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:	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*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier [redacted]

b. Agency Routing Number

2. DATE SUBMITTED 2015-06-25

Application Identifier

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **Organizational DUNS*:** [redacted]

Legal Name*: University of North Carolina at Chapel Hill
Department: Office of Sponsored Research
Division: Research
Street1*: 104 Airport Drive, CB 1350
Street2: Suite 2200
City*: Chapel Hill
County: Orange
State*: NC: North Carolina
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 27599-1350

Person to be contacted on matters involving this application
Prefix: First Name*: Pamela Middle Name: P Last Name*: Bordsen Suffix:
Position/Title: Grants Analyst/Reviewer
Street1*: CB:9525 1140-C Bioinformatics , 130 Mason Farm Road
Street2:
City*: Chapel Hill
County: Orange
State*: NC: North Carolina
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 27599-9525
Phone Number*: ([redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* H: Public/State Controlled Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

❍ New ● Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Enhance AAV Liver Transduction with Capsid Immune Evasion

12. PROPOSED PROJECT
Start Date* 04/01/2016
Ending Date* 03/31/2021

13. CONGRESSIONAL DISTRICTS OF APPLICANT
NC-004

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Chengwen Middle Name: Last Name*: Li Suffix:
Position/Title: Research Assistant Professor
Organization Name*: University of North Carolina at Chapel Hill
Department: Pediatrics
Division:
Street1*: 7113 Thurston Bowles;CB:7352
Street2:
City*: Chapel Hill
County: Orange
State*: NC: North Carolina
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 27599-7352
Phone Number*: [redacted] Fax Number: (919) 966-0907 Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	[redacted]
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	[redacted]
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Dr. First Name*: Barbara Middle Name: Last Name*: Entwisle Suffix: Ph.D.
Position/Title*: Vice Chancellor for Research
Organization Name*: University of North Carolina at Chapel Hill
Department: Office of Sponsored Research
Division: Research
Street1*: 104 Airport Drive, Suite 2200
Street2: CB 1350
City*: Chapel Hill
County: Orange
State*: NC: North Carolina
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 27599-1350
Phone Number*: [redacted] Fax Number: [redacted] Email*: [redacted]

Signature of Authorized Representative*
Barbara Entwisle

Date Signed*
06/25/2015

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:CoverLetter1022072232.pdf

424 R&R and PHS-398 Specific Table Of Contents

OMB Number: 4040-0010
Expiration Date: 06/30/2016

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:	The University of North Carolina at Chapel Hill
Duns Number:	[redacted]
Street1*:	104 Airport Drive, CB 1350
Street2:	Suite 2200
City*:	Chapel Hill
County:	Orange
State*:	NC: North Carolina
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	27599-1350
Project/Performance Site Congressional District*:	NC-004

File Name

Additional Location(s)

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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

RESEARCH & RELATED Other Project Information

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*	Abstract1022072214.pdf
8. Project Narrative*	Narrative1022072216.pdf
9. Bibliography & References Cited	Bibliography1022072258.pdf
10.Facilities & Other Resources	FacilitiesAndResources1022072218.pdf
11.Equipment	Equipment1022072219.pdf

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

ABSTRACT

Adeno-associated virus (AAV) vector has been successfully applied in phase I clinical trials in hemophilia B patients with liver targeting. However, these studies have suggested that AAV capsid specific cytotoxic T lymphocytes (CTL) have the potential to eliminate AAV transduced hepatocytes and result in the therapeutic failure. Our prior studies have demonstrated that AAV capsid antigen presentation is dose-dependent and requires capsid ubiquitination for proteasome mediated degradation. The contamination of empty virions in AAV preparation inhibits transduction from full particles of AAV vectors and potentially increases the risk of virus capsid antigen load. In this proposal we will investigate capsid antigen presentation from AAV empty virions and the effect of empty particles on antigen presentation from full virus transduction (**Aim 1**). To decrease antigen presentation on AAV transduced cells for avoiding capsid specific CTL-mediated elimination, it has been proposed to modify the AAV capsid surface or apply proteasome inhibitors to enhance AAV transduction while lowering the effective dose or to escape capsid ubiquitination. We will study the effect of AAV mutants and proteasome inhibitors on AAV capsid antigen presentation (**Aim 2**). It is well-known that the transduction of AAV vectors in mouse models does not always translate into the human. Finally, we will explore the directed evolution approach combined with a rational design strategy to isolate AAV vectors with human hepatocyte specific tropism and the ability to evade a capsid specific CTL response in humanized mice (**Aim 3**). Elucidation of AAV empty capsid antigen presentation *in vivo* and the development of an AAV vector with enhanced human liver transduction and CTL immune-evasion will allow us to design safer and more effective strategies that address the current clinical complications for human liver gene therapy using AAV.

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

Chengwen Li

Laboratory: The PI has 800 sq.ft. of lab space in the Gene Therapy Center with basic lab equipment including a biosafety hood, incubators, Benchtop PCR (Biorad), -80 and -20 freezers, refrigerator, and various small equipment for molecular biology. In addition, the lab is equipped with a Perkin Elmer spectrofluorometer, Sorvall benchtop centrifuge. The PI also has access to all common equipment in the Gene Therapy Center's 2,345 sq.ft core facility including: Perkin Elmer luminometer, Gel Documentation system, complete molecular biology wet lab, ultra speed centrifuges, scintillation counter, sonicator, controlled temperature rooms, dark room, cryostat, PCR machine, incubators, cold boxes, freezers and various small equipment for general molecular biology, viral tissue culture room and glassware washing facility. Also available is a P3 containment facility.

Animals: 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. The animal imaging facility is equipped with a Xenogen IVIS Lumina and a Xenogen Kinetic for live animal imaging studies.

Computers: The lab has three desktop machines equipped with molecular modeling, live animal image analysis, statistical and data analysis software. Access to center IBM and Macintosh computers and printers, laptops is also available.

Office: Over 150 square feet of administrative office space and additional office facilities is 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 CF Center cell culture, tissue histology and mouse model core facilities, 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, Flow Cytometry facility, 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.

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
Scintillation Counter
Fraction Collector
Spectrophotometer
Film Processor
Walk in Warm and Cold Rooms
Cryostat
Perkin Elmer Thermal Cycler (PCR)
Electrophoresis equipment
High Resolution Ion Exchange Column
Branson Sonifier II
Ranin HPCL apparatus
Fotodyne Gel Camera

MAJOR EQUIPMENT

R. Jude Samulski

High and low speed centrifuges
Tissue Culture Hoods
Air Jacketed C02 Incubators
Scintillation Counter
Fraction Collector
Spectrophotometer
Film Processor
Walk in Warm and Cold Rooms
Wave insect cell bioreactor
Cryostat
Perkin Elmer Thermal Cycler (PCR)
Electrophoresis equipment
High Resolution Ion Exchange Column
Branson Sonifier II
Ranin HPCL apparatus
Fotodyne Gel Camera
New Brunswick Sci. C25 Incubator Shaker

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

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

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

Position/Title*: Research Assistant Professor
Organization Name*: University of North Carolina at Chapel Hill
Department: Pediatrics
Division:
Street1*: 7113 Thurston Bowles;CB:7352
Street2:
City*: Chapel Hill
County: Orange
State*: NC: North Carolina
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 27599-7352

Phone Number*: [redacted] Fax Number: [redacted] E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: MD/PhD Degree Year: 1995

	File Name
Attach Biographical Sketch*:	Li_Biosketch1022072247.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT11947975 Funding Opportunity Number: PA-13-302 . Received Date: 2015-06-25T09:02:19.000-04:00

PROFILE - Senior/Key Person

Prefix:	First Name*: Richard	Middle Name J	Last Name*: Samulski	Suffix:

Position/Title*:	Dir, Gene Therapy Center, Prof
Organization Name*:	University of North Carolina at Chapel Hill
Department:	Pharmacology
Division:	
Street1*:	7119 Thurston-Bowles;CB:7352
Street2:	
City*:	Chapel Hill
County:	Orange
State*:	NC: North Carolina
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	27599-7352

Phone Number*: [redacted] Fax Number: [redacted] E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 1982

	File Name
Attach Biographical Sketch*:	Samulski_Biosketch1022072231.pdf
Attach Current & Pending Support:	

Tracking Number: GRANT11947975 Funding Opportunity Number: PA-13-302 . Received Date: 2015-06-25T09:02:19.000-04:00

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: **LI, CHENGWEN**

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

POSITION TITLE: **RESEARCH ASSISTANT 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
Tongji Medical University, Wuhan, Hubei, China	BS	07/87	Medicine
Peking Union Medical College (PUMC), China	MD/PhD	07/95	Immunotherapy
Tokai University School of Medicine, Japan	Postdoctoral	01/2000	Immunology, Cell Therapy
Gene Therapy Center, UNC at Chapel Hill, NC USA	Postdoctoral	05/04	Gene Therapy, Immunology

A. Personal Statement

My lab's research has focused on the study of the immune response to adeno-associated virus (AAV) vector *in vitro* and in animal models. The ultimate goal is to develop effective strategies to evade immune response mediated elimination of AAV transduced target cells and escape neutralizing antibody activity. During past years, we have demonstrated that AAV capsids elicit a CTL response via both the classical pathway and cross-presentation of capsid antigens after AAV administration; capsid antigen cross-presentation is dependent on proteasome mediated degradation of AAV capsid and requires AAV virion escape from the endosome. In addition, AAV capsid antigen presentation is dose-dependent and more effective at the earlier period after AAV transduction. In summary, our track record, technical expertise, resources are well-suited to successfully achieve the goals outlined in the current proposal. We anticipate that progress made over the next few years will lay the groundwork to design safer and more effective AAV vectors for clinical gene therapy studies.

1. **Li C,** Hirsch M, Asokan A, Zeithaml B, Ma H, Kafri T, Samulski RJ. Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid in vivo. J Virol. 2007 Jul;81(14):7540-7. Epub 2007 May 2.
2. **Li C,** Hirsch M, DiPrimio N, Asokan A, Goudy K, Tisch R, Samulski RJ. Cytotoxic-T-lymphocyte-mediated elimination of target cells transduced with engineered AAV type 2 vector in vivo. J Virol. 2009 Jul;83(13):6817-24.
3. **Li C**, He Y, Nicolson S, Hirsch M, Weinberg MS, Zhang P, Kafri T, Samulski RJ. Adeno-associated virus capsid antigen presentation is dependent on endosomal escape. J Clin Invest. 2013 Mar 1;123(3):1390-401
4. He Y, Weinberg MS, Hirsch M, Johnson MC, Tisch R, Samulski RJ, **Li C.** Adeno-associated virus capsid antigen presentation is dependent on endosomal escape. Hum Gene Ther. 2013 May;24(5):545-53.

B. Position and Honors

Positions and Employment

2004-2010 Research Associate, The University of North Carolina at Chapel Hill, NC,

2010-present Research Assistant Professor, The University of North Carolina at Chapel Hill, NC, Department of Pediatrics

Professional Societies

1997-2002 Member of the American Society of Hematology
2009-present Member of American Society of Gene and Cell Therapy

C. Contribution to Science

1. AAV Neutralizing antibody: Gene therapy using adeno-associated virus (AAV) as a delivery vehicle has been successful in phase I clinical trials in patients with blood diseases and blindness. A major restriction for systemic AAV vector application is the high prevalence of AAV neutralizing antibodies (NAbs) in the human population. Over 90% of the population is naturally infected by AAV and about half still have neutralizing antibodies. We have recently performed a kinetics study analyzing NAb prevalence in 62 children with hemophilia A, and demonstrated that i) if a low titer of NAbs exists, an alternative serotype or modified capsid should be considered and ii) the earlier in life that vector can be administrated, the better the chance of avoiding the likelihood of primary wt AAV infection and natural NAb levels we observed that increases with age. Additionally, our studies have demonstrated that A20 antibody (which only recognizes intact AAV2 virion) cannot block AAV2.5 (AAV2 capsid with 5 amino acids substitution from AAV1 capsid) transduction, and no obvious relationship is observed between muscle transduction from mutants and inserted amino acid properties, or NAb titer and cross-reactivity. This result suggests that the enhanced muscle transduction and immune profile change from mutants with 265 insertions are related to structural changes of the mutant virion.

a. **Li C**, Narkbunnam N, Samulski RJ, Asokan A, Hu G, Jacobson LJ, Manco-Johnson MJ, Monahan PE; Joint Outcome Study Investigators. Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. Gene Ther. 2012 Mar;19(3):288-94
b. Bowles DE, McPhee SW, **Li C**, Gray SJ, Samulski JJ, Camp AS, Li J, Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther. 2012 Feb;20(2):443-55.
c. **Li C**, Diprimio N, Bowles DE, Hirsch ML, Monahan PE, Asokan A, Rabinowitz J, Agbandje-McKenna M, Samulski RJ. Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles. J Virol. 2012 Aug;86(15):7752-9.

2. Immune response to transgene: The immune response has been implicated as a critical factor in determining the success of gene therapy in clinical trials. Generally, the CD8+ CTL immune response is crucial for the specific eradication of cells displaying foreign peptides (i.e., virus- or bacteria-infected cells). Epitopes recognized by CTLs likely arise from primary ORFs, particularly via the degradation of newly synthesized proteins. However, recent studies indicate that CTL epitopes can also be encoded by non-primary ORF sequences and other nontraditional sources in tumor- and virus-infected cells. We have demonstrated the previously uncharacterized finding that a therapeutic cassette currently being used for human investigation displays alternative reading frames (ARFs) that generate unwanted protein products to induce a cytotoxic T lymphocyte (CTL) response. Such unforeseen epitope generation warrants careful analysis of transgene sequences for ARFs to reduce the potential for adverse events arising from immune responses during clinical gene therapy protocols.

a. **Li C**, Goudy K, Hirsch M, Asokan A, Fan Y, Alexander J, Sun J, Monahan P, Seiber D, Sidney J, Sette A, Tisch R, Frelinger J, Samulski RJ. Cellular immune response to cryptic epitopes during therapeutic gene transfer. Proc Natl Acad Sci U S A. 2009 Jun 30;106(26):10770-4.
b. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, **Li C**, Galloway G, Malik V, Coley B, Clark KR, Li J, Xiao X, Samulski J, McPhee SW, Samulski RJ, Walker CM. Dystrophin immunity in Duchenne's muscular dystrophy. N Engl J Med. 2010 Oct 7;363(15):1429-37.

3. Optimization of transgene cassette: To develop AAV liver specific transgene expression, we have found that a small regulatory element from AAV2 integration site of the human chromosome 19 has enhancer

function only in liver but not in muscle. Many diseases result from accumulation of misfolded protein in the cytoplasm; addition of wild type gene product by gene delivery leads to improvement but may not completely correct the phenotype. Using alpha-1 antitrypsin (AAT) deficiency with the piZZ mutant phenotype as a model system, we demonstrated that a single AAV vector is able to deliver two gene products to both silence the piZZ transcript and restore circulating wild-type AAT expression from a resistant codon-optimized AAT (AAT-opt) transgene cassette. These results implicate that similar gene-therapy strategies could be considered for any diseases caused by accumulation of misfolded proteins.

a. **Li C**, Hirsch M, Carter P, Asokan A, Zhou X, Wu Z, Samulski RJ. A small regulatory element from chromosome 19 enhances liver-specific gene expression. Gene Ther. 2009 Jan;16(1):43-51.
b. **Li C**, Xiao P, Gray SJ, Weinberg MS, Samulski RJ. Combination therapy utilizing shRNA knockdown and an optimized resistant transgene for rescue of diseases caused by misfolded proteins. Proc Natl Acad Sci U S A. 2011 Aug 23;108(34):14258-63.
c. Monahan PE, Sun J, Gui T, Hu G, Hannah WB, Wichlan DG, Wu Z, Grieger JC, **Li C**, Suwanmanee T, Stafford DW, Booth CJ, Samulski JJ, Kafri T, McPhee SW, Samulski RJ. Employing a gain-of-function factor IX variant R338L to advance the efficacy and safety of hemophilia B human gene therapy: preclinical evaluation supporting an ongoing adeno-associated virus clinical trial. Hum Gene Ther. 2015 Feb;26(2):69-81.

Complete List of Published Work in MyBibliography:

http://www.ncbi.nlm.nih.gov/sites/myncbi/chengwen.li.1/bibliography/47955510/public/?sort=date&direction=ascending

D. Research Support

Ongoing Research Support

R01DK084033-03 (Li/Samulski MPI) 04/01/12-03/31/17
NIH/NIDDK
AAV Gene Therapy for AAT Deficiency
The proposed studies seek to develop novel AAV vectors with improved liver-specific targeting and explore the ability of these vectors to escape neutralizing antibody in an AAT deficiency mouse model (PiZZ mice).

P01 HL112761 (Samulski, PI) 2/08/2013 – 1/31/2018
NIH NHLBI
Neutralizing antibody and AAV FIX gene therapy
Role: Co-PI Animal Models Core
The goal of this program is overcome the major barrier to AAV gene therapy that exists in up to 50% of the population who have circulating AAV neutralizing antibodies (NAb).

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 specific expertise in optimization of AAV vector for clinical applications. AAV is the only known human DNA virus that is non-pathogenic, has broad tropism, and is able to establish both latent and lytic life cycle. Since our contribution of the original cloning of an infectious AAV genome, my lab has focused over 30 years on development of this unique virus as a gene delivery system. These efforts have led to the first long term gene delivery in muscle, brain and other target tissue. Development of novel AAV variants as alternative vectors and improvements in AAV production have allowed the lab to support the first clinical trial for gene delivery to brain and develop the first chimeric AAV for gene delivery for muscular dystrophy. One of my current goals is to continue to derive delivery systems for safe and efficient use in human gene therapy with the ultimate goal of facilitating the progression and translation of gene therapy research from the laboratory bench into Phase I clinical trials for the treatment of human disease. Recent clinical trials in patients with hemophilia B have demonstrated that an AAV capsid -specific CTL response eliminates AAV transduced hepatocytes resulting in therapeutic failure. To better understand this “rate-limiting” step, under NIH-funded grant, we observed and published that capsid antigen presentation in AAV transduced cells is dependent on proteasome mediated AAV capsid degradation as well as determined that capsid antigen presentation is dose-dependent. These observations laid the groundwork for our proposed research by development and testing of novel AAV vectors with CTL evasion potential. We have established working protocols, assays and animal models to facilitate our hypothesis of “evolving AAV capsid to avoid immune response in humanized liver model”.

1. Li C, Hirsch M, DiPrimio N, Asokan A, Goudy K, Tisch R, **Samulski RJ**. Cytotoxic-T-lymphocyte-mediated elimination of target cells transduced with engineered AAV type 2 vector in vivo. J Virol. 2009 Jul;83(13):6817-24.
2. Li C, He Y, Nicolson S, Hirsch M, Weinberg MS, Zhang P, Kafri T, **Samulski RJ**. Adeno-associated virus capsid antigen presentation is dependent on endosomal escape. J Clin Invest. 2013 Mar 1;123(3):1390-401
3. He Y, Weinberg MS, Hirsch M, Johnson MC, Tisch R, **Samulski** RJ, Li C. Adeno-associated virus capsid antigen presentation is dependent on endosomal escape. Hum Gene Ther. 2013 May;24(5):545-53.

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 Member, Recombinant DNA Advisory Committee, NIH
1993-present Director, Gene Therapy Center, University of North Carolina, Chapel Hill, NC
1999-present Professor, Department of Pharmacology, University of North Carolina, Chapel Hill, NC
2003–present Founder, Acting Chief Scientific Officer, Asklepios BioPharmaceuticals, Inc., Chapel Hill, NC
2009-2010 Vice President, American Society of Gene & Cell Therapy (ASGCT).
2010-2011 President-Elect, American Society of Gene & Cell Therapy (ASGCT).
2011-2012 President, American Society of Gene & Cell Therapy (ASGCT).

Ad hoc reviewer for National Science Foundation grant applications
Ad hoc reviewer for March of Dimes grant applications
Member, International Advisory Committee for the Xth International Congress of Virology

Honors and Awards

1990 Outstanding Young Men of America Award
1991 University of Pittsburgh, President's Distinguished Research Award
2008 ASGT Outstanding Achievement Award

C. Contribution to Science

1. AAV biology: AAV transduction involves many steps including AAV binding to the receptors or co-receptors on the cell surface, endocytosis, escape from the endosomes, trafficking to the perinuclear space, nucleus entrance, uncoating and transgene expression. A strong focus of my lab is basic understanding of AAV biology which has led to the first discovery of an AAV receptor (heparan sulfate) and co-recptor ($\alpha_v\beta5$), mechanism of viral trafficking and genome persistence. As pioneers, we have identified several primary receptors and co-receptors as well as elucidated the mechanism of AAV trafficking intracellularly, and maintain a robust effort studying AAV biology with both natural and chimeric AAV capsids.

a. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol. 1998 Feb;72(2):1438-45.
b. Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. Nat Med. 1999 Jan;5(1):78-82.
c. Xiao PJ, Samulski RJ. Cytoplasmic trafficking, endosomal escape, and perinuclear accumulation of adeno-associated virus type 2 particles are facilitated by microtubule network. J Virol. 2012 Oct;86(19):10462-73.
d. Nicolson SC, Samulski RJ. Recombinant adeno-associated virus utilizes host cell nuclear import machinery to enter the nucleus. J Virol. 2014 Apr;88(8):4132-44.

2. AAV capsid engineering: Reengineering of adeno-associated virus (AAV) isolates may yield variants with improved properties for clinical applications. We have performed the first clinical trial in patients with Duchenne muscular dystrophy using a chimeric adeno-associated virus (AAV) capsid variant (designated AAV2.5) derived from a rational design strategy. The novel chimeric vector has the improved muscle transduction capacity of AAV1 with reduced antigenic cross-reactivity against both parental serotypes, while keeping the AAV2 receptor binding. Also, we observed that reengineering AAV receptors change AAV tropism and enhance transduction. Using an AAV evolution approach, AAV mutants can be isolated with specific tissue tropism and the ability to evade neutralizing antibodies. This is a major focus of the lab.

a. Li W, Asokan A, Wu Z, Van Dyke T, DiPrimio N, Johnson JS, Govindaswamy L, Agbandje-McKenna M, Leichtle S, Redmond DE Jr, McCown TJ, Petermann KB, Sharpless NE, Samulski RJ. Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. Mol Ther. 2008 Jul;16(7):1252-60.
b. Asokan A, Conway JC, Phillips JL, Li C, Hegge J, Sinnott R, Yadav S, DiPrimio N, Nam HJ, Agbandje-McKenna M, McPhee S, Wolff J, Samulski RJ. Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. Nat Biotechnol. 2010 Jan;28(1):79-82.
c. Bowles DE, McPhee SW, Li C, Gray SJ, Samulski JJ, Camp AS, Li J, Wang B, Monahan PE, Rabinowitz JE, Grieger JC, Govindasamy L, Agbandje-McKenna M, Xiao X, Samulski RJ. Phase 1 gene therapy for Duchenne muscular dystrophy using a translational optimized AAV vector. Mol Ther. 2012 Feb;20(2):443-55.
d. 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.

3. AAV Neutralizing antibody: While AAV gene therapy continues to yield clinical results supportive of the hope for eventual treatment of many diseases, the presence of patient neutralizing antibodies (NAbs) remains a challenge. NAb-mediated elimination of AAV vectors has become a rate-limiting step in advancing the field and a determinant for repeat administration of AAV gene transfer. The fact that more than 90% of the population has been exposed to natural AAV2 infection, and half of those infected carry NAbs in their blood, highlights the significance of this problem. The study of interaction of neutralizing antibody with AAV virion allows us to develop effective strategies to evade NAbs activity in patients with positive NAb or for re-administration.

a. Li C, Narkbunnam N, Samulski RJ, Asokan A, Hu G, Jacobson LJ, Manco-Johnson MJ, Monahan PE; Joint Outcome Study Investigators. Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. Gene Ther. 2012 Mar;19(3):288-94
b. Li C, Diprimio N, Bowles DE, Hirsch ML, Monahan PE, Asokan A, Rabinowitz J, Agbandje-McKenna M, Samulski RJ. Single amino acid modification of adeno-associated virus capsid changes transduction and humoral immune profiles. J Virol. 2012 Aug;86(15):7752-9.
c. Gurda BL, DiMattia MA, Miller EB, Bennett A, McKenna R, Weichert WS, Nelson CD, Chen WJ, Muzyczka N, Olson NH, Sinkovits RS, Chiorini JA, Zolotutkhin S, Kozyreva OG, Samulski RJ, Baker TS, Parrish CR, Agbandje-McKenna M. Capsid antibodies to different adeno-associated virus serotypes bind common regions. J Virol. 2013 Aug;87(16):9111-24.

[redacted]

4. Optimization of transgene cassette: AAV is a single stranded virus; after uncoating in the nucleus, the single stranded AAV genome must be converted into double-stranded DNA intermediate for transcription. The requirement of second strand synthesis has led to the development of a new class of AAV vectors called "self complementary" (scAAV), which induces much higher and faster transgene expression. To further optimize scAAV vector for clinical trial, we have explored different elements in transgene cassettes for controlling tissue specific transgene expression, as well as optimization of coding and uncoding sequences that impact transgene expression.

a. McCarty DM, Monahan PE, Samulski RJ. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. Gene Ther. 2001 Aug;8(16):1248-54.
b. Wu Z, Sun J, Zhang T, Yin C, Yin F, Van Dyke T, Samulski RJ, Monahan PE. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. Mol Ther. 2008 Feb;16(2):280-9.
c. Li C, Hirsch M, Carter P, Asokan A, Zhou X, Wu Z, Samulski RJ. A small regulatory element from chromosome 19 enhances liver-specific gene expression. Gene Ther. 2009 Jan;16(1):43-51.
d. Gray SJ, Foti SB, Schwartz JW, Bachaboina L, Taylor-Blake B, Coleman J, Ehlers MD, Zylka MJ, McCown TJ, Samulski RJ. Optimizing promoters for recombinant adeno-associated virus-mediated

gene expression in the peripheral and central nervous system using self-complementary vectors. Hum Gene Ther. 2011 Sep;22(9):1143-53.

5. **Broad collaboration in gene therapy community:** I have actively participated in studies in AAV biology and pre-clinical/clinical trials in the gene therapy community by collaboration or providing support, especially in three clinical trials including Duchenne's muscular dystrophy, canavan disease and hemophilia.

   a. Mendell JR, Campbell K, Rodino-Klapac L, Sahenk Z, Shilling C, Lewis S, Bowles D, Gray S, Li C, Galloway G, Malik V, Coley B, Clark KR, Li J, Xiao X, Samulski J, McPhee SW, Samulski RJ, Walker CM. Dystrophin immunity in Duchenne's muscular dystrophy. N Engl J Med. 2010 Oct 7;363(15):1429-37.
   b. Leone P, Shera D, McPhee SW, Francis JS, Kolodny EH, Bilaniuk LT, Wang DJ, Assadi M, Goldfarb O, Goldman HW, Freese A, Young D, During MJ, Samulski RJ, Janson CG. Long-term follow-up after gene therapy for canavan disease. Sci Transl Med. 2012 Dec 19;4(165):165ra163.
   c. Ishikawa K, Fish KM, Tilemann L, Rapti K, Aguero J, Santos-Gallego CG, Lee A, Karakikes I, Xie C, Akar FG, Shimada YJ, Gwathmey JK, Asokan A, McPhee S, Samulski J, Samulski RJ, Sigg DC, Weber T, Kranias EG, Hajjar RJ. Cardiac I-1c overexpression with reengineered AAV improves cardiac function in swine ischemic heart failure. Mol Ther. 2014 Dec;22(12):2038-45.
   d. Ling C, Wang Y, Lu Y, Wang L, Jayandharan GR, Aslanidi GV, Li B, Cheng B, Ma W, Lentz T, Ling C, Xiao X, Samulski RJ, Muzyczka N, Srivastava A. Enhanced transgene expression from recombinant single-stranded D-sequence-substituted adeno-associated virus vectors in human cell lines in vitro and in murine hepatocytes in vivo. J Virol. 2015 Jan 15;89(2):952-61.

Complete List of Published Work in MyBibliography:

http://www.ncbi.nlm.nih.gov/myncbi/collections/bibliography/40585640

D. Research Support

Ongoing Research Support

R01DK084033 Li/Samulski (MPI) 04/01/12-01/31/17
NIH/NIDDK
AAV Gene Therapy for AAT Deficiency

The proposed studies test several alternative solutions including development of novel AAV virions with improved liver-specific targeting.

R01 EY005951 Campochiaro P (PI), Samulski RJ(Sub PI) 05/01/2012 – 04/30/2017
NIH/National Eye Institute – Subcontract to Johns Hopkins University
Oxidative damage and cone cell death in RP

The long-term objective of this collaborative effort is to evaluate protective gene therapy for the treatment and prevention of oxidative damage and cone cell death in human RP.

1P01HL112761 Samulski, RJ (PI) 02/08/13-01/31/2018
NIH/NHLBI
NEUTRALIZING ANTIBODY & AAV FIX GENE THERAPY
Project 1-FIX Gene Therapy and Role of AAV Nab PI

This proposal will investigate the relationship of HLA class II phenotype with AAV neutralizing antibody against different serotypes, and explore the novel approach to evade neutralizing antibody activity by using Nab specific aptamers.

R01 AI072176 Hirsch/Samulski (MPI) 05/15/2013 – 04/30/2018
NIH/NIAID

Rational and Combinatorial Engineering of AAV Vectors

The primary goal is to develop more efficient safe AAV vectors that overcome rate-limiting properties associated with current AAV vector transduction and provide a more desirable delivery reagent for future clinical trials.

R01 AR 064369 Hirsch/Samulski (MPI) 09/17/2013 – 08/31/2018
NIH/NIAMS
Overcoming our clinical complications: AAV vector design for the treatment of DMD

Explore the peptides from viruses to inhibit antigen presentation from transgene minidystrophin after AAV muscular delivery in canine model.

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)*

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

PHS 398 Cover Page Supplement

5. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ● No ❍ Yes

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

Cell Line(s): Specific stem cell line cannot be referenced at this time. One from the registry will be used.

6. Inventions and Patents (For renewal applications only)

Inventions and Patents*: ❍ Yes ❍ No

If the answer is "Yes" then please answer the following:

Previously Reported*: ❍ Yes ❍ No

7. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

First Name*:

Middle Name:

Last Name*:

Suffix:

❑ Change of Grantee Institution

Name of former institution*:

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]

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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	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]

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

PHS 398 Modular Budget

Budget Period: 3
Start Date: 04/01/2018 End Date: 03/31/2019

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]

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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	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]

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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]

Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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	

Tracking Number: GRANT11947975
Funding Opportunity Number: PA-13-302. Received Date: 2015-06-25T09:02:19.000-04:00

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 the experiments about effect of empty virions and AAV2 mutants as well as proteasome inhibitors on capsid antigen presentation *in vitro* and *in vivo*.

Karen Hogan, Research Specialist, (6 CM Years 1-5) will be responsible for animal experiments including animal care and breeding.

Chen Xiaojing, Research Assistant, (9 CM Years 1-2, 8.4 CM Year 3, 7.8 CM Year 4, 6 CM Year 5) will be primarily responsible for vector clone and production. She will also perform experiments to characterize AAV mutants isolated from humanized mice.

Fringe benefits: Fringe benefits are requested in accordance with institutional guidelines for each position. Fringes for PI's and Research Tech are calculated at [redacted] for retirement and social security with Medical Insurance calculated at [redacted]. prorated to percent effort. The Post Doc receives [redacted] fringe with [redacted] for Medical Insurance.

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	

INTRODUCTION

The current proposal is a revised grant submission based on the previous submission (R01 AI117408) entitled "Enhance AAV Liver Transduction with Capsid Immune Evasion". We would like to thank the reviewers for their critiques and for awarding the proposal a favorable score **(22 percentile)**. We also appreciate their encouraging comments that characterize the submission as "*highly significant because it addresses the issue of antigen cross-presentation, thought to be a critical barrier to the use of AAV gene therapy for hemophilia B",* and *"the logical plan for inhibiting the CTL response to the capsid and the outstanding investigators deemed to be leaders in the field*". More importantly, we thank the reviewers for identifying and articulating potential weaknesses with respect to specific experiments and overall goals of the proposal that we could specifically address to improve the re-submission. We have now addressed these concerns individually below and accordingly made relevant changes to improve the application. Key points were identified, namely: **1)** the approaches are not innovative and are likely to lead to only incremental progress, **2**) the rationale for the experimental design was poorly articulated and **3**) the applicants did not adequately consider the effect of pre-existing immune responses on antigen presentation.

Point 1) We agree with the reviewers about the novelty. In the resubmission application, we have combined Aim 1 and 2 from the previous submission and provided the new Aim 3 to isolate AAV mutants with human hepatocyte tropism and the ability to avoid capsid ubiquitination using innovative approaches (the combination of directed evolution and rational design).

Point 2) We appreciate the concern from the reviewers. In the resubmission, we have provided more careful rationale for the proposal. AAV capsid antigen presentation is dose-dependent and relies on capsid degradation by the proteasome, and some AAV mutants or proteasome inhibitors are able to increase AAV transduction and block capsid antigen presentation. We hypothesize that it is feasible to develop AAV mutants with avoidance of AAV capsid ubiquitination to decrease AAV capsid antigen presentation and enhance hepatocyte transduction.

Point 3) We acknowledge the concern from the reviewers about pre-existing immunity. Although antibody response can affect antigen presentation, prior studies have demonstrated that AAV antibody blocks AAV transduction *in vitro* and *in vivo* (e.g. no AAV genomes are detected in tissues including the liver after systemic administration of AAV in mice with pre-vaccination of AAV). These observations indicate that it is impossible to study AAV capsid antigen presentation in the setting of pre-existing antibody. Different AAV serotypes and mutants have been proposed to evade AAV neutralizing antibody; however, most epitopes from AAV serotypes and mutants are conserved. This implies that capsid specific CTL response is still able to clear AAV transduced cells from other serotypes or mutants. In this proposal, we propose to study the mutants with the ability to escape ubiquitination and degradation by the proteasome; these mutant capsids should mount no or low capsid antigen on the target cell surface for CTL recognition, and therefore avoid CTL mediated elimination. These mutants will be used to evade capsid specific CTL mediated elimination regardless of pre-existing cellular immune response.

In this submission, we have made every effort to address the minor points raised by the reviewers and to bolster the application with any reviewer-recommended suggestions. We thank the reviewers again for their constructive comments and have provided a point-by-point response to their critique below.

1. "*It is not clear how the observed data, particularly those from mutants whose transduction mechanisms have not been clearly understood, can be generalized or exploited to generate novel capsids that can avoid the CTL-mediated destruction of AAV transduced cells in Aim 3*" (Reviewer 2). We overall agree with the reviewer's comment; however, this is why we propose the study to investigate the effect of different mutants on AAV capsid antigen presentation. The majority of the concerns have been addressed in the "pitfall section". **2.** "*It is not clearly stated how the ultimate goal of the project "enhance AAV liver transduction with capsid immune evasion" will be achieved using the study outcomes*" (Reviewer 2). In short, A, with respect to enhancing AAV liver transduction, we will study the effect of AAV mutants and proteasome inhibitors; B, for capsid evasion, we focus on AAV mutants with decreased ubiquitination and proteasome inhibitors which should block proteasome mediated capsid degradation. We have modified the application to emphasize their roles. **3.** "*HepG2/H2-Kb/TAP-/- xenograft model may not be the best model for this project*" (Reviewer 2). We have deleted experiments related to the HepG2/H2-Kb/TAP-/- xenograft model in our resubmission. **4.** "*Insertion of the OVA SIINFEKL peptide into the HI loop of various AAV2 and AAV8 mutants might negatively affect the ability to transduce cells in vitro and in vivo"* (Reviewer 2). We agree with the reviewer. If the transduction ability is affected from these mutants with substitution of OVA peptide, we will study capsid antigen presentation from mutants using peptide specific CTLs induced by Ad/AAV capsid immunization even though the assay sensitivity is lower than OVA approach. It completely addresses the reviewer's concern. **5.** "*Biosafety consideration in the use of AAV and adenoviral vectors is not described in the application*" (Reviewer 2). We have provided biosafety consideration.

SPECIFIC AIMS

Adeno-associated virus (AAV) vector has been successfully applied to target the liver in clinical trials with hemophilia patients[1,2]. These trials have suggested that the AAV capsid specific cytotoxic T lymphocytes (CTLs) eliminate AAV vector targeted liver cells, following AAV2 or AAV8 transduction, and result in therapeutic failure. Our studies and others have demonstrated that both classical antigen presentation and cross-presentation pathways are involved in mounting an AAV capsid specific CTL response[3-7]. In clinical trials, ion exchange chromatography has been used to purify AAV vectors. Unlike CsCl purification approach, the chromatographic method cannot currently separate genome-containing AAV capsids (full particles) from empty particles. The contamination of empty virions potentially increases the AAV capsid antigen load in transduced cells and it has been demonstrated that empty virion contamination in vector preparations induces liver damage, which potentially enhances capsid antigen presentation from full virion transduction[8,9]. Although we have observed a lower capsid antigen presentation from AAV empty virion infection compared to full particles *in vitro*[10], our *in vivo* preliminary result demonstrated that AAV empty capsids still elicit capsid antigen presentation. In this proposal we will investigate the kinetics of capsid antigen presentation from empty virions and the effect of empty particles on antigen presentation from full particle transduction (**Aim 1a and 1b**). We have demonstrated that AAV capsid cross-presentation is dependent on virion endosomal escape and proteasome-mediated capsid degradation in AAV transduced cells *in vitro*[10]. However, the mechanistic insights of the work were largely elucidated *in vitro*, and over a limited time period (24 to 48 hrs), and therefore it remains unclear which aspects of our discoveries translate *in vivo* regarding long-term antigen processing and presentation. The mechanism of capsid antigen presentation from empty virions and full particles *in vivo* will be performed using mouse models deficient in the genes responsible for classical class-I antigen presentation (TAP -/- mice) or classical class-II antigen presentation (Cat S -/- mice) (**Aim 1c**).

Our data have shown that capsid antigen presentation is dose-dependent and requires capsid ubiquitination for proteasome mediated degradation[11,12]. To decrease antigen presentation on AAV transduced cells for avoiding capsid specific CTL-mediated elimination, it has been proposed to modify the AAV capsid surface in order to enhance AAV transduction while lowering the effective dose, or to escape capsid ubiquitination[13,14]. It is unclear whether the enhancement of liver transduction with AAV mutants or a decrease in capsid ubiquitination influences capsid antigen presentation *in vivo* (**Aim 2a**). Proteasome inhibitors have been shown to enhance AAV transduction and inhibit antigen presentation[12,15]. However, our further *in vitro* study demonstrated varying effects of the proteasome inhibitor on capsid antigen cross-presentation in a dose related manner. A high dose of the proteasome inhibitor bortezomib blocks capsid antigen presentation, while a lower dose of bortezomib increases capsid antigen presentation without enhanced transduction. We hypothesize that proteasome inhibitor treatment will change the profile of AAV antigen presentation *in vivo* and the combination of AAV mutants and proteasome inhibitors will further increase AAV transduction while inhibiting capsid antigen presentation (**Aim 2b and 2c**).

It is well-known that the transduction of AAV vectors in mouse models does not always translate into the human host. To address this, a mouse model xenografted with human hepatocytes has been used to develop AAV vectors for human liver targeting gene therapy[16]. In this proposal we will explore the directed evolution approach combined with a rational design strategy to isolate AAV vectors with human hepatocyte specific tropism and the ability to evade a capsid specific CTL response in humanized mice (**Aim 3**). Elucidation of AAV empty capsid antigen presentation *in vivo* and the development of an AAV vector with enhanced *human* liver transduction and CTL immune-evasion, will allow us to design safer and more effective strategies that address the current clinical complications for human liver gene therapy using AAV. To address these issues, we will execute the following specific aims:

1. **Study the effect of AAV empty particles on AAV capsid antigen cross-presentation *in vivo*.**
   - **a**. The kinetics and dose-response of AAV capsid antigen presentation from AAV empty virions *in vivo*.
   - **b.** The effect of empty particles on capsid antigen presentation from full-particle AAV transduction *in vivo*.
   - **c**. AAV capsid antigen presentation in TAP-/- and in Cat S-/- mice.
2. **Investigate AAV capsid antigen presentation following administration of AAV mutants and/or proteasome inhibitors for enhanced liver transduction *in vivo*.**
   - **a**. Capsid antigen presentation from AAV mutants with enhanced liver transduction in mice.
   - **b**. The effect of proteasome inhibitors (high vs low dose) on natural AAV capsid antigen presentation *in vivo*.
   - **c**. The effect of a combination of AAV mutants with proteasome inhibitors on antigen presentation *in vivo*.
3. **Isolate AAV chimeric capsids with human hepatocyte tropism and the capacity for CTL evasion.**
   - **a**. Verify AAV human liver transduction efficiency in xenograft mice.
   - **b**. Characterization of AAV mutants recovered from human liver xenografted mice.
   - **c**. Investigation of capsid CTL evasion from humanized AAV mutants.

RESEARCH STRATEGY

A. SIGNIFICANCE

Adeno-associated virus (AAV) vectors have been successfully used to transduce hepatocytes in Phase I clinical trials in patients with hemophilia B[1,2]. However, clinical results have suggested that capsid specific cytotoxic T lymphocytes (CTLs) eliminated AAV transduced hepatocytes thus resulting in the therapeutic failure[1,2]. These observations pose an outstanding concern regarding capsid antigen presentation in AAV transduced cells which are recognized and eliminated by capsid specific CTLs in clinical trials. Our prior studies have supported classical class I antigen presentation and cross-presentation of AAV capsid epitopes[3]. Most peptides to be loaded on MHC class I molecules are generated by proteasome degradation of newly synthesized ubiquitinated proteins. Exogenous protein can also be presented on MHC class I molecules through cross-presentation. Two main intracellular pathways for cross-presentation have been described: endocytic (TAP, proteasome-independent) and cytosolic (TAP, proteasome-dependent) MHC class I peptide loading[17]. In the TAP-independent pathway, exogenous antigens that have been endocytosed are degraded by proteases, and the resulting peptides bind to MHC class I molecules in late endosomes and lysosomes. In the TAP dependent pathway, the internalized exogenous antigens are transferred from the endocytic pathway to the cytosol and degraded by proteasomes; the resulting peptides are then transported to the ER via TAP. We have applied pharmacological agents and demonstrated that the classic MHC class I antigen presentation pathway plays a major role in AAV capsid antigen cross-presentation in AAV transduced cells *in vitro*[12]. However, the mechanism of capsid antigen cross-presentation from AAV transduced cells *in vivo* is perhaps different from that *in vitro* due to the far more complex environment.

AAV vectors purified from cesium chloride (CsCl) density gradients have been applied in clinical trials; however, this purification approach is not scalable. Recently, ion-exchange chromatography has been studied to purify AAV vectors[18-21]. Unlike the CsCl approach, the chromatographic method cannot separate genome-containing particles of AAV vectors (full particles) from empty particles as it relies on the charge of the capsid surface. The contamination of vector preparations by empty particles inhibits transduction of genome containing AAV vectors and potentially increases the virus capsid antigen load in transduced cells[21]. Although empty AAV particles contain the identical protein components required for trafficking as full particles, our preliminary results demonstrated that capsid antigen presentation was significantly reduced in AAV transduced cells infected with AAV2 empty virions compared to full particles *in vitro*[12]. This phenomenon may be interpreted as insufficient escape of these empty virions from the endosome[22]. However, our *in vivo* results demonstrated that capsid antigen presentation was induced from AAV empty virions. This finding is inconsistent with our *in vitro* observations and supports the likely scenario that a much more complex mechanism of antigen presentation occurs *in vivo*. Recently, empty AAV capsids were proposed to function as decoys in the clinical trials to allow AAV full particles to escape neutralizing antibodies[23], but liver damage was observed following systemic administration of AAV preparations contaminated with empty particles[9], which alludes to the possibility that empty particles enhance capsid antigen presentation. Elucidation of the efficiency of capsid antigen presentation from empty virions, and the effect of empty virions on capsid antigen presentation of full AAV capsids following *in vivo* administration, will address the concern of whether contamination of empty AAV particles is a potential risk that increases antigen presentation in AAV transduced liver cells *in vivo* (**Aim 1**).

Our previous study has demonstrated that capsid antigen presentation in AAV transduced target cells is dose-dependent[11]. To avoid capsid specific CTL mediated clearance of AAV transduced liver cells, a lower dose of AAV vector has been proposed to reduce the capsid antigen load in AAV transduced cells. To obtain similar transduction efficiencies with low vector doses, several approaches have been explored including transgene optimization[24,25], capsid alterations[26], and drug treatments to enhance transduction[27-35]. It has been demonstrated that modification of the AAV capsid can enhance liver transduction and modulate the immunogenicity of AAV capsid antigens[13,14,36-40]. Our recent study demonstrated that engraftment of the AAV9 galactose receptor binding residues into the AAV2 virion (dual receptors) induced stronger liver transgene expression[13]. After transduction, the AAV capsid is ubiquitinated for degradation and therefore, inhibition of capsid ubiquitination may also enhance AAV transduction. In fact, it has been demonstrated that modification of capsid tyrosine residues helps AAV vectors escape ubiquitination and subsequent proteasome-mediated degradation, thus enhancing transduction[14]. The administration of these tyrosine capsid mutants results in less capsid specific CTL mediated elimination of AAV transduced liver cells compared to the equivalent wild type AAV capsid, perhaps due to faster virus trafficking to the nucleus of hepatocytes, and therefore less capsid antigen presentation in transduced cells[41]. However, it is unclear whether capsid modifications that enhance transduction affect capsid antigen presentation (**Aim 2a**). A number of pharmacological agents have also been used for enhancing AAV transduction, including proteasome inhibitors, DNA synthesis inhibitors and topoisomerase inhibitors[27,28,30-34]. In particular, AAV transduction with proteasome inhibitors has been tested in large animals[32]. In spite of the enhanced effect on AAV transduction, our study also demonstrated inhibition of

capsid antigen presentation using a high dose of the proteasome inhibitor bortezomib[12], while increased antigen presentation was observed at a lower dose *in vitro*. It is still unclear whether proteasome inhibitor treatment at different doses or the combination of AAV mutants with a proteasome inhibitor affects capsid antigen presentation *in vivo* (**Aim 2b and c**). Investigating the effects of enhanced liver transduction or avoidance of ubiquitination of AAV capsid mutants and proteasome inhibitors on capsid-based antigen presentation will allow the community to understand the effective parameters for designing safer AAV vectors with enhanced liver transduction and long-term clinical efficacy.

It has been well known that the results from mouse studies rarely translate to larger animals and humans in hemophilia AAV gene therapy due to species-dependent hepatocyte tropism. To develop liver targeting AAV virus for human clinical trials, more authentic animal models are greatly needed. Recent studies have demonstrated that AAV serotypes 2 and 8 could not transduce human hepatocytes efficiently in human liver xenografted mice[42,1,2]. This finding is especially relevant because AAV8 transduces mouse liver quite readily and with much greater efficiency than AAV2. However, the substitution of an AAV8 capsid in place of AAV2 for factor IX delivery in human clinical trials did not lead to a similar degree of increased factor IX expression from human liver, as would have been predicted by the expression advantage seen in mouse liver *in vivo* [1,2,43,44]. Here, we propose to use directed evolution in combination with a rationally designed approach (mutations to avoid capsid ubiquitination) for selection of AAV mutants with enhanced human liver tropism and CTL evasion. Such enhanced reagents can be immediately transferred to clinical applications (**Aim 3**). The mutants isolated from humanized mice can also be applied in the clinical trials with pre-existing immune response but without neutralizing antibody (NAb). NAb blocks AAV transduction, so it is impossible to study capsid antigen presentation in the presence of pre-existing antibody. Different AAV serotypes and mutants have been proposed to evade AAV Nabs; however, most epitopes from AAV serotypes and mutants are conserved. This indicates that the capsid specific CTL response is still able to clear AAV transduced cells from other serotypes or mutants. In this proposal, we propose to develop mutants with the ability to escape ubiquitination and degradation by the proteasome; these mutant capsids will mount no or low capsid antigen on target cell surface for CTL recognition, and therefore avoid CTL mediated elimination. These mutants can be used to evade capsid specific CTL response regardless of pre-existing cellular immune response.

Our labs have recently completed numerous studies that demonstrate our capability for conducting these investigations, including the following observations: **1**) AAV capsid antigen presentation is dependent on proteasome-mediated capsid degradation *in vitro*[12], **2**) AAV capsid antigen cross-presentation is dose-dependent *in vivo*[11], **3**) although no capsid antigen presentation from empty virions was displayed *in vitro*, capsid antigen cross-presentation was observed in mice after systemic administration of AAV empty vectors (**Fig. 1**), **4**) modification of AAV virions increases liver transduction (**Fig. 5** and **6**), **5**) proteasome inhibitors enhance AAV liver transduction *in vivo* **(Fig. 7**), **6**) AAV mutants have been isolated with specific cell tropism or the ability to cross blood-brain barrier using directed evolution approach[45,46], and **7**) the humanized mouse has been successfully established with human hepatocytes. All of these preliminary results lay the groundwork to develop novel AAV mutants with human hepatocyte tropism and evasion of capsid specific CTL response. The long-term goal is to apply this information to design safer and more effective strategies for overcoming cell-based immunity to AAV vectors, thereby generating a long-lasting therapeutic product.

B. INNOVATION

AAV vector gene therapy is yielding clinical data supporting the hope for the eventual treatment of many diseases. However, capsid specific CTL responses have been documented in patients with hemophilia B in several Phase I clinical trials. Over 90% of the human population is infected by AAV, and some of them have memory capsid CTLs in circulation[47,48]. AAV empty virions have been used in clinical trials as decoys for AAV neutralizing antibodies; however, one of the clinical concerns is that the contamination of empty particles in AAV vector preparation may potentially increase the AAV capsid antigen load. The detailed elucidation of AAV capsid antigen presentation from empty particles and the effect of empty particles on full particle capsid antigen presentation is innovative and timely for the guidance of future clinical trials. To decrease capsid specific CTL mediated killing of AAV transduced target cells, it has been proposed to use lower doses of more potent AAV vectors, due to the dose-dependent nature of capsid antigen presentation[11]. To achieve this goal, capsid mutants and proteasome inhibitors have been explored to enhance AAV transduction and also to interfere with capsid antigen presentation. Elucidation of the effect of AAV mutants or proteasome inhibitors that enhance AAV transduction and decrease AAV capsid antigen presentation is both important and innovative. To explore effective approaches of successfully evading capsid CTL response for human clinical trial, the development of AAV mutants by rational design and directed evolution in the human hepatocyte xenografted mouse is novel and straightforward.

C. APPROACH

The primary objectives of the proposal are the following: (i) to study the antigen presentation from AAV empty particles *in vivo,* (ii) to investigate the effect of AAV mutants and proteasome inhibitors that enhance liver

transduction on capsid antigen cross presentation, (iii) to isolate AAV mutants with human hepatocyte tropism and the capacity to avoid capsid antigen presentation.

C1. Study the effect of AAV empty particles on AAV capsid antigen cross-presentation *in vivo*.

***Rationale*.** Ion-exchange chromatography has been used to purify AAV vectors at clinical quantities; however, this approach is unable to eliminate empty particle contamination as it relies on capsid charge and not overall particle density[49]. The empty particles inhibit AAV transduction and potentially increase the capsid antigen load in transduced cells[21,23]. A recent study demonstrates that the empty virions in AAV vector preparations induce liver damage, which may up-regulate antigen presentation following AAV transduction[9]. These observations pose an outstanding concern in the clinical trials using AAV vector preparations contaminated with empty particles. Our previous study in the HepG2 cell line demonstrated that far less antigen presentation was induced from empty virions compared to full particles [50]. However, the antigen presentation mechanism from AAV empty capsid *in vivo* is likely much more complicated than that in cell lines considering the shorter experimental time period *in vitro* and more environmental factors influencing antigen presentation *in vivo*. Thus, the studies from cell lines *in vitro* may not represent all the alternative processes of capsid antigen that can take place over extended periods of time *in vivo*. A recent study demonstrated that empty AAV8 virions do not induce strong antigen presentation in mice when compared to full particles[51]; in this study the marker antigen, OVA peptide SIINFEKL, was located at the N-terminal of VP2. These viruses were assembled from two AAV helper plasmids: one to encode AAV VP1 and VP3, the other one for VP2 with the OVA peptide insertion at N-terminal. The virus preparation may be composed of VP1/VP2/VP3 (intact virus), VP2/VP3, or VP3 only. The latter two viruses do not have infectivity since they cannot escape from endosome due to no PLA2 domain. Additionally it is unknown whether VP1 and VP2 are cleaved in the endosome after their exposure on the virion surface. In our preliminary studies, using AAV virions with OVA peptide integration in VP3, we found that AAV2-OVA empty virions induced comparable capsid antigen presentation to full particles after systemic administration (**Fig. 1**), which is inconsistent to our *in vitro* observation that much lower capsid antigen presentation is mounted from empty virions compared to full particles.

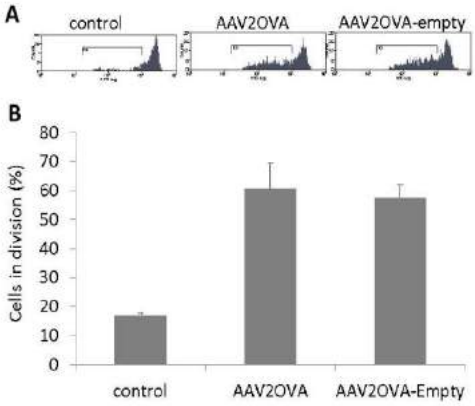

Fig. 1. Capsid antigen presentation after systemic administration of AAV2 empty virions. A. Representative flow cytometric histograms. **B.** Average T cell proliferation and standard deviation for four mice.

It has been shown that transgene expression gradually increases following AAV2 administration in mice, peaks at week 6, then remains stable for long-term[24]. To address whether the kinetics of antigen presentation of capsid epitopes *in vivo* corresponds with the dynamics of transgene expression, we injected $1x10^{11}$ particles of AAV2-OVA/AAT intravenously (IV). At various time points post-injection, splenic OT-1 T cells labeled with CFSE dye were administered to the treated mice. Ten days post-transfer, OT-1 T cell division was measured by flow cytometry. As shown in **Fig. 2**, over days 3-12 and days 21-30, OT-1 T cell division was significantly increased in AAV2-OVA/AAT vector-treated animals versus control recipients. However, no difference was observed for OT-1 cell proliferation between AAV2-OVA and the control groups over days 41-50 and days 61-70 ($p>0.05$). This result indicates that antigen cross-presentation from the AAV2 capsid occurs early after AAV2 systemic administration[11]. Similar kinetics and efficiency of antigen presentation was also observed from an AAV8-OVA vector after systemic administration.

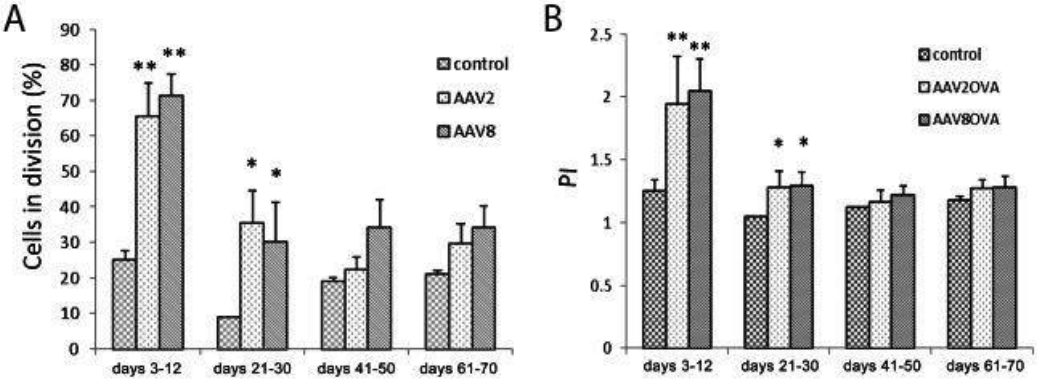

Fig. 2. The kinetics of capsid antigen presentation after AAV8OVA transduction in mice. Particles of AAVOVA/AAT virus (1×10^{11}) were injected intravenously into C57BL/6 mice, and at the indicated time points, 5×10^{6} CFSE-labeled OT-1 T cells were transferred. Ten days after transfer, proliferation 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.

To investigate the dose response of capsid antigen presentation *in vivo,* varying particle numbers of the AAV2-OVA vector were injected into mice to examine OT-1 T cell stimulation. When compared to the no treatment control, a dose of $5x10^{10}$ AAV2-OVA particles induced the proliferation of OT-1 T cells (**Fig. 3**). Importantly, there was no proliferation of spleen cells in groups with doses of AAV2-OVA less than or equal to $1x10^{10}$ particles. Together, these results demonstrate that efficient T cell stimulation occurs only at high doses of AAV vectors[11]. In this specific aim, we will further investigate the kinetics and dose-response of AAV (2 and 8) empty capsid antigen cross-presentation following systemic application of AAV empty virions in a mouse model (**C1.1**). Due to the mixture of full particles and empty virions in the vector preparations, we will also study the effect of their interactions on capsid antigen presentation (**C1.2**).

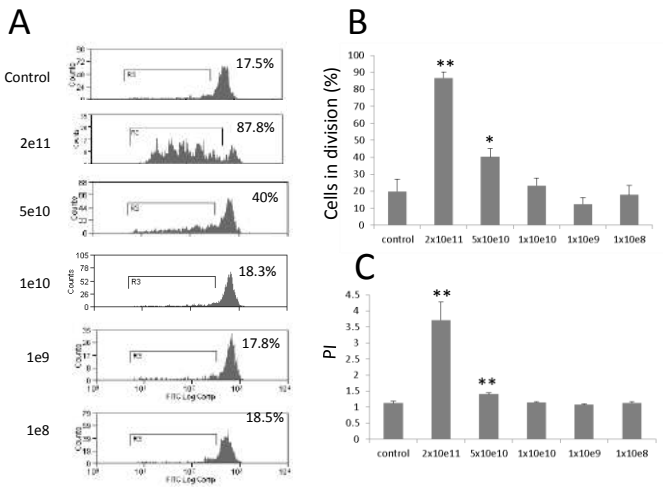

FIG. 3. Capsid antigen presentation after AAVOVA transduction is dose responsive *in vivo*. Various doses of AAV2OVA/AAT vector were injected intravenously into C57BL/6 mice and 3 days later, CFSE-labeled OT-1 T cells were transferred. On day 10 after transfer, OT-1 T cell proliferation in the spleen was assessed via flow cytometry. **(A)** Representative flow cytometric histograms. **(B)** Average T cell proliferation and standard deviation of four mice. **(C)** Average proliferation index (PI) and standard deviation. ***p*<0.01, **p*<0.05 compared with control mice without AAV treatment.

Effective AAV transduction is composed of several steps, including vector binding to the cell surface via a receptor-mediated mechanism followed by endocytosis through clathrin-coated pits. The particles then travel from early endosomes to late endosomes followed by escape to the lysosome or peri-nuclear localization. Once inside the nucleus, viral uncoating and the initiation of second-strand synthesis is proposed to occur, followed by transgene expression[52-55]. To address the mechanism of capsid antigen presentation after AAV transduction, we incubated HepG2/H2-kb cells with different chemicals at non-toxic concentrations for 1 hr before AAV2-OVA virions were added. After 24 hr, the spleen cells from OT-1 mice were co-cultured with AAV2-OVA transduced HepG2/H2-kb cells overnight. Then, activated OT-1 spleen cells were detected by flow cytometry for CD8 and CD69 staining, an early marker of T cell early activation. Consistent to prior work, both ammonium chloride and chloroquine (inhibitiors of acidification in the endosomes) inhibited AAV2 transduction[52]. Compared to antigen presentation from AAV2-OVA transduction without treatment, over 90% inhibition of antigen presentation was observed in groups treated with ammonium chloride and chloroquine (**Fig. 4A**). To analyze the participation of proteases in the processing of AAV capsids after transduction, two different protease inhibitors, leupeptin H and pepstatin A, were tested. Incubation of HepG2/H2-kb cells with the AAV2-OVA vector in the presence of leupeptin or pepstatin partially blocked OVA presentation (**Fig. 4A**), suggesting that OVA antigen processing from the AAV2-OVA virion required the participation of some proteases[12]. Two proteasome inhibitors, MG132 and Bortezomib, as with many previous studies, enhanced AAV2 transduction. However, OVA antigen presentation was completely diminished in the presence of these inhibitors (**Fig. 4A**). This result indicates that AAV2-OVA virions, or derived peptides, translocate to the cytosol of HepG2/H2-kb cells and are degraded by the proteasomes[12]. We also demonstrated that Brefeldin A (BFA) significantly suppressed AAV2 transduction, and inhibited capsid antigen presentation (**Fig. 4A**). This strongly suggests that AAV2-OVA derived peptides are transported to cell surface through the Golgi complex after translocation to ER[12]. Collectively from the above study with pharmacological agents, it is concluded that

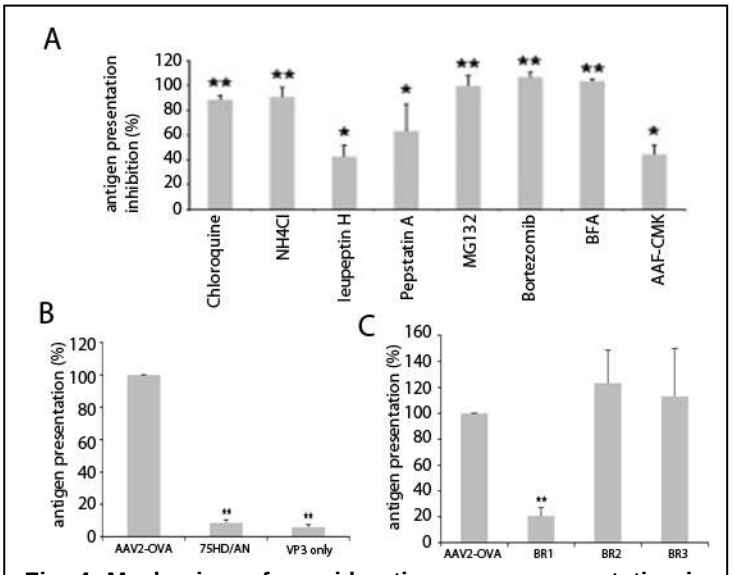

Fig. 4. Mechanism of capsid antigen cross-presentation in HepG2/H-2Kb cells. (**A**) Effect of inhibitors on antigen presentation. (**B**) Antigen presentation by transduced AAV2-OVA, 75HD/AN, and VP3-only virus. (**C**) Effect of AAV NLSs on capsid antigen presentation. **P* < 0.05 and ***P* < 0.01 when compared with AAV2-OVA.

capsid antigen presentation is dependent on proteasome-mediated degradation of the AAV capsid, although the acidic environment in endosomes is required for effective AAV vector escape.

The N termini of VP1 and VP2 in the AAV capsid are necessary for AAV vector subcellular trafficking. Two functional motifs have been identified: a phospholipase dominant (PLA_2) domain and putative nuclear localization signals (NLSs) BR1, BR2, and BR3[22,56-59]. The AAV PLA_2 region plays a role in disrupting the membrane of the vesicular compartment and allows the virus to escape into the cytosol from the endosome/lysosomes. It has been demonstrated that a mutant with a two residue substitution of PLA_2 (AAV2/75HD/AN) is unaffected in regards to cell surface binding and virus endocytosis, but greatly attenuates PLA_2 activity thereby decreasing vector transduction. To further confirm the role of AAV endosome/lysosome escape in capsid antigen presentation, we incubated AAV2-OVA/75HD/AN vectors with HepG2/H2-kb cells for capsid antigen presentation analysis. As shown in **Fig. 4B**, compared to the AAV2-OVA vector, significant reduction of antigen presentation was observed with the AAV2-OVA/75HD/AN mutant vector (**Fig. 4B**). Our previous study demonstrated that VP3 only virus, which does not contain the PLA_2 domain on its N-terminus, is not able to enter the nucleus and fails to transduce AAV2 permissive cells[30]. Similar to the AAV2-OVA/75HD/AN mutant, infection with AAV2-OVA VP3 only virus led to a reduction of antigen presentation in HepG2/H2-kb cells (**Fig. 4B**)[12]. We have demonstrated that mutations of three potential NLSs (BR1, BR2 and BR3) dramatically decreased transduction, due to defective nuclear entry of the mutant particles. To understand the relationship of AAV capsid antigen presentation in relation to the cytoplasmic localization of uncoated capsids, we infected HepG2/H2-kb cells with these mutants with OVA peptide substitution and examined antigen presentation on the cell surface. The BR1 mutant vector infection led to significantly decreased antigen presentation (**Fig. 4C**). Interestingly, both BR2 and BR3 mutants induced similar antigen presentation when compared to the AAV2-OVA vector (**Fig. 4C**). These results suggest that uncoating in the nucleus is not required for AAV capsid antigen presentation after infection[12]. Due to toxicity of chemical agents and inconsistency of antigen presentation from empty virion infection *in vitro* and in mice, it is impossible to study the mechanism of capsid antigen cross-antigen presentation *in vivo* using these chemicals and mutants. Therefore, we will study the mechanism of antigen presentation from empty capsids using our animal models deficient in genes responsible for classic class-I antigen presentation (TAP -/- mice) or class-II antigen presentation (Cat S -/- mice) in **Aim1.3**.

C1.1. The kinetics and dose-response of AAV capsid antigen presentation from AAV empty virions *in vivo.* AAV empty and full particles from AAV2-OVA and AAV8-OVA will be administered to mice at different doses. At different time points post-injection, CFSE-labeled spleen cells from OT-1 mice will be transfused into treated mice. The proliferation of OT-1 CD8 cells will be analyzed as a measure of OVA epitope presentation.

Empty AAV production. AAV empty virions are produced by transfection of 293 cells with the AAV-OVA helper and Ad helper plasmids without AAV transgene cassette plasmid, and then purified by CsCl gradient centrifugation. The titer of the produced empty particles will be determined using a Western blot with antibodies specifically detecting intact virions (A20 and ADK8 antibodies for AAV2 and AAV8, respectively).

Animal experiments for kinetics of antigen presentation from AAV empty capsids. Three groups of experiments will be designed: AAV empty capsids, AAV full particles and vehicle -treatment (PBS). After AAV administration ($1x10^{11}$ particles) to C57BL mice via tail vein injection, at different time points (days 3, 21, 41 and 61 post AAV administration), $5x10^6$ CFSE-labeled OT-1 spleen cells will be transfused into these treated mice via IV administration. Ten days after the OT-1 cell infusion, mice will be sacrificed and the proliferation of OT-1 CD8 cells will be analyzed. *Animal experiments for dose-response of AAV capsid antigen presentation from AAV empty capsids.* Escalating doses of AAV particles (10^8, 10^9, 10^{10}, 10^{11} and 10^{12}) will be injected into C57BL mice via tail vein. Three days later, CFSE-labeled OT-1 cells will be infused for an OT-1 CD8 proliferation assay[11]. Three groups of mice will be assigned: AAV empty, AAV full particle and vehicle treatment. *Statistics analysis:* Data are expressed as means ± standard error. A Student T-test (StatView, SAS institute, NC) will be used to determine significant differences between the different treatment groups.

C1.2. The effect of empty particles on AAV capsid antigen cross presentation from full particle AAV transduction *in vivo*. First, we will study the effect of AAV empty capsids on transgene production from AAV full virion transduction *in vivo* with different ratios of empty to full particles. Then, the effect of empty capsids on capsid antigen presentation from AAV full capsids will be explored. Also we will study the effect of full particles on capsid antigen presentation from empty particles *in vivo*.

Animal experiment for transgene production. $1x10^{11}$ particles of AAV/AAT spiked with different doses of AAV empty capsids (0, $1x10^{10}$, $3x10^{10}$, $1x10^{11}$, $3x10^{11}$, $1x10^{12}$) will be administered to C57BL mice via tail vein injection. At weeks 1, 2, 4, 6 post-injection, the AAT concentration in the blood will be measured. *Animal experiment for capsid antigen presentation.* To determine the effect of empty capsids on capsid antigen presentation from full virions, $1x10^{11}$ particles of AAV-OVA/AAT (2 and 8) spiked with different dose of AAV or AAV-OVA empty particles (0, $1x10^{10}$, $3x10^{10}$,$1x10^{11}$, $3x10^{11}$, $1x10^{12}$) will be administered into C57BL mice via tail vein injection. At 3 days post-injection, CFSE labeled OT-1 spleen cells will be transfused into mice for an

in vivo OT-1 CD8 cell proliferative analysis. To examine the effect of full particles on capsid antigen presentation from empty virions, $1x10^{11}$ particles of AAV-OVA empty virions spiked with different dose of AAV/AAT or AAV-OVA/AAT viruses (0, $1x10^{10}$, $3x10^{10}$,$1x10^{11}$, $3x10^{11}$, $1x10^{12}$) will be administered to C57BL mice via tail vein injection for a capsid specific CTL proliferation assay as described above.

C1.3. The mechanism of empty capsid antigen presentation *in vivo*. Transporter associated with Antigen Processing (TAP) is a member of the ATP-binding-cassette transporter family. It delivers cytosolic peptides into the ER for binding to nascent MHC class I molecules. Cathepsins are proteases located in the endosomes or lysosomes. Cathepsin S (Cat S) may participate in the degradation of exogenous antigenic proteins to peptides for presentation on MHC molecules. In this sub-Aim, we will use mice with TAP or Cat S deficiency to determine which pathway (endocytic or cytosolic) is involved in capsid antigen presentation in AAV transduced liver cells from full particles or empty virions *in vivo*. TAP-/- mice and Cat S -/- mice, as well as wild type C57BL controls, will receive AAV-OVA vector via tail vein injection. Transgene production will be measured using an ELISA for AAT. Antigen presentation following AAV-OVA administration will be analyzed via spleen T cell proliferation from OT-1 mice.

Transgene expression analysis. $1x10^{11}$ particles of AAV/AAT will be injected into mice (TAP-/-, Cat S-/- and C57BL) via the tail vein. AAT levels in blood will be determined by ELISA[3]. *In vivo T cell proliferation assay.* $1x10^{11}$ particles of AAV-OVA vectors will be injected into mice (TAP-/-, Cat S-/- and C57BL) via the tail vein. Three days later, $5x10^6$ CFSE labeled T cells from the spleen of OT-1 mice will be transferred via IV injection. Ten days later, T cells from the spleen and the liver are harvested and stained with antibodies specific for CD8. The frequency of proliferating T cells will be determined[12].

Anticipated results, potential pitfalls and alternative approaches: We expect that capsid antigen presentation from empty virions is dose-dependent and occurs early, similar to full particles of AAV *in vivo*. It is anticipated that antigen presentation from empty particles is relatively long after administration due to impaired nuclear trafficking compared to full virions. High levels of empty particle contamination are expected to inhibit AAV transgene production from the co-administered full particles. It is likely that empty particles will enhance antigen presentation from full particles or vice versa. Our preliminary data demonstrated that empty virions are deficient in endosomal escape and travel to the nucleus in cultured cells [30]. Therefore, it is possible that both endocytic and cytosolic MHC class I antigen presentation pathways are involved in capsid antigen presentation from empty AAV infection *in vivo*, which suggests that comparable or lower capsid antigen presentation from empty virions will be observed when compared to full particles in TAP -/- mice. If so, we will perform experiments to track empty particle trafficking in the liver using fluorescence Cy5 labeled virions to explore the difference of behavior between full and empty virions[60,61]. Although chemical inhibition of proteases can inhibit AAV capsid antigen presentation, the capsid antigen presentation from AAV transduction may not be impacted from the absence of the single protease, cathepsin S, in deficient mice. It is possible that effective AAV capsid antigen presentation is induced in TAP-/- mice since a TAP independent and proteasome dependent pathway for cross presentation has been identified[62]. If this is the case, we will study whether proteasome-mediated degradation of AAV virions plays a major role in capsid cross-presentation after AAV administration *in vivo* using proteasome inhibitors such as bortezomib or carflizomib. Considering the toxicity of proteasome inhibitors, the drugs' short half-life in mice (rapidly cleared following intravenous administration with the peak at about 30 minutes)[63,64], and the potential enhancement of antigen presentation observed when using low doses of such drugs (see **Fig. 8**), we will use the highest non-lethal dose of proteasome inhibitor with repeated drug delivery (three days) and analyze the antigen presentation over a very short time period after capsid specific CD8 cell infusion. One other concern is that TAP-/- mice may have lower overall MHC class expression; however, it has been shown that strong antigen presentation from endogenous or exogenous protein is nonetheless observed in these mice[65]. In general, the approaches proposed in this aim are straightforward and we have all the required reagents and extensive experience elucidating the CTL response to AAV capsids. As we perform these techniques on a regular basis, we don't foresee any problems in carrying out these experiments.

C2. Investigate effect of AAV mutants and proteasome inhibitors on AAV capsid antigen presentation.

***Rationale*.** Our preliminary data indicate that AAV capsid antigen presentation is dose-dependent, and that a lower dose of AAV vectors reduces the overall antigen presentation (**Fig. 3**). Therefore, it is reasonable to explore effective strategies to achieve the necessary therapeutic levels while using lower vector doses. To achieve this goal, several approaches have been exploited to enhance AAV liver transduction including utilization of different serotypes, genetic modification of AAV capsids, and optimization of AAV vector cassettes, as well as the application of chemical reagents. However, it is unclear how these strategies (especially for AAV mutants and pharmacological agents) impact AAV capsid antigen cross-presentation in target cells while having enhanced liver transduction.

AAV viruses exploit heparan sulfate (HS), galactose (Gal), or sialic acids (Sia) as primary receptors for cell surface binding[66,67]. Different AAV strains also require subsequent interaction with co-receptors for cellular

uptake[66,67]. Recently, key amino acid residues involved in Gal recognition by AAV9 capsids were identified[68]. Modification of receptor binding sites on the AAV capsid can either change transgene profile or transduction efficiency. We have pioneered rationale design studies of AAV capsids, for instance mutating the critical residues for primary receptor binding site between different serotypes and demonstrating that the AAV2/AAV8 chimera AAV2i8 displayed an altered transduction profile. AAV2i8 selectively transduces cardiac and whole-body skeletal muscles with high efficiency and loses liver tropism[38]. Further studies integrating AAV9 primary receptor Gal binding residues into the AAV2 capsid (AAV2G9) found that AAV2G9 has dual receptor function and exploits Gal and heparan sulfate receptors for infection. Of particular interest, AAV2G9 retains a similar tropism to AAV2 but confers more rapid onset and higher liver transgene expression in mice. Similarly, engraftment of the Gal footprint onto the AAV2i8 (AAV2i8G9) also induced higher transduction in muscles and liver, comparable with AAV9[13] (**Fig. 5**). In addition, we have demonstrated that the modifications at the 265 residue of the AAV2 capsid change AAV2 tissue tropism and the immune profile[69]. Insertion of an aspartic acid at the 265 residue of the AAV2 capsid (AAV2D) induced much higher muscle transduction than AAV2[69]. Similarly, systemic administration of AAV2D also induced higher liver transduction than AAV2 (**Fig. 6**). It is well known that residue 585 Arg contributes to the AAV2 heparin binding capacity and that mutation of the AAV2 heparin binding site (AAV2/585E) ablates AAV2 liver tropism[36]. However, insertion of Asp at residue 265 of AAV2/585E capsid increased the liver tropism to a similar transduction efficiency as observed using AAV8 (**Fig. 6**). These mutants (AAV2G9, AAV2D and AAV2D/585E) can be used at lower doses to achieve similar transgene gene expression in transduced liver to AAV2.

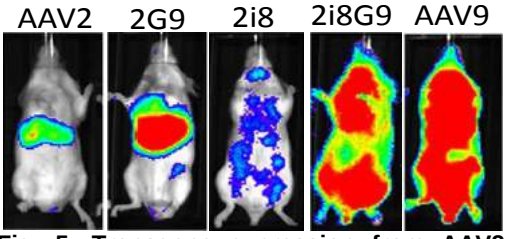

Fig. 5. Transgene expression from AAV2 mutants with modification of primary receptors. BALB/c mice were administered AAV vectors at a dose of 1 × 10^{11} particles via the tail vein, and bioluminescent images were collected at day 18 post-injection.

In addition to investigating AAV mutants with enhanced liver transduction, the AAV virion surface has also been modified to avoid ubiquitination for capsid degradation[14,39,40]. Ubiquitination is a post-translational modification of a substrate protein that can direct degradation via the proteasome. The process of ubiquitination initiates with ubiquitin binding to lysine residues on the protein substrate or to the amino group of the protein's N-terminus[70,71]. It has been shown that the mutants with surface-exposed tyrosine altered to phenylalanine reduce AAV capsid ubiquitination by elimination of potential capsid phosphorylation. Additionally, the triple mutant (AAV2 Y-F) with modification of three tyrosine residues (Y730+500+444F) induces 3 fold higher liver transduction[14]. However, decreased transgene expression with the triple mutant was still observed over time in the presence of capsid specific CTLs, which indicates that mutant capsid antigens are still able to be displayed on the surface of transduced liver cells for capsid specific CTL recognition, albeit at a low efficiency of presentation[41]. To reduce AAV capsid ubiquitination, some other studies have been performed following mutation of the lysine residues on the AAV capsid[39,40]. The mutants AAV2 K532R (lysine at residue 532 of AAV2 capsid was mutated to arginine) and AAV8 K137R demonstrated lower ubiquitination than AAV2 and AAV8[39,40], respectively, and both AAV mutants showed higher liver transduction in mice although a similar liver transduction efficiency from AAV8 K137R and AAV8 was observed from several groups including ours[72]. In spite of the decreased capsid ubiquitination for mutants with Y-F or K-R, further detailed studies are definitely needed to confirm the efficiency of antigen presentation from these mutants *in vivo*. In this study, we hypothesize that the mutants with multiple different modifications (AAV2G9, AAV2D, AAV2 Y-F and AAV2 K532R for AAV2, AAV8 Y-F and AAV8 K137R for AAV8) will further enhance liver transduction and decrease capsid antigen presentation (**Aim 2.1**).

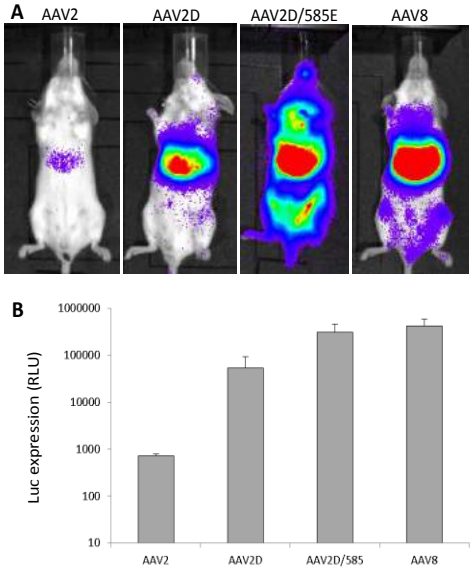

Fig. 6. Transgene expression from AAV2 mutants. A. representative images of vector induced luciferase activity. **B**. Fold increase of luciferase activity using AAV mutant capsids compared to AAV2.

A number of chemical reagents have been used for enhancing AAV transduction, including proteasome inhibitors such as MG-132 and bortezomib, DNA synthesis inhibitors such as hydroxyurea (HU) and aphidicolin, and topoisomerase inhibitors such as etoposide and camptothecin[27,28,30-34]. Thus far, the leading candidate for enhancing rAAV transduction *in vivo* is the proteasome inhibitor bortezomib, which has been demonstrated to increase transgene expression 3- to 6-fold in a large-animal study[32]. We have further

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 (**Aim 2.2**).

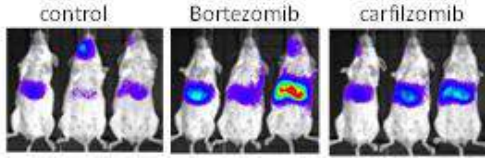

Fig. 7. The effect of proteasome inhibitors on AAV2 transduction. Balb/C mice received 1x10^{11} particles of AAV2/luc and 0.5 mg bortezomib/kg or 1 mg carfilzomib/kg at the same time. Transduction was assayed by live imaging at 7 days post AAV injection.

It is assumed that the mutants with several different individual modifications induce much higher liver transduction than mutants with a single modification, and the combination of proteasome inhibitor with AAV mutants will further enhance AAV liver transduction. Finally, we will explore the effect of the combination of proteasome inhibitors with AAV mutants described above on capsid antigen presentation *in vitro* and in mice (**Aim 2.3**).

C2.1. Study capsid antigen cross-presentation from AAV mutants.

First, we will clone OVA SIINFEKL peptide into the HI loop of AAV2 capsid mutants (AAV2G9, AAV2D, AAV2 Y-F and AAV2 K532R) or AAV8 mutants (AAV8 Y-F and AAV8 K137R) to generate the constructs pXR2G9-OVA, pXR2D-OVA, pXR2 Y-F-OVA, pXR2 K532R-OVA, pXR8 Y-F-OVA and pXR8 K137R-OVA. Next, we will make mutants with multiple mutations, then clone for the AAV2 mutant (defined as AAV2-MT4) which includes 4 different modifications: AAV2G9, AAV2D, AAV2 Y-F and AAV2 K532R, and for the AAV8 mutant (AAV8-MT2) which is composed of 2 modifications: AAV8 Y-F and AAV8 K137R, will be generated. These constructs will be used to package the AAT or luciferase transgene. After virus production, mutant vector characterization will be performed *in vitro* and *in vivo*. Then, these viruses will be used to examine the AAV capsid antigen presentation in HepG2/H2kb. Finally, AAV mutants will be injected into C57BL mice via the tail vein for a capsid antigen presentation analysis. Dose-response and kinetics of capsid antigen presentation will be determined.

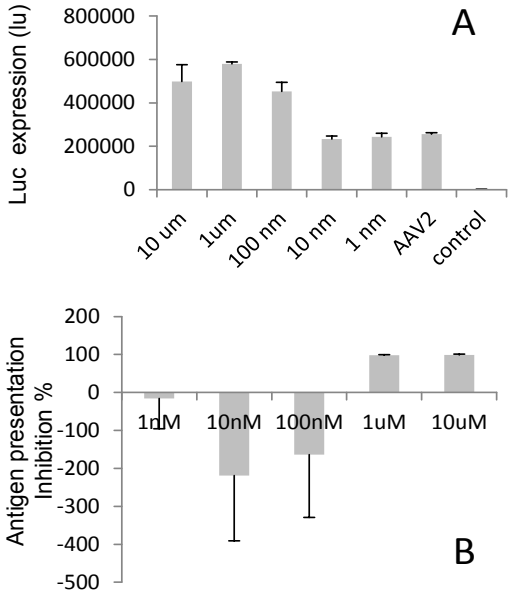

Fig. 8. The effect of bortezomib on capsid antigen presentation. A. Luciferase transgene expression. **B.** capsid antigen presentation. The x-axis indicates the concentration of bortezomib.

Clone of AAV mutants. Site-directed Mutagenesis and PCR will be used to generate AAV capsid mutants with single mutations. *Ubiquitin conjugation assay and immunoblotting.* A ubiquitination assay of viral capsids will be performed with a ubiquitin–protein conjugation kit (Boston Biochem, Cambridge, MA). Briefly, after conjugation fraction A, conjugation fraction B and ubiquitin are mixed with energy buffer; the conjugation reaction is then initiated by adding 3×10^8 heat-denatured AAV viral particles and incubated at 37°C for 4 hr. Equal volumes of sodium dodecyl sulfate (SDS)-denatured ubiquitinated samples are then resolved on a 4–20% gradient gel. The ubiquitination pattern for the various viral particles is detected by immunoblotting of the samples with mouse anti-ubiquitin monoclonal antibody (P4D1) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 (Cell Signaling Technology, Boston, MA). VP1, VP2, and VP3 capsid proteins are detected with AAV clone B1 antibody (Fitzgerald, North Acton, MA). *Luciferase imaging in mice.* Imaging will be performed with a Xenogen IVIS Lumina system after IP injection of the D-luciferin substrate. Bioluminescence imaging analysis is carried out with Living Image software (PerkinElmer, Waltham, MA). *Antigen presentation in mice:* C57BL mice will receive AAV mutant vectors (1x10^{11} particles) via tail vein injection. Antigen presentation will be analyzed for capsid specific T cell proliferation at different time points (day 3, 21, 41, 61) after AAV administration to examine the kinetics of antigen presentation. In the second experiment, different doses of AAV mutants (10^9, 10^{10}, 10^{11} and 2x10^{11} particles) will be administered into C57BL mice via tail vein injection, and 3 days after AAV injection, OT-1 spleen cells will be infused for CD8 T cell proliferation analysis as described above.

C2.2. The effect of proteasome inhibitors on AAV capsid antigen presentation *in vivo*. Proteasome inhibitors (bortezomib and carfilzomib) at different doses (10ug, 100ug, 1mg and 10mg/kg) will be co-administered to mice with AAV2-OVA/luc or AAV8-OVA/luc via tail vein injection, and transgene expression and antigen presentation (efficiency and kinetics) will be detected via live imaging of luciferase activity. Parameters for toxicities including body weight and blood chemistry will be monitored by the UNC animal clinical chemistry core facility (https://www.med.unc.edu/anclinic).

Antigen presentation in mice. C57BL mice will be treated with proteasome inhibitors at the above doses with co-administration of $1x10^{11}$ particles of AAV-OVA vector via tail vein injection. Antigen presentation will be analyzed for capsid specific T cell proliferation at different time points (day 3, 21, 41, 61) after AAV administration.

C2.3. The effect of proteasome inhibitors on antigen presentation *in vivo* using liver enhanced AAV mutants. After systemic administration of AAV mutants (AAV2-OVA-T4 or AAV8-OVA-T2) and proteasome inhibitors in mice (**C1.1** and **C1.2**), the transgene expression and antigen presentation will be examined.

Mouse experiments: we will administer the proteasome inhibitors with AAV2-OVA-MT4 or AAV8-OVA-MT2 into C57BL mice, and transgene expression will be examined to compare with AAV2-OVA and AAV8-OVA, respectively. For the antigen presentation assay, different doses (10^8, 10^9, 10^{10}, 10^{11}) of AAV2-OVA-MT4 and AAV8-OVA-MT2 mutants will be studied in the presence of proteasome inhibitors.

Anticipated results, potential pitfalls and alternative approaches: We have demonstrated that substitution of the HI loop in AAV2 and AAV8 virions with the immunodomain OVA SIINFEKL peptide does not change AAV infectivity or tissue tropism[11,73]. We expect that HI loop swapping with the OVA peptide in AAV2 or AAV8 mutants will neither influence transduction efficiency or tropism *in vitro* or in mice. Although higher transgene expression is expected, it is also anticipated that AAV mutants with Y-F or K-R change will induce lower capsid antigen presentation than wtAAV-OVA in HepG2, or similar capsid antigen presentation from other AAV mutants since our preliminary results suggest that uncoating of AAV virions in the cytoplasm plays a major role in AAV capsid antigen presentation[12]. Consistent with expected results in HepG2 cells, AAV2-OVA or AAV8-OVA mutants with Y-F or K-R modification will induce higher transgene expression than wtAAV-OVA after tail vein injection with lower antigen presentation. Despite the anticipated differences, it is possible that similar capsid antigen presentation will be induced between these mutants and wtAAV-OVA. Similar to wtAAV2-OVA and wtAAV8-OVA, stronger capsid antigen presentation is expected to occur early after AAV2 or AAV8 mutant administration and will gradually decrease over time. It is expected that the kinetics of capsid antigen presentation from AAV mutants' transduction is shorter than that from wtAAV2-OVA and wtAAV8-OVA. Our preliminary results have shown that the proteasome inhibitor bortezomib at a high dose has dual function: increased AAV transduction and decreased antigen presentation. However, when a relatively low dose is used, AAV transduction is increased or not changed, but antigen presentation is increased (**Fig. 8**). It is anticipated that the proteasome inhibitor carfilzomib will have a similar profile to bortezomib in affecting transgene expression and antigen presentation *in vitro*. *In vivo,* both proteasome inhibitors are hypothesized to decrease capsid antigen presentation at high-doses after AAV-OVA transduction, while antigen presentation will increase when low doses are administered, consistent with *in vitro* observations (**Fig. 8**). It is possible that high doses of proteasome inhibitors will also induce high capsid antigen presentation, perhaps because the antigen presentation assay requires a longer time *in vivo* and the effect of proteasome inhibition is short. When the AAV2-MT4 mutant which contains 4 different individual modifications (AAV2G9, AAV2D, AAV2 Y-F and AAV2 K532R) or AAV8-MT2 mutant which contains two modifications (AAV8 Y-F and AAV8 K137R) is administered in combination with proteasome inhibitors in mice, transgene expression is expected to be much higher than that from a single individual mutant or proteasome inhibitor alone. However, the capsid antigen presentation from the AAV2-MT4 or AAV8-MT2 mutant will be undetectable or much lower than that from the mutant alone or wt AAV2/AAV8 in the presence of proteasome inhibitors in mice. It is possible that the AAV2-MT4 mutant may not induce higher transgene expression than the single mutants. If this is the case, we will test the mutants with the combination of two or three individual modifications. The other concern is that the insertion of the OVA SIINFEKL peptide into the HI loop of various AAV2 and AAV8 mutants might negatively affect the transduction ability and tropism. If this is the case, we will study capsid antigen presentation from mutants using wild type capsid specific CTLs generated from mutant capsid immunized mice although this approach sensitivity is lower than that with OVA substitution. Several approaches will be used to generate wild type capsid specific CTLs in mice including: Ad vector delivery of mutant capsid, peptide pulsed dendritic cell immunization, or direct immunization with peptides since several peptides from the AAV capsid (AAV2 VP1 aa702-710, AAV8 VP1 aa517-525) have been identified as CD8 epitopes in C57BL mice[6]. While it is impossible to describe every potential outcome that we may encounter, we have strong preliminary data to support our experimental design. We also have extensive experience in adapting our protocols to unexpected outcomes.

C3. **Isolate AAV mutants with human hepatocyte tropism and immune-evasion capacity.**

***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[44,74]. This observation suggests that the results generated in murine tissues may not translate well into large animals or humans. It is imperative to develop AAV mutants with human hepatocyte tropism and ability to evade proteasome mediated degradation of capsid. Recently, a mouse model with a xenografted human liver has been used to study AAV vector transduction in human liver cells and to develop human liver-tropic AAV mutants[42]. However, the information is still lacking for a transduction profile of human hepatocytes from AAV serotypes and mutants which have demonstrated high mouse liver tropism. To address this concern, we will used a xenografted mouse model with human liver cells to examine the transduction efficiency of AAV serotypes and mutants developed from **Aim 2** in human liver cells and elucidate whether AAV mutants with immune evasion and mouse liver tropism from **Aim 2.1** also have higher transgene expression in human hepatocytes (**C3.1**).

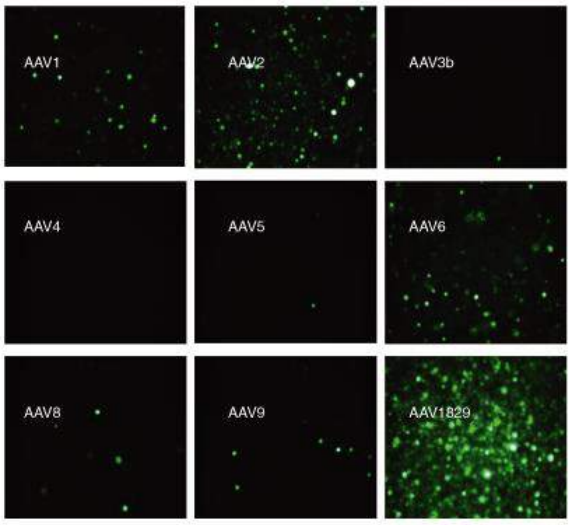

Fig. 9. Fluorescence micrographs of green fluorescent protein (GFP) transgene expression in CS1 cells transduced with AAV serotypes 1–9 (except 7) and the novel variant chimeric-1829 at a multiplicity of infection (MOI) of 1,000 for 48 hours.

DNA shuffling is a powerful process for directed evolution, which generates diversity by recombination, combining useful mutations from individual genes. Single and multigene traits that require many mutations for improved phenotypes can be evolved rapidly. We have utilized this approach to generate an AAV DNA shuffling library (from AAV capsid serotypes 1-6, 8 and 9) and isolated a cos-1 integrin minus specific variant (AAV1829) that had enhanced transduction in cos-1 cells compared to all parent serotypes (**Fig. 9**)[45]. Using the same approach, after IV administration of the AAV capsid library, we were able to isolate two mutants from neuron rich cells in rats with limbic seizure activity induced by kainic acid. These mutants transduced the cells that were localized to the piriform cortex and ventral hippocampus after IV administration, indicating that novel AAV vectors have been created that can selectively cross the seizure-compromised BBB and transduce cells[46]. Our *in vitro* studies have demonstrated that AAV capsid degradation via the proteasome plays a major role for effective capsid antigen presentation, and it is well-known that lysine residues are direct targets for host cell ubiquitination and therefore mutating them can reduce protein ubiquitination and subsequent proteasome-mediated degradation. Mutations of capsid lysine or tyrosine residues have led to low capsid ubiquitination to evade capsid specific CTL mediated elimination of AAV transduced cells[41]. From the above observations, we will apply the strategy of combining directed evolution with the AAV capsid library including rationally designed capsids with ubiquitination avoidance, to explore human liver-specific AAV capsids with the ability to avoid capsid CTLs in a xenograft mouse model (**C3.2** and **C3.3**).

C3.1. Verify AAV human liver transduction efficiency in xenograft mice. To study AAV transduction in human hepatocytes, we will first establish a xenografted mouse model with human liver cells. AAV serotypes and the mutants developed in **Aim 2** will be tested in this mouse model. Besides nude mice, some other mouse strains have been explored to establish human liver xenografts including immuno-deficient Rag$^{-/-}$/Ilrg2r$^{-/-}$ (NRG) mice with u-PA (Alb-uPA+/+), NRG mice with active Caspase 8 fused with FK506-binding domain (AFC8) and NRG mice with fumarylacetoacetate hydrolase deficiency (FRG)[75-78]. Compared to other strains, FRG mice have demonstrated high efficiency of human liver cell repopulation, no limitation with regard to age of the xenograft and easy breeding[77]. In this proposal, we will use FRG mice (from Yecuris™, Portland, OR) to perform human liver xenografts. In FRG mice, we have successfully grafted human liver cells from frozen fetal liver and fresh adult liver (**Fig. 10**).

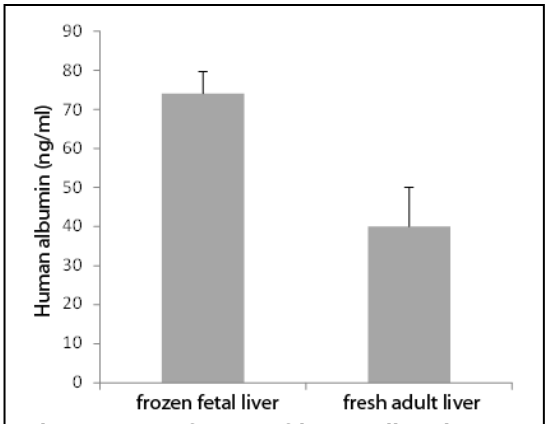

Fig. 10. Engraftment of human liver in FRG mice. FRG mice received 1x10^6 frozen fetal or fresh adult liver cells. The human albumin in the blood was measured by ELISA at 12 weeks and 5 weeks after xenograft. The data represent the average of 2 mice and SD.

Establishment of human liver xenografted mouse model. Hepatocytes isolated from normal human livers will be purchased from Triangle Research Labs. Following the Yecuris protocol, at 24

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.

C3.2. Selection of AAV mutants in human liver xenografted mice. High-throughput screening/ selection of capsid mutants from AAV libraries has been carried out earlier and appears to be a promising strategy to develop human liver specific AAV chimerics with the ability to evade capsid specific CTL mediated elimination of transduced cells. Several studies, including ours, have provided a solid rationale for carrying out directed evolution *in vivo* in xenografted mice[46,79,80]. First, we will rationally mutate AAV capsids from serotypes and mutants from Aim 2 which will be used for construction of the AAV capsid shuffling library based on the prediction of ubiquitination sites. Several available software programs will be used for this purpose including UbiPred, Composition of k-Spaced Amino Acid Pairs, and Prediction of Ubiquitination Sites with Bayesian Discriminant Method. Systematical analysis of scores from different programs will be performed for choosing top amino acids (lysine-K, serine-S and threonine-T) for mutation (K→R, S/T→A). Mutated AAV capsids will be digested with DNase, the digested capsid DNA fragments will be assembled into AAV capsid plasmid library, which will be used to generate an AAV capsid library. After injection of this AAV library into human liver xenografted mice in the presence of human adenovirus, human hepatocytes from mouse liver will be isolated using cell sorting to then isolate human hepatocyte tropic AAV mutants.

DNA shuffling and the generation of a new AAV capsid library. The techniques outlined herein were developed by our lab[46,80,81]. Briefly, lysine and serine/threonine in AAV capsids from serotypes 1-12 and 8 mutants (AAV2G9, AAV2D, AAV2 Y-F, AAV2 K532R, AAV8 Y-F, AAV8 K137R, AAV2-MT4, AAV8-MT2) from Aim 2 will be mutated into arginine and alanine, respectively, using random mutagenesis based on software predication and preliminary data from **Aim 3.1**. DNA shuffling will be carried out using a collection of capsid genes representing 12 different AAV serotypes and 8 mutants with lysine or serine/threonine modification. Following limited DNase digestion, the capsid fragments from these AAV serotypes will be assembled, amplified, and cloned into a wtAAV backbone to generate a shuffled AAV capsid plasmid library. This plasmid library then will be used to produce a shuffled AAV vector library that will be used for *in vivo* selection of liver-tropic AAV variants having the capacity to evade capsid specific CTL response. *Directed evolution in human liver xenografted mice.* The AAV vector library ($1x10^{11}$ particles) will be injected into human liver xenografted mice with adenovirus Ad5 dl309 ($1x10^{12}$ particles) via the tail vein. After 48 hrs, liver lysates will be generated by freeze-thaw cycles, heated to 56ºC for 30 min to inactivate adenovirus, and then clarified by centrifugation to remove cellular debris. The pooled supernatant will be injected IV, and this process is then repeated for three more cycles. After each cycle, low-molecular DNA (Hirt DNA) will be extracted from liver to evaluate the replication yield by qPCR using AAV *rep* gene-specific primers. Following this iterative selection, capsid DNA sequences from isolated liver DNA will be amplified through PCR and cloned into the pXR shuttle vector[82]. Individual clones, each potentially representing a chimeric capsid DNA sequence, will be sequenced and recombinant vectors packaged with the GFP will be generated for *in vitro* and *in vivo* characterization. Additionally, structural analysis of these mutants will be performed (as detailed below). *Isolation of human liver cells.* Mice will be perfused with cold PBS followed by collagenase to isolate liver cells. The liver cells labeled with FITC conjugated mouse anti-human HLA-ABC antibody will be sorted using flow cytometry. *In vivo characterization of AAV mutants in xenografted mice.* $1x10^{11}$ particles of 10 recovered mutant viruses encoding GFP, and the best serotype for human liver transduction based on result from **C3.1**), will be injected (IV) into xenografted mice to determine the transduction in human liver cells. *Structural analysis.* AAV mutants will be subjected to structural characterization by molecular modeling studies using Pymol® and SWISS-MODEL®. PDB files of the VP3 subunit of different AAV serotypes are now available and models can be generated as needed using other AAV strains as templates[45,79]. Sequence alignment analysis using Vector NTI® will carried out to determine which sequences are conserved in wild type AAV serotypes. Mapping of different domains onto the capsid surface will be carried out by generating 3D ribbon models of dimers, trimers and pentamers that represent the two-fold, three-fold, and five-fold axes of symmetry, which can be generated readily on the

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 with MG132, are infected with AAV mutants isolated from humanized mouse liver at $5x10^3$ particles per cell for 4 h at 37°C. Cells are harvested and lysed in 0.8 ml of radioimmunoprecipitation (RIPA) buffer. Cell lysates are precleared with 10 µl of protein G PLUS-agarose (Santa Cruz Biotechnology) and then are incubated with 10 µl of mouse anti-AAV monoclonal antibody clone B1at 4°C for 1 h, followed by the addition of 30 µl of protein G PLUS-agarose. After overnight incubation at 4°C, the beads are washed four times with 1 ml of ice-cold RIPA buffer and resolved by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE). After transfer to a nitrocellulose membrane, the blot is probed with mouse anti-ubiquitin monoclonal antibody P4D1, followed by horseradish peroxidase-conjugated secondary antibody. After the final washes, the ubiquitinated viral protein is visualized with an enhanced chemiluminescence system (Amersham Pharmacia). *Antigen presentation in mice:* C57BL mice will receive AAV mutant vectors ($1x10^{11}$ particles) or AAV2OVA via tail vein injection. Antigen presentation will be analyzed at different time points (day 3, 21, 41, 61) to examine the kinetics of antigen presentation. In the second experiment, different doses of AAV mutants (10^9, 10^{10}, 10^{11} and $2x10^{11}$ particles) will be administered into C57BL mice via tail vein injection, and 3 days later, OT-1 spleen cells will be infused to examine the dose-response of antigen presentation.

Anticipated result, potential pitfalls and alternative approaches: Synthesis of AAV capsid libraries has already been successfully achieved by our group. In addition, we have demonstrated the ability to isolate novel AAV mutants with the ability to cross the blood-brain barrier using an *in vivo* screening approach. We are confident that we will be able to extend these technical skills to obtain novel chimeric AAV capsids by directed evolution following injection of the AAV library in human liver xenografted mice. We do not anticipate any problems with harvesting organs and subsequent analysis of clones derived from multiple rounds of cycling. We anticipate that AAV mutants can be obtained in human liver xenografted mice. These mutants are expected to have decreased ability to bind ubiquitin and mount lower capsid antigen presentation, and also will be pre-disposed towards efficient human liver transduction. We carried out numerous studies using these protocols and have extensive experience; therefore, we do not have technical limitations in performing these experiments. Alternatively, we will isolate liver cells after AAV injection and amplify the AAV variants in the presence of Ad *in vitro*. It is also possible that the yield of AAV virus recovered from a xenografted liver will be too low for subsequent amplification. In this case, an intermediate step will be taken in which AAV genomes containing Hirt DNA prepared from the liver will be co-transfected into 293 cells with the helper plasmids pXR2 and pXX6-80. The chimeric AAV particles will then be used to infect 293 cells with wtAd, and the resulting variant AAV vector library will be injected into mice. As another alternative, variant capsid genes will be PCR amplified from Hirt DNA prepared from liver. The resulting library of amplicons will be subcloned into a wtAAV vector backbone, which will be used to produce variant AAV virus. It is possible that our strategy will not generate AAV capsids with improved transduction efficiencies compared with existing AAV capsids (e.g. AAV3). As an alternative approach to isolate AAV mutants with human hepatocyte tropism and the ability to escape capsid ubiquitination, we will make AAV shuffled library from 12 AAV serotypes and different mutants without any further substitutions of lysine and serine or threonine. This library will be injected into humanized mice for isolation of AAV mutants with human liver tropism. The isolated mutants will further modified at the residues of lysine, serine or threonine to decrease ubiquitination ability. However, the mutants derived from further modifications after selection may decrease its human hepatocyte tropism. The same concern as in Aim 2 is that the OVA peptide substitution in mutants might affect the transduction ability. Nevertheless, we believe that our approach proposed in this aim will at a minimum lead to the development of several AAV mutants that induce low capsid antigen presentation and have human hepatocyte tropism– a significant outcome.

Summary: The final objectives of this proposal are to explore novel AAV mutants with human hepatocyte tropism and the ability of avoiding proteasome mediated AAV capsid degradation. We anticipate that the overall proposed research will have a major impact on AAV vector development, and on the consideration of rational design of vectors. We feel our preliminary data are strong and very supportive of the experimental design presented in our 3 aims. We are well positioned with reagents and animal models to rigorously test these hypotheses. In short, AAV vector biology and the host immune response have now taken center stage following Phase I clinical studies. We hope to engage in pre-clinical studies that will better clarify this concern and the potential to explore effective approaches that may deflect future challenges of CTL responses to AAV transduced cells *in vivo*.

VERTEBRATE ANIMALS

Protocols for these projects are pending at the UNC-CH Institutional Animal Care and Use Committee and will be submitted using Just in time procedures. The Institutional Animal Care and Use Committee (IACUC) will review and approve the proposed use of animals before the activity is initiated. Details of the animal experiments can be found in the Research Design and Methods section. Regarding the use of vertebrate animals we will address the five points as required by the NIH instructions:

1) These studies will require the use of mice. The total number of 3490 mice is required for experiments proposed in this application. The experiments proposed will utilize mice with TAP deficiency, Cathepsin S deficiency, wild type C57BL mice, OT-1 mice and NOD-Scid-IL2Rγnull (Nod-Rag1null-IL2rγnull, NSG) with fumarylacetoacetate hydrolase deficiency (FRG) mice.

Aim 1. Study the effect of AAV empty particles on AAV capsid antigen cross-presentation.

Aim 1.1. The kinetics and dose-response of AAV capsid antigen presentation from AAV2 empty virions *in vivo*.

60 C57BL mice are required for kinetics = 5 mice/group x 3 groups (empty, full and no treatment) x 4 time points

90 C57BL mice are needed for dose-response = 5 mice/group x 3 groups x 6 doses (0, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12})

60 OT-1 are required for spleen cells.

For Aim 2.1, the total number of mice is 60 + 90 + 60 =210 including 150 C57BL mice and 60 OT-1 mice.

Aim 1.2. The effect of empty particles on AAV capsid antigen cross presentation from full-particle AAV transduction *in vivo*.

6 groups (0, $1x10^{10}$, $3x10^{10}$,$1x10^{11}$, $3x10^{11}$, $1x10^{12}$ empty or full spiked with $1x10^{11}$ full or empty particles AAV2/AAT, respectively) and C57 mice will used.

For effect of empty on transgene expression from AAV full virions, 5 mice x 6 groups = 30

For effect of empty on capsid antigen presentation from AAV full virions, 5 mice/group x 6 groups x 4 time points = 120

For effect of full virion on capsid antigen presentation from empty, 5 mice/group x 6 groups x 4 time points = 120

100 OT-1 mice are needed for spleen cells

For aim 2.2, the total number is 30 + 120 + 120 + 100 = 370 including 270 C57BL mice and 100 OT-1 mice.

Aim 1.3. AAV capsid antigen presentation in TAP-/- and in Cat S-/- mice.

For antigen presentation, 5 mice/group x 3 strains (TAP-/-, Cat S-/- and C57BL) x 2 serotypes (AAV2 and 8) x 2 (vector and control) x 2 (empty and full virion) = 120

20 OT-1 mice for OVA specific T cells.

For Aim 1.3, the total mice number is 120 + 20 = 140 including 40 TAP-/- mice, 40 Cat S -/- mice, 40 C57BL mice and 20 OT-1 mice.

For Aim 1, the total mice number is 210 + 370 + 140 = 720.

Aim 2. Investigate AAV capsid antigen presentation following administration of AAV mutants or proteasome inhibitors for enhanced AAV liver transduction.

Aim 2.1. Capsid antigen presentation from AAV mutants with enhanced liver transduction in mice.

For transgene expression: 95 C57BL mice are needed: 5 mice/group x 19 viruses (AAV2, AAV9, AAV2G9, AAV2G9-OVA, AAV2D, AAV2D-OVA, AAV2 Y-F, AAV2 Y-F-OVA, AAV2 K532R, AAV2 K532R-OVA, AAV8, AAV8 Y-F, AAV8 Y-F-OVA, AAV8 K137R, AAV8 K137R-OVA, AAV2-MT4, AAV2-MT4-OVA, AAV8-MT2, AAV8-MT2-OVA).

For *in vitro* antigen presentation, 30 OT-1 mice are needed.

For dose response, 300 mice are needed: 6 (10^9, $3x10^9$, $1x10^{10}$, $3x10^{10}$, $1x10^{11}$ particles of AAV vector and control) x 10 viruses (AAV2OVA, AAV2G9-OVA, AAV2D-OVA, AAV2 Y-F-OVA, AAV2 K532R-OVA, AAV8OVA, AAV8 Y-F-OVA, AAV8 K137R-OVA, AAV2-MT4-OVA, AAV8-MT2-OVA) x 5 mice/group.

For kinetics study, 400 mice are required: 4 time points (day3, day21, day 41 and day 61) x 2 (each mutant + control) x 10 viruses (AAV2OVA, AAV2G9-OVA, AAV2D-OVA, AAV2 Y-F-OVA, AAV2 K532R-OVA, AAV8OVA, AAV8 Y-F-OVA, AAV8 K137R-OVA, AAV2-MT4-OVA, AAV8-MT2-OVA) x 5 mice/group.

100 OT-1 mice are needed.

For Aim 2.1, the total mice number is 95 + 30 + 300 + 400 + 100 = 925 including 795 C57BL mice and 130 OT-1 mice

Aim 2.2. The effect of proteasome inhibitors on AAV capsid antigen presentation *in vivo*.

For transgene expression from single drug treatment, 100 C57BL mice are needed: 5 doses (including control without treatment) x 5 mice/group x 2 proteasome inhibitors (bortezomib and carfilzomib) x 2 serotypes (AAV2 and AAV8).

For antigen presentation from single drug treatment, 640 C57BL mice are needed: 4 time points (day3, day21, day 41 and day 61) x 2 (each dose of drug and control) x 4 dose (**Table 1**) x 5 mice/group x 2 proteasome inhibitors x 2 serotypes (AAV2 and AAV8).

200 OT-1 mice are needed to provided capsid specific CTLs.

For Aim 2.2, the total mice number is 100 + 640 + 200 = 940 including 740 C57BL mice and 200 OT-1 mice.

Aim 2.3. The effect of combination of AAV mutants with proteasome inhibitors on antigen presentation *in vivo.*

For dose response, 300 mice are needed: 5 (10^8, 10^9, 10^{10}, $1x10^{11}$ particles of AAV vector and control) x 4 viruses (AAV2OVA, AAV2-MT4-OVA, AAV8OVA, AAV8-MT2-OVA) x 5 mice/group x 3 (2 proteasome inhibitors and control).

60 OT-1 mice are needed.

For aim 2.3, the total number is 300 + 60 = 360 including 300 C57BL mice and 60 OT-1 mice.

For aim 2, the total number is 925 + 940 + 360 = 2325

Aim 3. **Isolate AAV mutants with human hepatocyte tropism and immune-evasion capacity.**

Aim 3.1. Verify AAV human liver transduction efficiency in xenograft mice.

For examination of human hepatocyte transduction efficiency from AAV serotypes and mutants, 100 mice are needed: 5 mice/AAV vector x 20 AAV vectors(12 serotypes, and 8 mutants AAV2G9, AAV2D, AAV2 Y-F, AAV2 K532R, AAV8 Y-F, AAV8 K137R, AAV2-MT4, AAV8-MT2).

For aim 3.1, the total number is 100 FRG mice.

Aim 3.2. **Selection of AAV mutants in human liver xenografted mice.**

For selection of AAV mutants with human hepatocyte tropism, 20 FRG mice will be required for isolation of AAV mutants: 4 cycles of amplification x 5 mice/cycle. 10 mutants will be tested in human liver xenograft mice. So 55 mice are needed: 5 mice/group x 11 (10 mutants isolated from humanized mouse and best serotype or mutant from **Aim 3.1**).

For aim 3.2, the total number is 20 + 55 = 75 FRG mice.

Aim 3.3. Investigation of immune-evasion from humanized AAV mutants

For investigation of antigen presentation from AAV mutants isolated from humanized mouse, 2 mutants with the best human hepatocyte transduction will be chosen for antigen presentation analysis *in vitro* and *in vivo*. For dose response, 90 mice are needed: 6 (10^9, $3x10^9$, $1x10^{10}$, $3x10^{10}$, $1x10^{11}$ particles of AAV vector and control) x 3 viruses (2 mutants and AAV2OVA) x 5 mice/group.

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.

2) The use of animal models in the arena of gene therapy is essential for vector development and for evaluation of safety about immune response to capsid and efficacy of planned protocols. It is clear that no human clinical trial can be considered prior to vector evaluation and its proven success in animal models. Employing animal model is essential for the ability to accurately predict the outcome of a gene therapy protocol. The mice with TAP deficiency, cathepsin S deficiency in this study are chosen since these mice are deficient in gene products which involve in antigen presentation and results from study in these mice will help us understand the mechanism of AAV capsid antigen presentation and explore the novel approaches to inhibit capsid specific CTL mediated elimination of AAV transduced target cells *in vivo*. OT-1 mice are chosen due to transgenic of specific TCRs to H-2^b OVA epitopes. C57BL mouse has H-2kb haplotype and presents OVA SIINFEKL peptide complex with H-2kb molecules on the cell surface, which can be recognized by OT-1 spleen cells and stimulates OT-1 CD8 cell proliferation. FRG mice has been successfully used to establish humanized model with liver due to deficiency of immune system and repopulation potential of xenografted liver cells in FRG mice. We will use FRG mice to engraft human liver to establish human liver xenograft mouse model.

3) The mice will be housed at the Department of Laboratory Animal Medicine (DLAM) facility located at the Genetic Medicine building on the UNC-CH campus. All animals are cared for by experienced technicians and veterinarians with special training in the handling of each of the aforementioned animal species.

4) All studies will be conducted in a manner to minimize all discomfort, distress, pain and injury. Animals that develop significant discomfort due to the procedures will be EUTHANIZED.

5) The methods of euthanizing animals will be consistent with the recommendations of the panel of Euthanasia of the American Veterinary Medical Association. All mice will be euthanized by CO2 asphyxiation.

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 and Samulski groups have been collaborating with one another for a number of years, bringing together expertise in the fields of immune response and AAV vector development for gene therapy applications, respectively. The new R01 further exploits this unique relationship to characterize a topic that has clinical significance, namely enhanced AAV liver transduction and decreased capsid antigen cross-presentation.

Drs. Li and Samulski will both provide oversight for the proposed work. These 2 investigators will be responsible for the implementation of the leadership plan, carrying out the specific aims, and ensuring that the work is in compliance with DHHS and NIH policies including those for biosafety and animal research. Specifically, Dr. Li will oversee experiments related to capsid antigen presentation. Dr. Samulski will be responsible for AAV mutant deveopment. Dr. Li will serve as contact PI and assume responsibility for: i) fiscal and administrative management, and ii) maintaining communication between the 2 groups through monthly meetings. Notably, the 2 groups are highly interactive and are both located within the UNC Gene Therapy Center in Chapel Hill, NC. Dr. Li will be responsible for communication with the NIH and submission of annual reports jointly prepared with Dr. Samulski. Publication authorship will be based on the relative scientific contributions of the PIs and key personnel.

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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, the UNC Vector Core has a standing policy regarding the mechanism of notification and distribution of new vector reagents for individuals in the research community at large. This policy includes the following components:
a. After new (AAV and Chimeric) viral vectors have been developed, the Core will assume the responsibility of maintaining the parental seed lot and will produce large quantities for the investigator's needs.
b. For individuals who may be interested in these reagents, we will place the description of the reagent on the Vector Core Website www.genetherapy.unc.edu/jvl.htm. Distribution to outside investigators will require written permission form the PI responsible for generating the viral vector.