Enhanced liver gene transfer and evasion of pre-existing humoral immunity with exosome-enveloped AAV vectors

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Supplemental materials and methods

AAV and exo-AAV vector production

Standard AAV and exo-AAV vectors were produced using adenovirus helper-free transfection method as described previously[1]. Briefly, human embryonic kidney cells (HEK293) grown in roller bottles were transfected with three plasmids containing adenovirus helper plasmid, the AAV Rep and Cap genes, and the ITR-flanked transgene expression cassette. The day following transfection, medium was changed to DMEM-containing 2% exosome-depleted FBS (made by overnight 100,000 x g ultracentrifugation to deplete bovine exosomes). 72 hours following transfection, medium was collected and exo-AAV vectors were isolated by differential centrifugation as descried earlier [2]. Briefly, cells were depleted at 300 x g for 5 min and 1,500 x g for 15 min. Next, larger extracellular vesicles (apoptotic bodies, microvesicles) were depleted by 20,000 x g for 60 min. The supernatant of 20,000 x g spin was subjected to 100,000 x g centrifugation for 1 hour using 70 Ti rotor in an Optima^{TM} L-90K ultracentrifuge (Beckman Coulter, Indianapolis IN, USA). The resulting exosome pellet was resuspended in PBS. From the same production, standard AAV vectors were purified from cells lysate using two successive ultracentrifugation rounds in cesium chloride density gradient[3]. Full capsids were then collected and formulated in PBS. Both exo-AAV and standard AAV vectors were stored at -80 °C. For the titration of the vectors, exo-AAV vectors were treated with detergent and then both standard AAV and exo-AAV vectors were subjected to DNase to remove plasmid DNA. Next, viral DNA from the samples was isolated using MagNA pure 96 DNA and viral NA small volume kit (Roche Diagnostics, Indianapolis IN, USA) according to manufacturer's instructions. AAV vector genomes in both standard AAV and exo-AAV vectors were quantified using a quantitative real-time polymerase chain reaction (qPCR).

Size characterization of exo-AAV vectors was performed using Nanosight NS300 (Malvern Instruments, Malvern, UK) following the manufacturer's protocol. The measurement of exosome size in this study was calculated by averaging the particles size of three 90-second videos. Data was analyzed with the NTA 3.0 software (Malvern Instruments. Malvern, UK)), which was optimized to first identify and then track each particle on a frame-by-frame basis.

Cell lines and serum specimens

Human hepatoma cell lines (HUH-7, HHL5 and HepG2 cells), HEK293 cells expressing the adenovirus E4 gene, and mouse muscle cell lines (C2C12 and NIH3T3 cells) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% of fetal bovine serum (Thermo Fisher Scientific) and 1% of antibiotics (penicillin 100U/mL and 100µg/mL streptomycin) (Thermo Fisher Scientific) at 37 °C in 5% CO_2.

All serum samples used in the study were from healthy adult donors and were purchased from the French Blood Bank (EFS). All required authorizations were obtained prior to acquiring the samples.

In vitro transduction experiments

For all in vitro experiments, cells were seeded in 96-well plates at a density of 20,000 cells/well. The following day, the cells were incubated with AAV8 or exo-AAV8 vectors encoding for luciferase at increasing multiplicity of infection (MOI, 250, 2500, 25000). For the receptor competition experiments, HUH-7 cells were first incubated with an excess of empty AAV8 capsids (1x, 5x, or 20x the MOI) for 5 minutes at +37 °C in 5% CO_2 to allow binding of empty particles to their surface receptor. Following the incubation, cells were treated with either AAV8 or exo-AAV8 vectors encoding for luciferase at multiplicity of infection of 250. Transduction efficiency was determined by measurement of luciferase expression using the Bright-Glo™ Fluc substrate (Promega, Madison, WI) and an Enspire luminometer (Perkin Elmer, Wellesley, MA).

ImageStream X Analysis

HUH-7 cells were seeded in 6-well plates 24 hours prior to infection. Either AAV8 or exo-AAV8 vectors were added to the cells at a multiplicity of infection of 25,000 and incubated for one hour at + 37 °C. Following infection, medium was removed and cells were washed three times with PBS to remove unbound vector. Cells were harvested, fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to manufacturer's instructions. Cells were then stained with anti-AAV8 capsid mouse IgG antibody (clone ADK8, Progen, Heiderlberg, Germany, at a dilution of 1:20) and incubated overnight at + 4 °C. The next day, cells were washed and stained with anti-mouse IgG conjugated to Alexa-Fluor 488 (Thermo

Fisher Scientific, at a dilution of 1:500). Following incubation, cells were washed twice and stained for nucleus using 7-AAD (Sigma-Aldrich, St. Louis, MO, USA). Analysis was performed using the ImageStream X flow imaging system (Merck-Millipore, Seattle, WA) at a 60X magnification. The data were analyzed with the IDEAS software version 6.1 (Merck-Millipore, Seattle, WA).

Mouse experiments

Mice were handled according to the French and European legislation of animal care and experimentation. All animal procedures were performed in accordance with the experimental authorization approved by the local intuitional ethical committees of the University Pierre and Marie Curie and the Centre d'Exploration et de Recherche Fonctionnelle Expérimentale (CERFE). Animals were allocated at random to treatment groups.

Hemostatically normal male or female mice C57BL/6 aged of 6-8 weeks old were injected intravenously via the tail vein with AAV or exo-AAV vectors encoding for human coagulation factor IX (hF.IX). For passive immunization experiments, 6-8 weeks old male C57BL/6 mice were injected intraperitoneally with human immunoglobulin (IVIg) at a dose of 0.5, 2, or 8 mg per animal. Twenty-four hours later, blood samples were collected for the determination of anti-AAV8 neutralizing antibodies (NAb) titer followed by the intravenous injection of AAV8-hF.IX or exo-AAV8-hF.IX vectors at dose of $5x10^{10}$ vg per mouse.

In order to induce antibodies to hF.IX antigen, hemostatically normal male mice C57BL/6 aged of 6-8 weeks old were treated with subcutaneous injection of 100 µg of recombinant hF.IX protein (BeneFix®, Pfizer, Paris, France) formulated in complete Freund's adjuvant (Sigma-Aldrich).

10 weeks old hemophilia B (*F9*$^{-/-}$) male mice on C57BL/6 background were injected intravenously via the retro-orbital vein with AAV8-hF.IX or exo-AAV8-hF.IX vectors at a dose of 10^9 vg per mouse.

In all experiments, peripheral blood was collected in sodium citrate and plasma was isolated and immediately transferred to tubes for storage at -80ºC. Spleen and inguinal lymph nodes were collected at necropsy in fresh RPMI medium for immunology studies and diverse organs were collected and were stored at -80°c for biodistribution analysis.

Anti-AAV antibodies assays

Antibody assays were performed as previously described[4,5]. Serum samples from treated animals were analyzed for anti-AAV IgG antibodies by ELISA. Briefly, nunc maxisorp plates (Thermo Fisher Scientific) were coated with AAV particles ($2x10^{10}$ particles/ml) and with serial dilution of purified mouse IgG (Sigma-Aldrich, St. Louis, MO) to generate a standard curve. After overnight incubation at +4°C, plates were blocked with PBS-0.05% Tween-20 containing 2% bovine serum albumin and appropriately diluted samples were plated in duplicate. Some selected serum samples were also analyzed for anti-AAV neutralizing antibody titer using a previously published protocol[4]. The neutralizing titer was determined as the highest sample dilution at which at least 50% inhibition occurred using a luciferase reporter assay. In some experiments, prior to the in vitro neutralization assay exo-AAV8 vectors were subjected to treatment with Triton X-100 (Sigma-Aldrich, St. Louis, MO) added at final concentration of 0.075%. To ensure complete disruption of lipid vesicles, samples were then vortexed for 30 seconds at room temperature. All vectors (AAV8, exo-AAV8, or exo-AAV8 treated with 0.075%) were retitered by qPCR before use in transduction experiments or NAb assays.

Factor IX transgene anti-human F.IX antibody determination

Plasma levels of human F.IX transgene and anti-human F.IX IgG antibodies were detected as previously described[5]. Quantification of anti-hF.IX IgG antibodies was performed as descried earlier[6]. Briefly, Nunc Maxisorp plates (Thermo Fisher Scientific Waltham, MA) were coated with 1 μg/well of purified human F.IX (Benefix®, Pfizer, Paris, France) and with serial dilution of mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) to generate a standard curve. Following overnight incubation at +4 °C, plates were washed and blocked with PBS-0.05% Tween-20 containing 2% bovine serum albumin and appropriately diluted samples were platted in duplicate. Detection was performed using anti-mouse IgG HRP-conjugated (Southern Biotech, Brimingham, AL).

Plasma samples from hemophilia B (*F9*$^{-/-}$ mice were also used to determine the anti-human F.IX inhibitory activity using a Bethesda assay as previously described[6].

AAV vector genome copy number (VGCN)

Tissue DNA was extracted from whole organ using the Magna Pure 96 DNA and viral NA small volume kit (Roche Diagnostics, Indianapolis IN) according to the manufacturer's instructions. In some experiments, liver non-parenchymal cells (NPC) were isolated via centrifugation in a Percoll solution (Sigma-Aldrich, St. Louis, MO) as previously described[7]. Vector genome copy number was quantified by TaqMan real-time PCR with the ABI PRISM 7900 HT sequence detector (Thermo Fisher Scientific, Waltham, MA). The mouse titin gene was used as normalizer. The primers and probes used for the quantification were as follow: hAAT promoter, forward 5'GGCGGGCGACTCAGATC-3', reverse 5'-GGGAGGCTGCTGGTGAATATT-3' and probe FAM 5'-AGCCCCTGTTTGCTCCTCCGATAACTG-3'. Titin; forward 5'-AAAACGAGCAGTGACGTGAGC-3', reverse 5'-TTCAGTCATGCTGCTAGCGC-3' and probe VIC 5'-TGCACGGAAGCGTCTCGTCTCAGTC-3'.

Immunohistochemistry

For hF.IX staining, livers were then snap-frozen in liquid nitrogen-cooled isopentene. Cryosections of livers tissue (8 μm) were fixed with 3% paraformaldehyde (PFA) for 10 minutes at room temperature (RT). Tissue sections were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) plus 4% goat serum and 0.1% Triton for 2 hours at RT. Samples were incubated with a monoclonal antibody against hF.IX (MA1-43012, Perbio Science, Villebon sur Yvette, France, dilution 1:100) overnight at +4 °C. Following washes in PBS with 0.05% Tween-20, samples were incubated with a secondary antibody conjugated to Alexa Fluor 555 (Thermo Fisher Scientific Waltham, MA, at a dilution of 1:200). Following incubation and washes, sections were counterstained with Dapi.

For capsid staining in HUH-7 cells, cells were stained with an anti-AAV capsid (clone ADK8/9, Progen, Heidelberg, Germany). Images were captured using Confocal Microscope Leica SP8 (Leica MicroSystems, Wetzlar, Germany). Analysis of liver section images was performed using the Cellprofiler software, the hF.IX staining signal was isolated as background signal (intensity 1 to 129), medium intensity (130 to 199) and high intensity (200 to 5000). A minimum of 5 fields per animal (n=5 animals/treatment group) were acquired and analyzed to generate the data on distribution of signal intensity.

RNA extraction and quantitative RT-PCR

Total RNA was isolated from livers using the Magna Pure 96 cellular RNA large volume kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. RNA samples were then pretreated with DNAse (Thermo Fischer Scientific, Waltham, MA) and reverse transcribed using RevertAid H minus first strand cDNA synthesis kit (Thermo Fischer Scientific). Quantitative RT-PCR was performed using absolute ROX mix and Taqman® arrays & plates containing the primer/probe specific for CD8, IFN-γ, FoxP3, IL-10 and GAPDH (Thermo Fischer Scientific). Gene expression was calculated using the $\Delta\Delta C_t$ method relative to GAPDH housekeeping gene and was calculated by normalizing to the mean expression of mice treated with standard AAV8 vectors.

Flow cytometry analysis

Single-cell suspensions from spleen and lymph nodes were prepared and stained for the surface markers with different fluorochrome combinations: anti-CD3 (APC-eFluor780, 17A2, BD Biosciences, San Jose, CA, dilution 1:300), anti-CD4 (PB, RM4-5, BD Biosciences, dilution 1:200), anti-CD25 (PE, PC-61, BD Biosciences, San Jose, CA, dilution 1:300) followed by cell viability staining using Fixable Live/Dead kit (Biolegend, San Diego, CA) according to manufacturer's instructions. Intracellular staining of FoxP3 (APC, FJK-16s, Thermo Fisher Scientific Waltham, MA, dilution 1:40) was performed after fixation and permeabilization using murine FoxP3 buffer kit (Thermo Fisher Scientific Waltham, MA) according to manufacturer's instructions. Intracellular cytokine staining was performed by incubating the cells with AAV8 peptides (1 μg/ml of each peptide, Mimotropes, Australia) or recombinant hF.IX protein (1 μg/ml, BeneFix®, Pfizer, Paris, France) or left untreated for 48 h at 37°C then treated with

monensin (GolgiStop, BD Biosciences, San Diego, CA) according to manufacturer's instructions. After additional 5 h of incubation, cells were harvested and stained for the surface markers, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences, San Diego, CA) and stained for intracellular IFN-γ (Alexa Fluor 647,XMG1.2, dilution 1:200, BD Biosciences, San Diego, CA). Samples were acquired using Sony Spectral cell analyzer SP6800 (Sony Biotechnology Inc, San Jose, CA) Data analysis was performed using the SP6800 software (Sony Biotechnology) and FlowJo software (Tree Star, Ashland, OR).

References

1. Matsushita T, Elliger S, Elliger C, et al. Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther. 1998;5(7):938-945.

2. Gyorgy B, Sage C, Indzhykulian AA, et al. Rescue of Hearing by Gene Delivery to Inner-Ear Hair Cells Using Exosome-Associated AAV. Mol Ther. 2017;25(2):379-391.

3. Ayuso E, Mingozzi F, Montane J, et al. High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. Gene Ther. 2010;17(4):503-510.

4. Meliani A, Leborgne C, Triffault S, Jeanson-Leh L, Veron P, Mingozzi F. Determination of anti-adeno-associated virus vector neutralizing antibody titer with an in vitro reporter system. Hum Gene Ther Methods. 2015;26(2):45-53.

5. Mingozzi F, Anguela XM, Pavani G, et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. Sci Transl Med. 2013;5(194):194ra192.

6. Fields PA, Arruda VR, Armstrong E, et al. Risk and prevention of anti-factor IX formation in AAV-mediated gene transfer in the context of a large deletion of F9. Mol Ther. 2001;4(3):201-210.

7. Merlin S, Cannizzo ES, Borroni E, et al. A Novel Platform for Immune Tolerance Induction in Hemophilia A Mice. Mol Ther. 2017.

Supplemental Tables

Table S1. Yields in production of standard AAV and exo-AAV vectors

Serotype (number of preparations)	**Yield (vg/RB*)**	
	Standard AAV	**exo-AAV**
AAV5 (n=2)	$2.3 \times 10^{12} \pm 2.5 \times 10^{11}$	$2.15 \times 10^{12} \pm 2.1 \times 10^{11}$
AAV6 (n=3)	$9.5 \times 10^{10} \pm 2.8 \times 10^{10}$	$1.4 \times 10^{12} \pm 8.7 \times 10^{11}$
AAV8 (n=2)	$1.8 \times 10^{12} \pm 2 \times 10^{12}$	$5.8 \times 10^{11} \pm 2.5 \times 10^{11}$
AAV9 (n=2)	$1.7 \times 10^{11} \pm 8.4 \times 10^{10}$	$4.5 \times 10^{12} \pm 5.5 \times 10^{12}$

*, vector genomes per roller bottle.

Table S2. Anti-hF.IX neutralizing antibodies (Bethesda assay)

Vector	**Bethesda unit**
Standard AAV8	0.1732
	0.1861
	0.1628
exo-AAV8	0.1560
	0.1623
	0.1709
	0.1869

Supplemental Figure Legends

Figure S1. Characterization of exo-AAV8 vectors preparations and in vitro transduction efficiency. (A) Cryo-TEM of exo-AAV8 vectors. Arrows indicate AAV particles; #, membrane of the exosomes with the characteristic lipid bilayer; *, carbon support film. (B,C,D) *In vitro* transduction at increasing multiplicity of infection (MOI) of HepG2 (B), C2C12 (C), and NIH3T3 (D) cell lines. Analysis performed 24 hours post transduction. Data are shown as mean of three independent experiments ± SEM. **, $p<0.01$; *** $p<0.001$; unpaired Student's *t*-test. RLU, relative light unit. (E,F) Flow imaging analysis of AAV entry and nuclear localization one hour post transduction. 10,000 events were acquired for each sample with magnification 60X. Cytoplasmic AAV8 capsid staining was determined by excluding the nucleus from the using a brightfield mask. The AAV8 capsid intensity in nucleus was performed using the co-localization wizard of the IDEAS v6 software. (E) Representative images of cells (bright filed, nucleus, AAV8 capsid). (F) Frequency of AAV8 capsid in cytoplasm and nucleus 1-hour post transduction.

Figure S2. Autophagy evaluation and receptor competition experiments. (A,B) HUH-7 cells were treated with either standard AAV8 or exo-AAV8 at an MOI of 25,000 for 24 hours. Untreated cells were used as negative controls. (A) Representative Western blot for LC3-II. (B) Densitometry quantification of LC3-II/actin. Data are represented as mean ± SD of two independent experiments. (C) HUH-7 cells were first incubated for 5 minutes at +37 °C in 5% CO_2 with excess of empty AAV8 capsids (1x, 5x or 20x the MOI) followed by the addition of

either standard AAV8 or exo-AAV8 encoding for luciferase at an MOI of 250 for 24 hours at +37 °C in 5% CO_2. Data are shown as % of max of luciferase expression control (no pre-incubation with an excess of empty AAV8 capsids). Data are shown as mean ± SD of two independent experiments. Statistical analyses were performed using one-way ANOVA with Bonferroni post-test (*, $p<0.05$; **, $p<0.01$; ns, not significant).

Figure S3. Exo-AAV8 vector biodistribution. Male C57BL/6 mice (n=5) injected intravenously with 10^9 vg/mouse of exo- or standard AAV8 vectors. Various tissues were collected at the time of sacrifice (21 days post vector injection) for quantification of vector genome copy number (VGCN)/diploid genome. Data are shown as mean ± SEM. Statistical analyses were performed using Mann-Whitney test (*, $p<0.05$; *,* $p<0.01$).

Figure S4. Characterization of exo-AAV5 vectors. Concentration and mean particle size of an exo-AAV5-hF.IX vector measured with the Nanosight ns300 analyzer. Representative plot generated from the average of three 90-second videos.

Figure S5. Anti-capsid and anti-transgene humoral responses following exo- and standard AAV vector gene transfer. (A) Anti-AAV8 IgG antibodies performed by ELISA in sera of animals treated with $5x10^{10}$ vg of either exo- or standard AAV8-hF.IX vectors (n=5 per group). (B) Anti-AAV5 IgG antibodies performed by ELISA in sera of animals treated with $1x10^9$ vg of either exo- or standard AAV5-hF.IX vectors (n=5 per group). (C,D) Analysis of T cell responses

to the AAV capsid and the hF.IX transgene by intracellular staining for IFN-γ. Splenocytes from mice injected with standard or exo-AAV8 were harvested at day 21 and restimulated in vitro for 48 hours with either medium only (negative control), hF.IX protein, or with a pool of AAV8 capsid-derived peptides. (C) Representative flow plots showing staining for $CD8^+$ T cells and IFN-γ. Cells were gated on $CD3^+$ T cells. (D) Summary of flow cytometry data showing the frequency of $CD8^+$IFN-γ^+ T cells (n=5, showed as average ± standard error). (E-H) Livers from animals treated with vector dose of 5 x 10^{10} vg of either standard AAV8 or exo-AAV8 vectors were collected 42 days post vector injection. Total RNA was extracted and mRNA expression levels of CD8 (E), IFN-γ (F), FoxP3 (G) and IL-10 (H) determined. mRNA expression was normalized against the GAPDH housekeeping gene. Data shown as mean ± standard error. Statistical analysis was performed using the Mann-Whitney test (*, $p<0.05$).

Figure S6. Anti-hF.IX transgene IgG responses. Antibodies were measured by ELISA in animals treated with exo- or standard AAV8-hF.IX vectors at high doses ($5x10^{10}$ vg, E) or low doses ($1x10^{10}$ vg, F). As positive control for anti-hF.IX IgG, a group of mice were injected subcutaneously with 100 μg of hF.IX protein formulated in complete Freund's adjuvant.

Figure S7. Readministration with standard or exo-AAV vectors. (A) C57BL/6 mice (n=5/group) were dosed at day 0 with standard or exo-AAV8 vectors expressing luciferase. Twenty one days later animals were re-injected with standard or exo-AAV8 vectors expressing hF.IX. Animals were sacrificed at day 42. (B) Human F.IX transgene expression levels in plasma measured by ELISA at day 42. Shown are individual animals, average levels, and standard error

of the mean. (C) Vector genome copy number (VGCN) in liver at day 42. The primers and probe used for the quantitative real-time PCR used in the assay were specific for hF.IX and therefore measured only the VGCN deriving from the second administration of vector. Shown are average values for the two treatment groups, error bars represent the standard error of the mean. Statistical analysis was performed using the Mann-Whitney test (*, $p<0.05$).

Supplemental Figures

Figure S1

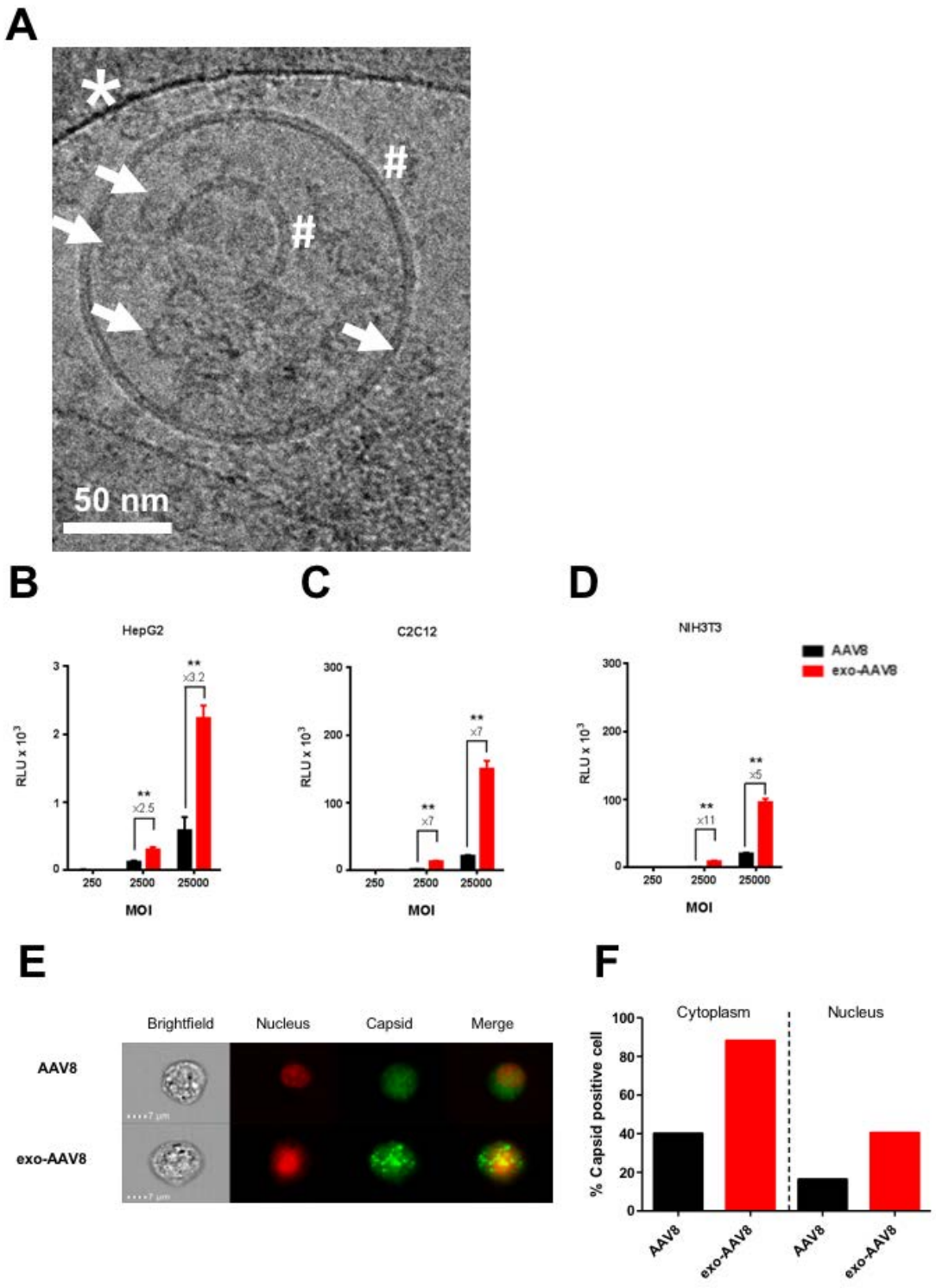

Figure S2

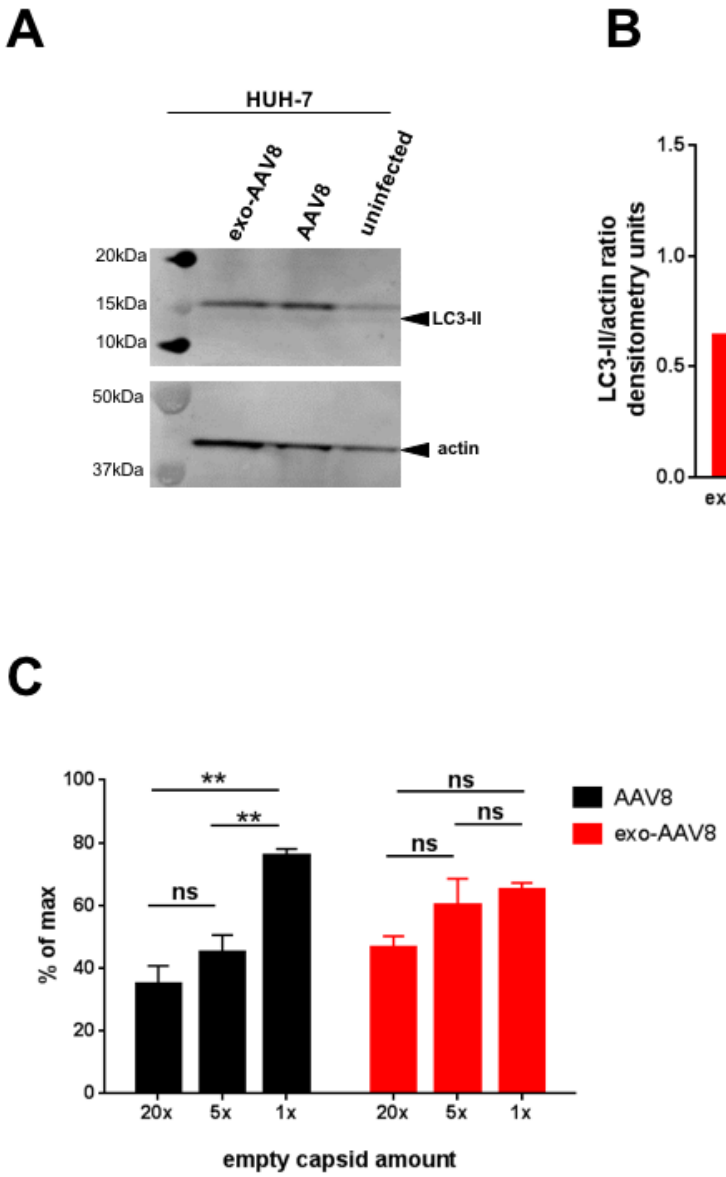
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Figure S3

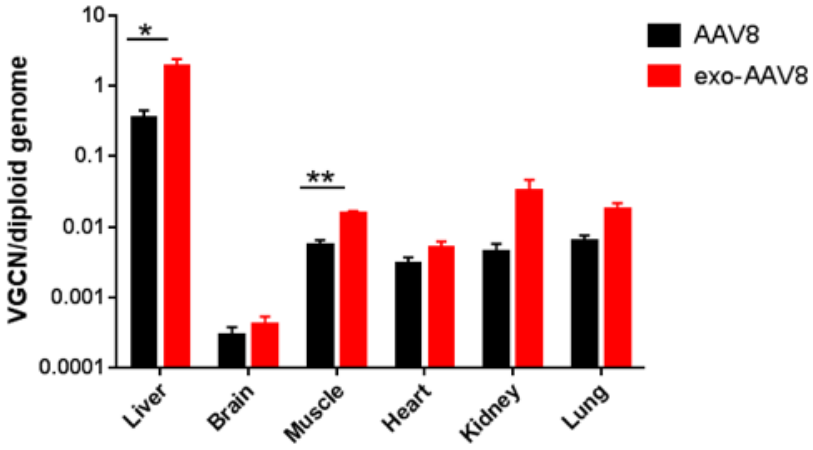
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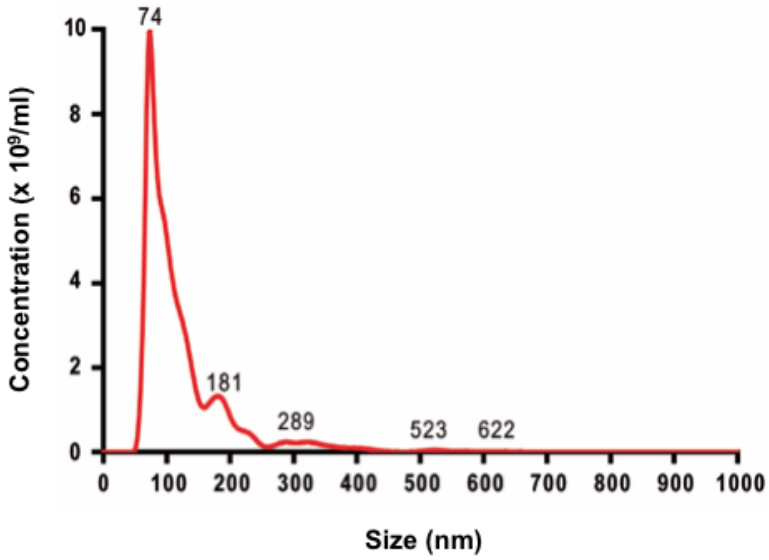
Figure S4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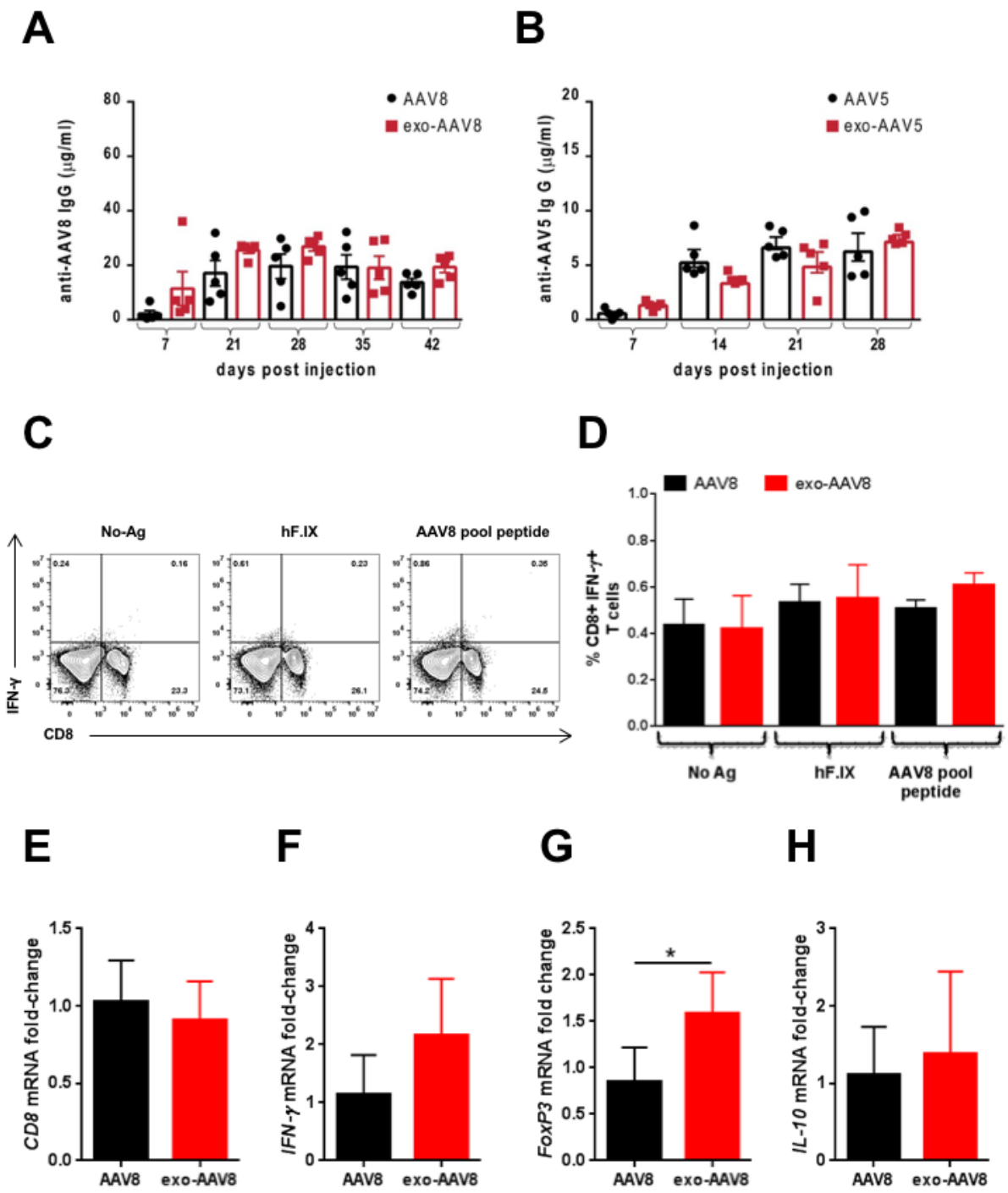
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Figure S6

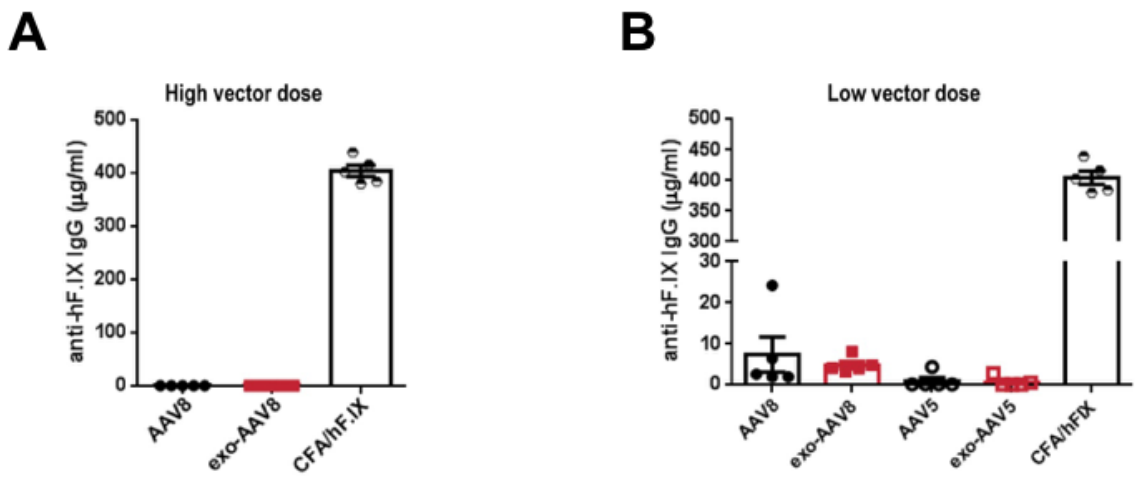
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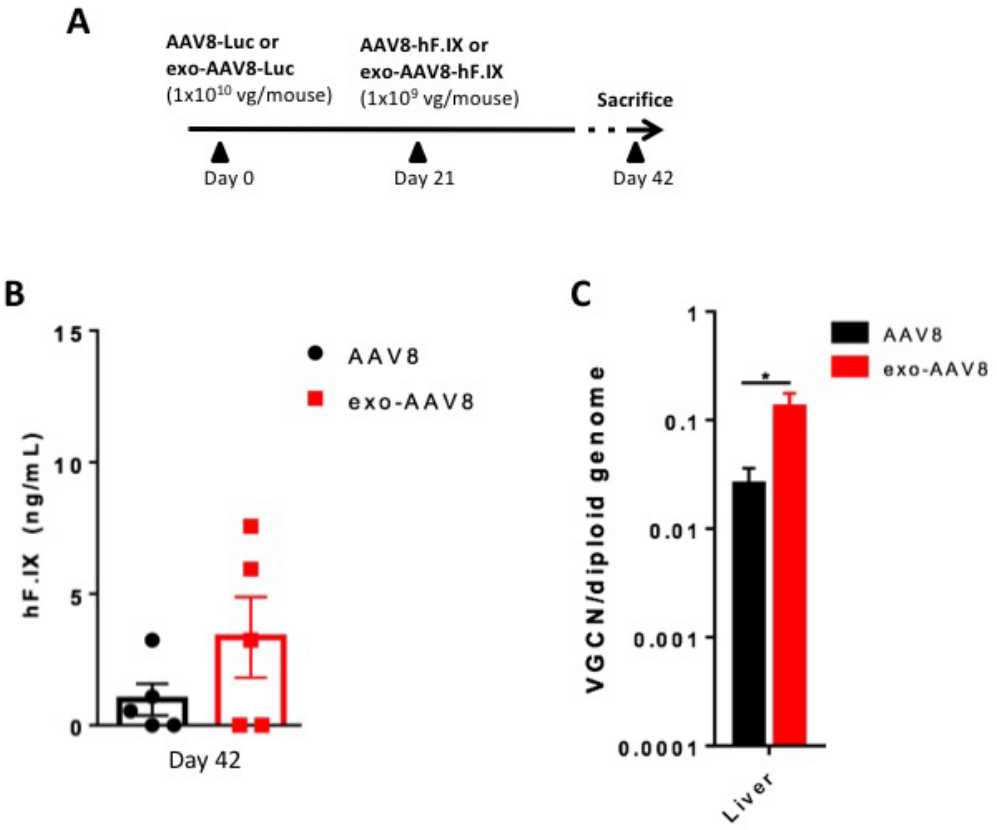
Figure S7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Sacrifice
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