Supplementary Methods

Adenovirus vectors

All generated **replication-deficient** and **oncolytic** vectors contain the firefly luciferase gene under control of the CMV IE promoter and poly A tail, and are based on a wild type Ad5 genome captured in a bacterial artificial chromosome (BAC). All subsequent modifications were introduced into the BACs by homologous recombination (Stanton et al., 2008) Replication-deficient vectors carry a complete deletion of the E1 and E3 regions, the LUC expression cassette replacing E1. For generation of oncolytic vectors, a 24-base pair deletion dl922–947 (Δ24) (Fueyo et al., 2000) was introduced into the E1A to restrict viral replication to proliferating, pRB-defective cells (Sherr, 1996). T1 mutation, a single adenine base addition at position 445 within the endoplasmic reticulum (ER) retention domain E3/19K, was incorporated to enhance viral release from ER and oncolytic potency was also introduced (Gros et al., 2008). The LUC cassette was inserted into the HpaI site following E1A.
The three detargeting mutations were KO1, HVR7 and RGD-RGE. KO1 is a double amino acid substitution in the AB fiber knob loop (S408E P409A) abolishing CAR binding. HVR7 mutation is a 5-amino-acid substitution in the hexon (I421G, T423N, E424S, L426Y, E451Q) which prevents interaction with FX. RGD to RGE involves a point mutation (D342E) in the penton abolishing binding of αvβ3/5 integrins. Heterologous A20 peptide sequence (NAVPNLRGDLQVLAQKVART) from FMDV was inserted into the fiber knob HI loop for targeting to αvβ6. Ad vectors were generated in T-REx-293 or HEK23-β6 cell lines (A20-modified vectors) and purified essentially as described previously (Uusi-Kerttula et al., 2015, Uusi-Kerttula et al., 2016).

Cell lines and primary EOC ex vivo cultures

SKOV3-β6 cell line was generated in-house for an αvβ6-expressing EOC cell line. pBABE puro plasmid with human itgb6 gene (2400 bp; GenBank ID: NM_000888) from (pBABE-β6 puro; #13596, Addgene) expresses the human integrin β6 subunit that pairs in cells with αv subunit, forming the αvβ6 heterodimer (Hynes, 2002). Plasmid was transfected into a 293Phoenix packaging cell line in a confluent T25 flask using Effectene transfection reagent (#301425; Qiagen, Manchester, UK) according to manufacturer's instructions. After 48 h, the retrovirus was harvested and filtered through a 0.4 µm syringe filter. A 6-well plate was coated with RetroNectin reagent (#T100B, Takara Bio Europe SAS, France) O/N at 4ºC, washed with PBS, 1.5 mL of virus added in the coated well, the plate sealed in a bag and centrifuged at 2800 rpm at 30°C for 2 h. The virus was removed and wells washed once with PBS and SKOV3 cells seeded at ~70% density (1×10^5 cells/mL). After 48 h, cells were detached from the plate using trypsin and seeded into a T25 tissue culture flask in DMEM medium supplemented with 5 µg/mL puromycin (#P8833, Sigma Aldrich, Gillingham, UK). After 3–4 days the only surviving cells were cells expressing αvβ6 integrin. The highest 10% αvβ6-expressing population was sorted for subsequent assays in two rounds of FACS by using a primary anti-αvβ6 clone 10D5 (1:100; #MAB2077Z; Millipore, Watford, UK) and a a secondary anti-mouse IgG/IgA/IgM-FITC conjugate (1:100; #0855499; MP Biomedicals, USA). Permission for the collection and cultivation of primary EOC cells from ascites was granted through a Wales Cancer Bank application for biomaterials, reference WCB 14/004. All patients gave written informed consent for the use of their samples, prior to collection. Ascites clinical samples were collected from patients undergoing treatment for advanced ovarian cancer at Velindre Cancer Centre, Cardiff, and anonymised. Cells were collected by centrifugation of 500 mL ascites, processed and sub-cultured as described previously (Uusi-Kerttula et al., 2015, Uusi-Kerttula et al., 2016).

In vitro assays

Cell surface receptor expression was assessed by flow cytometry as described previously (Uusi-Kerttula et al., 2016): cells were stained with primary anti-αvβ6 clone 10D5 (#MAB2077Z; Millipore, Watford, UK) or anti-CAR clone RmcB (#05-644; Millipore, Watford, UK) for 1 h on ice, followed by a secondary F(ab')2-goat α-mouse IgG (H+L) IgG AlexaFluor 647 (#A21237, Life Technologies, Paisley, UK) for 30 min on ice. 10 000 events were recorded and data analysed in BD Accuri C6 software.
Antibody qualification from ovarian ascites and serum was performed by ELISA essentially as previously reported (Stallwood et al., 2000). Viruses were diluted at 200 ng protein/mL in coating buffer ($NaHCO_3/Na_2HCO_3$, pH 9.6) and heat-inactivated at 56ºC for 30 min. 96-well flat-bottom ELISA plates (#44-2404-21, MaxiSorp, Nunc; Affymetrix eBioscience) were coated with 100 µL of virus / well and incubated O/N at 4ºC. Each well was washed 6 x in 200 µL of PBS-T (0.01% Tween-20/PBS). Two-fold dilutions (1:50–1:51 200) of ascites samples and serum control were prepared in 1% BSA/PBS-T and 100 µL of added into each well in duplicate for 2 h at room temperature (RT). The wash was repeated and wells incubated with 100 µL of anti-human IgG-HRP (1:10 000; goat α-human IgG-HRP, sc-2907, #J0914, Santa Cruz) in 1% BSA/PBS-T for 1 h at RT. After a wash, 100 µL of substrate (1:1 mix of BD OptEIA TMB substrate reagents; #555214, Benkton Dickinson, Oxford, UK) was added into each well and incubated for 20 min at RT. Reaction was stopped with 100 µL of 0.2 M H2SO4 and absorbance measured at λ450 nm.
Antigen specificity of the antibodies was performed by **Western blot**. Viruses were run in NuPAGE Novex® 10% Bis-Tris SDS-PAGE gels (#WG1201BOX; Life Technologies, Loughborough, UK) at 0.625, 1.25, 2.5 and 5 µg of protein/well (1 µg of protein corresponding to 4×10^9 viral particles (Von Seggern et al., 1998)) in a total volume of 40 µL (including 2 µL of DTT and 10 µL of 4 x NuPAGE LDS sample buffer) for 1 h 20 min at 150 V. Proteins were transferred onto an Amersham Protran 0.45 µm nitrocellulose blotting membrane (#10600002; GE Healthcare Life Science, Little Chalfont, UK) by semi-dry blotting in a Trans-blot SD Semi-dry Transfer Cell (Bio-Rad, Hertfordshire, UK) for 1 h at 20 V. Membrane was blocked in 5% milk/TBS-T for 90 min at RT or O/N at 4ºC on a rocker and incubated in ascites or serum at 1:500 in 5%/TBS-T for 90 min at RT on a rocker. After three washes for 10 min in TBS-T on a rocker at RT it was incubated in secondary anti-human IgG-HRP (goat α-human IgG-HRP sc-2907 #J0914, Santa Cruz) at 1:2000 in 5%/TBS-T for 1 h at RT on a rocker, and washed again 3 x 10 min in TBS-T at RT on a rocker. The proteins were then visualised by incubating the membrane in West Pico SuperSignal (#34078; Thermo Scientific, Paisley, UK) for 10 min, and membrane imaged in G:BOX Chemi XX6 imager (Syngene, Cambridge, UK).
Cell transduction efficiency in vitro was assessed using Luciferase Assay System kit (#1501; Promega, UK) essentially as described earlier (Uusi-Kerttula et al., 2015, Uusi-Kerttula et al., 2016). 20 000 cells/well were adhered in a 96-well plate overnight, then infected with 5000 (transduction and competition inhibition assays) or 200 000 (neutralisation assays) viral particles (vp)/cell in serum-free RPMI medium for 3 h at 4°C (competition inhibition) or 37°C (transduction and neutralisation assays), and media replaced with complete media. 48 h later cells were washed with 200 µL of PBS, lysed with 100 µL of cell culture lysis buffer and plates frozen at –70ºC. Luciferase activity was measured from 10 µL lysed cells on a multimode plate reader, and relative light units (RLU) normalised to total protein concentration in each well (RLU/mg). To assess the effect of FX on transduction efficiency, transduction media were supplemented with 10 µg/ml of purified human FX (#HCX0050, Haematologic Technologies, Cambridge Bioscience, Cambridge, UK). An anticoagulant X-bp, that binds to FX via its Gla-binding domain, was used at a molar excess of 3:1 to inactivate FX in transduction medium.
In **competition inhibition**/function blocking assays cells were pre-incubated with normal mouse IgG control (1 µg/mL) or anti-αvβ6 function-blocking antibody (1 µg/mL; clone 10D5) for 30 min on ice, and viruses added on the top for 2 h on ice.
Vector tropism for cellular receptors CAR, CD46 and αvβ6 integrin was assessed in competition inhibition assays, which is a variation of a standard luciferase transduction assay. Cells were pre-incubated in Ni-NTA purified recombinant His-tagged knob proteins kn5.wt, kn5.hCAR–, kn5.HI.A20, kn48.wt and kn48.DG.A20 at a final concentration of 10 µg/10^5 cells in triplicate. For binding inhibition with function-blocking antibodies, cells were pre-incubated in anti-αvβ6 antibody clone 10D5 (Abcam, Cambridge, UK), anti-CD46 clone MEM-258 (Abcam, Cambridge, UK) or normal anti-mouse control IgG (Santa Cruz Biotechnologies, Heidelberg, Germany) for 30 min on ice (all 10 µg/mL). Luciferase activity was measured as described above. Neutralisation assays were performed essentially as described above, with the exception of pre-incubation in 2-fold serial dilutions (1:40–1:2.5, corresponding to final concentration of 10–40%) of cell-free ovarian ascitic fluid for 3 h at 37ºC.

Supplementary Methods

In vivo studies

All animal experiments were performed at Mayo Clinic, Rochester, MN, USA. Permissions for the animal experiments were requested from Institutional Animal Care and Use Committee (IACUC) and permissions for the Ad vectors were requested from Centers for Disease Control (CDC). Immunocompromised (athymic) NOD/SCID mice were ordered from Jackson Laboratories (Bar Harbor, Maine, USA) and wild type albino B6 mice from Charles River (USA) and allowed to acclimatise for a minimum of 48 h prior to experiments. For consistency of results, all animals were sex-matched; female mice were chosen due to the ease of housing. All animal handling and injections were performed by a highly experienced veterinary technologist Mrs Jill M. Thompson.

Biodistribution study with replication-deficient vectors was performed in wild type B6 albino mice (B6N-Tyrc-Brd/BrdCrCrl) that is a spontaneous albino mutant co-isogenic C57BL/6 strain with an MHC haplotype H2b. This immunocompetent strain was chosen due to its white coat, which makes luciferase tracking feasible. 6-week-old mice (n=5/group) were ordered from Charles River Laboratories International (USA) and allowed to acclimatise for one week prior to experiments. Viruses were injected into the lateral tail vein at 1×10^{11} vp in a total volume of 100 µL of PBS. Animals were monitored daily for distress and vector-induced toxicities. All mice were sacrificed immediately after IVIS imaging at 72 h by inhalation of CO_2, and organs harvested for the assessment of vector distribution.

Oncolytic efficacy of replication-competent oncolytic vectors was studied in immunocompromised NOD/SCID white female mice. Treatment schedule was first optimised in a small pilot study in eight mice. 1×10^{7} SKOV3-β6 cells in PBS were implanted i.p. on day 0, and animals monitored daily for weight changes and build-up of malignant ascites. Two mice were sacrificed on days 7, 14, 21 and 48/49 (final endpoint). When the last two mice were sacrificed, they had 2.6 mL of ascites and ill health due to a widely-spread tumour mass. Cells were collected post-mortem and assessed for CAR and αvβ6 receptor expression by flow cytometry as described above. For the efficacy study, NOD/SCID mice were xenografted i.p. with 1×10^{7} of SKOV3 (n=20) on day 0, and allowed to grow for 14 days prior to the first virus treatment. Mice (n=5/group) were treated with an i.p. injection of 1×10^{10} vp of Ads (PBS, Ad5, Ad5.A20 and Ad_{NULL}-A20) on days 14, 16 and 18. Animals were monitored daily for general health and the build-up of ascites. The primary endpoint of this study was treatment-related overall survival. Statistical analysis of survival proportions was performed by Gehan–Breslow–Wilcoxon test.

IVIS imaging

Trafficking of luciferase-expressing vectors was monitored at 72 h (biodistribution study) or 48 h and 7 days (efficacy study) post-infection using a non-invasive In Vivo Imaging System Bioluminescent Imager (IVIS-200, PerkinElmer). 200 µL of 15 mg/mL D-luciferin (Gold Biotechnology, USA) was injected i.p., the mice were anaesthetised by inhalation of 2% isoflurane (1.5% for maintenance) and imaged 5 min later with 1 min exposure. In the biodistribution study, mice were sacrificed after IVIS imaging by inhalation of CO_2, and organs and tumours harvested immediately post-mortem. Organs were imaged ex vivo on a 12-well plate by addition of 2 mL of D-luciferin (300 µg/mL) in PBS. Images were adjusted in Living Image software on a normalised scale (photons/second/cm^2/steradian) to exclude background luminescence. Data were normalised, and analysed by one-way ANOVA and Sidak's multiple comparisons *post hoc* test in GraphPad Prism.

Viral genome copy number

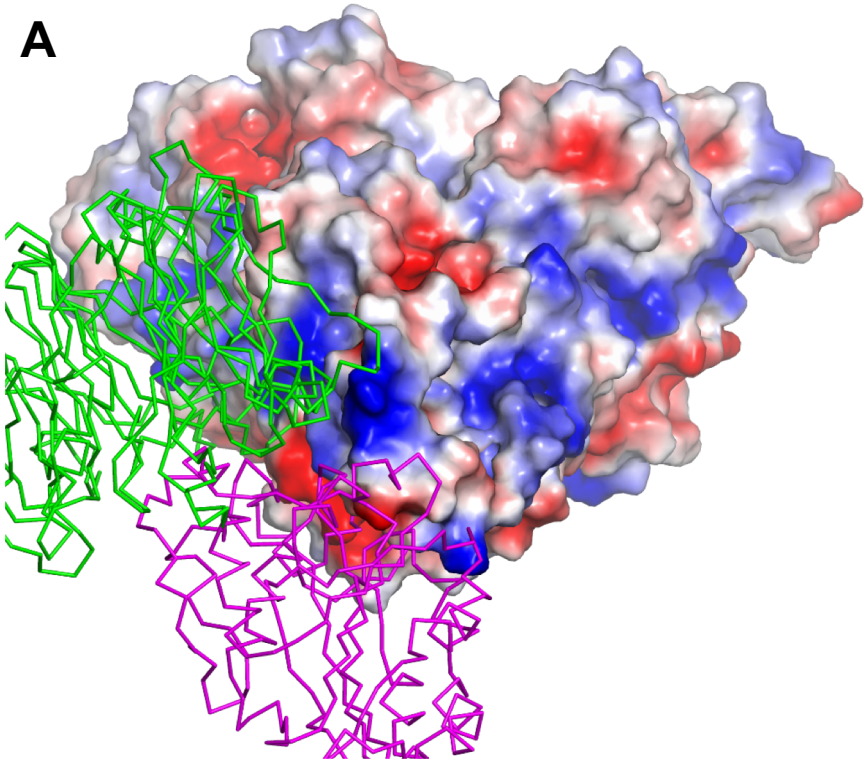
Viral genome numbers in lungs, liver, spleen and end-point tumours were quantified by qPCR. Viral and total genomic DNA was obtained from non-fixed murine tissues (~10 mg of spleen and ~25 mg of other organs) and tumours (10−30 mg) using DNeasy Blood & Tissue DNA extraction kit or AllPrep DNA/RNA Mini Kit according to the manufacturer's protocol (#69506 and #80204; Qiagen, Germantown, MD, USA). The concentration of purified DNA was determined using a NanoDrop ND-1000 photospectrometer (NanoDrop Technologies). 40 ng of total DNA was subjected to fluorogenic quantitative PCR using Fast SYBR Green Master Mix system (#4385612; Applied Biosystems, Life Technologies, Paisley, UK). Reactions were performed in triplicate, using primers for the hexon region of the genome, Forward: 5′-CGCGGTGCGGCTGGTG-3′ and Reverse: 5′-TGGCGCATCCCATTCTCC-3′ (Sigma Aldrich, Gillingham, UK). Negative controls were run to ensure no amplification occurred in the absence of DNA. Cycling conditions were as follows; 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. Total adenoviral genomes were calculated using a standard curve of 10^{1}–10^{7} viral genomes; separate standard curve was prepared for each virus. Data were normalised, and analysed by one-way ANOVA and Sidak's multiple comparisons post hoc test in GraphPad Prism.

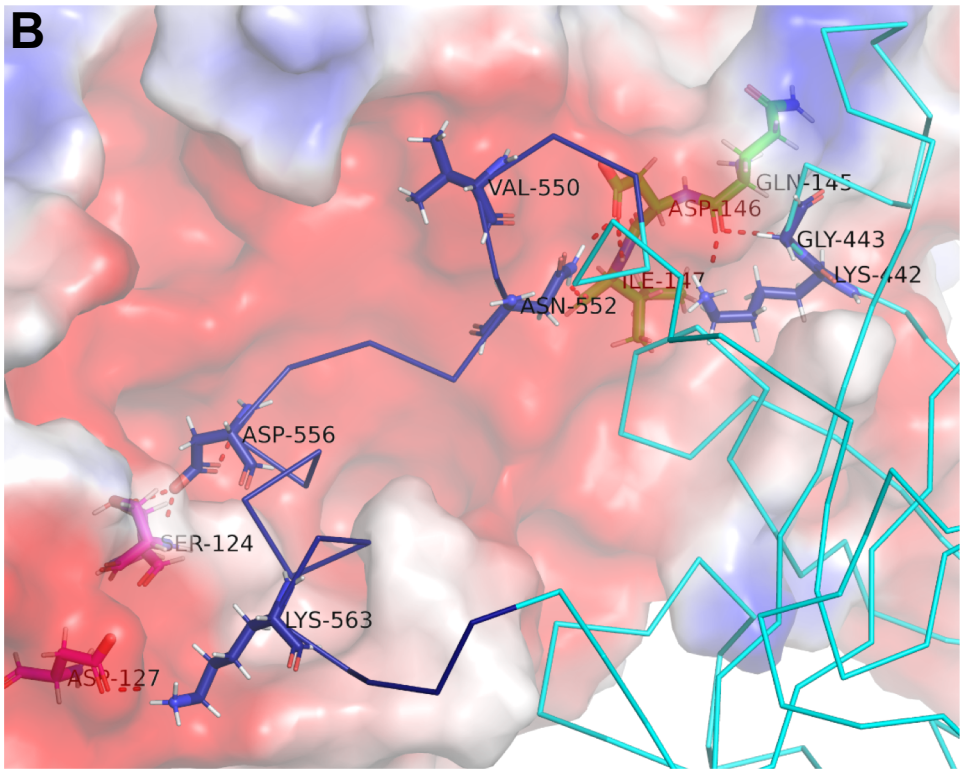
αvβ6 (ITGB6) gene expression in tumours

Relative gene expression was determined from end-point tumours by qPCR. RNA was obtained with AllPrep DNA/RNA Mini Kit according to the manufacturer's protocols. cDNA was generated and qPCR performed by standard methods using TaqMan probes for ITGB6 (αvβ6) gene and for ACTB (beta actin) as an endogenous control. Expression levels are shown relative to mouse #1 in the PBS treatment group in the SKOV3 cohort (= low αvβ6 expression).

Immunohistochemistry

Livers were harvested post-mortem, immersed in 10 mL volume of 10% formalin (#032-059; Protocol®, Fisher Scientific, MI, USA), and sent to Histology Core Laboratory, Mayo Clinic Arizona, Scottsdale, AZ, USA for preparation of formalin-fixed paraffin-embedded tissue slides. Tissues were first re-hydrated by incubating in three changes of xylene for 5 min. They were then washed twice in 100% industrial methylated spirit (IMS) for 3 min, once in 90% IMS for 3 min, distilled H_2O for 5 min and rinsed in PBS. The tissues were circled with ImmEDGE pen (#H-4000; Vector Laboratories, Peterborough, UK), and treated with proteinase K (#4333793; AB, Foster City, CA, USA) at 20 µg/mL in Tris/EDTA (TE) buffer for 10 min at RT. Tissues were then washed in PBS for 5 min and blocked in 2.5% horse serum (#MP-7401; ImmPRESS™ HRP anti-rabbit IgG-HRP polymer detection kit; Vector Laboratories, Peterborough, UK) for 1 h. After a 5-min wash in PBS, endogenous hydrogen peroxide activity was quenched by treating the tissues with 3% H_2O_2 (#21,676-3; Sigma Aldrich, Gillingham, UK) in PBS for 10 min, after which the tissues were washed in PBS for 5 min. Tissues were then incubated with primary polyclonal rabbit anti-adenovirus type 5 antibody (1 µg/mL; #ab6982, Abcam, Cambridge, UK), anti-ITGB6 antibody (1:10; #ab197672, Abcam, Cambridge, UK) or anti-CAR antibody (1:100; #ab153740, Abcam, Cambridge, UK) O/N at 4ºC in a humidified container. A rabbit IgG isotype control antibody (1 µg/mL; #GTX35035; GeneTex, Wembley, UK) was included as a negative control to detect any non-specific staining. Tissues were washed in 1 mL of PBS for 5 min and incubated with anti-rabbit secondary (#MP-7401; ImmPRESS™ HRP anti-rabbit IgG-HRP polymer detection kit, made in horse; Vector Laboratories, Peterborough, UK) for 30 min at RT. Tissues were washed in PBS for 5 min and incubated with DAB substrate for 3 min. They were then rinsed in tap water, counterstained in haematoxylin for 15 s and rinsed in running water for 15 min until the water was completely clear. The tissues were then de-hydrated by repeating the re-hydration sequel in reverse, starting from 90% IMS and proceeding to the xylene washes. Tissues were mounted using DPX Mountant for histology (#06522; Sigma Aldrich, Gillingham, UK), coverslip added on the top, and images acquired at 10 x, 20 x and 40 x magnification using a light microscope (Leica DMC 2900; Leica Microsystems Ltd, CH-9435 Heerbrugg, Switzerland).



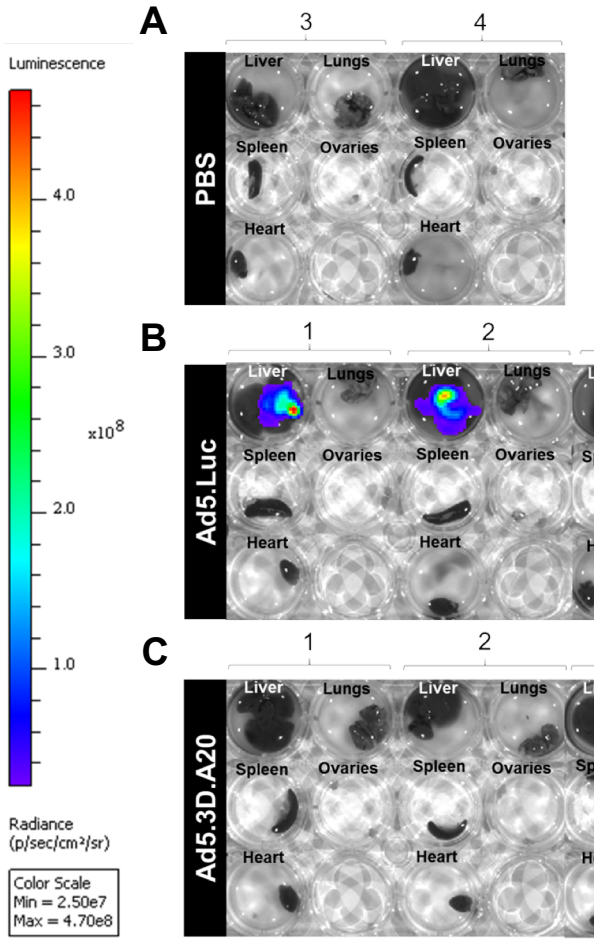


Supplemental Figure I. Homology modelling of the $Ad5_{NULL}$-A20 complex. Homology modelling of the $Ad5_{NULL}$-A20 vector in complex with the αvβ6 dimer suggests a mechanism for high affinity ligand-receptor interaction. (A) The αv (green) and β6 (purple) Cα trace in complex with the $Ad5_{NULL}$-A20 fiber-knob surface charge shows the regions of positive (blue) and negative (red) surface potential in the A20 loops. (B) shows the contact residues (labelled, shown as sticks) between $Ad5_{NULL}$-A20 and αvβ6 as they present through the semi-transparent surface (colour by charge) of αvβ6. The table in (C) shows the binding energies ($Kcalmol^{-1}$) of the $Ad5_{NULL}$-A20 in complex with the αv and β6 subunits individually and in total as calculated by PISA after energy minimisation by YASARA.

C

Binding Energy ($KcalMol^{-1}$)

	αV	β6	Total
5NEM	–1.3	–8.1	–9.4
Ad5.A20	–12.3	–11.4	–23.7
$Ad5_{NULL}$-A20	–12.9	–11.4	–24.3

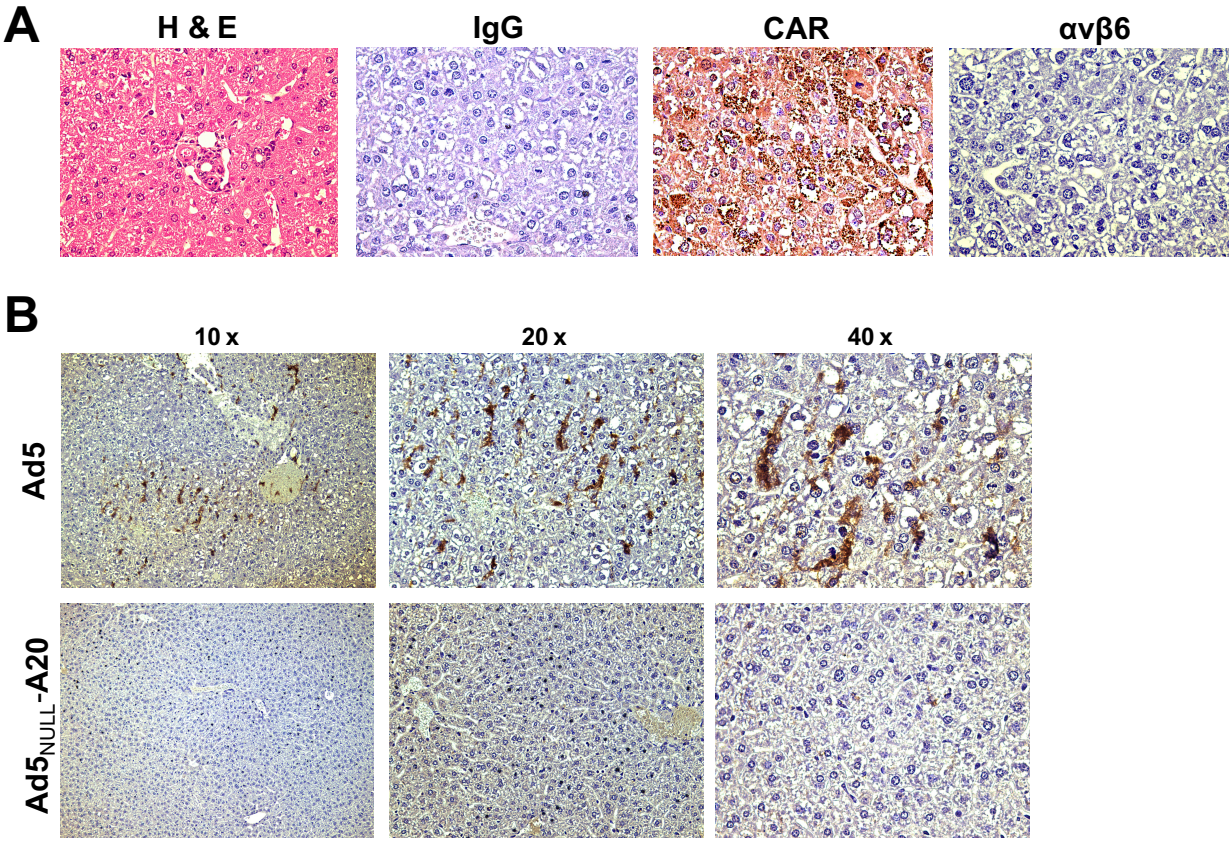


D

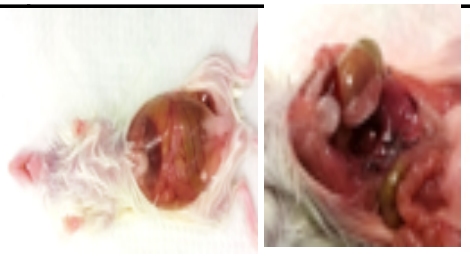
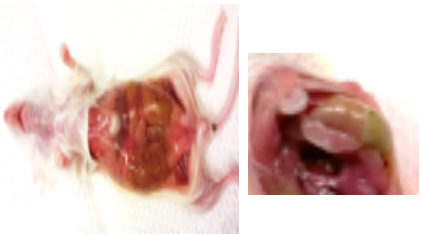
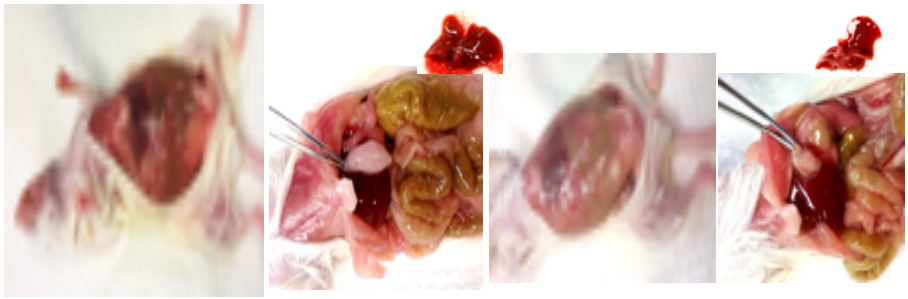
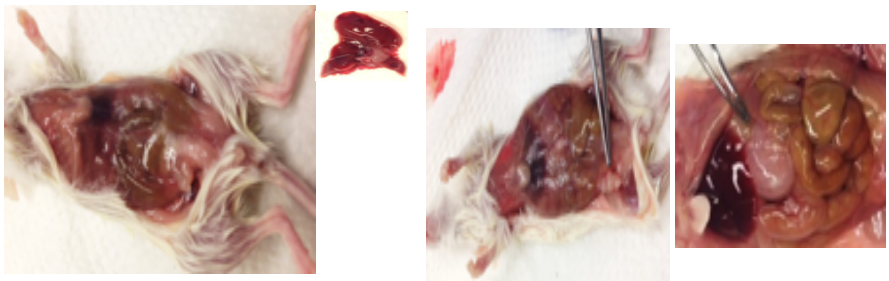
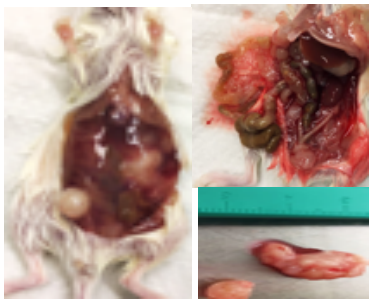
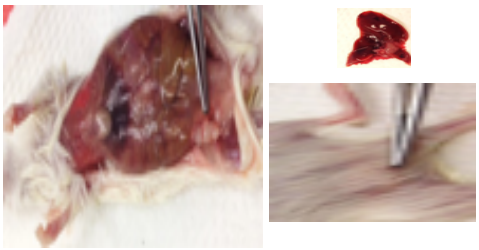
Fold decrease in ex vivo luminescence vs. Ad5 group

Group	Liver	Spleen	Lungs	Ovaries	Heart
$Ad5_{NULL}$-A20	13 300	9230	3700	1100	573

Supplement II. Biodistribution study: Heat-map images of luminescence intensity ex vivo. Liver, spleen, lungs, ovaries and heart were collected immediately post-mortem from animals that had been intravenously inoculated with (A) PBS (control), (B) replication-deficient Ad5 or (C) replication-deficient $Ad5_{NULL}$-A20 vectors. Organs were immersed in D-luciferin and imaged in IVIS imager. The colour of the tissue indicates the relative luminescence intensity emitted by luciferase transgene; scales were normalised to exclude background luminescence. (D) Fold decrease in total luminescence (photons/second) relative to Ad5. The mean luminescence intensity of the Ad5 group was divided by the mean luminescence in each organ in the $Ad5_{NULL}$-A20 group. The mean value of the PBS control group was subtracted from all values.



Supplement II. Biodistribution study: Immunohistochemistry on formalin-fixed, paraffin-embedded liver sections. (A) Haematoxylin-eosin staining for the visualisation of cellular structures, and mouse liver staining using a rabbit IgG isotype control antibody (1 µg/mL), a primary rabbit anti-CAR antibody (1:100) and a primary rabbit anti-ITGB6 (αvβ6) antibody (1:10). (B) Staining of Ad5-infected liver cells in animals infected with Ad5 or $Ad5_{NULL}$-A20 vectors, using a primary rabbit anti-Ad5 antibody (1 µg/mL). DAB was used as a substrate, sections counterstained in haematoxylin, mounted on coverslips and observed under a light microscope.

Time-point	Mouse #1	Mouse #2	Observations
Day 7			Small tumour at the injection site, a larger one by the pancreas (Ø ~1 cm) No ascites
Day 14			Tumour at injection site, Ø ~1 cm tumour by the pancreas, 4–5 micro tumours between the lobes of the liver No ascites
Day 21			Ø ~ 1cm tumour at injection site, Ø ~ 1.5 cm tumour by the pancreas, tumour mass between the lobes of the liver No ascites
Days 48 & 49			Ø ~1.5 cm tumour at injection site, Ø ~2 cm tumour by the pancreas, tumour mass spread all around the lining of abdomen, diaphragm, intestinal lining 2.6 mL of ascites

Supplementary Figure IV. Pilot study: Tumour localisation and take rate in NOD/SCID mice. 1×10^7 of SKOV3-β6 cells/animal were implanted i.p. on day 0, and two mice sacrificed on each time-point: on day 7, 14, 21 and 48/49 (final endpoint). The approximate tumour size was measured, and the volume of ascites quantified.