts from Amazonia. Testing of New World monkeys from Brazil showed the presence of neutralizing antibodies to chimpanzee adenoviruses in nearly all sera of common marmosets, which suggested spillover of these viruses from monkeys into the human population.<sup>46</sup> The same viruses tested against sera from different regions in China showed low prevalence rates for high-titer antibodies, although sera with low titers ( $\leq 1:40$ ) were found in up to 20% of adults.<sup>47</sup> Another study measured the prevalence rate of neutralizing antibodies in Caucasians residing in the US or Europe against a large panel of chimpanzee adenoviruses of family B, C, and E. The prevalence rate for the chimpanzee-origin adenoviruses with titers greater than 1:200 was in 5% of

sera with the exception of SAdV-3-neutralizing antibodies, which was found in about 10% of sera. One of the viruses, CAAdV-63, was tested for neutralization by sera from Kenyan children. Sera with high titers of CAAdV-63 neutralizing antibodies were rare (approximately 0.5%) whereas low titers were common (17.5%).<sup>48</sup>

Preexisting neutralizing antibodies to adenovirus cause rapid uptake of the virions by cells of the reticuloendothelial system. They also lead to activation of complement.<sup>49</sup> Specific neutralizing antibodies prevent cell transduction by adenoviruses and transcription and translation of the transgene product. This results in a reduction of the vaccine antigen and diminishes the vectors' ability to stimulate adaptive immune responses<sup>33,34,50</sup> while paradoxically increasing innate responses.<sup>51,52</sup> The latter presumably reflects that neutralizing antibodies target the vectors to Fc-receptor-positive cells. Binding antibodies or preexisting T cell responses to antigens of adenovirus do not appear to have a major effect on transgene product-specific B or T cell responses. The effect of preexisting neutralizing antibodies can be circumvented by increasing the vaccine dose; this is problematic in humans because, depending on titers of neutralizing antibodies, increases by 100- to 1000-fold are required,<sup>53</sup> which would likely result in unacceptable toxicity. Alternatively, adenovirus vectors could be combined with another vaccine modality such as a DNA vaccine or a poxvirus vector in a so-called prime-boost regimen.<sup>54–58</sup> Although this is known to increase transgene product-specific immune responses markedly, it also increases the cost and complexity of a vaccine. Repeated use of the same adenovirus vector has been tested to augment immune responses; this is relatively ineffective because neutralizing antibodies induced by the first vaccine dose impair uptake of repeat doses.<sup>59</sup> Loss of transduction in the presence of preexisting neutralizing antibodies can be circumvented by coating viruses with polyethylene glycol<sup>60</sup> or hydrophilic polymers,<sup>61</sup> or encapsidating them into microspheres.<sup>62</sup> It is not yet known whether these approaches also rescue adaptive immune responses to the transgene product. It is also possible to swap variable loops of the hexon of common human serotypes with those of rare human serotypes.<sup>63</sup> We used a straightforward approach by developing vectors that typically fail to infect humans, such as those derived from nonhuman primates.<sup>33,34</sup> By now a large number of vectors have been isolated from chimpanzees.<sup>48,64</sup> They are phylogenetically grouped within human serotypes<sup>65</sup>; their molecular organization, receptor usage, and growth characteristics are similar to those of human serotypes. Antibodies to human serotypes of adenoviruses fail to cross-react with chimpanzee-derived adenoviruses. Most important, as already mentioned, neutralizing antibodies to chimpanzee adenoviruses are only rarely found in humans. Prevalence rates of such antibodies are slightly increased in Africa, presumably owing to closer contact with infected chimpanzees. Chimpanzee adenovirus vectors are thus highly suited as vaccine carriers for use in humans.

## 5. Innate Immune Responses to Adenovirus Vectors

Adenoviruses and adenovirus vectors stimulate potent innate immune responses that cause dose-limiting toxicity. Innate responses, which lead to the release of proinflammatory cytokines and chemokines such as type I IFN, IL-6, IL-12, Regulated

on Activation, Normal T cell Expressed and Secreted, macrophage inflammatory protein-1 $\beta$ , IFN-gamma-induced protein (IP)-10, and others, are typically triggered through recognition of pathogen-associated molecular patterns by cellular pathogen recognition receptors located on the cell surface, on endosomes, or within the cytoplasm. Several motifs present on adenovirus vectors are recognized by innate sensor. The RGD motif on penton activates the nuclear factor-kappa light chain enhancer of activated B cells pathway, which triggers an inflammatory reaction.<sup>66</sup> The double-stranded adenovirus genome is recognized by Toll-like receptor (TLR)-9, one of the endosomal sensors.<sup>67</sup> Adenovirus vectors appear to be recognized by additional TLRs because innate responses are diminished in cells lacking TIR domain-containing adapter-inducing IFN-beta, which serves as an adaptor to TLR-4 and -3.<sup>68,69</sup> The adenovirus genome further activates retinoic acid-inducible gene-1, another intracellular sensor of double-stranded DNA.<sup>70</sup>

Adenoviruses applied to the airways interact with surfactant,<sup>71</sup> which appears to block inflammatory responses, presumably by causing opsonization of the virus particles. High doses of adenovirus vectors further activate the complement system through interactions between fiber knob with blood Factor IX and C4 binding protein. These interactions increase inflammatory responses.<sup>72,73</sup> Adenovirus vectors are sequestered by preexisting neutralizing antibodies; for unknown reasons, this interaction increases inflammatory responses to the vectors.<sup>51,52</sup>

The potency of innate immune responses partly depends on the vector serotype. For example, in mice, some of the chimpanzee-derived adenovirus vectors were shown to induce more potent type I IFN responses compared with HAdV-5 vectors.<sup>74,75</sup> This may relate to the frequency of CpG motifs, which tend to be lower in genomes of HAdV-5 (2168 CG motifs) and HAdV-26 (2206 CG motifs) viruses than in those of chimpanzee-origin adenoviruses (approximately 2400–2700 CG motifs). Whether more intense inflammatory reactions in mice translate into heightened toxicity in humans remains to be tested.

## 6. Humoral Immune Responses to Adenoviral Vectors

Adenoviral vectors induce potent B cell responses to the capsid antigens of the vector<sup>46,44</sup> and to foreign transgene products.<sup>45,76</sup> The latter allow for the use of adenovirus vectors as prophylactic vaccines to infections, which can be prevented by neutralizing antibodies. The advantages of adenovirus vectors for delivery of antigens from other pathogens for induction of protective antibody responses are numerous because they interact with innate sensor drive maturation of antigen-presenting cells and do not require addition of adjuvants. E1-deleted adenoviral vectors are safe at immunogenic doses. They can be given through multiple routes including intranasal, intramuscular, and oral, provided vectors are encapsidated. Similar to wild-type adenoviruses, E1-deleted adenovirus vectors persist in activated T cells<sup>5</sup> and induce sustained antibody responses. Adenoviruses use the host cell machinery for posttranslational modifications of antigens so that viral antigens are presented in their native form unless their structure depends on co-interactions with other antigens. We compared the magnitude



of transgene product-specific antibody responses to two chimpanzee-derived adenovirus vectors, both of which are family E members, with those induced by vectors derived from HAdV5, a family C member, and HAdV26, a family D member.<sup>45</sup> Vectors expressed the rabies virus glycoprotein as the transgene product. The HAdV-5 vector induced markedly higher rabies virus-specific antibody responses compared with the other vectors.

E3-deleted HAdV-5 vectors expressing the rabies virus glycoprotein have been licensed for immunization of wildlife such as foxes or skunks.<sup>77</sup> Neither of these species supports replication of HAdV-5 virus, so that in its intended target species the vaccine is replication-defective. E1-deleted adenovirus vectors based on simian serotypes also expressing the rabies virus glycoprotein are under development for human immunization.<sup>33</sup> These vectors are highly immunogenic and induce protective titers of rabies virus–neutralizing antibodies in nonhuman primates after a single low dose ( $10^9$  vp) given intramuscularly before challenge.<sup>78</sup> Antibody titers and protective immunity were shown to be sustained for at least 2 years. Ebola virus in the winter and spring of 2014 caused an outbreak in Guinea, which by fall of the same year had spread to Liberia and Sierra Leone with isolated cases imported to Europe and the US. The World Health Organization estimated that without improved interventions, the virus would continue to spread and potentially infect up to a million individuals by 2015.<sup>79</sup> Others voiced concern about viral mutation that may allow Ebola virus to be transmitted by aerosols, even further increasing infection rates. One of the first Ebola vaccines that underwent testing in humans was based on an E1-deleted HAdV-5 vector expressing the glycoproteins of Zaire and Sudan Ebola species.<sup>80</sup> The vaccine, which had been shown to protect nonhuman primates against Ebola virus infection,<sup>81</sup> was found to be safe at  $2 \times 10^{10}$  vp per dose and human subjects developed specific T and B cell responses. Because of concerns about preexisting HAdV-5 neutralizing antibodies,<sup>82</sup> which tend to be prevalent and robust in African human populations, a second set of vectors based on chimpanzee serotype 3 was constructed.<sup>83</sup> This vaccine is currently undergoing testing in human volunteers. Provided the vaccine is immunogenic and safe, it may then be used under an investigational new drug application in consenting individuals at high risk of contracting the virus. One potential setback of this Ebola vaccine is that although humoral responses to the viral glycoprotein developed rapidly in nonhuman primates, antibody titers were not sustained, but rather declined to baseline within less than a year. This could potentially be addressed by a booster immunization with an adenovirus vector based on a different serotype or an unrelated vaccine prototype such as a poxvirus vector.

A plethora of adenovirus vectors based on human and simian serotypes have been tested preclinically for induction of protective antibody responses to numerous pathogens including hepatitis B virus,<sup>84</sup> Dengue virus,<sup>85</sup> *Severe acute respiratory syndrome* coronavirus,<sup>86</sup> rotavirus,<sup>87</sup> respiratory syncytial virus,<sup>88</sup> rabies virus,<sup>33,77</sup> herpes simplex virus type 2,<sup>89</sup> Hantaan virus,<sup>90</sup> influenza virus,<sup>91</sup> and plasmodium vivax.<sup>92</sup> Results consistently showed that the vectors induced potent antibody responses dominated by isotypes linked to type 1 T helper cell responses upon systemic immunization whereas mucosal injections also led to local immunoglobulin A production.<sup>93</sup>

## 7. Cellular Immune Responses to Adenoviral Vectors

Although neutralizing antibodies are the primary correlate of protection against most pathogens for some of the more complex viruses, intracellular bacteria, or protozoa, protection can be provided by cellular immune responses that achieve accelerated clearance of infected cells. By the same token, chronic viral infections or cancer cells are best combated with vaccine-induced CD8<sup>+</sup> T cell responses. Adenoviruses acquired by natural infections induce CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to a number of viral proteins. Such T cells can be found in most human adults.<sup>94</sup> They cross-react between different adenovirus serotypes including those derived from chimpanzee-origin adenoviruses.<sup>39</sup> Adenovirus-specific CD4<sup>+</sup> T cells were found to be monofunctional; they largely belong to the memory subset. In contrast, adenovirus-specific CD8<sup>+</sup> T cells are more polyfunctional. They are highly activated and are phenotypically mainly grouped into effector/effector memory subsets. This may reflect repeated exposures to different serotypes of adenoviruses or internal reactivations of CD8<sup>+</sup> T cells by persisting viruses. Adenoviral vectors induce remarkably high transgene product-specific CD8<sup>+</sup> T cell responses and, at least in mice, only modest CD4<sup>+</sup> T cell responses.<sup>34,45</sup> Induction of T cells is triggered by cross-presentation of antigen.<sup>95</sup> Transgene product-specific CD8<sup>+</sup> T cells similar to those induced by natural infections to the adenovirus antigens are polyfunctional, and because of the vectors' persistence, a large proportion remains activated. Nevertheless, because levels of persistence are low, adenovirus-induced T cells in part transition into the memory pool, which allows for increased responses after booster immunizations.<sup>88,49</sup>

We compared the magnitude of transgene product-specific CD8<sup>+</sup> T cell responses to HAdV-5, HAdV-26, and chimpanzee-derived adenovirus vectors SAdV-24 and SAdV-25.<sup>45</sup> In mice, responses were largely comparable. Others compared T cell responses to different human and chimpanzee serotypes in mice and rhesus macaques. In both species, human serotypes adenoviruses such as HAdV-5 and -6 based on family C induced higher CD8<sup>+</sup> T cell responses compared with those from family D, such as HAdV-26 or HAdV-24. Adenovirus vectors based on family B, such as HAdV-35 and HAdV34, both of which use CD46 rather than CAR for cell entry, were the least immunogenic.<sup>48</sup> Similar trends were seen for chimpanzee-origin adenovirus vectors; those from group C, such as SAdV-3, SAdV-20, or SAdV-11, tended to be more immunogenic than those of group E, such as SAdV-4 or -5, whereas the one group B virus, SAdV-30, was the least immunogenic.<sup>48</sup> Nevertheless, some of the family E–derived vectors were as immunogenic as or even more so than some of the family C vectors.

Large numbers of different adenovirus vectors have undergone preclinical testing as T cell–inducing vaccines for a variety of pathogens, including HIV-1/SIV,<sup>34,50,68</sup> hepatitis C virus (HCV),<sup>58</sup> *Trypanosoma cruzii*,<sup>96</sup> *Mycobacterium tuberculosis*,<sup>97,98</sup> dengue virus,<sup>99</sup> human CMV,<sup>100</sup> influenza A virus,<sup>101,102</sup> Rift valley fever virus,<sup>103</sup> and Epstein Barr virus.<sup>104</sup> Results showed that adenovirus vectors induced exceptionally potent and sustained CD8<sup>+</sup> T cell responses in animals that were higher than those induced by other recombinant vaccines such as DNA vaccines or poxvirus vectors. T cell responses induced by an adenovirus vector can be enhanced by prime boost-regimens using serologically distinct adenovirus vectors or other vaccine platforms for priming or boosting.

## 8. Clinical Experience with Vaccines Based on Adenoviral Vector

Early-stage safety studies have been conducted with several human- as well as chimpanzee-derived adenovirus vectors expressing antigens of HIV-1,<sup>58</sup> *M. tuberculosis*,<sup>105</sup> *Plasmodium falciparum*,<sup>106</sup> Ebola virus,<sup>80</sup> influenza A virus,<sup>107</sup> and HCV.<sup>108</sup> Results showed that toxicity was dose-related and unaffected by preexisting neutralizing antibodies to the vectors.<sup>109,110</sup> At high doses of  $10^{11}$  vp, individuals mainly experienced mild to moderate flulike symptoms with fever, myalgia, fatigue, and headache. Injection-site reactions such as erythema and local pain were seen regardless of vector dose. Repeated injections of the same vector resulted in decreased systemic reactions upon sequential immunizations.<sup>111</sup> Vaccinated individuals did not develop significant changes in blood values. Overall, the vaccines were well tolerated. Analyses of vaccine-induced immune responses showed that in a dose-dependent manner, vaccine recipients developed T and B cells to the transgene product. They also had increases in immune responses to antigens of the adenoviral vector.

The first large-scale phase IIb trial for an adenoviral vector vaccine, the STEP trial, was conducted by Merck with HAdV-5 vectors expressing Gag, Pol, and Nef of HIV-1 clade B for induction of T cells. Individuals from North or South America, the Caribbean, or Australia at high risk for HIV-1 acquisition received three injections of  $5 \times 10^{10}$  vp of the vaccine or placebo on day 1 and weeks 4 and 26. The trial was designed to enroll 1500 individuals with HAdV-5-specific neutralizing antibody titers less than 1:200 at baseline and 1500 individuals with titers greater than 1:200. As expected, the vaccine elicited potent CD8<sup>+</sup> T cell responses that were slightly attenuated in individuals with high titers of preexisting neutralizing antibodies to HAdV-5. CD4<sup>+</sup> T cell responses to HIV-1 were observed in only about a third of vaccine recipients.<sup>112</sup> The trial was stopped prematurely after an interim analysis by the Safety Monitoring Board showed that the vaccine neither prevented HIV-1 infections nor reduced viral loads in individuals who became infected.<sup>113</sup> A trial conducted in parallel in South Africa, the Phambili trial,<sup>114</sup> was stopped shortly thereafter and in both trials participants were unblinded. In the STEP trial, male vaccine recipients, who were mainly homosexual and engaged in high-risk anal intercourse had increased rates of HIV-1 acquisition (49 of 941 male vaccine recipients) compared with placebo controls (33 of 922 participants). Increased acquisition was linked to high titers of HAdV-5-neutralizing antibodies at baseline as well as the lack of circumcision.<sup>115</sup> There was no link to differences in risk behavior.<sup>115</sup> This trend for increased HIV-1 acquisition, which was transient and waned after about 18 months,<sup>116</sup> was not seen in the Phambili trial, which enrolled mainly heterosexual men and women. Additional studies comparing HIV-1 acquisition rates of participants in other HIV-1 trials based on vaccines other than adenoviral vectors showed that titers of neutralizing antibodies to several adenoviral serotypes did not increase HIV-1 acquisition.<sup>117</sup> By the same token, the army failed to observe increases in HIV-1 infection rates in recruits who received the live attenuated adenovirus vaccines compared with those who did not.<sup>118</sup> High neutralizing antibody titers to adenovirus per se or vaccination with an adenovirus thus

do not appear to increase the risk of HIV-1 infection. The mechanisms underlying the increased acquisition of HIV-1 in HAdV-5–seropositive individuals of the STEP trial have been studied extensively but remain unexplained. One possibility that has been explored is that CD4<sup>+</sup> T cells induced by adenovirus vectors become highly susceptible to infection with HIV-1.<sup>119</sup> Such T cells also express homing markers for gut mucosa. The increased presence of HIV-1 target cells at the port of viral entry could explain increased HIV-1 acquisition after vaccination with an adenoviral vector; nevertheless, it does not explain why this increased acquisition was seen only in homosexual males with high baseline titers of HAdV-5–specific neutralizing antibodies.

The next large-scale efficacy trial was again based on an HAdV-5 vector combined with DNA vaccine priming to prevent HIV-1. The DNA vaccine expressed Gag, Pol, Nef, and Env from clade A, B, and C. The HAdV-5 vectors given as mixtures expressed a gag–pol fusion protein and Env of clades A, B, and C.<sup>120</sup> The HAdV-5 vector of HVTN505 differed from the vector used in the STEP trial by the additional deletion of E4 and by the inclusion of Env as a vaccine target antigen. The DNA vaccines were given three times on days 0 and weeks 4 and 8; the HAdV-5 vectors were given once on week 24. The study, conducted in the US, enrolled circumcised males and transgender women with preexisting neutralizing antibody titers to HAdV-5 below 1:18, who were at high risk for HIV-1 acquisition. A total of 1251 participants were enrolled into the vaccine arm; 1245 participants were enrolled into the placebo arm. End points were the prevention of HIV-1 acquisition or lowering of viral loads in infected vaccine recipients. An interim analysis showed that neither of these end points would be met and the trial was halted and unblinded. The vaccines did not increase HIV-1 acquisition rates. The vaccine induced HIV-1–specific CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells in about 60% of recipients, antibodies to gp41 in all recipients, and antibodies to gp120 in about 50% of vaccine recipients. Antibodies to the V1–V2 loop, which in another, more successful HIV-1 vaccine efficacy trial<sup>121,122</sup> had been identified as correlates of protection against viral acquisition, were detected in only about 20% of vaccine recipients. Response rates for neutralizing antibodies were low and only tier 1 HIV-1 virus could be neutralized.

The futility of three HAdV-5 vector-based HIV-1 vaccine efficacy trials combined with the increased risk for HIV-1 acquisition in a subcohort of the STEP trial raised questions about the use of adenoviral vectors as a vaccine platform for HIV-1 in general. The initial early-phase immunogenicity trials with the STEP and HVTN505 vaccines had shown that the breadth of HIV-1–specific T cell responses was limited because T cells from most vaccinated individuals only recognized one to four epitopes of HIV-1.<sup>123</sup> This limited epitope specificity was recapitulated in a small phase I trial with an HAdV-26 vaccine expressing Env. We tested chimpanzee-derived adenovirus vectors expressing HIV-1 clade B Gag in rhesus macaques, and after a single immunization the breadth of the response was also limited and most animals responded to only one or two epitopes. A boost with a serologically distinct adenovirus vectors markedly increased the breadth of the response and most animals responded after the boost to 15–30 different Gag epitopes.<sup>124</sup> The restricted epitope specificity observed in the clinical trials, which carries the risk that HIV-1 may escape cellular

immunosurveillance, can thus potentially be addressed by prime-boosting with two different serotypes of adenovirus vector vaccines. Also, the efficacy of the STEP and HVTN505 vaccines was limited in preclinical studies. The STEP vaccine protected rhesus macaques against challenge with the SIV/HIV chimera SHIV89.6P<sup>125</sup> but provided no protection against a more stringent challenge with SIVmac239.<sup>126</sup> By the same token, the HVTN505 vaccine failed to protect rhesus macaques from SIVmac251 challenges but conferred MHC class I allele-dependent protection against the less stringent SIVsmE660 challenge.<sup>127</sup> Others described induction of protective immunity in rhesus macaques immunized with adenovirus vectors from other human as well as chimpanzee serotypes<sup>15,128</sup> against SIVmac239 or SIVmac251, and the use of these alternative adenovirus vector for HIV-1 prophylaxis continues to be explored.

Other clinical efficacy trials explored HAdV-5 or CAAdV-63 as carriers for malaria vaccines. An HAdV-5 vector expressing the *P. falciparum* circumsporozoite protein and apical membrane antigen-1 failed to protect against a controlled malaria infection,<sup>129</sup> whereas some protection (about 27%) was achieved when this vaccine was combined with a DNA vaccine prime.<sup>130</sup> A similar level of efficacy was achieved with a CAAdV-63 expressing T cell-inducing antigens of *P. falciparum* followed by a modified vaccinia Ankara boost.<sup>131</sup>

Adenovirus vector-based vaccines for *M. tuberculosis* have thus far undergone only early-stage safety and immunogenicity trials. An HAdV-5 vector expressing Ag85A was shown to induce T cells in individuals who were naive to antigens of this pathogen; responses were more robust in previously BCG vaccinated trial volunteers. Immunogenicity was not affected by preexisting HAdV-5-neutralizing antibodies.<sup>105</sup>

One phase I trial tested HAdV-6 and CAAdV-3 vectors expressing the NS protein of HCV. Both vectors induced HCV-specific T cell responses that cross-reacted between heterologous HCV strains. Responses to either vaccine were polyfunctional and sustained. Responses were boosted when the two vectors were used sequentially.<sup>108</sup>

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