SUPPLEMENTARY MATERIAL AND METHODS

Sequencing of Viral Genomic DNA

Genomic DNA was isolated from 3 X 10^{12} vp of purified virus preparation by digestion with Proteinase K (0.5 mg/ml) in 1% SDS-TEN (2 hrs at 55°C). After a Phenol-Chloroform extraction and Ethanol precipitation, the genomic DNA was resuspended in water and submitted for genomic sequencing. For full genomic sequencing, a shot gun library with overlapping fragments of 1-3 Kb in size was created and sequenced. Primers were designed to directly sequence the adenoviral DNA for closing the gaps and determine the DNA sequence of both ends. Partial sequencing of selected region of viral genome (ITRs, pIX, hexon, fiber, E3 region) was obtained by primer walking technique.

Phylogenetic analysis of chimpanzee adenovirus

The phylogenetic tree was calculated using the neighbor-joining method as implemented in ClustalX (41) and displayed using drawtree from PHYLIP version 3.69 (42). Alignment positions containing gaps were excluded from the analysis. The alignment of hexon proteins was manually optimized taking into account structural restraints from the Ad5 hexon Xray structure (43). Bootstrap confidence values are reported at branch points (1000 bootstrap cycles).

Vector Construction and Rescue

Genomic viral DNAs were cloned into a standard plasmid vectors by homologous recombination in E.coli BJ5183 using a shuttle vector containing viral DNA sequences derived from both left and right end of viral genome. As described more in detail below, the high degree of sequence homology within each species was exploited to clone the

entire genome of different isolates using shuttle vectors specific for species B, C and E adenoviruses.

Construction of a species C shuttle vector

The ChAd3 viral genome was fully sequenced and then the sequence information was used to construct a shuttle vector to facilitate the cloning and construction by homologous recombination of species C chimpanzee adenovirus.

Briefly, the shuttle vector pChAd3EGFP was constructed as follows: a ChAd3 DNA fragment (nt 3542-4105) containing pIX coding region was amplified by PCR with the oligonucleotides 5'-TATTCTGCGATCGCTGAGGTGGGTGAGTGGGCG-3' and 5'-TAGGCGCGCCCTTAAACGGCATTTGTGGGAG-3' digested with SgfI- AscI then cloned in a standard cloning vector digested with SgfI and AscI, generating pARS-ChAd3D. ChAd3 right end (nt 37320-37441) was amplified by PCR with oligonucleotides 5'-CGTCTAGAAGACCCGAGTCTTACCAGT-3' and 5'-CGGGATCCGTTTAAACCATCATCAATAATATACCTTATT-3' digested with XbaI and BamHI then ligated to pARS-ChAd3D restricted with XbaI and BamHI, generating pARS-ChAd3RD. ChAd3 viral DNA left end (nt 1-460) was amplified by PCR with oligonucleotides 5'-ATGGAATTCGTTTAAACCATCATCAATAATATACCTC-3' and 5'-ATGACGCGATCGCTGATATCCTATAATAATAAAACGCAGACTTTG-3', digested with EcoRI and SgfI then cloned pARS-ChAd3RD digested with EcoRI and SgfI, thus generating pARS-ChAd3RLD. The HCMV-EGFP-bgh polyA cassette was amplified by PCR using the oligonucleotides 5'-TCAAGATATCCCATT GCATACGTTGTATCCATATC-3' and 5'-TCAAGATATCTAGAGCCCACCGCATC

CC-3’ then cloned into pARS-ChAd3RLD by standard cloning techniques generating pARSChAd3-EGFP.

Construction of species B shuttle vector

The species B shuttle vector pARSChAd30_EGFP designed to clone species B chimp adenovirus, was constructed as described below. ChAd30 right end was amplified by PCR with oligonucleotides 5’-ATCGTCTAGATAGCTTACCGCACAAATCAG-3’ and 5’-ATCCGGGATCCGTTTAAACCATCATCAATAATATACCTTATAG-3’, digested with XbaI and BamHI then ligated to pARSChAd3-RLD restricted with XbaI and BamHI, generating pARS-ChAd30R. A ChAd30 DNA fragment containing pIX coding region was amplified by PCR with the oligonucleotides 5’-ATGCGCGATCGC AGTGAGTAGTGGGGAATGCTG-3’and 5’GTTAGGCGCGCCCCTCTACAGGGTC CTGGAAA-3’ digested with SgfI-AscI then cloned into pARS-ChAd30R digested with SgfI-AscI, generating pARS-ChAd30RD. ChAd30 viral DNA left end (nt 1-457) was amplified by PCR with 5’-ATGGAATTCGTTTAAACCAT CATCATCAATAATATA CCTTATAG-3’ and 5’-TCAAGATATCTACGTAAAAACACAGGACTTTG-3’, digested with EcoRI and EcoRV then cloned into pARS-ChAd30RD digested with EcoRI and EcoRV, thus generating pARS-ChAd30RLD. The HCMV-EGFP-bgh polyA cassette was amplified by PCR using the oligonucleotides 5’-TCAAGATATCCCATT GCATACGTTGTATCCATATC-3’ and 5’-TCAAGATATCTAGAGCCCACCGCATC CC-3’ then cloned into pARS-ChAd30RLD by standard cloning techniques generating pARS-ChAd30-EGFP.

Construction of species E shuttle vector

The shuttle pARSChAd63-EGFP vector used to clone subgroup E chimpanzee adenovirus was based on ChAd63 was constructed as follows. ChAd63 right end (nt 36216-36643) was amplified by PCR with oligonucleotides 5'-CGTCTAGACAGCGTCCATAGCTTACCG-3' and 5'-CGGGATCCGTTTAAACCATCATCAATAATATACCTCAAAC-3' digested with XbaI and BamHI then ligated to pARSChAd3-RLD restricted with XbaI and BamHI, generating pARS-ChAd63R. A ChAd63 DNA fragment (nt 3422-3814) containing pIX coding region was amplified by PCR with the oligonucleotides 5'-ATGCGCGATCGCGTGAGTAGTGTTTGGGGGTG-3' and 5'-TAGGCGCGCCGCTTCTCCTCGTTCAGGCTGGC-3' digested with SgfI- AscI then cloned into pARS-ChAd63R digested with SgfI- AscI, generating pARS-ChAd63RD. ChAd63 viral DNA left end (nt 1-455) was amplified by PCR with oligonucleotides 5'-ATGGAATTCGTTTAAACCATCATCAATAATATACCTCAAAC-3' and 5'-TCAAGATATCCGTAAAAACACCGGACTTTGAC-3', digested with EcoRI and EcoRV then cloned pARS-ChAd63RD digested with EcoRI and EcoRV, thus generating pARS-ChAd63RLD. The HCMV-EGFP-bgh polyA cassette was amplified by PCR using the oligonucleotides 5'-TGATATCCCATTGCATACGTTGTATCCATATC-3' and 5'-TGATATCTAGAGCCCACCGCATCCC-3' digested with EcoRV then cloned into pARS-ChAd63RLD digested with EcoRV, generating pARSChAd63RLD-EGFP.

Construction of ΔE1 ChAd Vectors

ChAd vectors carrying the deletion of E1 region were constructed exploiting the same strategy. The species-specific shuttle vector pARSChAd30-EGFP for species B, pARSChAd3-EGFP for species C, pARSChAd63-EGFP for species E were linearized

between pIX and the Ad right end fragments. BJ5183 cells were then co-transformed with ChAd purified viral DNA and the linearized shuttle vector. Each species-specific shuttle vector was used in the recombination with viral DNA obtained from virus belonging to the same species. Homologous recombination between pIX genes, right ITR DNA sequences present at the ends of linearized pARSChAd EGFP shuttle and viral genomic DNA allowed the insertion of the viral genome in the plasmid vector, deleting at the same time the E1 region that was substituted by EGFP expression cassette finally generating pChAd EGFP vectors. PmeI restriction sites (not present in most of the ChAd virus genomes) were inserted between viral ITRs and plasmid DNA sequences to release viral DNA from plasmid DNA before the rescue of the vector by transfection in the packaging cell line.

Deletion of E3 Region

E3 region of chimpanzee adenoviruses was mapped by homology with human Adenoviruses. A strategy aimed to delete the entire E3 region was then designed. Since no information were available on the viability of chimpanzee adenovirus carrying the entire deletion of E3 region, the deletion strategy was first tested in the contest of species C ChAd3 and of species E ChAd63. To this end, a deletion of the entire E3 region of ChAd3 vector, spanning from nucleotide 28645 to nucleotide 32632 was introduced in pChAd3EGFP. The final recombination product was the pChAd3ΔE1,E3/EGFP preadeno plasmid. Similarly, ChAd63 E3 deletion spanning from nucleotide 27208 to nucleotide 31786 was introduced in pChAd63EGFP. The vector backbones were manipulated by following a multi-step strategy based on steps of PCR and homologous recombination in BJ5183 cells. The final recombination products were the pChAd63ΔE1,E3/EGFP and pChAd3ΔE1,E3/EGFP preadeno plasmid. Once both vectors were proven to be viable, the

E3 deletion was introduced in other members of species C and E chimpanzee/bonobo adenoviruses by following the same strategy. No E3-deleted vectors of species B adenovirus were generated. Detail on the strategy used will be provided upon request.

Deletion of E4 Region and Insertion of Ad5 E4orf6

To improve the productivity of chimp Ad vectors in human packaging cell lines expressing Ad5 E1 region, the native chimpanzee E4 region was substituted with Ad5 E4 ORF6. The details are reported below for ChAd3, ChAd63 and ChAd30 vectors. All other B, C and E vectors were constructed by following a similar strategy with minor modifications to be adapted to individual virus DNA sequences. The native ChAd3 E4 region was substituted with Ad5 E4orf6 in the contest of pChAd3ΔE1,E3 preadeno plasmid. To this end, Ad5 E4orf6 was cloned into a shuttle plasmid containing the last 393 bp derived from the right end of ChAd3 genome (bp 37349 to bp 37741). Subsequently, a DNA fragment of 144 bp derived from the fiber 3'end and including the E4 polyA (from bp 34491 to bp 34634 of ChAd3 sequence) was introduced downstream Ad5E4orf6 generating the plasmid pARSChAd3Ad5E4orf6-2. Finally, a DNA fragment from pARSChAd3Ad5E4orf6-2 containing at the boundaries the fiber 3'end/E4 polyA and the ChAd3 right end was introduced by homologous recombination into pChAd3 ΔE1,E3 linearized with PacI restriction enzyme by co-transforming E. coli strain BJ5183, thus generating pChAd3 ΔE1,3,4 Ad5orf6. Following this strategy, the entire ChAd3 E4 coding region was deleted and substituted with Ad5E4orf6 gene cloned 62 bp downstream the putative E4 TATA signal under the control of the native ChAd3 E4 promoter. The E4 deletion/Ad5Orf6 insertion was included in all species C vector by following the same strategy. In order to substitute the native ChAd30 E4 region, Ad5 E4orf6 was amplified

by PCR with oligonucleotides 5'-TAGCGGCGCGCCCATGGGGGTAGAGTCATAATC-3' and 5'-TAGCTCTAGAATGACTACGTCCGGCGTTCC-3' and cloned into pARS-ChAd30RLD at the 5' of the 364 bp derived from the right end of ChAd30 genome, generating the plasmid pARSChA30 Ad5E4orf6-1. Subsequently, a DNA fragment of 208 bp derived from the fiber 3'end and including the E4 polyA was amplified by PCR with oligonucleotides 5'-TAGCGGCGCGCCTGTACATGACAACATAAAATAAAGATAAC-3' and 5'-TAGCGGCGCGCCCATCACTGATCCAAGATTAGCC-3'then fused into pARSChA30 Ad5E4orf6-1, to Ad5E4orf6, generating the plasmid pARSChA30 Ad5E4orf6-2. Finally, pARSChAd30Ad5E4orf6-2 linearized by restriction enzyme digestion to leave at the boundaries the fiber 3'end/E4 polyA and the ChAd30 left end was recombined by co-transforming BJ5183 cells with pChAd30-EGFP digested with PmeI restriction enzyme to release ΔE1 viral DNA, thus generating pChAd30 ΔE1,4 Ad5orf6 EGFP.

In order to substitute ChAd63 E4 region with Ad5 E4orf6, Ad5 E4orf6 was fused to native E4 promoter into pARS-ChAd63RLD-EGFP. Subsequently, a DNA fragment of 200 bp derived from the fiber 3'end and including the E4 polyA (from bp 33624 to bp 33823 of ChAd63 map) was introduced downstream Ad5E4orf6 generating the plasmid pARSChAd63Ad5E4orf6-2. Finally, pARSChAd63Ad5E4orf6-2 was linearized by restriction enzyme digestion to leave at the boundaries the fiber 3'end/E4 polyA and the ChAd63 left end was recombined with ΔE1 ChAd63 backbone by co-transforming BJ5183 cells with pChAd63-EGFP digested with PmeI restriction enzyme to release ΔE1 viral DNA, thus generating pChAd63 ΔE1,3,4 Ad5orf6 EGFP.

Following this strategy, the entire ChAd E4 coding region was deleted and substituted with Ad5E4orf6 gene cloned downstream the putative E4 TATA signal thus under the control of the native ChAd E4 promoter.

Rescue and amplification of ΔE1 Vectors

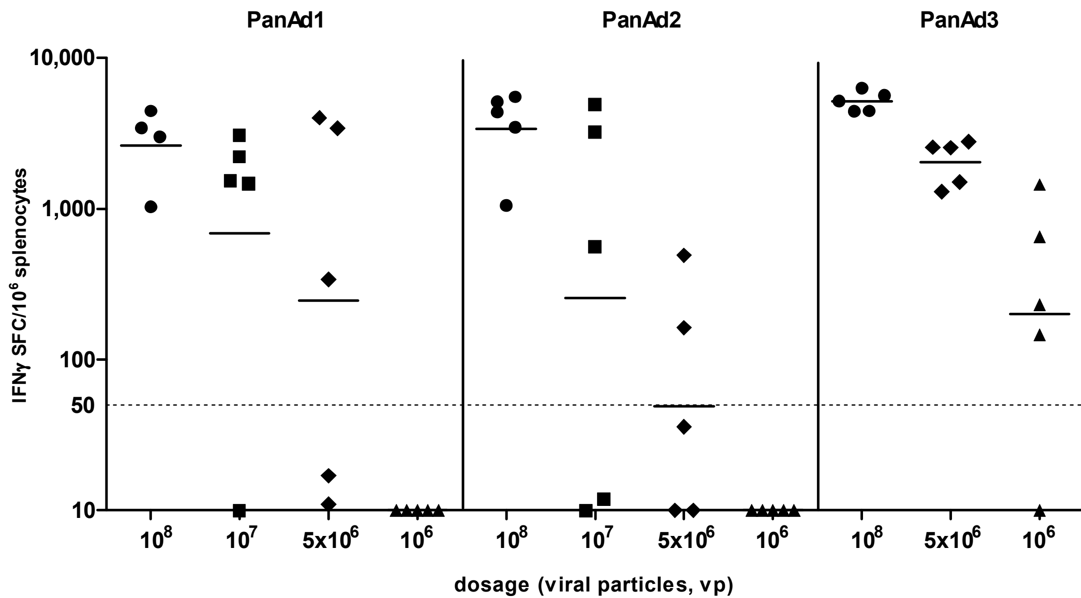
Chimpanzee preAd plasmid were first digested with PmeI to release the viral ITRs then $3\text{-}5x10^6$ HEK293/PER.C6 cells grown in DMEM, 10% fetal bovine serum (FBS), 1% Pen-Strep in 6 cm cell culture dishes were transfected with 10 micrograms of cloned viral vector released from plasmid sequences by endonuclease digestion. DNA transfection was performed using Lipofectamine (Invitrogen).

Transfected cells and culture medium were collected 5-10 days post-transfection and lysed by freeze-thaw. Rescued vectors were then amplified by serial passaging on HEK293 or PER.C6 cells. A large-scale amplification was performed by infecting cells planted on 5-10 cell-factories (NUNC, Inc.) on a total of $1\text{-}2x10^9$ cells. A purified vector preparation was obtained on cesium chloride gradient by two ultra-centrifuge runs, dialyzed against A195 buffer (44) (species C adenovirus; composition: 10mM Trizma base, 75mM NaCl, 1mM MgCl2, 0.02%, PS-80, 5% Sucrose, 0.1mM EDTA, 10mM L-Histidine, 0.5% Ethanol, pH 7.4) or A438 buffer (species B, E adenoviruses; composition: 10 mM Histidine, 7.5% sucrose, 35 mM NaCl ,1 mM $MgCl_2$, 0.1% PS-80, 0.1 mM EDTA, 0.5% (v/v) Ethanol pH 6.6 and stored at –70°C in aliquots.

Titration of Adeno Vector Preparations

Vector titration was performed by real-time quantitative polymerase chain reaction (PCR) employing TaqMan reporter-quencher dye chemistry. Primers and probe were designed on Human Cytomegalovirus (HCMV) promoter present in all vectors. The probe is labeled

with a reporter dye (6-FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end. Standard curves were generated with serial dilutions (from 10^7 to 10 copies) of preAd plasmid. Reactions were performed in a 50 µl reaction mixture containing 10 µl of DNA and 25 ul TaqMan Universal 2X PCR Master Mix (Applied Biosystems), 10 pmoles of each primer (Q-CMV forward primer GTACGGTGGGAGGTCTATAT AAGCA and Q-CMV reverse primer GGTGTCTTCTATGGAGGTCAAAACA) and
0.2 µM of fluorogenic probe (Q-CMV probe AGCTCGTTTAGTGAACCGTCAGATC GCC, (Applied Biosystem)). The reaction conditions were: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 60 s, 60°C for 60 s. The plate was loaded into the ABI PRISM 7900HT Sequence Detection System instrument according to the user manual instructions. Wild type viruses were quantified using a similar method with primers and probes annealing on conserved regions of hexon gene. Different sets of primers and probes were designed for each adenovirus species. Primers and probes for Species E, C and B are the following forward primer AGTGGGCGTACATGCACATC, reverse primer AAACTTGTTCCCCAGACTGAAGTAG, probe TACCTGAGTCCGGGTCTGGTGCAGTT; Species C chimpanzee adenovirus, forward primer CGCAGTGGTCGTACATGCA, reverse primer TTCCTAAACTTGTTACTCAGGCTGAAG, probe CTCGGGCCAGGACGCCTCG; Species B chimpanzee adenovirus, forward primer AGTGGGCATACATGCACATC, reverse primer AAACTTGTTCCCCAGATTGAAGTAG, probe TACCTGAGTCCGGGTCTGGTGCAGTT.



Supplementary Figure 1

Supplementary Fig.1 legend. Representative dose/response experiments with PanAdgag vectors. Groups of five BALB/c mice were immunized with escalating doses (indicated at the bottom of the graph) of PanAd1, PanAd2 or PanAd3 encoding HIV-1 gag and sacrificed three weeks later. Splenocyte IFNγ ELISpot responses to the CD8+ peptide AMQMLKETI are reported on the vertical axis and are expressed as IFNγ SFC per 10^6 splenocytes. Symbols correspond to individual mice responses, subtracted of the DMSO background (tipically not higher than 10 SFC/million spenocytes). Horizontal lines represent geometric mean of each group. A dashed line set at 50 SFC shows cut off to define a positive response. The immunological potency of each vector is defined as the minimal dose at which at least two out of five mice show a positive response, resulting in 10^6 vp for PanAd3 and $5x10^6$ vp for PanAd1 and PanAd2, as reported in Figure 1 panel C.