Video Article

Utilizing the Antigen Capsid-Incorporation Strategy for the Development of Adenovirus Serotype 5-Vectored Vaccine Approaches

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Abstract

Adenovirus serotype 5 (Ad5) has been extensively modified with traditional transgene methods for the vaccine development. The reduced efficacies of these traditionally modified Ad5 vectors in clinical trials could be primarily correlated with Ad5 pre-existing immunity (PEI) among the majority of the population. To promote Ad5-vectored vaccine development by solving the concern of Ad5 PEI, the innovative Antigen Capsid-Incorporation strategy has been employed. By merit of this strategy, Ad5-vectored we first constructed the hexon shuttle plasmid HVR1-KWAS-HVR5-His_6/pH5S by subcloning the hypervariable region (HVR) 1 of hexon into a previously constructed shuttle plasmid HVR5-His_6/pH5S, which had His_6 tag incorporated into the HVR5. This HVR1 DNA fragment containing a HIV epitope ELDKWAS was synthesized. HVR1-KWAS-HVR5-His_6/pH5S was then linearized and co-transformed with linearized backbone plasmid pAd5/ΔH5 (GL) , for homologous recombination. This recombined plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6 was transfected into cells to generate the viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6. This vector was validated to have qualitative fitness indicated by viral physical titer (VP/ml), infectious titer (IP/ml) and corresponding VP/IP ratio. Both the HIV epitope and His_6 tag were surface-exposed on the Ad5 capsid, and retained epitope-specific antigenicity of their own. A neutralization assay indicated the ability of this divalent vector to circumvent neutralization by Ad5-positive sera *in vitro*. Mice immunization demonstrated the generation of robust humoral immunity specific to the HIV epitope and His_6. This proof-of-principle study suggested that the protocol associated with the Antigen Capsid-Incorporation strategy could be feasibly utilized for the generation of Ad5-vectored vaccines by modifying different capsid proteins. This protocol could even be further modified for the generation of rare-serotype adenovirus-vectored vaccines.

Video Link

The video component of this article can be found at http://www.jove.com/video/52655/

Introduction

Human adenovirus (Ad) is a medium-sized, non-enveloped virus with an icosahedral nucleocapsid containing a double stranded DNA genome. Ad belongs to the Adenoviridae family, with a classification into seven groups (A through G). Each group contains virus of different serotypes. Of which, adenovirus serotype 5 (Ad5) from group C has been the most extensively studied and the most widely applied for vectored approaches like gene therapy and vaccinations.

The traditional transgene strategy has been developed and applied for Ad5 modifications, which is characterized by the displacement of the virus early genes with a gene-of-interest, and the focused expression of the gene-of-interest in a host. Examples are the construction of pENV9/Ad5hrΔE3 by replacing early gene 3 (E3) with rev gene of Simian Immunodeficiency Virus[1], the construction of AdCMVGag by replacing early gene 1 (E1) with the gag gene of Human Immunodeficiency Virus (HIV)[2], and the construction of Ad*lac*Z by replacing E1 with *lac*Z gene[3]. The broad application of the traditional transgene strategy on Ad5 depends on the following merits: the wide range of hosts for Ad5, the feasible gene engineering on virus and virus propagation, the large accommodation of foreign gene insert and the safety of Ad5[4,5]. However, the reduced efficacies of Ad5-vectored clinical therapies by use of this strategy has been a major bottleneck, which has been mapped to be primarily associated with Ad5 PEI, since Ad5 is so prevalent among the majority of children and adults[4,6].

To overcome the major bottleneck of Ad5, the primary objective is to develop an alternative strategy circumventing Ad5 PEI. Innate immunity[7], adaptive immunity such as neutralizing antibodies (NAbs)[8-10] and $CD8^+$ T cell responses[10] against Ad5 have been shown to contribute to Ad5 PEI, with Ad5 NAbs appearing to play the dominant role in the contributions to Ad5 PEI[10,11]. Moreover, Ad5 NAbs target epitopes located in capsid proteins, including the major protein hexon, fiber and penton base. Of which, hexon is the major target of Ad5 NAbs[8,11-13]. Based on these findings, an innovative Antigen Capsid-Incorporation strategy has been introduced. This novel strategy highlights the replacement or incorporation of proteins-of-interest on Ad5 capsid proteins, which shifts or masks the Ad5 neutralizing epitopes, leading to the decreased recognition by the NAbs and efficient Ad5 vector administrations. It is noteworthy that this strategy is competitive because it can also help hosts elicit robust humoral immunity and potent cellular immunity by directly presenting antigens-of-interest to the immune system[4,14,15]. Based on this

strategy, the molecular cloning and recombinant Ad viral vector rescue can be structurally divided into four main steps: (a) the preparation of gene-of-interest fragment by either polymerase chain reaction (PCR) or synthesis; (b) the ligation of gene fragment into a shuttle plasmid that contains the gene-of-interest fragment and homologous arms to an adenovirus backbone; (c) the homologous recombination by co-transforming the shuttle plasmid containing the gene-of-interest fragment with the linearized backbone plasmid pAd5/ΔH5 (GL)[16]; (d) the transfection of linearized recombinant adenoviral plasmid to rescue the recombinant Ad vector incorporated with antigens-of-interest.

Our group and some others have extended this alternative Ad incorporation strategy for Ad vectored vaccine development against different infectious pathogens. We reported the generation of a recombinant Ad vector Ad-HVR1-lgs-His_6-V3 by incorporating a His-tagged HIV-1 antigen V3 into the HVR1 locale of Ad5 hexon (hexon5). This generated vector triggered strong humoral immune response specific to the V3 epitope[4]. We also reported the development of Ad5/HVR2-MPER-L15ΔE1 by incorporating HIV-1 membrane proximal ectodomain region (MPER) into the HVR2 locale of hexon5[2]. In addition, Dr. Zhou's group has used the benefits of this Ad incorporation strategy to develop Ad serotype 3 (Ad3) vectored vaccines, *i.e.*, the generation of viral vector R1SP70A3 by incorporating a neutralizing epitope SP70 of Enterovirus 71 into the HVR1 of Ad3 hexon (hexon3). R1SP70A3 generated strong NAbs and IFN-γ production specific to the epitope SP70, which lead to the high rate of protection against Enterovirus 71 challenge[15].

For the purpose of technical reference, our study took advantage of the qualitative Antigen Capsid-Incorporation strategy to focus on the generation of a divalent Ad5 vector Ad5/H5-HVR1-KWAS-HVR5-His_6 by incorporating an HIV-1 antigen into HVR1 and a His tag into HVR5 of hexon5. The generated viral vector was also immunologically evaluated. The Antigen Capsid-Incorporation strategy could be utilized towards the development of Ad5-vectored vaccination approaches against different infectious diseases.

Protocol

The University of Alabama at Birmingham Institutional Animal Use and Care Committee approved the use of mice as described herein under the approved protocol number 101109272.

1. Genetic Construction of a Modified Plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6 with the Antigen Capsid-Incorporation Strategy

1. Construction of the shuttle plasmid HVR1-KWAS-HVR5-His_6/pH5S
    1. Order a plasmid containing the synthesized DNA sequence HVR1-KWAS between the restriction enzyme sites AgeI to AccI of hexon5 gene.
    2. Digest 6 μg of the synthesized fragment (HVR1-KWAS) with the enzymes AgeI (6 units) and AccI (6 units) for 3 hrs at 37 °C. Resolve the digested fragment in a 2% agarose gel by electrophoresis, and purify the fragment with a DNA gel extraction kit according to the manufacturer's protocol.
    3. Based on the molar ratio of insert to vector at 3 : 1, use T4 DNA ligase and ligase buffer to ligate 12 ng of the fragment (HVR1-KWAS) into 100 ng of a previously constructed shuttle plasmid HVR5-His_6/pH5S[17] in a 10 μl volume at RT for 2 hr, via the sites of AgeI and AccI.
    4. Transform 1 μl out of 10 μl of the ligation product into 50 μl of electrocompetent DH5α cells in an electroporator (at 1800 V). Add 950 μl of SOC medium to the transformed DH5α cells and incubate the mixture for 1 hr at 37 °C, with 300 rpm. Spread 100-200 μl of the 1 ml mixture on the Luria-Bertani (LB) agar containing kanamycin and incubate O/N at 37 °C.
        1. To screen ~ 10 colonies by PCR targeting the fragment HVR1-KWAS, mix the forward primer (TATGTGTGTCATGTATGCGT), reverse primer (GGAGGCCCACTTGTCCAGCTC) and a single DH5α colony into a PCR master mix solution (according to manufacturer's protocol). Run samples on a thermocycler by referring to the manual provided with the PCR master mix solution.
        2. Use the forward primer (TATGTGTGTCATGTATGCGT) to sequence the fragment HVR1-KWAS in the clones screened in the section 1.1.4.1.

2. Construction of the plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6
    1. Digest 6 μg of HVR1-KWAS-HVR5-His_6/pH5S with enzymes EcoRI (6 units) and PmeI (6 units) for 3 hrs at 37 °C, and purify the fragment containing homologous recombination arms and the dual modified hexon5 gene, as stated in step 1.1.2. Linearize the backbone plasmid pAd5/ΔH5 (GL)[16] with SwaI.
    2. Co-transform 100 ng of the target fragment from the shuttle plasmid and 100 ng of the linearized pAd5/ΔH5 (GL) backbone into 50 μl of electrocompetent BJ5183 cells for the homologous recombination in an electroporator (at 1800 V). Add 950 μl of SOC medium to the transformed cells and incubate the mixture for 1 hr at 37 °C, 300 rpm. Spread 100 - 200 μl of the 1 ml mixture on the LB agar containing kanamycin (final 50 μg/ml) for O/N incubation at 37 °C.
        1. Pick ~10 tiny colonies into 3 ml liquid LB containing kanamycin (final 50 μg/ml) for O/N incubation at a shaker (250 rpm, 37°C). Extract plasmids from the culture. Use PCR analysis to screen the 10 colonies by targeting the fragment HVR1-KWAS, as illustrated in step 1.1.4.1.
        2. To screen the same colonies by PCR analysis targeting the pIX fragment of the backbone genome, mix the forward primer (AGCTGTTGGATCTGCGCCAGCAGGTT), reverse primer (CCAAACAGAGTCTGGTTTTTTATTTAT) and a single BJ5183 colony into a PCR master mix solution (according to manufacturer's protocol). Run samples on a thermocycler by referring to the manual provided with the PCR master mix solution.
        3. Designate the PCR double-positive colonies as pAd5/H5-HVR1-KWAS-HVR5-His_6.
    3. Transform 100 ng of the plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6 into DH5α cells as illustrated in step 1.1.4.
        1. Use PCR analysis to screen ~ 10 colonies by targeting the fragment HVR1-KWAS, as illustrated in step 1.1.4.1.
        2. Use PCR analysis to screen the same colonies by targeting the pIX fragment of the backbone genome, as illustrated in step 1.2.2.2.

3. Use the forward primer (TATGTGTGTCATGTATGCGT) to sequence the fragment HVR1-KWAS in pAd5/H5-HVR1-KWAS-HVR5-His_6.

2. Preparation of Modified Ad5 Viral Vector Ad5/H5-HVR1-KWAS-HVR5-His_6

1. Constitute the complete medium by adding FBS (final 10%), 100X Non-Essential Amino Acids (final 0.1 mM), 200 mM L-glutamine (final 2 mM) and penicillin/streptomycin solution (final 1%) into DMEM with high glucose. Maintain human embryonic kidney (HEK293) cells in complete medium in a culture incubator (37 °C and 5% CO2 under 85% humidified conditions).
2. Rescue of viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6
    1. Seed 3.0 x 10^6 of the HEK293 cells in one T-25 flask containing 5 ml of complete medium and culture O/N to achieve a monolayer with 80% confluence.
    2. Linearize 15 µg of pAd5/H5-HVR1-KWAS-HVR5-His_6 in a 100 µl volume with restriction enzyme PacI (15 units) at 37 °C for 3 hrs.
        1. Extract linearized pAd5/H5-HVR1-KWAS-HVR5-His_6 by centrifuging the reaction twice (10,000 x g for 1 min) with an equal volume of phenol:chloroform:isoamyl alcohol (ratio at 25 : 24 : 1) in a fume hood, and by sequential centrifugation (10,000 x g for 10 min) with a mixed solution (300 µl of 100% ethanol and 10 µl of sodium acetate) and 700 µl of 70% ethanol. Discard supernatant at each centrifugation step.
    3. Resuspend the purified plasmid in ~ 40 µl of distilled water or Tris-EDTA (TE) buffer. Quantitate the plasmid DNA by measuring the optical density (OD) at 260 nm.
    4. Transfect 3 µg of the linearized plasmid with commercial liposomal transfection reagent in the T-25 flask, according to the manufacturer's manual. Change the transfection medium with complete medium at 6 hrs post-transfection and subsequent incubation in the culture incubator. Maintain transfected cells by replacing complete medium every two to three days, until individual viral plaques form.
    5. When plaques develop to full cytopathic effect (CPE), scrape the remaining cells off the flask in a sterile hood, and harvest cell lysate by centrifuging medium at 300 x g for 10 min at 4 °C. Suspend the cell pellet in ~1 ml of medium containing 2% FBS, and break cells by freeze-thawing four times. Collect the supernatant containing rescued virus after centrifuging lysate at 10,000 x g for 10 min at 4 °C.
3. Large scale propagation of the viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6
    1. Propagate the virus in a T-75 flask containing HEK293 cells in the sterile hood by infecting with 1/3 to full lysate from the T-25 flask. Allow full CPE to develop within two to three days in the culture incubator. Harvest the lysate supernatant as stated in step 2.2.5.
    2. Propagate the virus in one to three T-175 flasks in the sterile hood by infecting with 1/2 to full lysate from the T-75 flask, depending on the virus propagation conditions. Allow full CPE to develop within two to three days in the culture incubator. Harvest the lysate supernatant as stated in step 2.2.5.
    3. Propagate the virus in one dozen or more T-175 flasks in the sterile hood by infecting with 1/2 to full lysate from the previous T-175 flask(s). Determine the amount of flask usage based on the virus propagation conditions and the amount of virus in need. Harvest the lysate supernatant as stated in step 2.2.5 when full CPE occurs within two to three days in the culture incubator.
4. Purification of Ad5/H5-HVR1-KWAS-HVR5-His_6 by caesium chloride
    1. Prepare two densities of CsCl solutions in 5 mM HEPES: 1.33 g/ml and 1.45 g/ml, while avoiding contact with CsCl.
    2. Load 4 ml of CsCl (1.33 g/ml) in a sterile ultracentrifuge tube, and gently load 4 ml of CsCl (1.45 g/ml) against bottom of the tube. Load 4 ml of lysate supernatant slowly on top of the gradients in the sterile hood.
    3. Centrifuge the tube at 110,000 x g for 3 hrs at 4 °C to separate the mature/lower virus band from the defective/higher virus band. Collect the lower band using a 3 ml syringe armed with a 33 G needle and dilute the band with 5 mM HEPES to a 4 ml volume in the sterile hood.
    4. Load another tube with two densities of CsCl solutions and the diluted band of virus as described in step 2.4.2. Centrifuge the tube at 110,000 x g O/N at 4 °C. Collect the viral band as described in step 2.4.3.
    5. Inject the collected virus solution in a dialysis cassette by a 3 ml syringe armed with a 33 G needle in the sterile hood.
    6. Place the cassette in 700 ml of 1x dialysis buffer (1 L recipe: 100 ml of 10x PBS, 100 ml of 100% glycerol and 800 ml of distilled water; filter the buffer through a 0.22 µm filter) and replace the dialysis buffer every 3 hrs for 4 times. Aspirate out dialyzed virus solution using needled syringe.

3. Validation of the Rescued Viral Vector

1. Viral Vector Titrations
    1. For the virus physical titer, dilute both virus and dialysis buffer (background control) at 1 : 10 and 1 : 20 in virus lysis buffer (10% SDS in Tris-EDTA) in small tubes, and incubate all tubes at 56 °C for 10 min.
    2. Turn on a biophotometer and set the mode of absorbance at OD 260 nm. Use the diluted dialysis buffer (1 : 10) to balance the background signal by clicking the "blank" bottom. Type "10 + 90" in the biophotometer screen, and read the signal of the diluted virus (1 : 10).
    3. Read the signal of the diluted virus (1 : 20) by following the method in step 3.1.2, but instead type "5 + 95" in the biophotometer screen. Multiply the two virus readout numbers by $1.1x10^{12}$ and calculate the average titer with a unit as VP/ml, based on the two individual titers from the two dilutions.
    4. For the virus infectious titer (IP/ml), use the KARBER statistical $TCID_{50}$ method[14] to determine the infection depth on HEK293 cells at 10 days post infection (d.p.i.)
2. Evaluation of antigenic exposure display on the viral vector

1. Coat the viral vector in an ELISA plate and proceed with the rest of steps in a standard ELISA method[14]. Use human anti-gp41 (2F5) monoclonal antibody (mAb) and mouse anti-His tag mAb for the detection of corresponding incorporated antigens[14].
2. Resolve the viral vector in a denatured protein gel electrophoresis (SDS-PAGE) and proceed with the rest of steps in a standard western-blot method[14]. Use human anti-gp41 (2F5) monoclonal antibody (mAb) and mouse anti-His tag mAb for the detection of corresponding incorporated antigens[14].

3. *In vitro* evaluation of the vector on the ability to bypass Ad5-positve sera
    1. Maintain HeLa cells in complete medium in the culture incubator (37 °C and 5% CO_2 under 85% humidified conditions). Constitute the complete medium by adding FBS (final 10%), 200 mM L-glutamine (final 2 mM) and penicillin/streptomycin solution (final 1%) into Minimum Essential Medium Eagle (MEME).
    2. Seed HeLa cells in 6-well plates at $1x10^6$ cells/well, and incubate the plates in the culture incubator (37 °C and 5% CO_2 under 85% humidified conditions) for 2 hrs. Incubate Ad5-positive sera (0.1 µl or 0 µl) with 5 IP/cell of viral vector (Ad5 or Ad5/H5-HVR1-KWAS-HVR5-His_6) in the culture incubator for 1 hr before adding the mixtures onto the 6-well plates containing cells.
    3. At 24 hrs post infection (h.p.i.), rinse cells once with PBS and lyse cells in 0.5 ml of lysis buffer. Centrifuge at 15,000 x g for 5 min and collect the supernatant. Mix 20 µl of the supernatant with 100 µl of luciferase substrate for the luciferase signal reading, since viruses contain the luciferase gene.

4. Immunological Evaluation of the Rescued Viral Vector

1. Prepare two immunization groups: Ad5 and Ad5/H5-HVR1-KWAS-HVR5-His_6.
    1. Inject mice (n = 8/group) intramuscularly with viruses at $1x10^{10}$ VP/mouse, in a homologous “prime-boost” immunization regimen, with an interval of 2 weeks.
    2. At 2 weeks post every injection, bleed mice without anesthesia by cheek bleeding with animal lancets (5 mm tip length) to collect blood in 1.5 ml tubes.

2. Mix the blood in the tubes by inverting up and down, and incubate the blood at RT for 30 min. Spin the tubes at 10,000 x g for 5 min at 4 °C and transfer sera (top layer) to new 1.5 ml tubes.
    1. Spin the new tubes at 10,000 x g for 5 min at 4 °C and transfer sera to newly marked 1.5 ml tubes for storage at -80 °C.

3. Evaluation of antigen-specific humoral immunity by sera-based ELISA
    1. Dilute His peptide (stock at 1 mM) and HIV-1 peptide (stock at 1 mM) separately in 100.
    2. mM carbonate buffer (pH 9.5) to achieve the peptide coating concentration at 10 µM. Coat the ELISA plates with the diluted peptides separately by adding 100 µl per well and incubating the plates O/N at 4 °C.
    3. Wash the plates with PBST for four times at 200 µl/well and block for 1 hr in 5% BSA in PBST. Apply mice sera to the plates at 1 : 100 dilution in the blocking buffer, 100 µl/well. Incubate for 2 hr incubation at RT.
    4. Wash the plates with PBST for four times. Block the plates by incubating at RT for 30 min in the blocking buffer at 100 µl/well.
    5. Apply goat anti-mouse IgG-HRP (1:5000 in the blocking buffer) at 100 µl/well to both the plates coated with His peptide and HIV-1 peptide individually. Incubate for 2 hr at RT. Wash the plates with PBST.
    6. Read the plates at OD450 nm after incubating with HRP substrate for 30 min.

Representative Results

The Antigen Capsid-Incorporation strategy (**Figure 1A**) was utilized to generate the divalent Ad5 viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6. Firstly, the shuttle plasmid HVR1-KWAS-HVR5-His_6/pH5S was constructed by subcloning HVR1-KWAS fragment into the previous constructed shuttle plasmid HVR5-His_6/pH5S[17]. Secondly, the plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6[14] was constructed by a homologous recombination between the fragment of “EcoRI-HVR1-KWAS-HVR5-His6-PmeI” and SwaI-linearized pAd5/ΔH5 (GL)[16] (**Figure 1B**). Transfection of PacI digested pAd5/H5-HVR1-KWAS-HVR5-His_6 in a T-25 flask lead to the rescue of the viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6[14]. The rescued viral vector was then propagated in a large scale and purified by two cycles of CsCl gradient ultracentrifuge.

In order to demonstrate the viability/fitness of the rescued viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6, the physical titer was determined at 1.3×10^{11} VP/ml by measuring the physical copies of viral genome, and the infectious titer was determined at 1.4×10^9 IP/ml by titrating the actual infectious viral particles. The VP/IP ratio was subsequently calculated at 92[14]. Normally, an Ad with a VP/IP ratio below 1,000 indicates a qualitative fitness of this virus. In order to demonstrate whether this rescued virus displays the antigenic HIV antigen and His tag respectively on the surface of the viral capsid major protein hexon5, ELISA was undertaken with human 2F5 mAb and mouse anti-His tag mAb, respectively. Results indicated that both the human 2F5 mAb (**Figure 2A**)[14] and mouse anti-His tag mAb (**Figure 2B**)[14] showed binding to Ad5/H5-HVR1-KWAS-HVR5-His 6,whereas there was no binding to the negative control vector Ad5. Western-blot analysis was used to confirm the data in the ELISA, as both human 2F5 mAb (**Figure 2C**)[14] and mouse anti-His tag mAb (**Figure 2D**)[14] detected specific bands around 117 kDa only in the virus Ad5/H5-HVR1-KWAS-HVR5-His_6, which corresponds to the size of hexon5 protein. In order to evaluate the potential of Ad5/H5-HVR1-KWAS-HVR5-His_6 in circumventing neutralization by Ad5-positive sera, neutralization assay analysis was carried out as stated in the protocol section 3.3. The results showed that the relative luminescent unit (RLU) signal from the viral vector Ad5 treated with 0 µl of Ad5-positve sera is 12 times higher than that from the same vector treated with 0.1 µl of Ad5-positve sera (p value < 0.001). This suggested the neutralization of the vector Ad5 by the Ad5-positvive sera. In contrast, there was little difference from the viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6 treated with 0 µl and 0.1 µl of Ad5-positve sera (**Figure 3**). Since hexon is the major target of Ad NAbs, and hexon-specific neutralizing epitopes reside in all HVRs of hexon[12,18], the data above demonstrated that Ad5/H5-HVR1-KWAS-HVR5-His_6 escaped neutralization by Ad5-positve sera *in vitro* and suggested the potential of Ad5/H5-HVR1-KWAS-HVR5-His_6 on circumventing Ad5 PEI in the host.

To investigate whether the modified divalent Ad5 viral vector with the Antigen Capsid-Incorporation strategy could elicit robust humoral immunity specific to the incorporated antigens-of-interest, the "prime-boost" immunization regimen was applied to immunize mice with control viral vector Ad5 and the divalent vector Ad5/H5-HVR1-KWAS-HVR5-His_6. Sera-based ELISA coated with HIV-1 peptide showed that sera from immunization group of Ad5/H5-HVR1-KWAS-HVR5-His_6 had significant binding to the HIV-1 peptide at dilutions between 40 and 640, when compared to the sera from the control group (p value < 0.001) (**Figure 4A**)[14]. Sera-based ELISA coated with His peptide showed that sera from the immunization group of Ad5/H5-HVR1-KWAS-HVR5-His_6 had significant binding to the His peptide when compared to the sera from the control group, as statistical p value < 0.001 at the sera dilutions of 40 and 80, p value < 0.01 at the dilution of 160 and p value < 0.05 at a 320 dilution (**Figure 4B**)[14]. The above results indicated the generation of humoral immune response against the incorporated antigens on the divalent viral vector.

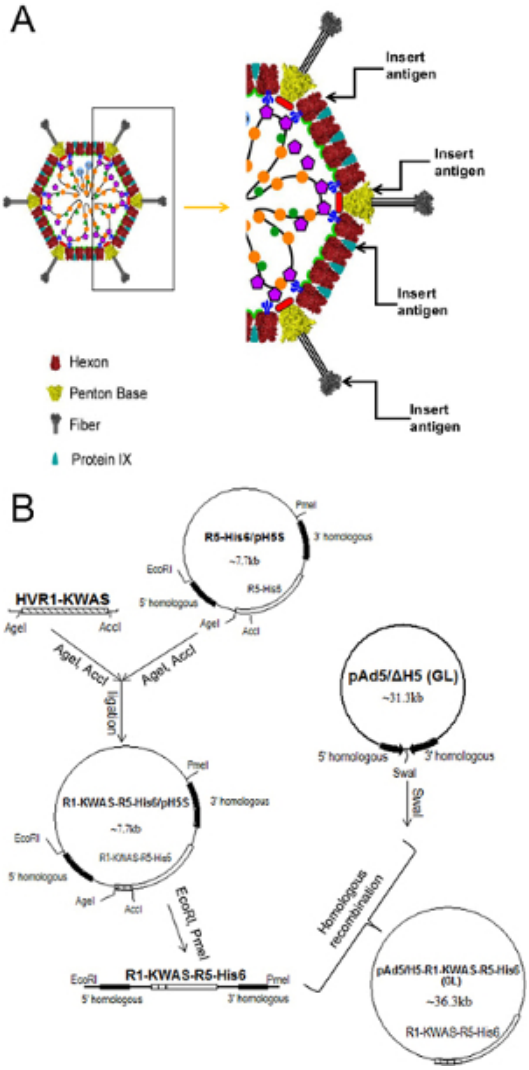

Figure 1. Schematic illustration of the genetic construction of plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6 with the Antigen Capsid-Incorporation strategy. (A) The Antigen Capsid-Incorporation strategy is characterized by directly display antigen-of-interest on the capsid proteins (hexon, penta base, pIX and/or fiber) of adenovirus. This figure was adapted from Nemerow *et al*., 2009. Virology 384 (2009) 380–388, copyright Elsevier. **(B)** Gene fragment (HVR1-KWAS) was synthesized and cloned into a pUC vector by a company. This fragment was subcloned into the previously constructed shuttle plasmid HVR5-His_6/pH5S by the restriction enzymes AgeI and AccI to generate HVR1-KWAS-HVR5-His_6/pH5S. The resulting shuttle plasmid was digested with enzymes EcoRI and PmeI, and co-transformed with SwaI-linearized backbone plasmid pAd5/ΔH5 (GL) for the homologous recombination, resulting in the generation of the recombined backbone plasmid pAd5/H5-HVR1-KWAS-HVR5-His_6. In this figure, R1 denotes HVR1 and R5 denotes HVR5, and GL denotes green fluorescence protein (GFP) and luciferase, separately. Please click here to view a larger version of this figure.

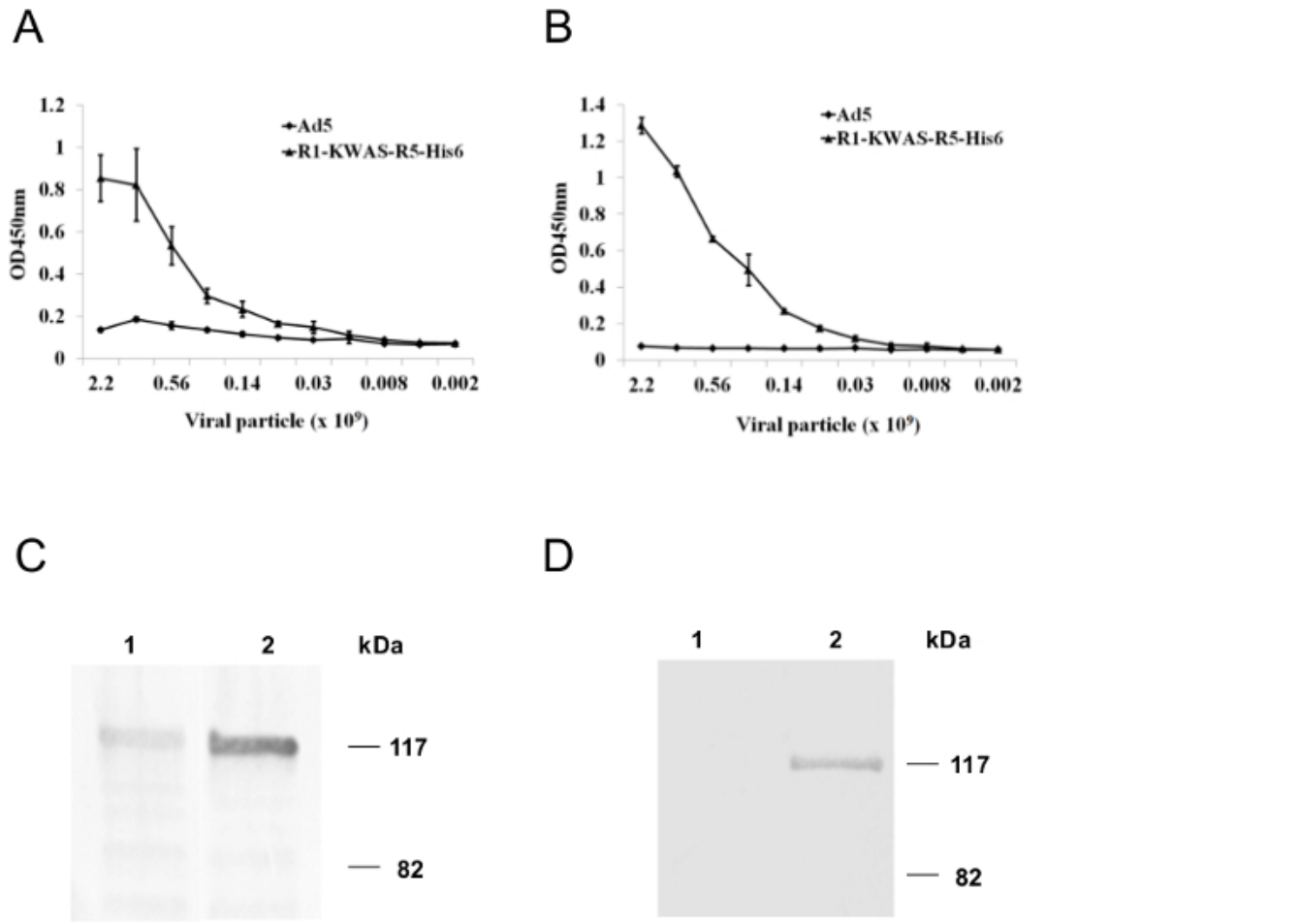

Figure 2. Evaluation of the antigenic exposure of incorporated antigens on the capsid surface of the divalent viral vector Ad5/H5-HVR1-KWAS-HVR5-His$_6$. Whole viral vector ELISA was carried out to determine if the incorporated antigens were exposed on vector surface while maintaining antigenic characteristics of their own. ELISA plates were coated with the divalent vector, and incubated with human 2F5 mAb **(A)** or mouse anti-His mAb **(B)**. Data indicated the significant binding of the antibodies to the divalent vector, but not to the control vector Ad5. R1-KWAS-R5-His$_6$ denotes Ad5/H5-HVR1-KWAS-HVR5-His$_6$. Western-blot analysis was performed to confirm the antigenic binding of the human 2F5 mAb **(C)** or mouse anti-His mAb **(D)** to the incorporated antigens on the divalent vector. In both **(C)** and **(D)**, lane 1 indicates Ad5, and lane 2 indicate Ad5/H5-HVR1-KWAS-HVR5-His$_6$. Please click here to view a larger version of this figure.

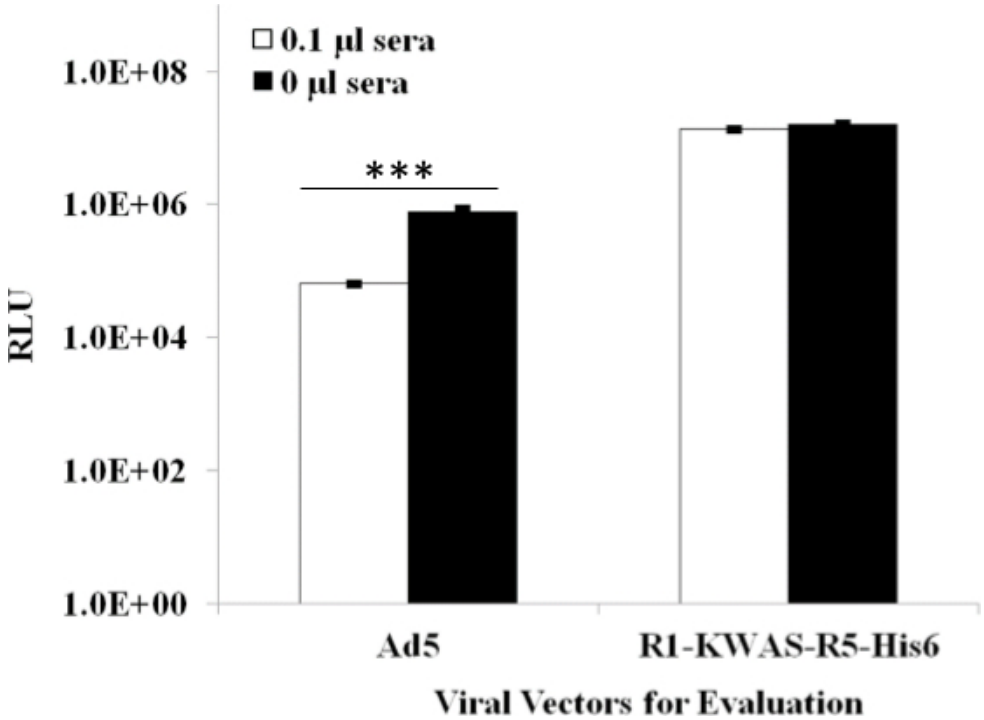

Figure 3. Evaluation of the divalent viral vector's ability to escape the neutralization by Ad5-positve sera *in vitro*. Neutralization assay was performed to determine if the divalent vector could escape the neutralization. Results indicated the neutralization of Ad5 virus by the sera, but the escape of neutralization of the divalent virus *in vitro*. In this figure, R1-KWAS-R5-His_6 denotes Ad5/H5-HVR1-KWAS-HVR5-His_6. The asterisks *** denotes the p value < 0.001.

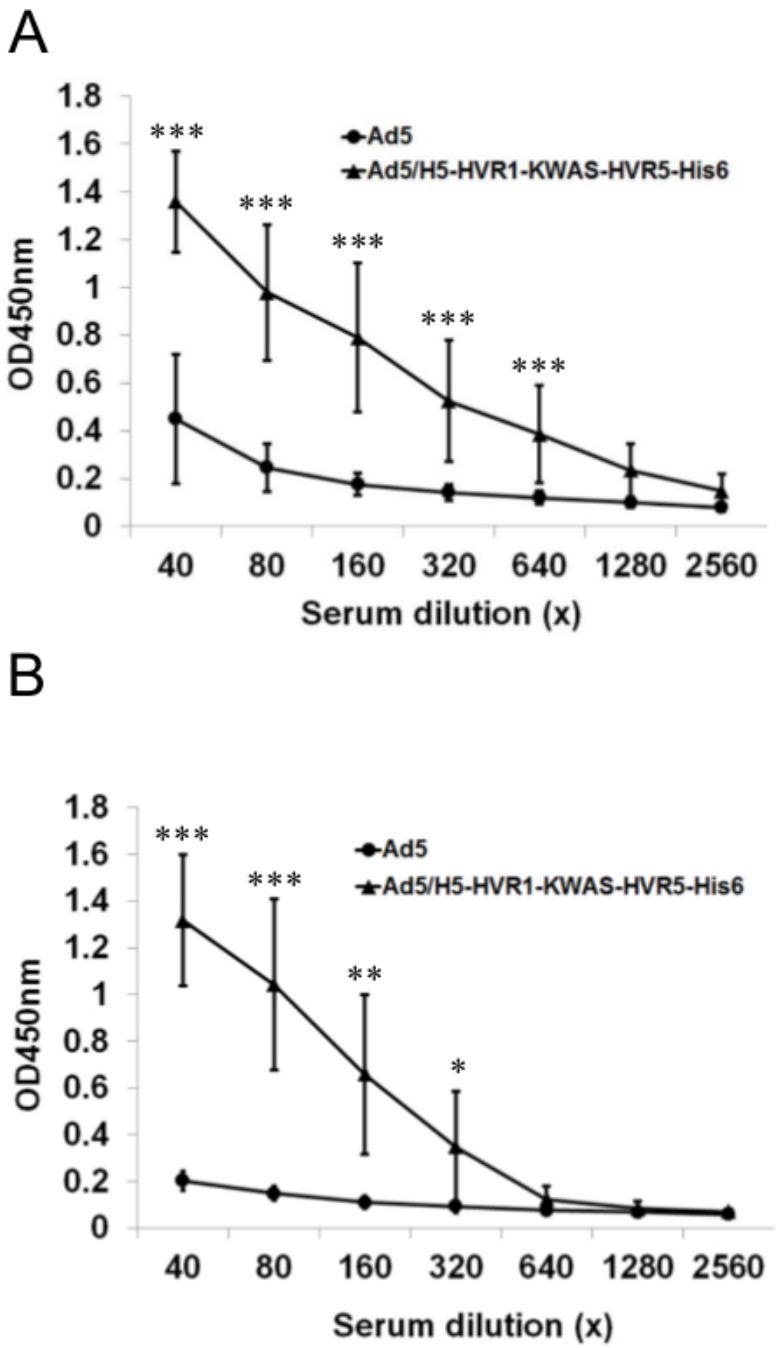

Figure 4. *In vivo* generation of humoral immunity by the divalent viral vector in the mice model. Sera-based ELISA was used to evaluate the humoral immunity against the incorporated proteins on the divalent vector Ad5/H5-HVR1-KWAS-HVR5-His_6. HIV-1 peptide **(A)** and His peptide **(B)** were coated in the ELISA plates separately, followed by the incubation with the mice sera from the two immunization groups (Ad5 and Ad5/H5-HVR1-KWAS-HVR5-His_6). Data indicated the significant generation of humoral immune responses against the HIV-1 antigen in HVR1 **(A)** and the His tag in HVR5 **(B)**. Each data point represents means and SD from eight replicates. The asterisks *** denotes the p value < 0.001, ** denotes the p value < 0.01, and * denotes the p value < 0.05.

Discussion

The application of the traditional transgene strategy on Ad5 modification for the development of vaccines has been diminished primarily due to the bottleneck associated with the Ad5 PEI[4,6]. This bottleneck can be partially diminished by application of the alternative Antigen Capsid-Incorporation strategy (**Figure 1A**), since this strategy can evade neutralization by Ad5 NAbs by replacing neutralizing epitopes of Ad5 with antigens-of-interest, and facilitate the generation of robust immunity to the incorporated antigens-of-interest. In this study, the generation of the modified divalent viral vector Ad5/H5-HVR1-KWAS-HVR5-His_6 was introduced as a conceptual example (**Figure 1B**). Corresponding to the protocol section 1.1.1, the synthesized fragment in the ordered plasmid contains an HIV-1 gp41 short gene substitution in the HVR1 locale of

hexon5. The partial gp41 gene encodes an epitope ELDKWAS, which substitutes partial HVR1 from the amino acids 139 to 144 of hexon5[14]. ELDKWAS is a potent neutralizing epitope in HIV-1 gp41[19].

During the cloning, there were two critical steps. One step was the selection of two restriction enzymes when synthesizing the gene fragment interest in the protocol section 1.1.1. The selection of the two enzymes conditionally depends on the location of Ad capsid to be modified, *i.e.*, in this study, gene fragment containing KWAS was designated to incorporate into HVR1 of hexon5 protein. Restriction enzymes AgeI and AccI exist separately at N-terminal and C-terminal flanking areas of the HVR1 locale, and do not exist in the fragment HVR1-KWAS, thus AgeI and AccI were chosen to be individually put at N-terminus and C-terminus of the synthesized fragment HVR1-KWAS. Since hexon is the major target of Ad NAbs, and hexon-specific neutralizing epitopes reside in all HVRs of hexon[12,18], it is feasible that the hexon modified Ad5 vectors with the Antigen Capsid-Incorporation strategy could gain the ability to bypass the neutralization by Ad5-positive sera *in vitro*, and even the Ad5 PEI in host. In this study, both HVR1 and HVR5 on the divalent viral vector could contribute to the escape of neutralization by the Ad5-positive sera. The other critical step was in the protocol section 1.2.2. After the O/N incubation of co-transformed mixture on LB agar, the grown BJ5183 colonies need to be immediately cultured in liquid LB for O/N, and plasmids need to be immediately extracted from the BJ5183 cells as well. Delaying the procedures will likely lead to potential recombination and mutations, since the recombined plasmid in the BJ5183 cells is not stable. The correct plasmid from the homologous recombination needs to be transformed into DH5α in order to maintain a stable and correct genome species.

After the cloning, there were two more critical steps. The first step in the protocol section is about transfection. It is necessary to transfect the recombined Ad backbone plasmid into cells that express Ad5 E1 (HEK293), if the backbone plasmid is E1 gene deleted. The other step in the protocol section 2.3 is about virus upscaling. The basic procedure for virus upscaling follows the principle of standard virus upscaling from a T-25 flask to T-75 flasks and to T-175 flasks. However the number of the flasks (T-75 and/or T-175) for use depends on the growth speed of the virus, the CPE development caused by the virus infection, and the amount of virus needed.

The Antigen Capsid-Incorporation strategy allows for broad application on Ad modifications. With this strategy, it is feasible to modify or incorporate heterologous antigens on different capsid major proteins, such as hexon[2,4,20], penton base[21] and fiber[22]. The capsid minor protein pIX C terminus also showed promise for the heterologous antigen/peptide incorporation[21,23]. Besides the single modification, multiple modifications on Ad with this strategy also achieved success. Examples are the generation of divalent Ad5/H5-HVR1-KWAS-HVR5-His_6 in this study by incorporations in both HVR1 and HVR5 of hexon5, and the incorporation of two neutralizing epitopes of enterovirus type 71 into either HVR1 and HVR2 or HVR1 and HVR4, or HVR1 and HVR5 of hexon3[24]. These examples imply the feasibility of hexon modifications in other serotypes of Ad, for the purpose of either gene therapy or vaccination approach. Our data also demonstrated the incorporations of two *Trypanosoma cruzi* epitopes into hexon and pIX (unpublished data). This strategy has also been extended to make chimeric Ad vectors for either vaccination or gene therapy approaches. Examples are the generation of chimeric Ad5H3 by switching hexon5 with hexon3[16], the generation of chimeric Ad3H7 by switching hexon3 with hexon7[25], and the generation of chimeric Ad5F4 by replacing Ad5 fiber with Ad4 fiber[26].

In this study, the divalent Ad5/H5-HVR1-KWAS-HVR5-His_6 demonstrated the eliciting of humoral immune responses specific to the incorporated antigens-of-interest. Our current project demonstrates that the ASP2-specific CD8 T cell response is significantly primed by immunization with a vector Ad5-pIX-ASP2, which was generated by incorporating ASP2 peptide into protein IX (pIX) of Ad5, with the Antigen Capsid-Incorporation strategy (data not shown). ASP2, also named amastigote surface protein 2, is an immunodominant antigen of the digenetic intracellular protozoan parasite *Trypanosoma cruzi* (*T. cruzi*)[27]. This new experimental finding extends our knowledge that the application of Antigen Capsid-Incorporation strategy on Ad vectored vaccines can elicit not only humoral immune responses, but also the cellular immune responses specific to the antigens-of-interest.

Although comparatively advantageous to the traditional transgene strategy, the Antigen Capsid-Incorporation strategy also has three disadvantages. First, it cannot allow the incorporation of gene-of-interest as long as traditional strategy does. It has been reported that the Antigen Capsid-Incorporation strategy on hexon5 allows for insertions of a maximum 80 amino acids (aa) in HVR1, 77 aa in HVR5 and 57 aa in HVR2[2]. Of which, HVR1 is the most permissive locale for the incorporation. However, the minor capsid protein pIX can accommodate an insertion of 1,000 amino acids[28]. Second, some antigens-of-interest in nature fail in incorporating into the Ad capsid proteins, due to the factors such as charges and forces of amino acids, which seem to disrupt the structural arrangement of Ad virus. In this case, the introduction of appropriate spacers linked to the antigens-of-interest may help the successful incorporation[4]. Third, incorporations in some HVR locales, *i.e.*, HVR6 or HVR7 could lead to failed generation of viable viral vectors[12]. Given the advantages and disadvantages of both strategies, a wise selection from the two strategies or a combination of both strategies will depend on the specific conditions/needs. An example of combing both the strategies is the generation of Ad5/HVR2-MPER-L15(Gag), whereby the membrane-proximal external region (MPER) of HIV-1 gp41was incorporated into HVR2 of hexon5 with the Antigen Capsid-Incorporation strategy, and HIV-1 Gag gene was inserted to replace the E1 gene locale of Ad5 with the traditional transgene strategy[2].

Disclosures

The authors have nothing to disclose.

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