TITLE

Improved adenoviral vectors and uses thereof

FIELD OF THE INVENTION

The invention relates to the field of medicine, more in particular to the field of therapeutic and prophylactic treatment, by using recombinant chimeric adenoviral vectors comprising a therapeutic nucleic acid in vaccine compositions.

BACKGROUND OF THE INVENTION

Recombinant adenoviral vectors are widely applied for gene therapy applications and vaccines. To date, 51 different adenovirus serotypes have been identified. The subgroup C adenoviruses have been most extensively studied for applications such as gene therapy; especially serotype 2 and 5 (Ad2 and Ad5) are widely used in the art. Recombinant Ad5 is used in a variety of different purposes, including vaccination. Importantly, Ad5 vector-based vaccines have been shown to elicit potent and protective immune responses in a variety of animal models. Moreover, large-scale clinical trials for HIV vaccination are ongoing in which Ad5-based recombinant vectors are being used (WO 01/02607; WO 02/22080; Shiver et al. 2002; Letvin et al. 2002; Shiver and Emini. 2004). However, the utility of recombinant Ad5 vector-based vaccines for HIV and other pathogens will likely be significantly limited by the high seroprevalence of Ad5-specific neutralizing antibodies (NAbs) in human populations. The existence of anti-Ad5 immunity has been shown to suppress substantially the immunogenicity of Ad5-based vaccines in studies in mice and rhesus monkeys. Early data from phase-1 clinical trials show that this problem may also occur in humans (Shiver 2004).

One promising strategy to circumvent the existence of pre-existing immunity in individuals previously infected with the most common human adenoviruses (such as Ad5), involves the development of recombinant vectors from adenovirus serotypes that do not encounter such pre-existing immunities. Human adenoviral vectors that were identified to be particularly useful are based on serotypes 11, 26, 34, 35, 48, 49, and 50 as was shown in WO 00/70071, WO 02/40665 and WO 2004/037294 (see also Vogels et al. 2003). Others have found that also adenovirus 24 (Ad24) is of particular interest as it is shown to be a rare serotype (WO 2004/083418).

A similar strategy is based on the use of simian adenoviruses since these do typically not infect humans. They exhibit a low seroprevalence in human samples. They are however applicable for human use since it was shown that these viruses could infect human cells in vitro (WO 03/000283; WO 2004/037189).

It was shown that adenovirus serotype 35 (Ad35) vector-based vaccines could elicit potent cellular immune responses that were not significantly suppressed by anti-Ad5 immunity (Barouch et al. 2004; Vogels et al. 2003). Similarly, chimpanzee adenoviruses have been shown to elicit immune responses that were minimally affected by anti-Ad5 immunity (Farina et al. 2001; Pinto et al. 2003). It was recently demonstrated that neutralizing antibodies (NAbs) and $CD8^+$ T lymphocyte responses both contribute to anti-Ad5 immunity, whereas Ad5-specific NAbs appear to play the primary role (Sumida et al. 2004). Although this development appears to be a very useful approach, it was also demonstrated in mice that Ad35 vector-based vaccines proved less immunogenic than Ad5 vector-based vaccines in studies in which there was no pre-existing Ad5-immunity (Barouch et al. 2004).

Clearly, there is a need in the field for alternative adenoviral vectors that do not encounter pre-existing immunities in the host, but that are still immunogenic and capable of inducing strong immune responses against the proteins encoded by the heterologous nucleic acids inserted in the nucleic acid carried by the vector.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (A) shows the percentage of seroprevalence of Ad5, Ad11 and Ad35 in samples from the United States, Haiti, Botswana, Zambia and South Africa; (B) shows the neutralizing antibody titer against Ad5, Ad11 and Ad35 in the samples from the US and those from Africa. Figure 1C shows the neutralizing antibody titer against Ad5, Ad5f35, Ad5f35p35, Ad35f5 and Ad35 in these samples.

Figure 2 (A) shows the nucleic acid sequence encoding the Ad35k5 fiber protein (SEQ ID NO:1; the part encoding the Ad5 fiber knob is underlined); (B) shows the amino acid sequence of the Ad35k5 fiber protein (SEQ ID NO:2; the part representing the Ad5 fiber knob is underlined); (C) shows the nucleic acid sequence encoding the Ad35f5 fiber protein (SEQ ID NO:4; the part encoding the remaining Ad35 fiber fragment is underlined, while the BsiWI cloning site is represented in small caps); (D) shows the amino acid sequence of the Ad35f5 fiber protein (SEQ ID NO:5; the part representing the remaining Ad35 fiber fragment is underlined); (E) shows the nucleic acid sequence encoding the Ad35k26 fiber protein (SEQ ID NO:80; the part encoding the Ad26 fiber knob is underlined); (F) shows the amino acid sequence of the Ad35k26 fiber protein (SEQ ID NO:81; the part representing the Ad26 fiber knob is underlined); (G)

shows the nucleic acid sequence encoding the Ad35k49 fiber protein (SEQ ID NO:82; the part encoding the Ad49 fiber knob is underlined); (H) shows the amino acid sequence of the Ad35k49 fiber protein (SEQ ID NO:83; the part representing the Ad49 fiber knob is underlined).

Figure 3 shows the T cell response induced towards SIV gag upon injection of naïve mice with Ad5, Ad35fib5.BSU, Ad35.BSU and Ad35 vectors carrying the SIV gag-encoding gene in comparison to mice injected with empty vectors (Ad5.empty and Ad35.empty). The y-axis depicts SFC/10^6 splenocytes.

Figure 4 shows the immunogenicity of Ad5, Ad35k5, and Ad35 vectors expressing SIV Gag in naïve mice, with 10^9 vp (A) and 10^8 vp (B), or with pre-immunization with one (C) or two (D) injections of 10^{10} vp Ad5-Empty prior to immunization with 10^8 vp of the respective vectors. In all cases, Gag-specific $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization.

Figure 5 shows the anti-vector immunity elicited by Ad35k5 vectors in mice. (A) Naïve C57/BL6 mice primed at week 0 with 10^9 vp Ad35k5-Gag and boosted at week 4 with 10^9 vp Ad5-Gag or Ad35-Gag. (B) Serum samples from mice injected with 10^9 vp Ad5-Gag, Ad35k5-Gag, or Ad35-Gag assessed in Ad5 and Ad35 luciferase-based virus neutralization assays.

Figure 6 shows the immunogenicity of Ad5, Ad35k5, and Ad35 vectors expressing HIV-1 Env in mice. Naïve Balb/c mice were immunized with 10^8 vp Ad5-Env, Ad35k5-Env, or Ad35-Env. (A) Env-specific cellular immune responses

assessed by pooled peptide and MI10 epitope peptide-specific IFN-γ ELISPOT assays. (B) Env-specific humoral immune responses assessed by ELISA.

Figure 7 shows the immunogenicity of Ad5, Ad35k5, and Ad35 vectors in rhesus monkeys primed at week 0 with 10^{11} vp Ad5-Gag (A,B), Ad35-Gag (C,D), and Ad35k5-Gag (E,F). At week 12, all monkeys received a homologous boost immunization. Env- and Gag-specific cellular immune responses were assessed by pooled peptide IFN-γ ELISPOT assays at multiple time points following immunization (A,C,E). Vector-specific NAb titers were assessed by Ad5 and Ad35 virus neutralization assays (B,D,F).

Figure 8 shows the $CD4^+$ and $CD8^+$ T lymphocyte responses in monkeys in the study described in Figure 7. Pooled peptide IFN-γ ELISPOT assays were performed using CD8-depleted and CD4-depleted PBMC's from monkeys at week 16 following immunization. Monkeys received 10^{11} vp (A) Ad5-Gag, (B) Ad35-Gag, or (C) Ad35k5-Gag at week 0 and week 12.

Figure 9 shows the immunogenicity of Ad5-Gag, Ad5HVR48(1)-Gag and Ad5HVR48(1-7) vectors in naïve mice receiving (A) 10^9 vp, (B) 10^8 vp, or (C) 10^7 vp. Gag-specific $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays at multiple time points following immunization.

Figure 10 shows the immunogenicity of Ad5-Gag, Ad5HVR48(1)-Gag and Ad5HVR48(1-7) vectors in mice receiving (A) 10^9 vp, (B) 10^8 vp, or (C) 10^7 vp, pre-immunized with two injections of 10^{10} vp Ad5-Empty 8 and 4 weeks prior to immunization.

Figure 11 shows the amino acid sequence (SEQ ID NO:12) of the Ad5-based hexon protein, with the incorporation of the seven HVR's for the seven corresponding HVR's (underlined) from Ad48 (Ad5HVR48(1-7)).

Figure 12 shows the amino acid sequence (SEQ ID NO:13) of the Ad5-based hexon protein, with the incorporation of the seven HVR's for the seven corresponding HVR's (underlined) from Ad35 (Ad5HVR35(1-7)).

Figure 13 shows the amino acid sequence (SEQ ID NO:14) of the Ad5-based hexon protein, with the incorporation of the seven HVR's for the seven corresponding HVR's (underlined) from Ad11 (Ad5HVR11(1-7)).

Figure 14 shows the amino acid sequence (SEQ ID NO:15) of the Ad5-based hexon protein, with the incorporation of the seven HVR's for the seven corresponding HVR's (underlined) from Ad26 (Ad5HVR26(1-7)).

Figure 15 shows the amino acid sequence (SEQ ID NO:16) of the Ad5-based hexon protein, with the incorporation of the seven HVR's for the seven corresponding HVR's (underlined) from Pan9 adenovirus (Ad5HVRPan9(1-7)).

Figure 16 shows the intracellular gag-staining in a background setting (no infection, graph 1° & 2° Ab) and after infection with Ad35k5 (two batches: #4, #5), with Ad35k26 (two batches: #3 twice, # 28) and with Ad35k49 (three batches: #4, #5, #9).

Figure 17 shows the amino acid sequence of the hexon protein in Ad5HVR48(1-7)* (SEQ ID NO:84) in which the

HVR1 sequence as defined in Table IV is deleted, the HVR2 sequence according to Table IV is replaced with a QG linker and HVR3 - HVR7 according to the definition of Table IV have been replaced between Ad5 and Ad48.

Figure 18; as figure 17, now for Ad5HVR35(1-7)* (SEQ ID NO:85).

Figure 19; as figure 17, now for Ad5HVR26(1-7)* (SEQ ID NO:86).

Figure 20; as figure 17, now for Ad5HVR49(1-7)* (SEQ ID NO:87).

Figure 21 is a graph indicating the CD8+ T-lymphocyte response in mice after a double pre-immunization with Ad5-empty, followed by a priming on day 0 with Ad35-Gag and a boost on day 28 with three different vectors as indicated.

SUMMARY OF THE INVENTION

Newly developed recombinant adenoviral vectors for improved gene delivery, vaccination, and gene therapy are disclosed herein. In one preferred embodiment the vector is a recombinant adenovirus based on adenovirus serotype 35 (Ad35) wherein at least the Ad35 fiber knob has been replaced by the fiber knob of a serotype that binds to the Coxsackievirus and Adenovirus Receptor (CAR). More preferably, this serotype is an Ad5 fiber knob (resulting in a vector termed Ad35k5, wherein only the knob has been replaced, or in a vector termed Ad35f5, wherein the knob, shaft and part of the tail have been replaced). The shaft and tail of the fiber may be of the carrying backbone serotype, i.e. Ad35, whereas the invention also relates

to vectors in which the shaft domain is of the same serotype as fiber knob serotype. In Ad35f5 the part of the tail region left from the backbone serotype ensures a proper interaction with the remaining part of the capsid for the production of stable vectors. The vectors of the present invention comprise a therapeutic nucleic acid of interest, preferably a nucleic acid that is applicable for vaccination purposes.

The invention relates to recombinant adenoviral vectors that encounter low pre-existing immunity in most of the human population, while still being able to induce a strong immune response against the antigen encoded by a nucleic acid comprised by the vector. This is achieved by producing recombinant vectors based on adenovirus serotypes, such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49, Ad50, or simian adenoviruses, carrying a chimeric capsid. The chimeric capsid comprises fiber proteins that are partly based on fiber proteins from human adenovirus serotypes different from the backbone vector, and that in a most preferred embodiment bind (through their knob domain) to the CAR receptor. Many different adenoviruses that preferentially bind CAR can be used for providing the fiber knob, such as human adenoviruses from subgroup A, C, D, E, and F, and ovine adenoviruses that also bind CAR. Preferred adenovirus serotypes that are used for their fiber knob domains are the human adenoviruses from subgroup C, more preferably Ad2 and Ad5.

In another embodiment the virus produced according to the invention is a recombinant adenovirus based on a subgroup C adenovirus, preferably Ad5, which is made replication-defective by a functional deletion of the E1 region and wherein the hexon protein in the viral capsid is a chimeric protein such that one or more of the hyper variable regions (HVR's) have been replaced by the HVR's

derived from a rare adenovirus serotype. Such rare serotypes do not encounter NAbs in most of the individuals in the human population. Preferred serotypes that are used to provide the HVR's are Ad35 and Ad48. Preferably, the recombinant virus comprises a heterologous nucleic acid of interest that is to be delivered to the host for prophylactic or therapeutic purposes.

DETAILED DESCRIPTION

As discussed above, Ad5-based recombinant vectors are hampered in their use by the existence of neutralizing antibodies (NAbs) that are present in most of the human individuals due to a prior infection with wild type virus. Although the different capsid proteins present in the viral coat induce antibodies in the host, it has been demonstrated that the primary target of Ad5-specific NAbs is the Ad5 hexon protein (Sumida et al. 2005). This led to the idea that, by changing the hexon protein such that it could no longer be seen by the pre-existing NAbs, improved adenoviral vectors could be produced that would be beneficial in humans that either already have NAbs against Ad5, or that were previously immunized with previous vaccines based on Ad5, or that were primed with Ad5 based vectors in a prime/boost vaccination regime. However, altering the hexon protein turned out not to be an easy task. Complete hexon changes have generally only been possible between adenoviruses within the same Ad subgroup and resulted in poorly viable virus (Youil et al. 2002; Gall et al. 1998; Roy et al. 1998). If this would be the limitation then a vector based on Ad5 had to contain a hexon protein from another subgroup C adenoviruses. However, most of these, if not all, are useless in the sense that those serotypes are

not considered rare. Most individuals in the human population have once encountered the serotypes from subgroup C. It would be preferred to use a hexon from a serotype that should not encounter NAbs already present. Those rare serotypes are predominantly found in subgroup B (Ad11, Ad34, Ad35, Ad50) and D (Ad24, Ad26, Ad48 and Ad49).

The inventors of the present invention have now for the first time shown that by (re-)defining specific parts of the hexon and by using a conservative approach and available structure and sequence data, certain regions could be identified and could be swapped resulting in producible recombinant viruses that are viable and could be produced to high enough titres. The preferred serotypes that are used to provide their hexon protein, or the relevant parts thereof, are Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50, as these serotypes are known to encounter low pre-existing immunity (see WO 00/70071). More preferred are Ad11, Ad26, Ad35 and Ad48, whereas Ad48 is the most preferred serotype. Also non-human adenoviruses are interesting in this respect. One preferred example is the chimpanzee adenovirus Pan9.

The regions that were identified are the 7 surface loops also known as the hexon Hypervariable Regions (HVR's). The hexon variability among adenovirus serotypes is concentrated in these 7 loops (Crawford-Miksza and Schnurr. 1996). It is to be understood that the invention is not limited to the use of the HVR's of Ad48 as outlined in the examples. This is further substantiated by the identification of HVR's in Ad5, Ad11, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50 in a somewhat broad definition (see Table II) and in a somewhat more limited, a more minimalistic definition (see Table IV). Ad48 serves as an example for all other serotypes that also encounter low-

pre-existing immunity and that are also useful in making chimeric adenovirus that benefit from the known advantages of the subgroup C adenovirus, exemplified by Ad5 (strong immunogenicity, easy to produce, etc.) with the benefits of the rare serotypes (low pre-existing immunity). Clearly, if one would contemplate the use of a prime/boost vaccination regimen in which it is preferred to use another serotype backbone, such as Ad35, then it would be beneficial to boost with a vector that does not encounter the raised NAbs against the priming vector. Thus, combinations such as Ad11, Ad24, Ad26, Ad35, Ad48 Ad49, Ad50, etc. with the HVR's of another rare serotype (such as any of the rare serotypes mentioned above) are also part of the present invention. Non-limiting examples of such vectors are Ad5HVR48, Ad5HVR35, Ad35HVR11, Ad35HVR48, Ad11HVR35, and Ad11HVR48, having at least one, more preferably six, and most preferably seven HVR's exchanged between serotypes. So, preferably at least one HVR from a rare serotype is taken and inserted into the hexon of the backbone serotype. It has been shown by the inventors of the present invention that replacing all seven HVR's from Ad5 by the corresponding HVR's from Ad48 resulted in a viable and producible vector that encountered hardly any pre-existing immunity in mice immunized with empty Ad5 viruses. However, if only the first HVR (seen from the left ITR to the right ITR in the viral genome) was replaced no effect was seen. This does not mean that one replacement could not be enough. It is thought that one region is more immunogenic than others, and it remains to be investigated which of the 7 identified regions contributes most and which of the HVR's do not have to be replaced in a certain setting or for a specific application. It may be that certain individuals raise different immune responses towards

different HVR's in comparison to other individuals. Moreover, chimeric hexon containing vectors may prove beneficial in settings where there is only a moderate NAb activity in the host. The pre-existing immunity raised according to the provided examples is very high, due to 2 consecutive doses of 10^{10} vp of the empty Ad5 vector. Nevertheless, it is most preferred that all HVR's within the hexon protein are replaced as this would provide the best chance of yielding a vector not detected by the pre-existing NAbs present in the host.

To develop improved adenoviral vectors, first the Ad5-specific neutralizing antibody (NAb) epitopes were mapped. It was revealed that Ad5 seroprevalence and titers were very high in human populations (approximately 50% in the United States and 90% in parts of the developing world). Moreover, as disclosed herein, functionally significant Ad5-specific NAbs were found to be directed almost exclusively against the hexon protein (see also Sumida et al. 2005). In contrast, Ad5-specific antibodies directed against the fiber protein do not significantly suppress Ad5 vaccine immunogenicity under relevant natural existing circumstances, i.e. which circumstances relate to anti-Ad5 NAbs titers found in humans. In contrast to Ad5, Ad35 and Ad11 seroprevalence and titers are found to be very low in human populations in many different parts of the world.

The present invention relates to novel adenoviral vectors that carry the hexon of a rare (or at least rarely neutralized) Ad serotype such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50 and preferably at least the fiber knob domain from a serotype that interacts with the CAR receptor, such as most human adenovirus serotypes from subgroup A, C, D, E, and F and the ovine

adenoviruses. Preferred serotypes that are used as a backbone vector and thus carry their own hexon protein towards which rarely NAbs are found in the human population are Ad11 and Ad35. A preferred serotype that is used as a backbone vector and for its fiber is Ad5. It is well recognized in the art that cells that play a major role in immune responses are dendritic cells, since these are cells that are able to efficiently present immunogenic peptides on their surface thereby eliciting a strong immune response against such a peptide. Vectors that have been preferred in the art are because of their ability to infect dendritic cells very efficiently are those from adenovirus subgroup B, or vectors that carry fiber proteins from adenoviruses that recognize and infect dendritic cells in an efficient manner, see for example WO 02/24730 and WO 00/70071. These vectors presumably use CD46 as receptor. Surprisingly, as shown herein, it was now found by the inventors of the present invention that a vector carrying at least a fiber knob of an adenoviral vector known to interact through CAR, namely Ad5, when cloned on top of a vector that encounters low levels of pre-existing immunity, behaves such that immune responses are improved. This is highly unexpected taking the desired immune response route via dendritic cells into account.

It should be understood that the knob domain, which domain can easily be distinguished from the rest of the fiber protein by those of general skill in the art (through sequence comparisons and general knowledge about receptor interactions) is the domain that is predominantly involved in the recognition of specific cellular receptors to which the adenoviral vector binds. It was previously shown that the Ad5 fiber knob interacts with CAR on the surface of cells and mediates efficient

viral attachment prior to viral entry that is further facilitated by the penton base and cellular integrins (Bergelson et al. 1997; Bewley et al. 1999; Roelvink et al. 1998 and 1999; Wickham et al. 1993), while the B group viruses, such as Ad11 and Ad35 interact through CD46 (Gagger et al. 2003). The present invention relates to adenoviral vectors in which at least the receptor-binding domain (defined by the knob domain) is swapped. The latter vectors thus still may comprise fiber domains such as parts of the shaft and/or the tail from the backbone vector. It is actually desired to use at least a part of the tail region of the backbone vector to ensure a proper stable interaction with other capsid proteins, resulting in stable recombinant vectors.

The chimeric adenoviral vectors of the present invention are more efficient for vaccines and gene therapy than vectors solely based on Ad5 or Ad35 without chimeric fibers. Chimeric adenoviruses carrying chimeric Ad5-Ad35 fibers are known in the art. Ad5 vectors carrying chimeric fibers in which at least the knob domain was derived from Ad35, were disclosed (WO 00/31285, WO 00/52186; WO 02/24730; Shayakhmetov et al. 2003; Rea et al. 2001). Smith et al. (2003) disclosed a chimeric fiber in which the tail and shaft are from Ad35, while the knob domain is from Ad5. All the vectors from these cited references are based on Ad5, implying the fact that the vectors would still encounter pre-existing immunity due to the presence of the Ad5 hexon in the viral vector capsid. The data provided by Ophorst et al. (2004) confirmed this phenomenon. Recombinant replication-defective Ad35 vectors and methods for producing them are also known in the art (WO 00/70071; WO 02/40665), whereas Ganesh et al. (2003) disclosed the use of an Ad35-based vector which comprises a fiber in which

the fiber knob of Ad35 has been replaced by that of Ad5. However, the vector carried a marker gene (Green Fluorescence Protein, GFP) and apparently exhibited low transduction efficiencies. Consequently, Ganesh et al. do not disclose or suggest vaccines based on vectors wherein at least the knob of Ad35 was replaced by the knob of Ad5, let alone advantages associated with their use. Ganesh et al. disclose also a vector based on Ad35 with a complete Ad5 fiber. It must be noted that this vector comprises a full Ad5 fiber and not a chimeric fiber and thus differs from the vectors disclosed herein. Ganesh et al. warn that low transduction efficiencies were found with the partial fiber swap. The vectors according to the invention have good transduction efficiencies, while the chimeric fibers ensure a stable anchoring in the remaining capsid. The person skilled in the art would not have an incentive for vaccination purposes to replace the fiber knob of serotypes that are known to infect dendritic cells very efficiently (such as those of subgroup B, for instance Ad11, Ad34 and Ad35) by a fiber knob from a serotype that infects cells primarily through CAR.

Because the neutralizing activity from the host towards the adenoviral vectors is primarily due to NAbs against the hexon protein, adenoviral vectors may also be based on highly neutralized serotypes, exemplified by Ad5, wherein the hexon is swapped against the hexon of a rarely neutralized serotype, such as Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50. An example of such a vector is referred to as Ad5h35 (Ad5-based vector comprising the hexon protein from Ad35 in place of the Ad5 hexon). A person skilled in the art is able to envision and to make the different possible combinations based on the information disclosed herein and based on

knowledge available in the art. It became clear that exchanging entire hexons proved difficult (Gall et al. 1998; Youil et al. 2002). One new and useful way of constructing chimeric hexon protein is disclosed in the examples. Another way of cloning vectors comprising chimeric capsid using hexon proteins from other serotypes has been disclosed in example 8 of WO 00/03029.

The recombinant adenoviral vectors of the present invention, namely those based on serotypes that encounter generally low pre-existing activities in the human population (Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50, as well as certain simian adenoviruses) carrying the knob domain of an adenovirus that generally elicit a high immune response, represent an improvement over both the adenovirus that generally elicits a high immune response as well as over the adenovirus that evades pre-existing neutralizing activity because it carries a hexon protein that is not detected by the pre-existing immunity in a high percentage of the world population. In other words and as exemplified herein, Ad35fib5 and Ad35k5 both represent improvements over both the Ad5 and the Ad35 vector.

Given the high levels of anti-Ad5 immunity in human populations, Ad5 vectors are likely to be substantially suppressed by anti-vector immunity, at least based on experiments done in mice and monkeys. Given the low levels of anti-Ad35 immunity in human populations, Ad35 and Ad35-related vectors have a substantial advantage over Ad5 vectors in this regard. In other words, Ad35, Ad35fib5 and Ad35k5 vectors are not substantially suppressed by anti-vector immunity. However, Ad35 vector-based vaccines are less immunogenic than Ad5 vector-based vaccines in terms of antibodies against the antigenic insert.

Importantly, it is now for the first time shown by the inventors of the present invention that low-neutralized vectors carrying at least a (heterologous) knob domain of a different, but highly immunogenic vector are substantially more potent in inducing an immune response than the backbone vector with its native fiber. Thus, such vectors represent a technical and practical advance over currently available adenoviral vectors. A person skilled in the art will be able to utilize the knowledge herewith provided to clone other adenoviral vectors based on the same principle. For instance, the invention also relates to recombinant simian adenoviruses that carry a knob domain of a fiber of an immunogenic human adenovirus. Besides recombinant simian adenoviruses other non-human adenoviruses, such as recombinant bovine and canine adenoviruses are encompassed by the present invention. It also relates to other low-neutralized human adenoviruses that can be employed as backbone vectors that carry at least a knob domain of other immunogenic adenoviruses. The different combinations are numerous, but limited to the extent that at least the hexon protein of the adenovirus should not encounter a high level of pre-existing immunity, while at least the knob domain delivers the recognition of the CAR receptor, thereby ensuring a high immune response in the host to which the vector is administered. A person of general skill in the art will be able to distinguish the described features by using the general knowledge in the art for determining pre-existing immunity, determining the level of immune responses elicited and the strategies for obtaining recombinant (chimeric) vectors as outlined by the present invention.

The potent immunogenicity of Ad35k5 vaccine vectors suggests a functionally relevant role of the Ad5 fiber

protein. The Ad5 fiber knob determines binding to the high-affinity receptor CAR and also plays an important role in the efficient intracellular trafficking of viral particles to the nucleus. The enhanced immunogenicity of Ad35k5 vectors likely reflects the known biologic functions of the Ad5 fiber knob, although the precise mechanisms have not yet been determined. The recombinant Ad35k5 vectors exhibit primarily the neutralization profile of the recombinant Ad35 vector and effectively evaded low/moderate levels of anti-Ad5 immunity, as shown herein. These observations are consistent with previous studies demonstrating that Ad5-specific NAbs are primarily directed against the Ad5 hexon protein. Supporting this model is also the observation that Ad5 vectors containing the Ad35 fiber protein were unable to circumvent anti-Ad5 immunity (Ophorst et al. 2004). However, high levels of anti-Ad5 immunity were in fact able to reduce the immunogenicity of Ad35k5 vectors, as shown herein, likely reflecting the lower titer but clearly detectable NAbs directed against the Ad5 fiber protein. It is to be noted that the reduction of Ad35k5 immunogenicity by anti-Ad5 immunity was only partial and was detectable only at particularly high Ad5-specific NAb titers.

Another limitation of Ad35k5 vectors may be their relatively high vp/pfu ratios, which were approximately 10-fold higher than those typically observed with Ad35 vectors. This observation suggests that viral integrity and stability may be compromised by the inclusion of a chimeric fiber protein, although this yet needs to be investigated and confirmed.

As outlined herein, the rhesus monkey studies comparing the immunogenicity of Ad5, Ad35k5, and Ad35 vectors confirmed the results obtained in mice. This is

relevant since mice lack the optimal Ad35 receptor CD46 and may therefore underestimate the immunogenicity of Ad35 vectors. In rhesus monkeys, Ad5 vectors elicited rapid and high titers of vector-specific NAbs, which effectively prevented a homologous boost immunization as expected. In contrast, both Ad35 and Ad35k5 vectors elicited lower vector-specific NAb titers as compared with Ad5 vectors, which facilitated boosting of these responses following re-administration of homologous vectors. It is speculated that the robust immune responses observed in the monkeys that received Ad35k5 vectors likely reflected the fact that these vectors both primed robust responses and could be boosted effectively.

The present invention demonstrates that capsid chimeric recombinant Ad vectors can be constructed to combine beneficial immunologic and serologic properties of different Ad serotypes. The generation of chimeric Ad vectors represents a novel strategy that leads to improved second-generation Ad vectors for both vaccination and gene therapy.

According to a preferred embodiment, the present invention relates to a replication-defective recombinant adenovirus serotype 35 (Ad35) vector comprising a chimeric fiber protein comprising at least a knob domain from a CAR-binding adenovirus serotype, wherein said recombinant vector further comprises a therapeutic nucleic acid of interest. A therapeutic nucleic acid is defined as a nucleic acid, which encodes a therapeutic proteinaceous substance, such as a protein, a peptide or a polypeptide, which is useful in the diagnostic, therapeutic and/or prophylactic treatment of mammals, preferably humans. Examples of therapeutic proteins are proteins that elicit immune responses in tumor

vaccination. Other examples are proteins that are useful in therapy of genetic disorders, such as those used in gene therapy. Preferred therapeutic proteins are proteins derived or based on or (directly) cloned from bacteria, parasites or infectious entities such as viruses. Adenoviral vectors are highly applicable for vaccination purposes against viruses such as Human Immunodeficiency Virus (HIV), SIV, Ebola virus, rabies virus, Herpes Simplex Virus (HSV), Hepatitis C virus (HCV), etc. Examples of HIV-derived antigens that may be encoded in the adenoviral nucleic acid are nef, gag, pol and env. An example of an HSV antigen is antigen 9D. Therapeutic proteins from parasites such as those that cause malaria may also be used in the vectors of the present invention. Particularly preferred proteins that may be cloned in these vectors are those from *Plasmodium falciparum*, such as the circumsporozoite (CS) protein and LSA-1. Other preferred proteins may be those from Mycobacterium strains, particularly those that cause tuberculosis, such as *Mycobacterium tuberculosis*. Preferred antigens from this bacterium are the 10.4, 85A, 85B and 85C antigens, which may thus also be used in the vectors of the present invention. Proteins used as markers for in vitro studies are typically not seen as therapeutic proteins, so GFP, luciferase, or CAT are not regarded therapeutic.

Suitable promoters and poly(A) sequences that may be used to express the antigens are well known in the art and include, but are not limited to CMV, Ad2 MLP, SV40, etc. Suitable transcription termination sequences may for example be derived from SV40 or BGH. The coding sequences of the antigens may be codon-optimised for optimal expression in mammals, preferably humans. Methods for codon-optimisation are well known in the art.

The invention relates to a replication-defective recombinant adenovirus selected from the group consisting of adenovirus serotypes 11, 24, 26, 34, 35, 48, 49 and 50, said adenovirus comprising a chimeric fiber protein comprising at least a knob domain from a CAR-binding adenovirus serotype, and wherein said recombinant vector further comprises a heterologous nucleic acid coding for a therapeutic or an antigenic protein of interest. A preferred serotype is a subgroup B serotype, more preferably serotype 11, 34 and 35, most preferably serotype 35 (Ad35). The invention relates to these serotypes since they encounter neutralizing pre-existing immunity in a significantly low percentage of human sera samples that are taken from healthy individuals worldwide. Chimeric fiber proteins as used herein refers to fibers that comprise parts from at least two different adenovirus serotypes, wherein the knob domain is from an adenovirus that binds to CAR. It is preferred that the tail domain of the fiber protein is of the backbone vector as this adds to the stability of the vector through a proper interaction with the capsid of the vector. In a preferred embodiment the knob domain that binds to CAR is from a fiber of an adenovirus serotype of subgroup C, more preferably from serotype Ad5.

A therapeutic protein of interest as used herein relates to proteins that are useful in mammalian therapeutic treatment, such a gene therapy. An antigenic protein of interest as used herein relates to a protein of interest towards which an immune response is invoked upon expression in the host, or in the host cells. This immune response is required for different kinds of vaccination settings: One example is tumour vaccination in which the immune response towards the antigenic protein of interest adds to the removal of tumour cells

that express the protein, while another preferred application is in prophylactic treatment such as vaccination to prevent or to significantly inhibit the infection of the host by pathogens, such as viruses, bacteria, yeast, or parasites. Thus, preferably, said antigenic protein of interest comprises a protein from a virus, a bacterium, a parasite or yeast. The use of recombinant adenoviruses according to the invention may also be used in invoking immune responses towards the antigenic proteins of interest in the course of a treatment of an infection that has already occurred, thus to prevent replication, packaging, etc. In other words, the vectors may also be used to prevent the spreading of the virus from the already-infected host to the next.

In one embodiment, the recombinant adenovirus according to the invention comprises a heterologous nucleic acid, which is under the control of a heterologous promoter.

In a preferred aspect of the invention, said antigenic protein comprises a protein from a virus, wherein said virus is a retrovirus, HSV or an Ebola virus, whereas it is preferred that if the antigenic protein is of a retrovirus, said virus is a simian or a human immunodeficiency retrovirus, wherein said heterologous nucleic acid preferably comprises one or more genes selected from the genes encoding the group of immunodeficiency virus proteins consisting of: gag, pol, env and nef.

In another aspect of the invention, said antigenic protein of interest is from a malaria-causing parasite, wherein said protein is preferably a circumsporozoite protein, or a Liver Specific Antigen (LSA-1, LSA-3), or an immunogenic part thereof, from a *Plasmodium* species, more preferably *Plasmodium falciparum*.

The invention relates to novel replication-defective recombinant adenoviruses comprising a heterologous nucleic acid and a chimeric fiber, wherein said adenovirus is selected from the group consisting of adenovirus serotypes 11, 24, 26, 34, 35, 48, 49 and 50, for use as a medicament.

The invention furthermore relates to the use of a recombinant adenoviral vector according to the invention in the manufacture of a medicament for the prophylactic or therapeutic treatment of a malaria-, an Ebola-, an HSV-, an HCV-, or an HIV infection.

In one particularly preferred embodiment, the invention relates to a chimeric replication-defective recombinant adenovirus comprising the knob domain from the fiber of Ad5, having the amino acid sequence as underlined in figure 2B, and a hexon protein from a serotype different from Ad5, wherein it is further preferred that said hexon protein is from a low-neutralized adenoviral serotype. Low-neutralized serotypes are those serotypes that encounter pre-existing neutralizing activity in the form of NAbs in the minority of samples from healthy individuals. In one specific embodiment, the invention relates to a chimeric replication-defective recombinant Ad35 vector comprising an Ad35 hexon and a chimeric fiber protein, said chimeric fiber protein having the amino acid sequence of SEQ ID NO:2.

Hexon HVR swaps

Since dominant Ad5-specific NAbs are primarily directed against the Ad5 hexon protein, it was reasoned that novel recombinant Ad5 vectors containing targeted mutational swaps in hexon may be able to evade dominant Ad5 hexon-specific NAbs. This is not a new thought and

several attempts in the art were made to produce such 'stealth' adenoviral vectors. However, none of them proved successful, as outlined below.

More than 99% of amino acid variability among hexon proteins from different serotypes seems to be concentrated in seven relatively short hyper variable regions (HVR's) that are located on the exposed surface of the hexon as identified by Crawford-Miksza and Schnurr (1996). These authors compared the hexon proteins of 15 different adenoviruses (Ad1, Ad2, Ad5, Ad6, Ad8, Ad9, Ad12, Ad15, Ad16, Ad31, Ad40, Ad41, Ad48, BAV3 and MAV1). The seven HVR's were identified among the 250 variable residues in loops 1 and 2 (also referred to as *l*1 and *l*2), wherein HVR1-HVR6 are in loop 1 and HVR7 is in loop 2. Crystal and co-workers (see WO 98/40509; US 6,127,525; US 6,153,435) replaced the entire loop 1 and 2 of the backbone vector (Ad5) with loops 1 and 2 of Ad2 (Gall et al. 1998) based on the findings of Crawford-Miksza et al. (1996). Also a vector was made in which only loop 2 was replaced. Viable viruses were produced. Ad2 and Ad5 are both subgroup C adenoviruses and cross-neutralization was still present, even after replacement of both loops, indicating that such swaps would not result in vectors that would have a decreased ability or inability to be recognized by a neutralizing antibody directed against the wild type adenovirus hexon protein, at least not when the swaps are within the same subgroup. To nevertheless try to obtain such vectors, they also tried to exchange the loops of Ad5 and replace them with the loops of Ad7. These attempts failed, no viable virus was produced, indicating that swaps from one subgroup to another were impossible to make, at least based on the identification of HVR's by Crawford-Miksza. Of course, with the knowledge of the viral genomic structure at hand, the

necessary genetic modification can be achieved via general molecular biology techniques, resulting in plasmids/cosmids that should encode for the entire recombinant (chimeric) virus. However, for unknown reasons, but most likely due to the rather critical aspects of the complex hexon structure and its role in capsid formation, viable viruses were not obtained when the hexon-coding region was modified (Rux et al. 2003). Gall et al. (1998) suggested exchanging only the external loops instead of exchanging the entire hexon. It was also mentioned that the other capsid proteins, such as penton may have significant neutralization epitopes to explain the failure with the Ad5-Ad2 swaps.

The inventors of the present invention also tried to generate chimeric adenoviruses based on Ad5 carrying chimeric hexon proteins comprising the HVR's of Ad35 or Ad48 in place of the Ad5 HVR's, as guided by Crawford-Miksza and Schnurr (1996) and as further outlined by Rux and Burnett (2000). These attempts failed. This was in concert with the findings of Crystal et al. who also could not show any production of recombinant viruses, wherein the hexon parts from different subgroups were exchanged (see above).

To tackle this matter, Rux et al. (2003) showed new high-resolution crystallographic refinements that were made of the Ad2 and Ad5 hexon structures to resolve earlier found differences in Ad2 and Ad5 hexon structures. This resulted in a new definition of the regions within the hexon protein, indicating nine HVR's instead of seven. Rux et al. (2003) also identified parts of the hexon protein that should not be violated when designing novel adenovirus-based vectors.

The inventors of the present invention also tried to produce chimeric adenoviruses comprising chimeric hexon

proteins with swapped HVR's, based on the definitions provided by Rux et al. (2003). However, again, no viable viruses could be produced. Clearly, based on the definitions provided in the art regarding the hexon HVR's, recombinant adenoviruses having one or more of the HVR's exchanged between a backbone vector and another serotype, at least between adenovirus serotypes of different subgroups, could not be produced.

The inventors of the present invention have now identified seven HVR's within human adenoviruses that differ from the seven HVR's identified by Crawford-Miksza and Schnurr (1996) and that also differ from the nine HVR's identified by Rux et al. (2003). The broad definition according to the invention is indicated in Table II, whereas an even more minimalistic definition is provided in Tabel IV. Replacement of these HVR's alone resulted in the production of viable virus. To the best of the knowledge of the inventors, this is the first time that anyone has been able to generate chimeric adenoviruses comprising chimeric hexon proteins, wherein not entire loops are exchanged, but wherein the distinct HVR's are exchanged. It is also held that this is the first successful attempt in obtaining recombinant adenoviruses comprising chimeric hexon proteins, wherein the hexons comprise parts from adenovirus serotypes from two different subgroups. The conceptual basis for the re-definition of the HVR's was to use conserved amino acids as junction points. It can nevertheless not be excluded that by shifting one or two or perhaps even three amino acids towards the N-, and/or C-terminus may yield in viable vectors. However, the definitions as previously provided in the art, were not sufficient to provide viable vectors with chimeric hexons. The definitions as provided herein are nevertheless not to be taken too

strictly, as slight shifts may also provide good results (see Table II in comparison with Table IV). Preferably, the identified HVR sequences (as represented by SEQ ID NO:17-79 and 88-150) are the regions that are swapped between serotypes.

Ad5-based vectors containing one or more HVR's, exchanged from Ad35 (subgroup B) or Ad48 (subgroup D) were constructed as disclosed in the examples. Clearly, one or more HVR's can be exchanged. As it is likely that NAbs are directed to any of the HVR's it is preferred to have most, if not all HVR's from a rare serotype in place of the wild type HVR's of the backbone vector. On the other hand, such large swaps might result in more-difficult-to-produce viable vectors. Herein, it is now disclosed that all seven identified HVR's within the backbone vector (exemplified by Ad5) can be replaced by the seven corresponding HVR's of a rare serotype (exemplified by Ad48). This also suggests that swaps based on the HVR* definitions of Table IV will also result in vialble viruses.

The spacer regions between the HVR's preferably remain to be of the backbone serotype to ensure proper folding of the protein. The preferred backbone is Ad5, although other-, generally used and widely applied serotypes form subgroup C, such as Ad2 can also be used. When HVR's are swapped, preferably at least one, more preferably two, even more preferably three, even more preferable four, even more preferably five, even more preferably six and most preferably seven HVR's are swapped.

The Ad5HVR35 and Ad5HVR48 vectors that were generated as disclosed herein are substantially more immunogenic than recombinant Ad5 vectors in the presence

of anti-Ad5 immunity, because NAbs, which are primarily directed against the hexon protein of Ad5 in Ad5-infected individuals, are no longer able to neutralize through the HVR's of hexon of Ad35 and Ad48. This feature holds true for all HVR's taken from all known rare serotypes, which may be selected from Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50.

As shown below, Ad5HVR48(1-7) viruses, containing all seven HVR's of Ad48 in place of the seven HVR's of Ad5, results in a virus that is not hindered by a pre-existing immunity induced by a pre-injection with Ad5 virus. This now enables one to use Ad5-based vectors in numerous settings, for instance in prime-boost regimens wherein it is required that the receptor recognition between the priming vector and the boosting vector remains the same. Another application would be the vaccination or therapeutic application with an Ad5-based vector in individuals that have encountered wild type Ad5 infection previous to the treatment.

Based on the identification of the HVR's as disclosed herein, numerous combinations between backbone vectors and other adenovirus serotypes are now feasible. The knowledge can be extrapolated to the hexons of all known human and non-human adenoviruses. Whenever a prime-boost regimen is required (not only for settings in which Ad5-based vectors are required), the knowledge provided herein can be applied to construct a vector with stealth-capacity, namely circumvention of pre-existing immunity against the hexon HVR's present in the previous applied virus. It is to be understood that the chimeric HVR vectors as disclosed herein can be further modified by methods known in the art. For instance, the fiber may be altered to yield a specific targeting ability (e.g., fiber knobs from subgroup B viruses, placed on subgroup C

based vectors may be targeted to smooth muscle cells, primary fibroblasts, dendritic cells, etc.). An example of such a vector would be an Ad5 based vector comprising the HVR's from for instance Ad48 and at least the fiber knob (the main determinant of receptor recognition) from for instance Ad35. Such vectors all fall within the scope of the present invention related to adenoviral vectors comprising hexons that are chimeric with respect to their HVR's.

The person skilled in the art will now be able, with the knowledge provided herein, to construct and to produce viable recombinant replication defective adenoviruses, which have one or more HVR's replaced by HVR's from other adenoviruses. Moreover, the sequences of the HVR's provided herein will now enable one to remove those sequences and to use these positions within the hexon to introduce other substances, such as targeting ligands to target the adenoviruses to cells of interest (in vivo and in vitro), radioactive binding sites for tracking adenoviruses in vitro, and certain B-cell epitopes (such as described by Worgall et al. 2005).

The present invention relates to a batch of a recombinant replication-defective adenovirus based on a serotype from a first subgroup, said adenovirus comprising a chimeric hexon protein wherein said hexon protein comprises sequences from the serotype of said first subgroup and at least one hyper variable region (HVR) sequence from a serotype from a second subgroup, wherein said first and said second subgroup are not the same. Preferably, said first subgroup is subgroup A, C, D, E, or F. Also preferred are batches according to the present invention wherein said second subgroup is subgroup B or D. More preferred are batches, wherein said

first subgroup is subgroup C, and the serotype from said subgroup C is Ad5, and wherein said serotype from subgroup B or D is selected from the group consisting of: Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50.

In a preferred embodiment of the present invention, said chimeric hexon protein comprises the first six HVR sequences (HVR1-HVR6), or all seven HVR sequences (HVR1-HVR7) from a serotype from said second subgroup.

In another preferred embodiment, said hexon protein retains the amino acid sequences of the backbone virus between the HVR sequences. Thus, the one or more HVR sequences as defined herein may be deleted, mutated, replaced by a linker or replaced by an HVR sequence from another serotype, highly preferably, the sequences linking the HVR's are maintained and are unchanged. This enables one to produce viruses that are stable due to their hexon-backbone structure provided by the non-HVR sequences. It is thus preferred that the sequences between the HVR sequences are from the serotype from said first subgroup.

In a highly preferred embodiment, the present invention provides batches of adenoviruses according to the present invention, wherein the first subgroup is subgroup C and wherein the at least one HVR sequence from the serotype of said second subgroup is selected from SEQ ID NO:24-79 and SEQ ID NO:95-150. In an even more preferred embodiment, the HVR1 sequence is selected from SEQ ID NO:17, 24, 31, 38, 45, 52, 59, 66, and 73, wherein the HVR2 sequence is selected from SEQ ID NO:18, 25, 32, 39, 46, 53, 60, 67, and 74, wherein the HVR3 sequence is selected from SEQ ID NO:19, 26, 33, 40, 47, 54, 61, 68, and 75, wherein the HVR4 sequence is selected from SEQ ID NO:20, 27, 34, 41, 48, 55, 62, 69, and 76, wherein the HVR5 sequence is selected from SEQ ID NO:21, 28, 35, 42,

49, 56, 63, 70, and 77, wherein the HVR6 sequence is selected from SEQ ID NO:22, 29, 36, 43, 50, 57, 64, 71, and 78, and wherein the HVR7 sequence is selected from SEQ ID NO:23, 30, 37, 44, 51, 58, 65, 72, and 79. In yet another preferred embodiment, the HVR1 sequence (HVR1*) is selected from SEQ ID NO:88, 95, 102, 109, 116, 123, 130, 137, and 144, wherein the HVR2 sequence (HVR2*) is selected from SEQ ID NO:89, 96, 103, 110, 117, 124, 131, 138, and 145, wherein the HVR3 sequence (HVR3*) is selected from SEQ ID NO:90, 97, 104, 111, 118, 125, 132, 139, and 146, wherein the HVR4 sequence (HVR4*) is selected from SEQ ID NO:91, 98, 105, 112, 119, 126, 133, 140, and 147, wherein the HVR5 sequence (HVR5*) is selected from SEQ ID NO:92, 99, 106, 113, 120, 127, 134, 141, and 148, wherein the HVR6 sequence (HVR6*) is selected from SEQ ID NO:93, 100, 107, 114, 121, 128, 135, 142, and 149, and wherein the HVR7 sequence (HVR7*) is selected from SEQ ID NO:94, 101, 108, 115, 122, 129, 136, 143, and 150. More preferably, the HVR1 sequence as represented by either the HVR1 sequences in Table II or by those in Table IV are deleted from the backbone vector. The reason for deleting the HVR1 region is that according to the crystal structure of the adenovirus hexon protein, the flanking sequences of HVR1 are very close together, indicating that these flanking sequences may be directly linked without loss of structure, except for the deletion. In another preferred embodiment, the HVR2 sequence as represented by either the HVR2 sequences in Table II or by those in Table IV are replaced by a short linker, more preferably by a two-amino acid linker, most preferably by a QG linker. Also the HVR2 flanking sequences are close together, following the crystal structure. However, as they are slightly more apart than seen for HVR1, it is preferred to include a small

bridging linker, preferably a linker of 1, 2, 3, or 4 amino acids, more preferably 2. Removal of HVR's from the hexon might be the ideal solution in preventing an attack by NAbs against the immunogenic determinants presented by the HVR's. However, in view of the crystal structure it does not seem feasible to delete HVR3-7 from the hexon protein without destroying its overall structure and thus possibly its function and role in capsid formation.

Although the hexon of Ad24 is also preferred for its HVR's, the sequence of the hexon protein of this specific 'rare' serotype was unknown to the inventors at the time of filing. Nevertheless, with the teaching as provided herein, the skilled person will now be able to determine the HVR's within any adenovirus hexon protein, including the preferred serotype Ad24. The use of any one of the HVR's of Ad24 is also part of the present invention.

The invention also relates to the use of an isolated nucleic acid encoding at least one HVR sequence of an hexon of a human adenovirus serotype from subgroup B in the construction of a chimeric hexon protein, which hexon further comprises sequences from an adenovirus from subgroup A, C, D, E, or F. Furthermore, the invention relates to the use of an isolated nucleic acid encoding at least one HVR sequence of an hexon of a human adenovirus serotype from subgroup D in the construction of a chimeric hexon protein, which hexon further comprises sequences from an adenovirus from subgroup A, B, C, E, or F. Preferably, the at least one HVR sequence is selected from SEQ ID NO:24-79 and 95-150.

Table III summarizes the various embodiments of the invention. The table indicates that the invention relates to a chimeric replication defective adenovirus based on a backbone adenovirus comprising a penton and hexon protein

of serotype X, wherein n HVR's in the hexon protein are of serotype Y, further comprising a fiber comprising a tail and a shaft of serotype F and a knob of serotype K, wherein:

- X is an adenovirus serotype selected from the group consisting of human adenoviruses 1-51, a simian-, a canine-, an ovine-, a porcine-, and a bovine serotype;
- Y is an adenovirus serotype selected from the group consisting of human adenoviruses 11, 24, 26, 34, 35, 48, 49 and 50, wherein X and Y may be the same or different, and wherein n represents any number of HVR's from 0 to 7, provided that if $X \neq Y$, that $n = 1-7$;
- F is an adenovirus serotype selected from the group consisting of human adenoviruses 1-51, a simian-, a canine-, an ovine-, a porcine-, and a bovine serotype, wherein F, X and Y may the same or different; and
- K is an adenovirus serotype that primarily uses CAR as cellular receptor, wherein K, F, X and Y may be the same or different. Preferably, at least part of the tail of the fiber protein that interacts with the penton protein is of serotype X in the event that F is not X.

It is to be understood that the backbone adenoviral vector may be any adenovirus (made recombinant such that it does not replicate in normal, non-packaging, cells) that is applicable for use in humans. Any of the human adenoviruses known (serotypes 1-51) within the six subgroups A, B, C, D, E, and F, as well as known simian-, canine-, bovine-, ovine-, and porcine- adenoviruses may be used as a backbone vector, as long as it is replication-defective and as long as it is applicable for human treatment. The backbone vector is replication-defective by deletion of at least one functional part of the E1 region, preferably by deletion of the entire

functional E1 region. Although the backbone vector typically comprises the other early and late regions, the skilled person is aware of the possibilities provided in the art to complement certain required adenoviral proteins by other means, for instance through complementation with helper viruses or by having the packaging cell transformed such that it comprises the required nucleic acids stably integrated in its genome. Typically, the viruses of the invention comprise an adenoviral genome with an E1 deletion, and preferably also an E3 deletion to provide space for heterologous nucleic acids of interest that may be cloned in the E1 region or in the E3 region, or both. The remaining regions, such as E2, E4, the ITR's and the late regions are generally present, although these may be complemented separately during production in a packaging cell. The vectors preferably comprises an E4orf6 region (possibly from another serotype) that is compatible with the E1B-55K protein present in the packaging cell line, as disclosed in WO 03/104467. This is done to enable production on known packaging systems such as PER.C6® cells or 293 cells, when the recombinant vector has an original E4orf6 region which is not compatible with E1B-55K of Ad5 present in such cells.

The backbone serotype is indicated above as 'X'. Further, the backbone virus may be engineered such that it contains HVR's from one or more of the adenovirus serotypes selected from human Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49, and Ad50 (indicated by serotype 'Y'). Clearly, when X is one of the Y serotypes, the HVR's may not have to be engineered as these serotypes are considered 'rare' and encounter only low pre-existing immunity is most individuals in the human population. So, when X is one of the Y serotypes, the number of swapped

HVR's (the number indicated by 'n'), may be zero, but also 1-7, whereas, when X is not one of the Y serotypes, at least one of the HVR's is from a Y serotype, wherein it is increasingly preferred to have more HVR's from a Y serotype, and wherein it is most preferred to have all seven HVR's exchanged for the corresponding regions from a Y serotype.

The backbone virus of the present invention comprises a penton protein of the same serotype as the backbone virus. The backbone virus is 'chimeric' in the sense that either the hexon is chimeric (having one or more HVR's exchanged or substituted) and/or in the sense that the fiber protein is chimeric and/or in the sense that the fiber protein is from a different serotype than the backbone virus. The possibility exists that X=Y=F=K, for example a recombinant Ad48 vector, as Ad48 binds CAR. However, such vectors are not 'chimeric' in the sense as described above, when n=0. Not-chimeric recombinant Ad11, Ad34, Ad35 and Ad50 do not fall within the above given definition, as the fiber knob should always be from a serotype that preferentially binds CAR, which these B-subgroup viruses do not.

The fiber is from a serotype indicated as 'F', comprising a tail from serotype F, comprising a shaft from serotype F and comprising a knob from a serotype indicated as 'K'. Clearly, when K is the same serotype as F, the fiber protein is not chimeric. K is a serotype that preferentially interacts with the CAR receptor and K may be the same serotype as X or different. Clearly, certain human serotypes, such as those from subgroup B, do not preferentially interact with CAR, but preferably interact with another cellular receptor, such as CD46. So, when K is the same as X, X is not one of the subgroup B adenoviruses. Also, when K is of a different serotype

than F (and the fiber is thus chimeric), at least part of the tail that interacts with penton in the capsid is of serotype X, as that would result in proper and stable capsid formation. As most of the capsid protein-encoding genes of the known adenoviruses are now available in the art, it is within the skill of the skilled artisan to identify the tail, shaft and knob region for every known and yet to be discovered adenovirus.

Preferred, but not limiting examples of chimeric replication-defective adenoviruses according to the present invention are: Ad5HVR11(1-7), Ad5HVR24(1-7), Ad5HVR26(1-7), Ad5HVR34(1-7), Ad5HVR35(1-7), Ad5HVR48(1-7), Ad5HVR49(1-7), Ad5HVR50(1-7), Ad5HVRPan9(1-7), Ad5HVR11(1-6), Ad5HVR24(1-6), Ad5HVR26(1-6), Ad5HVR34(1-6), Ad5HVR35(1-6), Ad5HVR48(1-6), Ad5HVR49(1-6), Ad5HVR50(1-6), Ad5HVRPan9(1-6), Ad5HVR11(1), Ad5HVR24(1), Ad5HVR26(1), Ad5HVR34(1), Ad5HVR35(1), Ad5HVR48(1), Ad5HVR49(1), Ad5HVR50(1), Ad5HVRPan9(1), Ad11k5, Ad24k5, Ad26k5, Ad34k5, Ad35k5, Ad48k5, Ad49k5, Ad50k5, Pan9k5, Ad11f5, Ad24f5, Ad26f5, Ad34f5, Ad35f5, Ad48f5, Ad49f5, Ad50f5, Pan9f5, Ad11k26, Ad34k26, Ad35k26, Ad50k26, Pan9k26, Ad11f26, Ad34f26, Ad35f26, Ad50f26, Pan9f26, Ad11k49, Ad34k49, Ad35k49, Ad50k49, Pan9k49, Ad11f49, Ad34f49, Ad35f49, Ad50f49, Pan9f49, Ad2HVR11(1-7), Ad2HVR24(1-7), Ad2HVR26(1-7), Ad2HVR34(1-7), Ad2HVR35(1-7), Ad2HVR48(1-7), Ad2HVR49(1-7), Ad2HVR50(1-7), Ad2HVRPan9(1-7), Ad2HVR11(1-6), Ad2HVR24(1-6), Ad2HVR26(1-6), Ad2HVR34(1-6), Ad2HVR35(1-6), Ad2HVR48(1-6), Ad2HVR49(1-6), Ad2HVR50(1-6), Ad2HVRPan9(1-6), Ad2HVR11(1), Ad2HVR24(1), Ad2HVR26(1), Ad2HVR34(1), Ad2HVR35(1), Ad2HVR48(1), Ad2HVR49(1), Ad2HVR50(1), Ad2HVRPan9(1), Ad11k2, Ad24k2, Ad26k2, Ad34k2, Ad35k2, Ad48k2, Ad49k2, Ad50k2, Pan9k2, Ad11f2, Ad24f2, Ad26f2, Ad34f2, Ad35f2, Ad48f2, Ad49f2, Ad50f2, and Pan9f2.

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EXAMPLES

Example 1. International seroprevalence and NAb titers to Ad5, Ad35, and Ad11

Ad-specific neutralizing antibody (NAb) responses were assessed by luciferase-based virus neutralization assays generally as described by Sprangers et al. (2003). Briefly, A549 human lung carcinoma cells were plated at a density of $1x10^4$ cells per well in 96-well plates and infected with E1-deleted, replication-incompetent Ad-Luciferase reporter constructs at a multiplicity of infection (moi) of 500 with 2-fold serial dilutions of serum in 200 µl reaction volumes. Following 24 h incubation, luciferase activity in the cells was measured using the Steady-Glo Luciferase Reagent System (Promega). 90% neutralization titers were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

Further to the studies disclosed in WO 00/70071, experiments were performed to assess the seroprevalence and NAb titers to Ad5 and alternate Ad serotypes in the developing world. The luciferase-based virus neutralization assays from Sprangers et al. (2003) was applied by using serum samples obtained from healthy adults in the United States (N=19), Haiti (N=67), Botswana (N=57), Zambia (N=29), and South Africa (N=59). As shown in figure 1A, the Ad5 seroprevalence was 50% with relatively low median titers in the United States. In contrast, the Ad5 seroprevalence was 82% in Haiti, 93% in Botswana, 93% in Zambia, and 88% in South Africa. Moreover, the median Ad5-specific NAb titers in these samples were >10-fold higher than the median titers found in the United States (fig. 1B). These data extend the previous findings (Kostense et al. 2004; Vogels et al.

2003) and demonstrate that Ad5-specific NAbs are nearly universal and present in high titers in the developing world. In contrast, the Ad11 and Ad35 seroprevalence and titers were substantially lower in these populations.

Example 2. Immunodominant targets of Ad5-specific neutralizing antibodies

The samples outlined in the previous example were further utilized to determine the dominant antigenic targets of Ad5-specific NAbs in both the USA and sub-Saharan Africa. Given the lack of detectable NAb cross-reactivity between Ad5 and Ad35, the capsid chimeric Ad5/Ad35 viruses expressing luciferase as targets in virus neutralization assays were used. These vectors consist of various combinations of Ad5 and Ad35 hexon, penton, and fiber proteins in the context of intact virus particles with wild-type growth kinetics (Havenga et al. 2002; Rea et al. 2001). The chimeric vectors used in this study included Ad5f35 (Ad5 containing the Ad35 fiber knob, -shaft and an Ad35-Ad5 chimeric tail), Ad5f35p35 (Ad5 containing the Ad35 fiber and -shaft, the Ad35-Ad5 chimeric tail, and penton), and Ad35f5 (Ad35 containing the Ad5 fiber knob, -shaft and an Ad5-Ad35 chimeric tail).

As shown in figure 1C, comparable NAb titers were observed against Ad5, Ad5f35, and Ad5f35p35; all based on Ad5. Since the capsid of the Ad5f35p35 vector contains the Ad5 hexon but the Ad35 derived fiber and Ad35 derived penton, these data suggest that the majority of Ad5-specific NAb activity was directed against the Ad5 hexon. Lower but clearly detectable titers were also measured against recombinant Ad35f5 (herein also referred to as Ad35fib5), demonstrating that Ad5 fiber-specific NAbs were present at 5- to 10-fold lower titers than Ad5

hexon-specific NAbs in these samples. No Ad5 penton-specific NAbs were measured using these viruses, but the similar NAb titers against Ad5f35 and Ad5f35p35 suggested that penton-specific NAbs played at most a relatively minor role in this neutralization.

Example 3. Generation of Ad35f5 and Ad35k5

Recombinant Ad35-based vaccines are immunogenic in the presence of anti-Ad5 immunity. However, recombinant Ad35 vaccines are intrinsically less immunogenic than recombinant Ad5 vaccines in the absence of anti-Ad5 immunity (Barouch et al. 2004). This problem has now been overcome by constructing capsid chimeric recombinant Ad35 vectors in which at least the Ad35 fiber knob is replaced with the Ad5 fiber knob (referred to as Ad35k5 for the knob replacement and Ad35f5 for most of the fiber replacement).

Recombinant Ad35k5 vectors were produced by replacing the Ad35 fiber protein-encoding gene with a chimeric fiber protein-encoding gene consisting of the Ad35 fiber tail and shaft (amino acids 1-132) linked to the Ad5 fiber knob (amino acids 133-314). Ad5 fiber-specific antibodies do not blunt recombinant Ad5 vaccine immunogenicity. The Ad35 fiber knob does not interact with CAR since it is a subgroup B adenovirus (Roelvink et al. 1998) but instead utilizes CD46 as its receptor (Gagger et al. 2003). The different fiber proteins direct these viruses into different intracellular trafficking pathways. Previous studies have shown that the Ad5 fiber knob facilitates rapid viral escape from early endosomes into the cytosol, leading to efficient translocation of viral genomes into the nucleus, whereas the fiber knobs from subfamily B adenoviruses, including Ad11 and Ad35, lead to retention of virus particles in late endosomes

and recycling of a large fraction back to the cell surface (Shayakhmetov et al. 2003).

The cloning of the recombinant nucleic acid encoding the Ad35k5 vector was as follows. The region encoding the Ad5 fiber knob was synthesized by PCR and was cloned as a BsiWI-NheI fragment into the pBR.Ad35.PacI-rITR.dE3 plasmid (= pBr.Ad35.PRnΔE3 in WO 2004/001032) to replace the corresponding region encoding the Ad35 fiber knob. The mutant pBR.Ad35k5.PacI-rITR.dE3 plasmid together with the pWE.Ad35.pIX-EcoRV (WO 2004/001032) cosmid and the adaptor plasmid pAdApt35-SIVGag, comprising the SIVmac239 Gag gene, were then co-transfected into PER.C6/55K cells (see WO 00/70071 and WO 02/40665), and double homologous recombination yielded the recombinant Ad35k5-SIVGag vector. This vector was plaque-purified, sequenced, expanded, and purified by CsCl gradient centrifugation following general purification methodology known to the skilled person. The nucleotide sequence of the chimeric fiber is shown in figure 2A (SEQ ID NO:1), while the amino acid sequence is shown in figure 2B (SEQ ID NO:2).

The Ad35f5 vector was typically made as outlined for Ad35fib16 in WO 00/70071, and Ad5fibXX chimeras as outlined in WO 00/03029; wherein a PCR product encoding a partial tail of Ad5 fiber linked to the Ad5 fiber shaft and Ad5 fiber knob were placed on the remaining part of the Ad35 fiber tail. The cloning procedure was also performed using an E3 deleted backbone vector and using BsiWI and NheI as restriction/cloning sites. For vector details see WO 03/104467 and WO 2004/001032.

In detail, the following procedures were performed to construct pBr/Ad35.pacI-rITRfib5: A PCR was performed on the plasmid pBr/Ad35.PacI-rITRNotI (= pBr/Ad35.PRn see WO 2004/001032) with primer DF35-1: 5′- CAC TCA CCA CCT CCA ATT CC-3′ (SEQ ID NO:6) and DF35-2: 5′- CGG GAT CCC

GTA CGG GTA GAC AGG GTT GAA GG-3′ (SEQ ID NO:7) containing a BamHI and a BsiWI site. This PCR resulted in a DNA fragment of approximately 650 bp, starting in the fiber 35 tail region, by which both a BsiWI site and a BamHI site was introduced. Next, a PCR was performed on plasmid pBr/Ad35.PRn with primer DF35-3: 5′- CGG GAT CCG CTA GCT GAA ATA AAG TTT AAG TGT TTT TAT TTA AAA TCA C-3′ (SEQ ID NO:8) containing a BamHI and a NheI site and DF35-4: 5′- CCA GTT GCA TTG CTT GGT TGG- 3′ (SEQ ID NO:9). This PCR resulted in a DNA fragment of approximately 1400 bp, starting after the stop codon of fiber 35 to the E4 region. pBr/Ad35.PRn was digested with MluI and NdeI, removing a 2850 bp DNA fragment from the Ad35 backbone (thus removing most of the fiber 35 region). The PCR fragment from DF35-1 + DF35-2 was digested with MluI and BamHI and the PCR fragment from DF35-3 + DF35-4 was digested with BamHI and NdeI. Both PCR fragments were ligated into the cut pBr/Ad35PRn plasmid, resulting in the pBr/Ad35PRndFIB construct. Then, the newly introduced BsiWI and NheI sites were used to introduce the Ad5 fiber sequence into the Ad35 backbone, by producing a PCR product using primers 35F5-5-F: 5′-CGG GAA CGT ACG ACA CGG AAA CCG GTC CTC C-3′ (SEQ ID NO:10) and 35F5-R: 5′-CGG CTA GCT AGC TTA TTC TTG GGC AAT GTA TGA AA-3′ (SEQ ID NO:11) on Ad5 DNA as a template. After this, the E3 region was deleted as performed for de pBr/Ad35PRNdE3 construct.

A person skilled in the art will be able, by applying general common knowledge with respect to molecular cloning and adenoviral production, to generate these clones, to insert fragments, to generate PCR products and to delete certain fragments and to eventually produce viruses in packaging cell lines. Moreover, production and purification methods for

obtaining adenoviral batches that can be used in vivo and in vitro are also well known in the art.

All vectors discussed herein were obtained using general CsCl purification methods and were found to be stable over at least 5 passages on PER.C6/55K packaging cell lines (data not shown). Growth rates and yields of Ad35k5 vectors were comparable with parental Ad35 vectors (data not shown). However, ratios of viral particles (vp) to plaque-forming units (pfu) were approximately 10-fold higher for Ad35k5 vectors (100-1000) as compared with Ad35 vectors (10-100).

It was also investigated whether the vectors were still able to recognize their respective receptors through their natural- or replaced fiber knobs (Ad5 fiber binding to CAR and Ad35 fiber binding to CD46). These specific interactions were still established with the chimeric vectors as well as with the original Ad5 and Ad35 based vectors (data not shown).

Following the same strategies, chimeric replication-defective Ad35-based viruses were made comprising the knob of Ad26 or Ad49. These viruses are named Ad35k26 and Ad35k49 and use the CAR-binding capacity of the fiber knob of the rare serotypes Ad26 and Ad49 in combination with the hexon protein present in the capsid of the backbone vector, which is also a rare serotype, exemplified herein by Ad35. The nucleic acid and amino acid sequences representing the respective Ad35k26 and Ad35k49 chimeric fibers are given in figure 2E-2H.

Ad35k5, Ad35k26 and Ad35k49 viruses were compared in their ability to provide transgene expression in vitro. All vectors carried the SIVGag transgene. 7.5 x 10^4 A549 cells were infected with master virus seed stock viruses (unknown concentration of virus) for 2 h in a 2 ml

volume. Cells were then washed and cultured for 48 h. Gag staining was evaluated by intracellular staining using the 2F12 anti-p27 monoclonal antibody followed by analysis by flow cytometry. The limitation of this experiment is that a specific number of viral particles was not used, and thus it cannot be concluded definitively whether Ad35k49 grows to higher titer than the others or whether it has higher specific activity. In any way, this vectors seems to have certain advantages over the other vectors, either in replication/growth or in expression levels. Figure 16 shows that three representative batches of Ad35k49 showed a significant higher intracellular expression as compared to representative batches of Ad35k5 and Ad35k26. The recombinant adenoviral vector Ad35k49 is a preferred embodiment of the invention.

For a good production of adenoviral vectors on packaging cells it is realized in the art that the E1B-55K protein, generally available by a stable integration of its gene in the genome of the packaging cell, should be compatible with the E4orf6 protein produced by the E4 region of the viral vector. It was found previously that the E4orf6 protein of Ad35 was not compatible with the E1B-55K protein generally available in known packaging cells such as 293 cells and PER.C6® cells (see WO 03/104467; WO 2004/001032; WO 2004/018627; WO 95/34671). To circumvent using new cell lines with E1B-55K from Ad35 integrated in the genome, and thus to enable one to use established production platforms as those provided by PER.C6 cells, further constructs were made in which the E4orf6 region of the Ad35 backbone was replaced with the E4orf6 region from Ad5, in line as what has been described in detail in WO 03/104467. The replacement of

the E4orf6 region was applied in all Ad35-based vectors, but not further used in the immunogenicity studies and targeting studies described herein.

Example 4. Immunogenicity of recombinant Ad35f5 and Ad35k5 in comparison with Ad5 and Ad35

The immunogenicity of the vectors was assessed by comparison of the chimeric fiber carrying vectors to Ad5-SIVGag and Ad35-SIVGag.

Naïve mice (N=5/group) were immunized intramuscularly with 10^{10} vp Ad5-SIVGag, Ad35-SIVGag, Ad35.BSU.SIVGag and Ad35fib5.BSU.SIVGag. The term BSU relates to the use of the endogenous pIX promoter in the adenoviral genomic nucleic acid, which ensures a proper expression of the pIX gene during the production of Ad35-based recombinant viruses. For details relating to the BSU constructs, see WO 2004/001032. Control mice (N=3/group) were immunized with empty Ad5 and Ad35 vectors. After 28 days a blood sample was taken and immune responses were determined using a SIV gag ELIspot assay as described below.

Figure 3 shows the results of these experiments and indicates that all vectors were able to induce a proper T cell response towards the SIV gag protein in comparison to the empty vectors, indicating that the insert provides an immunogenic protein upon injection in mice.

Another set of naïve mice (N=8/group) were immunized with 10^9 vp or 10^8 vp of the Ad5-Gag, Ad35-Gag, and Ad35k5-Gag vectors (all encoding the SIV gag protein). Vaccine-elicited $CD8^+$ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays using the following method: Tetrameric H-$2D^d$ complexes folded around the dominant SIVmac239 Gag AL11 epitope peptide (AAVKNWMTQTL;

SEQ ID NO:3; Barouch et al. 2004) were prepared and utilized to stain P18-specific $CD8^+$ T lymphocytes from mice using methods known in the art. Mouse blood was collected in RPMI 1640 containing 40 U/ml heparin. Following lysis of the Red Blood Cells, 0.1 µg of PE-labeled D^d/P18 tetramer in conjunction with APC-labelled anti-CD8alpha mAb (Ly-2; Caltag, Burlingame, CA, USA) was utilized to stain P18-specific $CD8^+$ T lymphocytes. The cells were washed in PBS containing 2% FBS and fixed in 0.5 ml PBS containing 1.5% paraformaldehyde. Samples were analysed by two-color flow cytometry on a FACS Calibur (Becton Dickinson). Gated $CD8^+$ T lymphocytes were examined for staining with the D^d/P18 tetramer. $CD8^+$ T lymphocytes from naïve mice were utilized as negative controls and exhibited <0.1% tetramer staining.

As shown in figure 4, all three vaccines were comparably immunogenic at the high dose of 10^9 vp (A). Importantly, at the lower dose of 10^8 vp (B), immune responses elicited by recombinant Ad35k5-Gag were significantly more potent than those elicited by recombinant Ad35-Gag ($p<0.01$ comparing mean tetramer responses among groups of mice on day 14 using analyses of variance with Bonferroni adjustments to account for multiple comparisons) and nearly comparable in magnitude to those elicited by Ad5-Gag (p=ns). This is consistent with prior observations (Barouch et al. 2004; Lemckert et al. 2005). These data demonstrate that engineering the Ad35 vector such that it carries the knob domain of a subgroup C adenovirus (e.g., Ad5) provides a substantial improvement of its immunogenicity towards the antigen inserted into the vector.

These experiments were performed in the absence of anti-Ad5 immunity, and it is shown here that Ad5 and Ad35k5 raised comparable immune responses, while these

were significantly higher than the Ad35 vector. It is important to note that the Ad35k5 vector carries the Ad35 hexon, which adds to the higher efficacy in raising immune responses in individuals that do harbour neutralizing activity against the hexon protein of Ad5. Thus, the Ad35k5 vector is significantly more efficient in the presence of anti-Ad5 immunity than both Ad5- and Ad35-based vectors; Ad5 is less immunogenic under these conditions because of its native hexon protein, and Ad35 is less immunogenic in general because of its native fiber knob domain.

In cannot be excluded that neutralizing antibodies against the fiber protein may be raised upon infection. However, it is held that under natural conditions such neutralizing activities are minimal, while the major part of the immune response of the host will be against the hexon protein. It is important to note that experiments wherein the recombinant vectors are compared with pre-immunisation with parental vectors are to be performed under conditions that mimic the natural (human) situation, wherein wild type viruses infect a natural host once or twice, inducing naturally occurring levels of neutralizing antibodies. In mice in a laboratory setting one can raise extreme immune responses by multifold injections and high titer administrations. Experiments mimicking the natural situation indicate whether neutralizing antibodies that may be directed to the fiber protein indeed are important for neutralizing the administered adenoviral vector. They also reveal the importance of the presence of the shaft region of the fiber, which part is typically also available for neutralizing activities.

We postulate that vectors comprising at least the knob from adenoviruses that recognize and infect host

cells via CAR (e.g., Ad2, Ad5, etc), and further comprising a major immunogenic determinant of the capsid (i.e., the hexon protein) from a least neutralized serotype (e.g., Ad11, Ad24, Ad26, Ad34, Ad35, Ad48, Ad49 and Ad50) are excellent vaccination vectors because they combine the hitherto unknown advantages of low vector pre-existing immunity in the human population, high antigenic insert immune response, reproducible production, efficient transduction and good stability.

Example 5. Immunogenicity of Ad5, Ad35k5 and Ad35 in mice with pre-existing immunity.

Next, the impact of anti-Ad5 immunity on the immunogenicity of these vectors was evaluated. Groups of C57/BL6 mice (N=4/group) were pre-immunized once with 10^{10} vp Ad5-Empty 4 weeks prior to vaccination to generate low/moderate levels of anti-Ad5 immunity. Ad5-specific neutralizing antibody (NAb) titers in these mice were 64-128 (Barouch et al. 2004; Lemckert et al. 2005; Sprangers et al. 2003). As shown in figure 4C, tetramer$^+$CD8$^+$ T lymphocyte responses elicited by 10^8 vp Ad5-Gag were essentially ablated in these mice. In contrast, 10^8 vp Ad35-Gag and Ad35k5-Gag responses were not substantially affected by this level of anti-Ad5 immunity. Importantly, Ad35k5-Gag proved more immunogenic than both Ad5-Gag ($p<0.001$) and Ad35-Gag ($p<0.05$) in these mice on day 14 following immunization.

The ability of Ad35k5-Gag to evade low/moderate levels of anti-Ad5 immunity is consistent with the previous findings that Ad5-specific NAbs are directed primarily against the Ad5 hexon protein (see above; Sumida et al. 2005). However, low levels of NAbs directed against the Ad5 fiber protein in this prior study were also detected. This experiment was therefore repeated in

mice with high levels of anti-Ad5 immunity. Mice were pre-immunized twice with 10^{10} vp Ad5-Empty 8 weeks and 4 weeks prior to vaccination. Ad5-specific NAb titers in these mice were 8,192-16,384 (Barouch et al. 2004; Lemckert et al. 2005; Sprangers et al. 2003), comparable with the highest titers found in individuals in sub-Saharan Africa (Kostense et al. 2004; Sumida et al. 2005). As shown in figure 4D, tetramer$^+$CD8$^+$ T lymphocyte responses elicited by Ad35k5-Gag were partially reduced in these mice and were comparable with those induced by Ad35-Gag (p=ns). Thus, high levels of anti-Ad5 immunity partially suppressed the immunogenicity of Ad35k5 vectors.

To evaluate in further detail vector-specific immunity elicited by the chimeric Ad35k5-Gag vectors, heterologous prime-boost studies as well as virus neutralization assays were performed. Groups of naïve C57/BL6 mice (N=4/group) were primed at week 0 with 10^9 vp Ad35k5-Gag and then boosted at week 4 with 10^9 vp Ad5-Gag or Ad35-Gag. As shown in figure 5A, tetramer$^+$CD8$^+$ T lymphocyte responses elicited by Ad35k5-Gag were efficiently boosted by Ad5-Gag but not by Ad35-Gag. These data suggest that Ad35 and Ad35k5 induced substantial cross-reactive anti-vector immunity, whereas Ad5 and Ad35k5 were largely immunologically distinct. Consistent with these observations, mice immunized once with Ad35k5-Gag generated Ad35-specific NAbs comparable with those induced by Ad35-Gag but substantially lower Ad5-specific NAbs (Fig. 5B). Thus, Ad35k5 vectors exhibited serologic profiles more similar to Ad35 vectors than Ad5 vectors.

To assess the generalizability of these results, the immunogenicity of Ad35k5 vectors expressing another antigen (Env: HIV-1 89.6P Env gp120, for cloning details,

see herein and Vogels et al. 2003) in a different strain of mice was evaluated. Groups of naïve Balb/c mice (N=4/group) were immunized once intramuscularly with 10^9 vp Ad5-Env, Ad35k5-Env, or Ad35-Env. Cellular and humoral immune responses elicited by Ad35k5-Env vectors were intermediate between those induced by Ad5-Env vectors and Ad35-Env vectors, as measured by IFN-γ ELISPOT assays (Fig. 6A) and Env-specific ELISA's (Fig. 6B).

The ELISA's were performed as follows. Serum antibody titers from immunized mice specific for HIV-1 Env or SIV Gag were measured by a direct ELISA as described (Barouch et al. 2003; Sumida et al. 2005). 96-well plates coated overnight with 100 µl/well of 1 µg/ml recombinant HIV-1 MN Env gp120 or SIVmac239 Gag p27 polypeptide (ImmunoDiagnostics) in PBS, were blocked for 2 h with PBS containing 2% BSA and 0.05% Tween-20. Sera were then added in serial dilutions and incubated for 1 h. The plates were washed three times with PBS containing 0.05% Tween-20 and incubated for 1 h with a 1:2000 dilution of a peroxidase-conjugated affinity-purified rabbit anti-mouse secondary antibody (Jackson Laboratories, USA). The plates were washed three times, developed with tetramethylbenzidine, stopped with 1% HCl, and analyzed at 450 nm with a Dynatech MR5000 ELISA plate reader.

Example 6. Immunogenicity of Ad5, Ad35k5 and Ad35 vectors in rhesus monkeys.

To confirm the immunogenicity studies in mice, a pilot study to evaluate the immunogenicity of these vectors in rhesus monkeys was performed. Nine *Mamu-A*01*-negative rhesus monkeys (N=3/group) were immunized intramuscularly with 10^{11} vp Ad5, Ad35 or Ad35k5 vectors expressing SIV Gag and HIV-1 Env. Monkeys were primed at

week 0 and received a homologous boost immunization at week 12. Figure 7 depicts antigen- and vector-specific immune responses in these animals. Gag- and Env-specific cellular immune responses were quantified by pooled peptide IFN-γ ELISPOT assays, and vector-specific NAb titers were determined by the luciferase-based virus neutralization assays at multiple time points following immunization. The neutralization assay was performed as described above.

The Ad5 vectors elicited high frequency ELISPOT responses following the primary immunization as expected (mean Gag+Env responses of 538 SFC/10^6 PBMC at week 12), but these responses were not substantially increased following the homologous boost immunization (mean 608 SFC/10^6 PBMC at week 16; Fig. 7A), presumably reflecting the rapid generation of high titers of Ad5-specific NAbs in these animals (Fig. 7B). In contrast, the Ad35 vectors elicited antigen-specific ELISPOT responses that were approximately 2-fold lower than those induced by the Ad5 vectors following the initial immunization (mean 248 SFC/10^6 PBMC at week 12). Interestingly, these responses increased substantially following the homologous boost immunization (mean 876 SFC/10^6 PBMC at week 16; Fig. 7C), consistent with the lower titers of vector-specific NAbs initially generated in these animals (Fig. 7D). These data suggest that Ad35 vectors elicited both lower antigen-specific immune responses as well as lower vector-specific immune responses as compared with Ad5 vectors in rhesus monkeys.

The Ad35k5 vectors elicited antigen-specific ELISPOT responses comparable with those induced by the Ad5 vectors following the primary immunization (mean 578 SFC/10^6 PBMC at week 12; Fig. 7E). Importantly, these responses were substantially enhanced following the

homologous boost immunization (mean 1736 SFC/10^6 PBMC at week 16), presumably reflecting the relatively low vector-specific NAbs initially generated in these monkeys (Fig. 7F). In fact, following the boost immunization, the Ad35k5 vectors elicited 2-3 fold higher Gag- and Env-specific ELISPOT responses than both the Ad5 and Ad35 vectors. Moreover, the Ad35k5 vectors elicited potent fractionated $CD4^+$ and $CD8^+$ T lymphocyte responses at week 16 following immunization as determined by ELISPOT assays using CD8-depleted and CD4-depleted PBMCs (Fig. 8A, B, C: Ad5, Ad35, Ad35k5 respectively).

Example 7. Generation of recombinant Ad5 vectors containing chimeric hexon proteins.

The HVR's of Ad2 according to Crawford-Miksza and Schnurr (1996), of Ad5 according to Rux and Burnett (2000), of Ad5 according to Rux et al. (2003) and of Ad5 according to the present invention are depicted in table I. Table II provides the seven HVR sequences of human adenoviruses Ad5, Ad11, Ad26, Ad35, and Ad48, and chimpanzee adenovirus 68 (Pan9) according to the HVR definition of the present invention. Specific positions within the respective hexon sequences are also depicted.

Ad5-based vectors containing one or more HVR's, exchanged from Ad35 (subgroup B) or Ad48 (subgroup D) were constructed.

In a more 'minimalistic' embodiment of the present invention, the HVR's of the above mentioned adenovirus serotypes are defined in a somewhat shortened fashion. These HVR's are depicted with an asterisk in Table IV as HVR(1-7)*. Using the minimalistic definition in recombinant vectors, HVR1* is deleted from the backbone, HVR2* is replaced with a short two-amino acid spacer (QG), whereas HVR3*, HVR4*, HVR5*, HVR6*, and HVR7* are

replaced by their respective shorter (*) counterparts from the other serotypes. Most significantly, HVR7 is redefined much narrower (compare Table II and Table IV).

Partial hexon genes containing the desired sequences were synthesized by GeneArt (Germany) and cloned as ApaI-HpaI fragments into a shuttle plasmid containing the complete Ad5 hexon gene. A larger AscI-AscI fragment was then excised from this shuttle plasmid and utilized to replace the corresponding AscI-AscI fragment in the Ad5 cosmid pWE.Ad5.AflII-rITR.dE3 (vector carrying a deletion of the E3 region and based on cosmid pWE.Ad5.AflII-rITR, see WO 02/40665). The mutant Ad5 cosmids together with the adaptor plasmid pAdApt-Gag (encoding the gag protein of Simian Immunodeficiency Virus (SIV)) were then co-transfected into PER.C6/55K cells (harbouring the 55K E1B gene from Ad35 in PER.C6® cells, see WO 02/40665), and homologous recombination yielded recombinant Ad5HVR35(1)-Gag, recombinant Ad5HVR48(1)-Gag and recombinant Ad5HVR48(1-7)-Gag viruses, wherein the '1' indicates the replacement of only HVR1 and wherein '1-7' indicates the replacement of all seven separate HVR's. These vectors were plaque-purified, sequenced, expanded, and purified by CsCl gradient centrifugation according to general procedures known in the art. The sequence of the hexon in Ad5HVR48(1-7)-Gag is given in figure 11.

Besides the three viruses mentioned above, the following recombinant vectors are also made: Ad5HVR35(1-6), Ad5HVR35(1-7) (fig. 12), Ad5HVR11(1-6), Ad5HVR11(1-7) (fig. 13), Ad5HVR26(1-6), Ad5HVR26(1-7) (fig. 14), Ad5HVRPan9(1-6) and Ad5HVRPan9(1-7) (fig. 15). '1-6' indicates the replacement of HVR1-HVR6, leaving HVR7 of the parent vector. Clearly, based on the several parental vectors available (such as Ad2 and Ad5) and the several

known rare serotypes, more combinations are possible to create, using the teaching of the present invention. Other rare human adenovirus serotypes that may be used to provide HVR's to produce 'stealth'-like vectors are Ad24, Ad34, Ad49 and Ad50.

Example 8. Immunogenicity of Ad5, Ad5HVR48(1) and Ad5HVR48(1-7) viruses in naïve mice and in mice with anti-Ad5 immunity

As outlined above, viable recombinant Ad5-Gag, Ad5HVR48(1)-Gag and Ad5HVR48(1-7)-Gag viruses were produced on packaging cells. The yield of Ad5HVR48(1)-Gag was comparable to the recombinant Ad5-Gag virus, whereas the growth rate, yield and vp/pfu ratio of Ad5HVR48(1-7)-Gag virus was approximately 2-fold lower than the Ad5-Gag virus. The Gag expression was first checked on A549 cells infected with 10^9 or 10^{10} vp of each vector. HPLC data indicated that intracellular Gag expression was sufficient and comparable between the different vectors (data not shown), although the expression from Ad5HVR48(1-7)-Gag was somewhat lower than from Ad5-Gag. This could be related to the somewhat slower growth-rate.

The immunogenicity of the viruses was first investigated by immunizing naïve C57/BL6 mice (4 mice per group) with 10^9, 10^8 and 10^7 vp of each vector. The vaccine-elicited CD8+ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays as described above for two weeks. The results are shown in figure 9. Clearly, all three vectors resulted in comparable immune responses in these naïve mice, despite the small differences in growth rate and transgene expression.

Subsequently, C57/BL6 mice were pre-immunized with 2 injections of 10^{10} vp Ad5-empty, respectively 8 and 4 weeks prior to immunization with the viruses of interest

(Ad5 Nab titers 8, 192-16, 384) to yield a pre-existing immunity against the Ad5 base vector. Then (at 8 weeks from the first pre-immunization) the mice were immunized as above with 10^9, 10^8, and 10^7 vp of recombinant Ad5-Gag, Ad5HVR48(1)-Gag and Ad5HVR48(1-7)-Gag. Again, the vaccine-elicited CD8+ T lymphocyte responses were assessed by D^b/AL11 tetramer binding assays as described above, for two weeks. The results are shown in figure 10. ELISPOT results mirrored the tetramer assays and provided similar results (data not shown).

As expected the Ad5-Gag vector encountered pre-existing immunity in these pre-immunized mice which resulted in an hardly detectable immune response. The Ad5HVR48(1)-Gag virus also failed to circumvent the high levels of anti-Ad5 immunity, suggesting that pre-existing immunity is at least not limited to HVR1 alone. Importantly, the immunogenicity of the Ad5HVR48(1-7)-Gag virus was not influenced by the Ad5-induced pre-existing immunity, indicating that by mutating the 7 hexon HVR's (as identified herein) of Ad5 and replacing them with the corresponding HVR's of a rare serotype (exemplified by Ad48) results in a vector that is not hampered by pre-existing immunity against the wild type protein. Similar results were obtained in experiments wherein mice were primed twice with an empty Ad5 vector, representing a situation with high levels of pre-existing immunity (figure 21). This experiment was done with 4 C57/BL6 mice per group. Groups of mice were pre-immunized with two injections of 10^{10} vp Ad5-empty to induce anti-Ad5 immunity. Mice that were pre-immunized with Ad5-empty had Ad5 NAb titres of 8,192 - 16,384. Mice were then primed on day 0 intramuscularly with 10^9 vp Ad35-Gag and then boosted on day 28 with either 10^9 vp Ad5-Gag, 10^9 vp Ad35-Gag, or 10^9 vp Ad5HVR48(1-7)-Gag. All injections utilized

a volume of 50 µl. The arrows on the x-axis indicate immunizations. Blood samples were obtained at days 0, 7, 14, 21, 28, 35, 42, 49, 56 for Db/AL11 tetramer staining assays to quantitate vaccine-elicited CD8+ T lymphocyte responses. At day 56, IFN-gamma ELISPOT assays were also performed and showed comparable results (data not shown). Ad5-Gag failed to boost, presumably due to the pre-existing anti-Ad5 immunity. Also Ad35-Gag failed to boost, presumably due to the anti-Ad35 immunity induced by the priming immunization. Ad5HVR48(1-7) effectively boosted the responses, confirming that this vector functions as some sort of novel 'serotype'. It is concluded that Ad5HVR48(1-7) thus can serve as an effective boosting vector in settings where Ad5 fails, and in settings where a heterologous vector (such as Ad35) is used as a prime vector. Combined with the prior studies in the presentation, we conclude overall that vectors as represented by Ad5HVR48(1-7) are both effective priming vectors and effective boosting vectors in settings of pre-existing anti-Ad5 immunity where Ad5 fails.

These results now enable one to use Ad5-based vectors in prime-boost settings, while the receptor recognition (mainly brought about by fiber and penton proteins) remains unchanged.

Table I. Hyper-variable region definitions within the hexon protein of human adenoviruses Ad2 (according to Crawford-Miksza and Schnurr, 1996), Ad5 (according to Rux and Burnett, 2000); Rux et al. 2003) and Ad5 (according to the present invention). The HVR definitions of Ad5 of Rux and Burnett (2000) correspond exactly to the Crawford-Miksza definitions based on the Ad2 sequence. Note that these HVR definitions are all changed by 1 position in this table due to the absence of the initial Methionine residue in the Rux and Burnett (2000) definitions (HVR1: 137-181, etc.).

	Crawford -Miksza (1996)	Rux (2000)	Rux (2003)	Rux (2003)	Present invention
	Ad2	**Ad5**	**Master**	**Ad5**	**Ad5**
HVR1	137-188	138-182	146-181	139-167	136-165
HVR2	194-204	188-194	199-221	184-198	188-194
HVR3	222-229	212-219	264-270	none	212-220
HVR4	258-271	248-261	288-293	254-258	248-258
HVR5	278-294	268-283	307-330	272-280	268-281
HVR6	316-327	305-316	358-366	308	305-310
HVR7	433-465	422-450	482-489	420-422	418-451
HVR8			503-513	435-440	
HVR9			518-522	445-446	

Table II.

Ad5	Position	Sequence	
HVR1	136-165	DEAATALEINLEEEDDDNEDEVDEQAEQQK	SEQ ID NO:17
HVR2	188-194	VEGQTPK	SEQ ID NO:18
HVR3	212-220	YETEINHAA	SEQ ID NO:19
HVR4	248-258	GILVKQQNGKL	SEQ ID NO:20
HVR5	268-281	STTEATAGNGDNLT	SEQ ID NO:21
HVR6	305-310	TIKEGN	SEQ ID NO:22
HVR7	418-451	GGVINTETLTKVKPKTGQENGWEKDATEFSDKNE	SEQ ID NO:23
Ad48			
HVR1	136-150	EEKKNGGGSDANQMQ	SEQ ID NO:24
HVR2	173-185	IDATKEEDNGKEI	SEQ ID NO:25
HVR3	203-210	QDSDNYYG	SEQ ID NO:26
HVR4	238-251	AKFKTPEKEGEEPK	SEQ ID NO:27
HVR5	261-277	DIPSTGTGGNGTNVNFK	SEQ ID NO:28
HVR6	301-306	GKEDAS	SEQ ID NO:29
HVR7	414-446	DGAGTNAVYQGVKVKTTNNTEWEKDTAVSEHNQ	SEQ ID NO:30
Ad35			
HVR1	136-160	IAKGVPTAAAAGNGEEEHETEEKTA	SEQ ID NO:31
HVR2	164-175	LEISAENESKPI	SEQ ID NO:32
HVR3	193-203	TDLDGKTEEYG	SEQ ID NO:33
HVR4	231-243	AKPKNSEPSSEKI	SEQ ID NO:34
HVR5	253-262	DNSSQRTNFS	SEQ ID NO:35
HVR6	286-291	GTEDTS	SEQ ID NO:36
HVR7	399-431	DGIGVPTTSYKSIVPNGEDNNNWKEPEVNGTSE	SEQ ID NO:37
Ad11			
HVR1	136-157	IAEGVKNTTGEEHVTEEETNTT	SEQ ID NO:38
HVR2	181-191	LEVSDEESKPI	SEQ ID NO:39
HVR3	209-219	TDLDGKTEKYG	SEQ ID NO:40
HVR4	247-259	AKQKTTEQPNQKV	SEQ ID NO:41
HVR5	269-278	DAASQKTNLS	SEQ ID NO:42
HVR6	302-307	GTEDTS	SEQ ID NO:43
HVR7	415-447	DGIGVPTTSYKSIVPNGDNAPNWKEPEVNGTSE	SEQ ID NO:44
Ad26			
HVR1	146-163	ETKEKQGTTGGVQQEKDV	SEQ ID NO:45
HVR2	186-197	TDETAENGKKDI	SEQ ID NO:46
HVR3	215-222	QENEAFYG	SEQ ID NO:47
HVR4	250-262	AKFKPVNEGEQPK	SEQ ID NO:48
HVR5	272-288	DVPGGSPPAGGSGEEYK	SEQ ID NO:49
HVR6	312-317	GTSDNS	SEQ ID NO:50
HVR7	425-461	NGTGTNSTYQGVKITNGNDGAEESEWEKDDAISRQNQ	SEQ ID NO:51
pan9			
HVR1	136-147	TYKADGETATEK	SEQ ID NO:52
HVR2	170-177	TDTDDQPI	SEQ ID NO:53
HVR3	195-205	HDITGTDEKYG	SEQ ID NO:54
HVR4	233-243	ANVKTGTGTTK	SEQ ID NO:55
HVR5	253-263	DNRSAAAAGLA	SEQ ID NO:56
HVR6	287-292	GTDDSS	SEQ ID NO:57
HVR7	400-432	DAVGRTDTYQGIKANGTDQTTWTKDDSVNDANE	SEQ ID NO:58

Table II. Continued

Ad34	Position	Sequence	
HVR1	136-160	LDKGVTSTGLVDDGNTDDGEEAKKA	SEQ ID NO:59
HVR2	184-194	LEVSTEGPKPI	SEQ ID NO:60
HVR3	212-222	TDLDGKTEEYG	SEQ ID NO:61
HVR4	250-263	AKVKPKEDDGTNNI	SEQ ID NO:62
HVR5	273-282	DLRSQRSELK	SEQ ID NO:63
HVR6	306-311	GVSDAS	SEQ ID NO:64
HVR7	419-450	DGVGPRTDSYKEIKPNGDQSTWTNVDPTGSSE	SEQ ID NO:65
Ad49			
HVR1	136-147	DAKENNGQGEAK	SEQ ID NO:66
HVR2	170-183	IDENKEEDEEGREI	SEQ ID NO:67
HVR3	201-208	QNTENFYG	SEQ ID NO:68
HVR4	236-248	AVFKTGENGKPTE	SEQ ID NO:69
HVR5	258-270	DLRQNDTGGNNNQ	SEQ ID NO:70
HVR6	294-299	GTSDDS	SEQ ID NO:71
HVR7	407-442	DGSGSSTAYQGVEPDTTVAGTNDKWKVNAKVAQHNQ	SEQ ID NO:72
Ad50			
HVR1	136-150	LNKGDEEDGEDDQQA	SEQ ID NO:73
HVR2	174-185	LEVPSEGGPKPI	SEQ ID NO:74
HVR3	203-213	TDTDGTDEKYG	SEQ ID NO:75
HVR4	241-251	AKVKKEEEGKV	SEQ ID NO:76
HVR5	261-270	DLRSQMTGLK	SEQ ID NO:77
HVR6	294-299	GASDAS	SEQ ID NO:78
HVR7	407-439	DGVGPRIDSYKGIETNGDETTTWKDLEPKGISE	SEQ ID NO:79

Table III. The chimeric replication-defective adenovirus vector according to the present invention, including the identification of the different elements within said vector.

Part of the chimeric adenovirus / vector	Characterising feature
Backbone serotype X (Elements of the vector not complemented by packaging cell and excluding the fiber and hexon protein, including the penton protein)	Wherein X is selected from the group consisting of the adenoviruses applicable for human treatment (including human adenovirus serotypes 1-51, chimpanzee-, bovine- and canine adenoviruses)
Hexon HVR(n) Y	Wherein Y is selected from the group consisting of human adenovirus serotypes 11, 24, 26, 34, 35, 48, 49 and 50, and wherein n=0-7, provided that if X≠Y, n=1-7.
Fiber delta knob F, comprising **tail T** and **shaft S**	Wherein F is selected from the group consisting of serotypes 1-51, chimp, ... provided that if F≠X, at least the part of T of F that interacts with penton is X
Knob K	Wherein K is selected from the group consisting of adenovirus serotypes that primarily use the CAR receptor on a cell for infection (human adenovirus serotypes from subgroup A, C, D, E and F) and K may be X or different.

Table IV.

Ad5	Position	Sequence	
HVR1*	137-165	EAATALEINLEEEDDDNEDEVDEQAEQQK	SEQ ID NO:88
HVR2*	188-193	VEGQTP	SEQ ID NO:89
HVR3*	213-215	ETE	SEQ ID NO:90
HVR4*	252-258	KQQNGKL	SEQ ID NO:91
HVR5*	269-281	TTEATAGNGDNLT	SEQ ID NO:92
HVR6*	306-308	IKE	SEQ ID NO:93
HVR7*	432-445	KTGQENGWEKDATE	SEQ ID NO:94
Ad48			
HVR1*	137-150	EKKNGGGSDANQMQ	SEQ ID NO:95
HVR2*	173-184	IDATKEEDNGKE	SEQ ID NO:96
HVR3*	204-206	DSD	SEQ ID NO:97
HVR4*	241-251	KTPEKEGEEPK	SEQ ID NO:98
HVR5*	262-277	IPSTGTGGNGTNVNFK	SEQ ID NO:99
HVR6*	302-304	KED	SEQ ID NO:100
HVR7*	428-440	KTTNNTEWEKDTA	SEQ ID NO:101
Ad35			
HVR1*	137-160	AKGVPTAAAAGNGEEEHETEEKTA	SEQ ID NO:102
HVR2*	164-174	LEISAENESKP	SEQ ID NO:103
HVR3*	194-196	DLD	SEQ ID NO:104
HVR4*	234-243	KNSEPSSEKI	SEQ ID NO:105
HVR5*	254-262	NSSQRTNFS	SEQ ID NO:106
HVR6*	287-289	TED	SEQ ID NO:107
HVR7*	414-425	NGEDNNNWKEPE	SEQ ID NO:108
Ad11			
HVR1*	137-157	AEGVKNTTGEEHVTEEETNTT	SEQ ID NO:109
HVR2*	181-190	LEVSDEESKP	SEQ ID NO:110
HVR3*	210-212	DLD	SEQ ID NO:111
HVR4*	250-259	KTTEQPNQKV	SEQ ID NO:112
HVR5*	270-278	AASQKTNLS	SEQ ID NO:113
HVR6*	303-305	TED	SEQ ID NO:114
HVR7*	430-441	NGDNAPNWKEPE	SEQ ID NO:115
Ad26			
HVR1*	147-163	TKEKQGTTGGVQQEKDV	SEQ ID NO:116
HVR2*	186-196	TDETAENGKKD	SEQ ID NO:117
HVR3*	216-218	ENE	SEQ ID NO:118
HVR4*	253-262	KPVNEGEQPK	SEQ ID NO:119
HVR5*	273-288	VPGGSPPAGGSGEEYK	SEQ ID NO:120
HVR6*	313-315	TSD	SEQ ID NO:121
HVR7*	439-455	TNGNDGAEESEWEKDDA	SEQ ID NO:122
pan9			
HVR1*	137-147	YKADGETATEK	SEQ ID NO:123
HVR2*	170-176	TDTDDQP	SEQ ID NO:124
HVR3*	196-198	DIT	SEQ ID NO:125
HVR4*	236-243	KTGTGTTK	SEQ ID NO:126
HVR5*	254-263	NRSAAAAGLA	SEQ ID NO:127
HVR6*	288-290	TDD	SEQ ID NO:128
HVR7*	414-426	NGTDQTTWTKDDS	SEQ ID NO:129

Table IV. Continued

Ad34	Position	Sequence	
HVR1*	137-160	DKGVTSTGLVDDGNTDDGEEAKKA	SEQ ID NO:130
HVR2*	184-193	LEVSTEGPKP	SEQ ID NO:131
HVR3*	213-215	DLD	SEQ ID NO:132
HVR4*	253-263	KPKEDDGTNNI	SEQ ID NO:133
HVR5*	274-282	LRSQRSELK	SEQ ID NO:134
HVR6*	307-309	VSD	SEQ ID NO:135
HVR7*	434-444	NGDQSTWTNVD	SEQ ID NO:136
Ad49			
HVR1*	137-147	AKENNGQGEAK	SEQ ID NO:137
HVR2*	170-182	IDENKEEDEEGRE	SEQ ID NO:138
HVR3*	202-204	NTE	SEQ ID NO:139
HVR4*	239-248	KTGENGKPTE	SEQ ID NO:140
HVR5*	259-270	LRQNDTGGNNNQ	SEQ ID NO:141
HVR6*	295-297	TSD	SEQ ID NO:142
HVR7*	421-436	DTTVAGTNDKWKVNAK	SEQ ID NO:143
Ad50			
HVR1*	137-150	NKGDEEDGEDDQQA	SEQ ID NO:144
HVR2*	174-184	LEVPSEGGPKP	SEQ ID NO:145
HVR3*	204-206	DTD	SEQ ID NO:146
HVR4*	244-251	KKEEEGKV	SEQ ID NO:147
HVR5*	262-270	LRSQMTGLK	SEQ ID NO:148
HVR6*	295-297	ASD	SEQ ID NO:149
HVR7*	422-433	NGDETTTWKDLE	SEQ ID NO:150

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WHAT WE CLAIM IS:

1. A recombinant replication-defective adenovirus based on Ad5, characterized in that said recombinant replication-defective adenovirus comprises a chimeric hexon protein wherein the seven hyper variable regions HVR1-7 of Ad5 as defined in SEQ. ID. NO: 17-23 have been replaced by all seven hyper variable region sequences HVR1 to HVR7 from Ad48 (SEQ. ID. NO. 24-30), or from Pan9 (SEQ. ID. NO. 52-58), and wherein the hexon protein sequences between the HVR sequences are from Ad5.

2. A recombinant replication-defective adenovirus according to claim 1, which is an adenovirus based on Ad5 wherein HVR1-7 of Ad5 as defined by SEQ. ID. NO: 17-23 have been replaced with corresponding HVR1-7 of Ad48 as defined by SEQ. ID. NO: 24-30.

3. A recombinant replication-defective adenovirus according to claim 1, which comprises a chimeric hexon sequence of SEQ. ID. NO. 12 or of SEQ. ID. NO 16.

4. A recombinant replication-defective adenovirus according to claim 3, comprising the chimeric hexon sequence of SEQ. ID. NO. 12.

5. A recombinant replication-defective adenovirus according to any one of the preceding claims, comprising a heterologous nucleic acid of interest that is to be delivered to the host for prophylactic or therapeutic purposes.