PI: **Li, Chengwen**	Title: Enhance AAV Liver Transduction with Capsid Immune Evasion	
Received: 06/25/2015	FOA: PA13-302	Council: 01/2016
Competition ID: FORMS-C	FOA Title: RESEARCH PROJECT GRANT (PARENT R01)	
1 R01 AI117408-01A1	Dual: DK,HL	Accession Number: 3838759
IPF: 578206	Organization: UNIV OF NORTH CAROLINA CHAPEL HILL	
Former Number:	Department: Pediatrics	
IRG/SRG: GDD	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: [redacted] Year 2: [redacted] Year 3: [redacted] Year 4: [redacted] Year 5: [redacted]	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: N Early Stage Investigator: N
Senior/Key Personnel:	*Organization:*	*Role Category:*
Chengwen Li	University of North Carolina at Chapel Hill	PD/PI
Richard Samulski	University of North Carolina at Chapel Hill	MPI

OMB Number: 4040-0001
Expiration Date: 06/30/2016

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**
1. TYPE OF SUBMISSION*	**4.a. Federal Identifier** [redacted]
❍ Pre-application ● Application ❍ Changed/Corrected Application	**b. Agency Routing Number**

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

[redacted]

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:
Duns Number: [redacted]
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:
Project/Performance Site Congressional District*:

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W

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number [redacted]

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	>(J4*3G4.-;;-K;;.,L1MI
8. Project Narrative*	%3**342N).-;;-K;;.8L1MI
9. Bibliography & References Cited	$2(025P*31DQ.-;;-K;;RSL1MI
10.Facilities & Other Resources	T3G20242)J>6M<)J5&*G)J.-;;-K;;.SL1MI
11.Equipment	/U&21')64.-;;-K;;.VL1MI

NARRATIVE

Having demonstrated that AAV capsid antigen presentation is dose-dependent and requires proteasome mediated degradation, and modification of the AAV capsid surface induces enhanced AAV transduction while lowering the effective dose or decreases capsid antigen presentation, we will explore to develop AAV mutants with the ability to evade capsid specific CTL mediated elimination and with human hepatocyte tropism. This study will allow us to design safer and more effective strategies for human liver gene therapy using AAV.

FACILITIES AND RESOURCES

R. Jude Samulski

Laboratory: Total laboratory space is approximately 2,300 square feet, equipped with chemical hoods, super speed centrifuges (Sorvall RC5C), cryostat, PCR machine, incubators, cold boxes, freezers and various small equipment for general molecular biology. Also available is a P3 containment facility, 500 square feet tissue culture facility with five tissue culture hoods, refrigerators, water bath, dual chamber incubators, and Centra-08R table top centrifuge, and a bench with a small sink and microscope dedicated to our vector production. WE also have access to all common equipment in the Gene Therapy Center's 2,345 square foot core facility, including: complete molecular biology wet lab, ultra speed centrifuges, scintillation counter, sonicator, controlled temperature rooms, dark room, viral tissue culture room and glassware washing facility.

Clinical: N/A

Animal: A virus-free animal facility for the Center is located on the ground floor of the Thurston Bowles building. This room is equipped with a tissue culture hood, and animal isolator and laminar flow hood. Basic animal care is provided by qualified animal technicians and a certified veterinarian is on duty at all times.

Computer: Access to departmental IBM and Macintosh computers and printers, Ethernet link to the UNC Chapel Hill mainframe, which includes access to Duke University, NC State and Wake Forest library databases.

Office: 170 square feet of administrative office space and additional office facilities available for laboratory personnel with administrative and secretarial support available for all personnel in the Gene therapy Center.

Other: Support facilities on campus include the Lineberger Comprehensive Cancer Center, UNC Hospitals, and the School of Public Health. Available within these facilities are a DNA sequencing facility, Confocal and Electron Microscopy facility, Histocompatibility, Flow Cytometry and Clinical Immunology Laboratories, Oligonucleotide facility as well as Phosphor Imaging, Tissue Culture, Transgenic, Biohazard/Virus Containment and Drug Screening facilities. Off campus facilities include: NIEHS, Duke University, NC State University, Research Triangle Park and Wake Forest University, all a short drive away.

MAJOR EQUIPMENT

Chengwen Li

High and low speed centrifuges
Tissue Culture Hoods
Air Jacketed C02 Incubators
Cryostat
Perkin Elmer Thermal Cycler (PCR)
Electrophoresis equipment

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BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person.

[redacted]

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: **SAMULSKI, RICHARD JUDE**

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: **Director, Gene Therapy Center Professor**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Clemson University - Clemson, SC	BS	05/76	Microbiology
University of Florida – Gainesville, FL	PhD	05/82	Molecular Biology
SUNY - Stony Brook, NK	Postdoctoral	05/84	Microbiology
Princeton University - Princeton, NJ	Postdoctoral	05/86	Molecular Biology

A. Personal Statement

I have the expertise, leadership, and motivation necessary to successfully carry out the proposed research project. I have a broad background in gene therapy related to adeno-associated virus (AAV) vector, with

4. Shen S, Horowitz ED, Troupes AN, Brown SM, Pulicherla N, **Samulski RJ**, Agbandje-McKenna M, Asokan A. Engraftment of a galactose receptor footprint onto adeno-associated viral capsids improves transduction efficiency. J Biol Chem. 2013 Oct 4;288(40):28814-23

B. Position and Honors

Positions and Employment

1986-1992 Assistant Professor, Department of Biological Sciences, University of Pittsburgh, Pittsburgh PA
1992-1993 Associate Professor, Department of Biological Sciences, University of Pittsburgh, Pittsburgh PA
1993-1999 Associate Professor, Department of Pharmacology, University of North Carolina, Chapel Hill
1994-1997

[redacted]

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

1. Project Director / Principal Investigator (PD/PI)

Prefix:
First Name*: Chengwen
Middle Name:
Last Name*: Li
Suffix:

2. Human Subjects

Clinical Trial? ❍ No ❍ Yes
Agency-Defined Phase III Clinical Trial?* ❍ No ❍ Yes

3. Permission Statement*

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

● Yes ❍ No

4. Program Income*

Is program income anticipated during the periods for which the grant support is requested? ❍ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

Budget Period*	Anticipated Amount ($)*	Source(s)*

PHS 398 Modular Budget

OMB Number: 0925-0001

Budget Period: 1

Start Date: 04/01/2016 End Date: 03/31/2017

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Organized Research_On Campus	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Darryl Mayes, 202-401-2808

Indirect Cost Rate Agreement Date: 05/16/2012

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

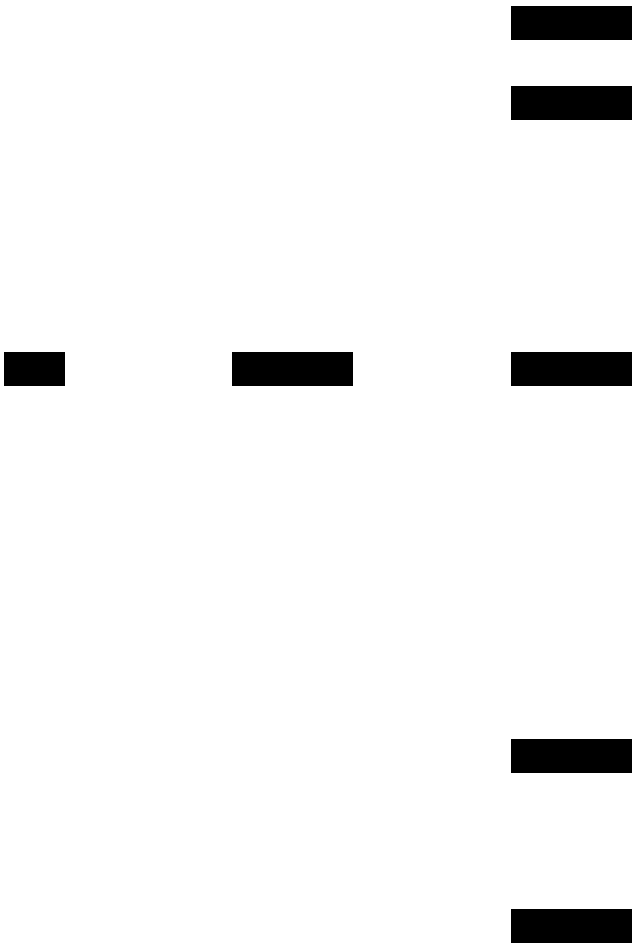
PHS 398 Modular Budget

Budget Period: 2	
Start Date: 04/01/2017 End Date: 03/31/2018	
A. Direct Costs	Funds Requested ($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	
	[redacted]

[redacted]

PHS 398 Modular Budget

Budget Period: 3



PHS 398 Modular Budget

Budget Period: 4	
Start Date: 04/01/2019 End Date: 03/31/2020	

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

Indirect Cost Type			
[redacted]	[redacted]	[redacted]	[redacted]

[redacted]

[redacted]

PHS 398 Modular Budget

Budget Period: 5	
Start Date: 04/01/2020	End Date: 03/31/2021

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium F&A*	[redacted]
Consortium F&A	0.00
Total Direct Costs*	[redacted]

B. Indirect Costs

	Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)
1.	Organized Research_On Campus	[redacted]	[redacted]	[redacted]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Darryl Mayes, 202-401-2808

Indirect Cost Rate Agreement Date: 05/16/2012

Total Indirect Costs: [redacted]

C. Total Direct and Indirect Costs (A + B)

Funds Requested ($): [redacted]

PHS 398 Modular Budget

Cumulative Budget Information

1. Total Costs, Entire Project Period

Section A, Total Direct Cost less Consortium F&A for Entire Project Period ($)	[redacted]
Section A, Total Consortium F&A for Entire Project Period ($)	0.00
Section A, Total Direct Costs for Entire Project Period ($)	[redacted]
Section B, Total Indirect Costs for Entire Project Period ($)	[redacted]
Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period ($)	[redacted]

2. Budget Justifications

Personnel Justification	PersonnelJustification1022072246.pdf
Consortium Justification	
Additional Narrative Justification	

PERSONNEL JUSTIFICATION

Chengwen Li, Ph.D., Principal Investigator, (3.6 CM Years 1-5) will have responsibility for the execution and completion of the proposed research and will oversee immunological analysis. In addition, he will also analyze experimental data and apply the results obtained towards further studies.

Richard J. Samulski, Ph.D., Principal Investigator (0.6 CM Years 1-5) will oversee work performed by all personnel involved in the research proposed and be the guiding force behind all work executed. He will provide his expertise and knowledge in the field of AAV biology and supervise vector construction and characterization.

Maxim Salganik, Post-doctoral Fellow, (6 CM Years 1-5) will carry out he[illegible] et

PHS 398 Research Plan

Please attach applicable sections of the research plan, below.

OMB Number: 0925-0001

1. Introduction to Application (for RESUBMISSION or REVISION only)	Introduction1022072221.pdf
2. Specific Aims	SpecificAims1022072222.pdf
3. Research Strategy*	ResearchStrategy1022072259.pdf
4. Progress Report Publication List	
Human Subjects Sections	
5. Protection of Human Subjects	
6. Inclusion of Women and Minorities	
7. Inclusion of Children	
Other Research Plan Sections	
8. Vertebrate Animals	VertebrateAnimals1022072224.pdf
9. Select Agent Research	SelectAgentResearch1022072227.pdf
10. Multiple PD/PI Leadership Plan	MultiplePI_LeadershipPlan1022072225.pdf
11. Consortium/Contractual Arrangements	
12. Letters of Support	
13. Resource Sharing Plan(s)	ResourceSharingPlan1022072245.pdf
Appendix (if applicable)	
14. Appendix	

SPECIFIC AIMS

Adeno-associated virus (AAV) vector has been successfully applied to target the liver in clinical trials with hemophilia patients[1,2]

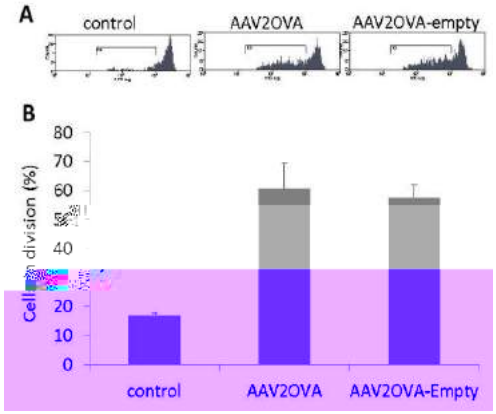


Fig. 1. Capsid antigen presentation after systemic administration of AAV2 empty virions. A. Representative flow cytometric histograms. **B.** Average T cell proliferation and standard deviation for four mice.

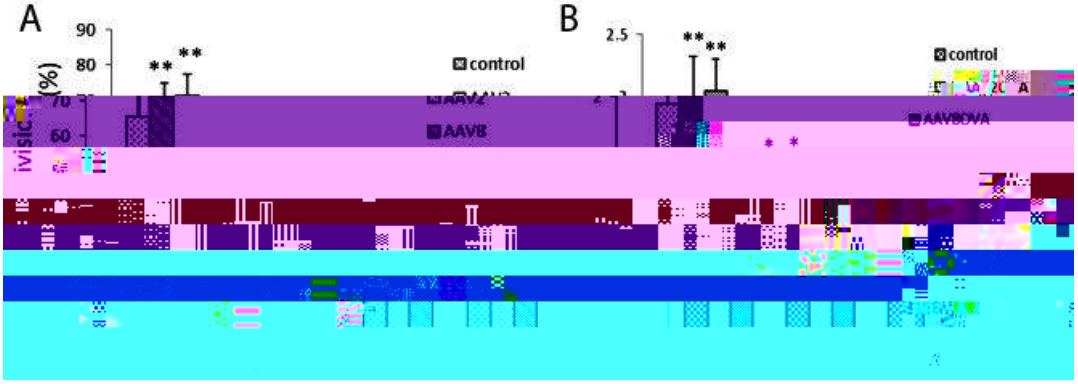


Fig. 2. The kinetics of capsid antigen presentation after AAV8OVA transduction in mice. Particles of AAVOVA/AAT virus (1×10^{11}) were injected intravenously into C57BL/6 mice, and at the indicated time points, 5×10^{6} CFSE-labeled OT-1 T cells were transferred. Ten days after transfer, proliferation of $CD8^{+}$ OT-1 T cells was measured by flow cytometry. **(A)** Average T cell proliferation and standard deviation for four mice. **(B)** Average proliferation index (PI) and standard deviation. $^{**}p<0.01$, $^{*}p<0.05$ compared with control mice without AAV treatment.

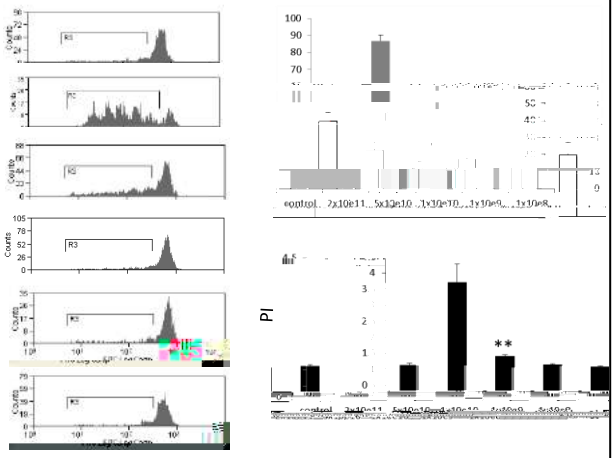


FIG. 3.

capsid antigen presentation is dependent on proteasome-

in vivo OT

identified another proteasome inhibitor, carfilzomib, which also augments AAV liver transduction (**Fig. 7**). As bortezomib enhances AAV transduction the greatest, we also studied the effect of this proteasome inhibitor on AAV capsid antigen presentation, and found that a high concentration of bortezomib inhibits capsid antigen presentation with enhanced transgene expression (**Figs. 4** and **7**). In contrast, a lower concentration of bortezomib (10nM) increased antigen presentation from AAV2-OVA transduced HepG2 cells without increasing transgene expression (**Fig. 8**), and an intermediate dose (100 nM) enhanced both transduction and antigen presentation. This *in vitro* result brings up the concern of whether the utilization of proteasome inhibitors to enhance AAV transduction may impart reduced, or perhaps *enhanced,* AAV capsid antigen presentation in patients. To address the effect of proteasome inhibitors on capsid antigen presentation, we will study AAV capsid antigen cross-presentation *in vivo* using proteasome inhibitors bortezomib and carifilzomib e exborm107(f)-1m998(r Td58(-26.341(7(A)3.92 Tf33.24 0 Td( ) Tj8)-89.004-19.48 0 Td6

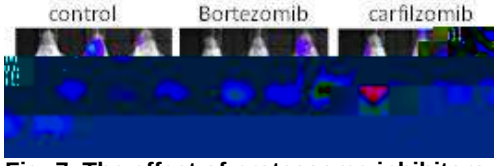


Fig. 7. The effect of proteasome inhibitors on AAV2 transduction. Balb/C mice received $1x10^{11}$ particles of AAV2/luc and 0.5 mg bortezomib/kg or 1 mg carfilzomib/kg at the same time. Transduction was assayed by live imaging at 7 days post AAV injection.

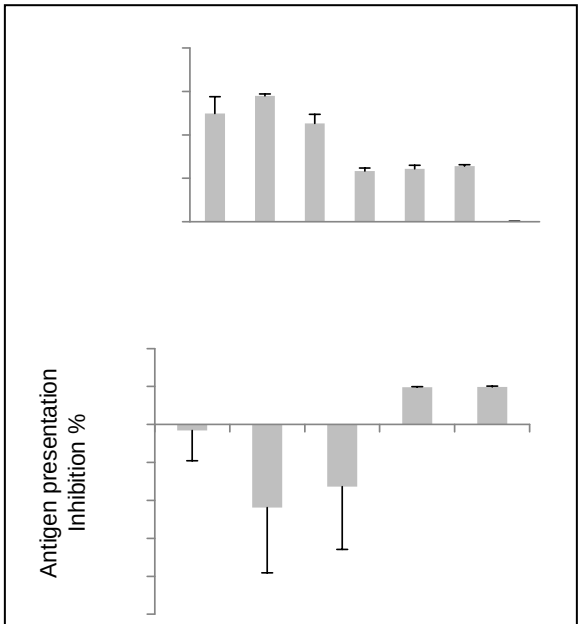


Fig. 8. The effect of bortezomib on capsid antigen presentation. A. Luciferase transgene expression. **B.** capsid antigen presentation. The x-axis indicates the concentration of bortezomib.

C2.2

***Rationale*.** Numerous studies have demonstrated that AAV8 is the most efficient and specific vector to transduce mouse liver among the known serotypes. However, the relative efficiency of AAV8 transduction in the liver of the mouse was not observed in primates and dogs when the same vector dose/kilogram body weight was used; much lower liver transduction was achieved in these larger animals than in mice

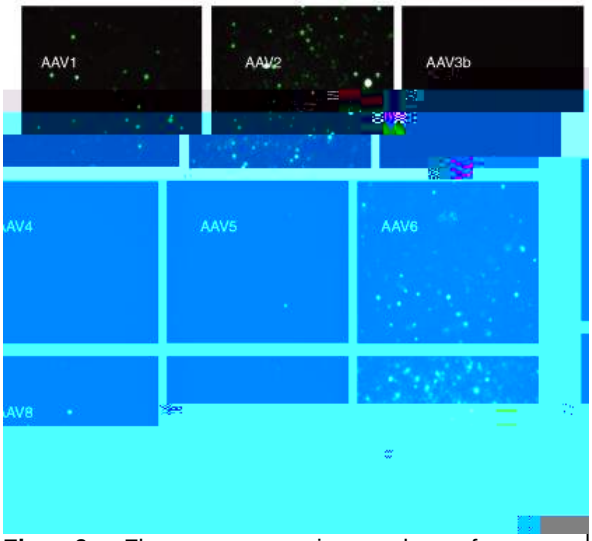


Fig. 9. Fluorescence micrographs of green fluorescent protein (GFP) transgene expression in CS1 cells transduced with AAV serotypes 1–9 (except 7) and the novel variant chimeric-1829 at a multiplicity of infection (MOI) of 1,000 for 48 hours.

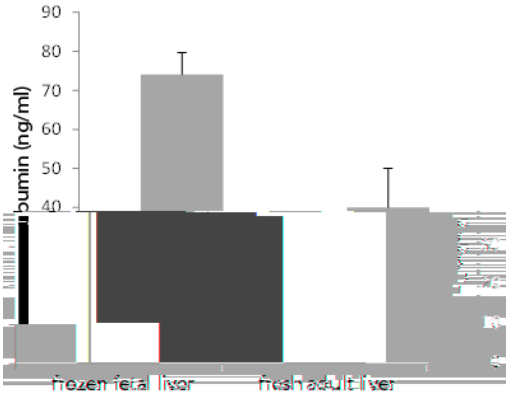


Fig. 10. Engraftment of human liver in FRG mice. FRG mice received 1x10^6 frozen fetal or fresh adult liver cells. The human albumin in the blood was measured by ELISA at 12 weeks and 5 weeks after xenograft. The data represent the average of 2 mice and SD.

hrs prior to transplant, $1.25X10^9$ pfu/25g of Ad/uPA virus will be administered to each mouse by retro-orbital injection, and drinking water containing SMX/TMP, but no nitisinone will be provided. $1X10^6$ hepatocytes in 100 ml will be injected into the inferior pole of the spleen. Animal body weights and survival rates will be closely monitored. The concentration of human albumin will be detected by ELISA using a commercial kit at months 2, 6, 12, 18 to determine the engraftment efficiency of human hepatocytes. *AAV transduction in human liver cells in xenografted mice.* Since self-complementary (sc) AAV induces much higher transduction than conventional single-stranded AAV vectors, scAAV/GFP from 12 serotypes and 8 mutants (AAV2G9, AAV2D, AAV2 Y-F, AAV2 K532R, AAV8 Y-F, AAV8 K137R, AAV2-MT4, AAV8-MT2) from Aim 2 will be administered to xenografted mice via systemic application at the dose of $1x10^{11}$ particles ($5x10^{12}$/kg). At week 6 after AAV injection, mice will be perfused and fixed, and the liver will be separated and sectioned. The antibodies against human albumin will be used to identify human cells using a PE-labeled secondary antibody. Double positive liver cells, GFP+ and PE+, will be observed using fluorescent microscopy and indicate human cell transduction.

viper database (viperdb.scripps.edu). Molecular modeling facilities are available to us through the Structural Bioinformatics facility at UNC-CH.

C3.3. **Investigation of immune-evasion from humanized AAV mutants.** Mutants isolated from humanized mice will be further characterized for their immune evasion ability including ubiquitination capacity *in vitro,* and for antigen presentation in AAV mutant -transduced cells *in vitro* and *in vivo*. For antigen presentation, the HI loop of 2 mutants with high human liver tropism from **C3.2** will be substituted for the OVA SIINFEKL peptide. *In vitro* antigen presentation is performed in HepG2/H2kb cells and *in vivo* analysis in C57 mice.
Detection of AAV capsid ubiquitination in AAV mutant transduced cells. $2x10^6$ cells, mock-treated or treated

VERTEBRATE ANIMALS

Protocols for these projects are pending at the UNC-CH Institutional Animal Care and Use Committee and will

For kinetics study, 120 mice are required: 4 time points (day3, day21, day 41 and day 61) x 2 (each mutant + control) x 3 viruses (2 mutants and AAV2OVA) x 5 mice/group.

60 OT-1 mice are needed.

For aim 3.3, the total number is 90 + 120 + 60 = 270 including 210 C57BL mice and 60 OT-1 mice.

For aim 3, the total number is 100 + 75 + 270 = 445

The total number of mice required for this proposal is 720 (**Aim 1**) + 2325 (**Am 2**) + 445 (**Aim 3**) = 3490.

BIOHAZARDS

Adeno-associated virus (AAV) is known not to cause any diseases in human or animals, so AAV vector is non-infectious and not hazardous materials.

Adenovirus can cause respiratory illness and various other illnesses such as gastroenteritis, conjunctivitis, cystitis and rash. The production of adenovirus and experiments with adenovirus (mice experiments) will be performed in Biosafety Level 2.

MULTIPLE PI LEADERSHIP PLAN

The Li

LITERATURE CITED

1 Manno, C. S. *et al.*

[redacted]

22 Johnson, J. S. *et al.* Mutagenesis of adeno-associated virus type 2 capsid protein VP1 uncovers new roles for basic amino acids in trafficking and cell-specific transduction. *J Virol* **84**, 8888-8902, doi:10.1128/JVI.00687-10 (2010).
23 Mingozzi, F. *et al.* Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci Transl Med* **5**, 194ra192, doi:10.1126/scitranslmed.3005795 (2013).
24 Wu, Z. *et al.* Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Molecular therapy : the journal of the American Society of Gene Therapy* **16**, 280-289, doi:10.1038/sj.mt.6300355 (2008).
25 Binny, C. *et al.* AAV-mediated gene transfer in the perinatal period results in expression of FVII at levels that protect against fatal spontaneous hemorrhage. *Blood* **119**, 957-966, doi:10.1182/blood-2011-09-377630 (2012).
26 Mitchell, A. M., Nicolson, S. C., Warischalk, J. K. & Samulski, R. J. AAV's anatomy: roadmap for optimizing vectors for translational success. *Curr Gene Ther* **10**, 319-340 (2010).
27 Douar, A. M., Poulard, K., Stockholm, D. & Danos, O. Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol* **75**, 1824-1833, doi:10.1128/JVI.75.4.1824-1833.2001 (2001).
28 Duan, D., Yue, Y., Yan, Z., Yang, J. & Engelhardt, J. F. Endosomal processing limits gene transfer to polarized airway epithelia by adeno-associated virus. *J Clin Invest* **105**, 1573-1587, doi:10.1172/JCI8317 (2000).
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RESOURCE SHARING PLAN

The PI of the application will implement the reagent and data sharing plan. We anticipate that several types of reagents will be developed that will be of broad interest to the gene transfer community nation-wide. The DNA sequences for mutated capsid and data from patients that appear in articles will be made available through the UNC Vector Core via the internet or through the **National Center for Biotechnology Information (NCBI)**, National Library of Medicine (NLM), NIH, national resource for molecular biology information. We anticipate that deposition of such data would be done at the time of publication, but early deposition, e.g., at the time of manuscript acceptance, will be encouraged. Published reviews and manuscripts will be posted with a link to the PubMed library (when available) on our website www.med.unc.edu/genether and will submit electronic copies of final peer-reviewed manuscripts, upon acceptance of publication, the National Library of Medicine's PubMed Central (PMC). The searchable PMC archive will provide greater public access and permanent preservation of NIH-supported research, as well as provide free, full-test publications on the internet as stated in the NIH policy to promote immediate public access to research findings (Feb, 2005).

VIRAL VECTORS: With respect to new viral vectors