Linking the Tropism and Transduction Efficiency of Porcine-Derived Adeno-Associated Viruses to their Transgene-Mediated Protective Efficacy

By

Alexander Juanito Arquillano Bello

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Medical Microbiology

University of Manitoba

Winnipeg, MB

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree of Doctor of Philosophy

ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor, Dr. Gary P. Kobinger. Thank you for being an outstanding leader and mentor. I appreciate all the opportunities you have given me and for providing me such a unique and memorable experience in your lab. Thank you for your guidance and for making my journey an enjoyable one.

Thanks also to my committee members: Dr. Keith Fowke (Medical Microbiology), Dr. Sam Kung (Immunology), and Dr. Xiao-Jian Yao (Medical Microbiology) for their valuable input and guidance throughout the years. As well, I would like to thank my external examiner, Dr. Elizabeth Simpson (UBC, Medical Genetics), for taking the time to review my thesis and attend my defense.

I am also thankful to the staff, faculty, and students of the Department of Medical Microbiology at the University of Manitoba for all the help they provided me, especially Angela Nelson. Thanks to the members of Special Pathogens at the National Microbiology Laboratory, Public Health Agency of Canada for all your help and support. Special thanks to members of the Kobinger lab, especially Allan Chand, Jenna Aviles, Kaylie Tran, Jonathan Audet, and Hughes Fausther Bovendo, for helping a great deal with experiments.

Finally, I would like to thank my family and my loving wife, Levilynn Bello, for always being understanding and patient with me as I pursued my degree. I am very grateful for your support throughout my endeavors.

ABSTRACT

Adeno-associated virus (AAV) is a small, non-pathogenic virus, which is not known to cause illness in infected hosts. Following infection of AAV into a host, the immune responses are limited mainly to neutralizing antibodies, in the absence of cell-mediated immunity. AAV and its tolerogenic qualities has been exploited as a vector for gene therapy applications, and many recombinant AAVs have been utilized in various clinical trials with great success. However, these vectors are based on human and non-human primate AAVs, to which there exists pre-existing cross-reactive immunity in the general population, increasing the risk of toxicity with increased doses. We hypothesized AAVs having low seroprevalence in the human population can be isolated from alternate sources, such as pigs, and may be used as alternatives to those used in current clinical trials. We also hypothesized that the close homology between pig and human tissues would allow AAVs isolated from pigs to transduce human cells efficiently. To address this hypothesis, several new AAVs from porcine tissues were isolated and a majority of these isolates did not react to pooled human immunoglobulin. Prior studies have determined that each AAV vector displays its own unique tissue tropism. This phenomenon was exemplified in porcine-derived AAVs, which preferred specific tissue targets, such as AAV1 in the muscle or AAV6 in the lungs, when injected *in vivo* in mice. As well, we determined that porcine-derived AAVs successfully transduced cells derived from humans. Apart from tissue tropism, immune responses generated against the AAV capsid are important for determining the safety profile of the vectors. Clinical trials utilizing AAV have produced promising results; however, there still exists the possibility of the host mounting adverse immune responses against transduced cells, as seen in clinical trials for the treatment of hemophilia. Although the transduction efficiency of

AAV gene transfer has been extensively studied in animal models, the host's immune response towards the gene product is still poorly understood. This thesis addresses the issue by providing a link between protective efficacy against lethal challenge and tissue tropism. Here, AAVs carrying an immunogenic transgene were developed, with the goal to identify those that can protect against lethal challenge of avian influenza or Ebola virus in mice, and those that had poor protective efficacy. It was observed that the protective efficacy afforded by an AAV was serotype specific. The protective efficacy and immune responses were compared to the biodistribution and cellular targets of each AAV. Overall, AAVs sharing broad tropism in biodistribution studies protected mice better against lethal challenge then those AAVs not found systemically. As well, those AAVs eliciting protective efficacy against lethal challenge were able to transduce antigen-presenting cells such as dendritic cells and B cells. The main hypothesis of this thesis states that AAVs, which have a high protective efficacy against a lethal infection model in mice, share a common tissue tropism. The link between tissue tropism and host immune responses has been poorly understood and this thesis contributes to the AAV field by highlighting the significance of the cellular targets of AAV and this relationship to protective efficacy.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
ABST	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulphonic acid]-diammonium salt
AD	agglutinating dose
AEC	3-Amino-9-Ethylcarbazole
ANOVA	analysis of variance
β-gal	beta-galactosidase
bGH	bovine growth hormone
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSL4	biosafety level 4
CIP	calf intestinal alkaline phosphatase
CFSE	carboxyfluorescein succinimidyl ester
CMV	cytomegalovirus
CPE	cytopathic effect
°C	degrees Celcius
DNA	deoxyribonucleic acid
EBOV	*Zaire ebolavirus*
EBOVGP	Ebola virus glycoprotein
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot analysis
EM	electron microscopy
gc	genome copies
GP	glycoprotein
FACS	fluorescence-activated cell sorting

FIX	factor IX
HA	hemagglutinin
HEK	human embryonic kidney
HRP	horseradish peroxidase
Hz	hertz
H05	A/Hanoi/30408/2005 H5N1
H05HA	avian influenza A/Hanoi/30408/2005 H5N1 hemagglutinin antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
IN	intranasal
ITR	inverted terminal repeat
IVTV	intravenous tail-vein
kb	kilobases
L	RNA-dependent RNA polymerase
MA-EBOV	mouse adapted Ebola virus
MDCK	Madin-Darby canine kidney
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
microRNA	micro ribonucleic acid
ml	milliliters
NA	neuraminidase
NAb	neutralizing antibody
NP	nucleoprotein
NHP	non-human primate
nm	nanometer

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PB1	polymerase basic 1
PB2	polymerase basic 2
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFU	plaque forming units
PMA	phorbol 12-myristate 13-acetate
polyA	polyadenylation
qPCR	quantitative real-time PCR
rAAV	recombinant Adeno-associated virus
RCF	relative centrifugal force
RDE	receptor destroying enzyme
RIPA	radioimmunoprecipitation
RPM	revolutions per minute
sc	self-complementary
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SFU	spot forming units
ss	single-strand
TAIL	thermal asymmetric interlaced
TPCK	Tosyl phenylalanyl chloromethyl ketone
VP	viral protein
wt	wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

1.0 INTRODUCTION

1.1 HISTORY OF AAV

In the 1960's, small contaminating particles were found in a human adenovirus preparation (Casto, Atchison, & Hammon, 1967). These particles were found to be non-infectious and were given the name adeno-associated virus (AAV). AAV particles encapsidate a 4.7 kilobase (kb) single-stranded (ss) deoxyribonucleic acid (DNA) genome of either positive or negative polarity (Siegl et al., 1985). The presence of helper virus such as adenovirus or herpes simplex virus has been shown to be important for replication and production of progeny AAV particles (Chang, Shi, & Shenk, 1989; Weindler & Heilbronn, 1991). In the absence of helper virus, wild-type (wt) AAV will integrate into the host chromosome and establish a latent infection. In the 1980s, AAV was developed as a gene transfer vector by replacing the viral genes with a gene-of-interest and providing this DNA in *cis*; transfection of this plasmid and plasmid encoding the Rep and Cap proteins of AAV in *trans* was carried out in adenovirus infected cells, resulting in production of viable recombinant AAV (rAAV) vectors. This process was refined to exclude helper virus, but rather, include the helper virus genes necessary to carry out replication of progeny rAAV vectors (Xiao, Li, & Samulski, 1998a). Gene transfer mediated by rAAV vector is stable and can last for up to years (H. Jiang et al., 2006). As well, AAV has preferential tissue tropisms and appears to subvert the host immune response when administered at the right conditions (Martino et al., 2009). The safety profile of AAV combined with its ability to efficiently transfer genetic material into a host makes AAV an attractive modality for gene therapy and vaccine purposes.

1.2 TAXONOMY

AAV belongs to the *Parvoviridae* family in the genus *dependovirus*, where members have a heavy reliance on coinfection with helper virus such as adenovirus or herpes simplex virus in order to replicate (Berns & Giraud, 1996). Species in *dependovirus* are generally designated with AAV followed by a number. For example, the first AAV was named 'AAV1', while succeeding AAVs were sequentially named 'AAV2', 'AAV3', etc. However, some AAVs isolated from different animal species are identified as the species from where they originated. For example, an AAV isolated from canine was labelled 'canine adeno-associated virus', whereas an AAV from bovine was called 'bovine adeno-associated virus'.

1.3 CAPSID STRUCTURE

1.3.1 CAPSID PROTEINS

AAV capsid is composed of three proteins: VP1, VP2, and VP3, having molecular weights of 87, 72, and 62 kiloDaltons, respectively (Jay, Laughlin, & Carter, 1981). 60 copies of the VP1, VP2, and VP3 proteins make up the capsid particles in a ratio of 1:1:10. All AAV capsid proteins arise from the same DNA as VP3, with additional amino acid residues at the N terminus for VP1 and VP2. The capsid proteins self-assemble in the host nucleus to form an icosahedral particle, encapsidating the single-stranded AAV genome. The size of the capsid is generally 20-26 nm in diameter, with a buoyant density in cesium chloride between 1.39 to 1.42 g/cm^3 and molecular weight of 5.5 to 6.2x10^6 Daltons (Siegl et al., 1985). The capsids are largely resistant to heat and proteinase K inactivation, as well as withstanding conditions between pH 3 and

9 at 56°C for 1 hour. However, the capsid can be chemically inactivated by β-propiolactone, hydroxylamine, formalin, and oxidizing agents.

1.3.2 SURFACE PROFILE

The AAV capsid is a non-enveloped icosahedral approximately 20 nm in diameter. The major structural proteins of AAV are made up of an eight-stranded, antiparallel β-barrel sheet. The surface of the AAV capsid is made up of large loops, also serving to connect the β-barrel sheets together (DiPrimio, Asokan, Govindasamy, Agbandje-McKenna, & Samulski, 2008). There is no evidence of AAV capsid proteins being glycosylated, but variable phosphorylation can occur (Zhong et al., 2008). The capsid may also include three smaller spike protrusions around the icosahedral threefold axis of symmetry, a pitted depression at the fivefold axis of symmetry, and dimple-like depressions at the twofold axis of symmetry (Xie et al., 2002). The putative receptor-binding sites are positively charged areas located at the side of each threefold peak, also having the largest buried surface area and the highest contact energy (Xie, Somasundaram, Bhatia, Bu, & Chapman, 2003a). As well, surface loops at the threefold axis contribute to the tropism of AAV capsid as they determine receptor binding and serve as epitope binding for antibodies. The twofold axis of symmetry has low contact energy and weaker amino acid interactions, whereas the fivefold axis of symmetry has intermediate amino acid interactions (Xie, Somasundaram, Bhatia, Bu, & Chapman, 2003b).

When assembled as a pentamer, the VP3 subunit forms 12 pores at the fivefold axis of symmetry. The pore is hypothesized to partake in assembly and packaging, and is likely necessary for Rep protein binding, capsid assembly, DNA packaging, and

exposure of the N terminus of VP1 for release from the endosome (Bleker, Sonntag, & Kleinschmidt, 2005; Grieger, Johnson, Gurda-Whitaker, Agbandje-McKenna, & Samulski, 2007).

1.3.3 AAV CAPSID FUNCTION

There are two main functions of the AAV capsid: receptor binding and release from the endosome. As mentioned in the previous section, surface loops on the AAV capsid are responsible for receptor binding and as targets for generation of neutralizing antibodies by the host immune system. For the second function, the VP1 protein contains a unique region on the N terminus having calcium-dependent phospholipase A_2 enzymatic activity. This sequence is buried deep within the AAV capsid and is exposed during cell entry in a pH dependent manner (Farr, Zhang, & Tattersall, 2005). The phospholipase A_2 activity helps facilitate release of the AAV particle from the endosome upon cell entry. Also found on the N-terminus of the VP1 protein is a basic amino acid motif, proposed to function as a nuclear localization signal for both cell entry and capsid assembly (Hoque et al., 1999). Nuclear localization can also be found on the VP2 capsid, thought to associate with VP3, facilitating transport of both proteins to the nucleus. Once transported to the nucleus, AAV capsid proteins can self-assemble (Sonntag, Schmidt, & Kleinschmidt, 2010). The smaller Rep proteins seem to sequester the single-stranded DNA to the capsid particles. Packaging of DNA into the capsid is thought to be carried out via the helicase activity of the larger Rep proteins (Berns & Giraud, 1996).

1.4 AAV GENOME

The genomic DNA of AAV is single-stranded of either negative sense or positive sense polarity, approximately 4.7 kb in length. The packaging of negative or positive

sense strands into AAV capsids are of equal proportions. The ends of the AAV genomes contain inverted terminal repeats (ITRs), that base-pair and form T-shaped, hairpin structures. Replication and packing of AAV genomes into capsids is initiated by *cis*-elements found within the AAV ITRs, which is recognized by AAV Rep proteins. The AAV genome consists of two genes: *rep* and *cap*. The *rep* gene encodes for four non-structural proteins (Rep78, Rep68, Rep52, and Rep40), while the *cap* gene encodes three structural proteins, which make up the capsid (VP1, VP2, and VP3). Three viral promoters are used for transcription: p5, p19, and p40. All transcripts contain a single intron, resulting in production of Rep78 and Rep52 for unspliced RNAs, and Rep68 and Rep40 for spliced transcripts. For capsid proteins, VP1 results from unspliced messages, whereas VP2 and VP3 arise from spliced RNAs. Translation for VP2 occurs from the weak start codon ACG, while VP3 arises from an ATG codon.

1.5 LIFE CYCLE

1.5.1 RECEPTOR BINDING AND TRAFFICKING

Our current understanding on the receptor binding and trafficking of AAVs is based on recombinant AAV vectors. As such, each AAV is observed to infect specific tissues, suggesting that they utilize different receptors or co-receptors. To name a few, AAV2, -3, -8, and -9 bind to the 37/67 Kilodalton Laminin receptor, AAV9 binds N-linked galactose, AAV2 and -3 bind heparin sulfate proteoglycan, AAV1, -4, -5, and -6 bind to different variations of O- or N-linked sialic acid, and AAV5 transduction is mediated by platelet derived growth factor receptor (Di Pasquale et al., 2003; Kaludov, Brown, Walters, Zabner, & Chiorini, 2001; Seiler, Miller, Zabner, & Halbert, 2006; Summerford & Samulski, 1998; Wu, Miller, Agbandje-McKenna, & Samulski, 2006).

Currently, many co-receptors for AAV2 binding have been identified and include: αvβ5 integrin, αvβ1 integrin, hepatocyte growth factor receptor, and CD9 (Asokan, Hamra, Govindasamy, Agbandje-McKenna, & Samulski, 2006; Kashiwakura et al., 2005; Kurzeder et al., 2007; Summerford, Bartlett, & Samulski, 1999). Here, it is evidenced that AAV transduction is not solely based on a single receptor, but different receptors or co-receptor. As well, many AAVs can bind more than one receptor, and bind similar receptors as other AAVs. Although the receptors for binding have been identified, little is known about the downstream effects of this binding. There still remains uncertainty on the signaling pathways for the role of AAV on enhancement of viral uptake and trafficking once it binds to the receptor. Once AAV binds to its receptor, it is thought to enter the cell via clathrin-coated vesicles (Bartlett, Wilcher, & Samulski, 2000). Via actions of the phospholipase A2 activity of VP1, AAV is able to escape from the endosome and is released into the cytoplasm, then trafficked to the nucleus by an unknown mechanism. Other parvoviruses have been shown to be actively transported to the vicinity of the nucleus via tubulin and dynein structures, suggesting that AAV may undergo this process as well (Vihinen-Ranta, Yuan, & Parrish, 2000). The N terminus of VP1 contains a nuclear localization signal, which is thought to facilitate entry into the nucleus via the nuclear pore complex. Following entry into the nucleus, intact AAV particles can be found where uncoating occurs, followed by processing of AAV DNA for second-strand synthesis (Grieger, Snowdy, & Samulski, 2006).

1.5.2 AAV DNA REPLICATION

Following uncoating, AAV DNA replication occurs within the nucleus. Before replication occurs, AAV must undergo second strand synthesis to make the DNA double-

stranded. Here, AAV relies upon host factors such as DNA polymerase d for replication. In the absence of helper virus, there is very limited expression of Rep 68/78; thus, preventing AAV gene expression, resulting in integration of AAV into the host chromosome. When helper virus is present, AAV DNA replication will occur. Adenovirus coinfection has been shown to activate the p5 (Rep68/78) and p19 (Rep40/52) promoter (Pereira, McCarty, & Muzyczka, 1997; Redemann, Mendelson, & Carter, 1989), resulting in expression of the Rep proteins essential for AAV DNA replication.

The AAV genome is flanked by ITRs containing motifs for the viral origin of replication, which are the terminal resolution site and Rep binding site. As the ITR is self-complementary and able to base-pair with itself, it provides a base-paired 3' hydroxyl group, acting as a primer for DNA replication via the host polymerase d (Nash, Chen, & Muzyczka, 2008; Slanina, Weger, Stow, Kuhrs, & Heilbronn, 2006; Ward, Falkenberg, Elias, Weitzman, & Linden, 2001). Extension occurs and the AAV template is copied. Essentially, the larger Rep proteins are able to recognize and bind to the Rep binding site and in turn, nick the terminal resolution site via its helicase activity, providing a 3' hydroxyl at the end of the ITR. This serves as a priming site for future AAV DNA replication.

1.5.3 AAV GENE EXPRESSION

As mentioned previously, the rate-limiting steps of AAV gene expression are: entry into the nucleus, uncoating and release of AAV DNA, and second strand synthesis. As well, the presence or absence of helper virus will affect the levels of gene expression. Three promoters are present in the AAV genome named: p5, p19, and p40. A

polyadenylation signal sequence is located downstream of the AAV genes, and an intron donor and two acceptor sites are found just after the p40 promoter (Berns & Giraud, 1996). Two mRNAs arise from the p5 promoter producing: Rep68 (spliced) and Rep78 (unspliced), whereas two mRNAs result from the p19 promoter, producing: Rep52 (unspliced) and Rep40 (spliced). The p40 promoter results in: VP1 (spliced product binds to first acceptor), VP2 (spliced product binds to second acceptor and splices out VP1 initiator codon; has a weak ACG start codon), and VP3 (same spliced product as VP2; has a strong ATG start codon). When helper virus is absent, Rep68/78 can repress AAV gene expression by binding to a site near the p5 promoter (Kyöstiö, Wonderling, & Owens, 1995). Limited Rep expression allows for establishment of a latent infection and integration of AAV DNA into the host chromosome. However, in the presence of adenovirus, Rep can activate all three of the AAV promoters (Pereira et al., 1997). The inhibitory properties of Rep68/78 on the p5 promoter can be suppressed by the adenovirus E1a transactivator or the herpes simplex virus ICP0, allowing for expression of AAV genes (Chang et al., 1989; Geoffroy et al., 2004). Other labs have shown that the AAV ITR itself can act as an initiator and enhancer of transcription, suggesting that host cellular proteins may recognize it since AAV DNA replication is reliant on host polymerase (Haberman, Mccown, Samulski, & Cown, 2000; Hölscher, Kleinschmidt, Bürkle, & Bu, 1995). Overall, AAV represses its own transcription and integration is established in healthy cells, whereas during co-infection with helper virus, AAV replication is enhanced.

1.5.4 HELPER VIRUS FUNCTION

AAV belongs to the genus *dependovirus* and as the name suggests, is dependent on other viruses in order to establish a productive infection. A productive infection occurs during co-infection with helper virus such as adenovirus or herpes simplex virus. The genes important for a productive infection from Adenovirus include E1a, E1b55k, E2a, E4orf6, and the viral associated RNA. E1a activates promoters in adenovirus, as well as the p5 promoter of AAV. E2a can bind single-stranded DNA and found at AAV replication centers; E2a has been shown to increase AAV replication *in vitro*. E1b55k and E4orf6 work in unison to enhance AAV replication and extension for second-strand synthesis (Fisher et al., 1996). It is thought that the viral associated RNA prevents phosphorylation of the eIF2alpha translocation factor, which in turn stimulates AAV protein expression (Nayak & Pintel, 2007). The main contributing factor of the helper virus function of adenovirus is to boost production of AAV proteins and modify the cellular milieu, supporting replication of AAV. By identifying the genes of adenovirus responsible for promoting AAV virus production, researchers have incorporated these genes in *trans*, allowing for production of recombinant AAV vectors without the presence of the helper virus itself (Xiao et al., 1998a).

Apart from adenovirus helper functions, many genes from herpes simplex virus 1 were also shown to provide helper virus function, including: UL5, UL8, UL52, and UL29. UL5, UL8, and UL52 make up the helicase/primase complex, and UL29 encodes for the DNA binding protein ICP8 (Weindler & Heilbronn, 1991). Other proteins from herpes simplex virus 1 not sufficient, but capable of enhancement of AAV replication, include: ICP0 transactivator that activates expression of Rep, the herpes simplex virus 1

DNA polymerase complex (the polymerase UL30 and co-factor UL42), and ICP4 and ICP22 proteins that enhances activity (Alazard-Dany et al., 2009; Geoffroy et al., 2004).

AAV replication has also been induced in the absence of helper virus and helper virus functions using treatments that cause genotoxic and cellular stress (Yalkinoglu, Heilbronn, Bürkle, Schlehofer, & zur Hausen, 1988). Such factors include UV irradiation, topoisomerase inhibitors, and hydroxyurea. However, the mechanism is unclear as to how these agents induce an environment supporting AAV replication.

1.5.5 AAV REP PROTEIN FUNCTION

The Rep proteins of AAV make up the non-structural proteins and consist of Rep40, Rep52, Rep68, and Rep78. The amount of Rep protein is proportional to the amount of transcript produced. The larger Rep68 and Rep78 are found primarily in the nucleus, while Rep52 and Rep40 can be found in the cytoplasm. Rep78/68 are required for all steps of the viral lifecycle including DNA replication, site-specific integration, production of virus from integrated genomes, and regulation of cellular and viral promoters (Hölscher et al., 1995; Kyöstiö et al., 1995; Labow, Hermonat, & Berns, 1986; Linden, Winocour, & Berns, 1996; Pereira et al., 1997; X. S. Wang, Ponnazhagan, & Srivastava, 1995). The smaller Rep52 and Rep40 seem to play a role in the sequestering and packaging of the AAV genomes, which may explain why an abundance of these proteins are seen during productive infection of AAV (Mouw & Pintel, 2000). Three functional domains are present in Rep78: DNA binding and endonuclease activity at the amino terminus, ATPase, helicase, and nuclear localization activity in the central domain (the most conserved among parvoviral non-structural proteins), and a Zinc-finger domain at the carboxy terminus. The remaining Rep68, Rep52, and Rep40 have various

combinations of these domains, which arise from alternative splicing and different promoters. All four Rep proteins contain the helicase and nuclear localization signal domain (Smith & Kotin, 2000).

1.5.6 AAV SITE-SPECIFIC INTEGRATION

Currently, wild-type AAV2 is the only eukaryotic virus shown to integrate into a specific location in the human genome (Linden et al., 1996). Following integration, AAV2 establishes a latent infection, allowing it to persist until a suitable environment permits progeny virus to be produced. AAV2 has been shown to integrate into a specific site known as AAVS1 on the q arm of human chromosome 19 (q13.4), which was found to contain a Rep binding site and terminal resolution site (Kotin, Linden, & Berns, 1992). The sequences in this site were found to include a 33-nucleotide sequence that contains the Rep binding site and terminal resolution site separated by 11 nucleotides. Recently, studies have shown that AAVS1 is not the only integration site existing for AAV; other sites have been identified also containing Rep binding sites located on chromosome 5p.13.3 and chromosome 3p24.3 (Hüser et al., 2010). AAV integration hotspots were found to be in open chromatin regions containing Rep binding site homologues. AAV generally integrates as a concatemer and is dependent on the helicase and endonuclease activity of the larger Rep proteins for site-specific integration. When Rep is absent, AAV DNA integration occurs randomly or may persist extrachromosomally (Mclaughlin, Collis, & Hermonat, 1988).

1.6 AAV TRANSMISSION

1.6.1 AAV PATHOGENESIS

Very little is known about the AAV natural infection, despite the fact that it has been studied for over 50 years. AAV is considered relatively safe as pathology and tumorigenesis is non-existent in AAV infections. In healthy cells, AAV is replication deficient. However, helper virus such as adenovirus, herpes simplex virus, or human papilloma virus can create an environment suitable for AAV replication numbering in the hundreds of thousands to one million copies per cell. The infected cells will die due to the AAV replication, and in this sense, AAV can have a protective effect on its host. Here, AAV may have evolved to be beneficial to both itself and its host (Weitzman & Linden, 2011).

Recently, it has been suggested that AAV may have a protective role in the prevention of cervical cancer (Agorastos et al., 2008). Studies have shown that infectious AAV can exist in human cervical biopsies, and it was proposed that AAV may be sexually transmitted with herpes or papilloma viruses (Walz et al., 1998). In addition, correlative studies have shown that women who seroconverted and had higher titers against AAV were less likely to develop cervical cancer (Georg-Fries, Biederlack, Wolf, & zur Hausen, 1984). 85% of healthy women had antibodies against AAV, while only 14% of cervical cancer patients had seroconverted (Mayor, Drake, Stahmann, & Mumford, 1976). However, it has also been proposed that AAV may lead to male sterility, as infectious AAV particles were found in semen samples from males who were diagnosed with infertility (Rohde et al., 1999).

Recently, studies have shown that AAVs can translocate between two different tissues. For example, AAV9 was suggested to pass thru endothelial cells, allowing for efficient transduction of cardiac and muscle cells when administered systemically. (Inagaki et al., 2006). As well, AAV serotypes 1, 2, 5, 6, 8, and 9 were shown to undergo axonal transport in the mouse brain (Aschauer, Kreuz, & Rumpel, 2013). Here, AAV is transported to different sites by an unknown mechanism. Viruses such as herpes simplex virus undergo retrograde transport, taking advantage of cellular proteins such as actin and dynein (Bearer, Breakefield, Schuback, Reese, & LaVail, 2000). In recent studies, there is evidence that AAV can associate with dynein and microtubules in the cytoplasmic environment (Kelkar et al., 2006). AAV can undergo retrograde transport, which may be due to mechanisms similar to that observed with other viruses.

1.6.2 PRE-EXISTING IMMUNITY

Approximately 80% of the human population has seroconverted against AAV. Antibodies against AAV1, -2, -5, -6, -8, and -9 have been found in many human subjects (Boutin et al., 2010a). This is quite significant as these serotypes are being utilized in clinical trials. In order for AAV vectors to be efficacious, subjects will have to be pre-screened for seroconversion. Otherwise, increased vector dose will have to be administered to overcome pre-existing immunity, increasing the risk of toxicity. In infants, neutralizing antibodies for AAV2 and AAV8 were present in 59% and 36% of newborns, respectively (Calcedo et al., 2011a). By the age of 10, 60% of children have neutralizing antibodies against AAV, with levels progressively increasing into adulthood. In addition to studies in humans, the presence of neutralizing antibodies has also been found in large animal models. Antibodies generated against AAV1, -2, -6, and -9 were

found in sera from sheep, rabbits, pigs, and dogs (Rapti et al., 2012). Here, the authors recommend that animals be pre-screened for AAV neutralizing antibodies before being considered for gene transfer experiments.

1.7.1 AAV SEROTYPES AND CELL AND TISSUE TROPISMS

AAV has been isolated as a contaminant in adenovirus preparations from sources including avian, bovine, human, non-human primate, rat, mouse, and snake (Atchison, Casto, & Hammon, 1965; Bossis & Chiorini, 2003; Farkas, 2004; G.-P. Gao et al., 2002; Lochrie et al., 2006; Schmidt, Katano, Bossis, & Chiorini, 2004). Apart from adenoviral stocks, a novel AAV called AAV5 was isolated from a human condylomatous wart (Bantel-Schaal & zur Hausen, 1984a). In total, 11 serotypes of AAV have been isolated from human and non-human primates, and hundreds of other isolates have been described (G. Gao et al., 2003, 2004a). Previously, AAV isolated from pig has never been described, and it was not until recently that our group was able to isolate novel AAVs from pig tissues (Bello et al., 2009; Puppo et al., 2013). The significance of the isolation of AAVs from pig tissues will be discussed in 5.1 and 5.2. AAV serotypes 2, 3, 5, and 6 were isolated from human cells, whereas AAV serotypes 1, 4, and 7-11 originated from non-human primates (G. Gao et al., 2003, 2004b). Tissue tropism of the AAV serotypes was found to be determined by 12 hypervariable regions on the capsid surface, with the most variability seen among the threefold axis peaks (G. Gao et al., 2003).

Although AAVs share similar structure, genome size, and organization, each AAV is considered to be unique in that each expresses specific tissue tropisms from one another (Wu, Asokan, & Samulski, 2006). As mentioned previously, the surface topology plays an important role in receptor binding and trafficking within cells, likely

contributing to the unique tropisms observed between AAVs. Current AAV vectors are being developed using rationale design; properties determining tropism of AAV vectors are being exploited in the development of next generation vectors, which are more efficient and have tightly regulated tissue specificity (Asokan, Schaffer, & Samulski, 2012; Asuri et al., 2012; Dalkara et al., 2013). However, the use of rationale design is limited to AAVs isolated from natural sources, and there is still much to be learned from the isolation of AAVs from alternative, natural sources. One of the main objectives of this thesis is the isolation of novel AAVs from pig tissues, which may form new clades and have unique tropisms to that of published AAVs.

AAV tropism has been studied in both cell lines and *in vivo*. When comparing AAV tropism in these two settings, there is no direct relationship between the transduction efficiency in immortalized cell lines and transduction in a related organ. For example, AAV8 was previously thought to be a poor vector due to its inefficiency in cell lines; but when injected *in vivo*, AAV8 was found to transduce muscle and liver cells quite efficiently (Ellis et al., 2013; G.-P. Gao et al., 2002). It was also suggested that factors responsible for modification of receptors *in vivo* are not present *in vitro*, highlighting the marked differences in transduction efficiencies between the two milieus (Bell et al., 2011).

The specific tissue tropisms of AAVs has been critically studied in many pre-clinical and clinical trials for treatment of ailments affecting the brain, muscle, liver, eyes, or lungs (Asokan et al., 2012; Limberis, Vandenberghe, Zhang, Pickles, & Wilson, 2009; Zhang & Duan, 2012). To name a few, AAV8 is tropic for the liver, central nervous system, and eye, AAV1, -6, and -9 for the heart and skeletal muscle, and AAV5

for the lung and central nervous system (Asokan et al., 2012). Despite development of these vectors for clinical trials, there still remains the risk of pre-existing immunity, as well as potential for adverse host immune responses. To prevent adverse effects of treatment, researchers have used glucocorticoids to immunosuppress the host's response (Nathwani et al., 2011). As well, the use of novel AAVs not circulating in the human population can be used to circumvent pre-existing immunity. The implications of the host immune response against AAV vectors and its transgene will be discussed in 1.10. One question not been closely looked at and posed by our lab is: what role does AAV tissue tropisms play in regards to the host's immune response? This thesis addresses the issue by examining the novel porcine-derived AAV tissue tropisms and providing its relationship to the host's immune response and protective efficacy against lethal challenge.

1.8 AAV VECTOROLOGY

Since the first account of naked DNA delivery to mammalian tissues, numerous methods for gene therapy have been established including gene gun, electroporation, sonoporation, viral vectors, etc. A good gene therapy vector should be designed to target specific tissues for a particular application and have low immunogenicity. Among these gene transfer methods being examined for *in vivo* delivery, viral vectors derived from AAV have shown promising results. The development of these vectors for gene therapy applications is attributed largely to their lack of pathogenicity, low immunogenicity, ability to establish stable long-term transgene expression in both dividing and post-mitotic cells, as well as their ability to target specific tissues. For example, AAV1, AAV8 and AAV9 specifically target muscle, liver and brain tissue, respectively, more

efficiently than most other serotypes. AAV serotypes with more pronounced tropisms would help to optimize therapeutic dosing in addition to decreasing undesirable side-effects associated with non-specific transduction.

1.8.1 ISOLATION OF NOVEL AAVS FROM NATURAL SOURCES

Initial AAVs were identified as contaminating particles in an Adenoviral preparation and since then, AAV has been isolated from numerous other sources including cell lines, human condylomatous genital warts, or other animal species such as birds, cows, mouse, and non-human primates. The isolation of novel AAV from different animal species has been performed to identify AAVs improving upon the properties of current AAV vectors. Recently, more than a hundred new AAV serotypes from non-human primates (NHP) were isolated (G. Gao et al., 2003). However, AAVs isolated from other natural sources have been overlooked in the past decade, with very few naturally occurring AAVs being described over the last five years. In clinical trials, the use of AAVs where pre-existing immunity exists could have an unfavorable impact on the host. Evidence of seroconversion against NHP AAVs within the general population will most likely reduce the efficacy should these vectors be used in human clinical trials. In the first AAV clinical trial to treat hemophilia B, relatively low neutralizing antibody titers completely neutralized large dose of the vector (Manno et al., 2006). As well, evidence of adverse host immune responses have been observed in a few of these trials (Boutin et al., 2010b; Halbert et al., 2006; Mingozzi & High, 2013; Mingozzi et al., 2007). Preceding our studies, the isolation of novel AAV from pigs had never been described. We believe that because of the high similarity between human and pig tissues, AAVs isolated from pigs may transduce human cells efficiently. Another favorable

feature of novel AAVs would be low seroprevalence in the human population. In our studies, we successfully isolated AAVs from pig tissues and show that there is little or no pre-existing immunity present against pig AAVs in the general population.

1.8.2 HOST IMMUNE RESPONSES TO AAV AND ITS TRANSGENES

AAVs currently used in clinical trials have been successful to various degrees. The host immune system appears to tolerate the transgene well, although adverse immune responses were seen, resulting in ablation of transduced cells and reduced transgene expression (Brantly et al., 2009; Manno et al., 2006). In these trials, it was not the transgene, but the AAV capsid itself, to which the immune response was mounted. Recent studies suggest that the route of administration and dosing plays an important role in the type of immune response mounted against the capsid or transgene (Brockstedt et al., 1999; Martino et al., 2009; Mingozzi et al., 2009). Essentially, AAVs injected intraperitoneally, intravenously, or intrahepatically seem well-tolerated; if the same vectors were administered intramuscularly, an immune response was observed against the transgene. To add to this, the amount of vector administered plays a role in the type of immune responses encountered: a low dose resulted in ignorance of transgene, an intermediate dose caused immunity against transgene, and a higher dose resulted in anergy of T cells via T cell exhaustion (Bharadwaj, Kelly, Kim, & Chao, 2010; Mingozzi et al., 2009). Here, a delicate balance between route and dose of vector is required in order to elicit the proper desired immune response. For example, a high or low dose via intrahepatic route would be good for gene therapy in the liver, whereas an intermediate dose via intramuscular injection would benefit for vaccination purposes.

Apart from the host immune response against AAV vectors, the mechanisms behind the induction of tolerance or stimulation of robust immune responses has been studied. Research has shown that the TLR9-MyD88 pathway is critical for the induction of adaptive immune responses against AAV(Zhu, Huang, & Yang, 2009). AAVrh32.33, a highly immunogenic AAV, stimulates robust immune responses against its transgene when injected into mice, resulting in abolishment of transgene expression (Lin et al., 2009). To show that the TL9 pathway could be circumvented, AAVrh32.33 was depleted of CpG sequences in its genome, resulting in sustained transgene expression (Faust et al., 2013). Aside from the immunogenicity of the AAV genome, the capsid structure is also important for driving CD4-dependent CD8+ T cell responses of AAV vectors (Mays et al., 2009). Further analysis of the AAVrh32.33 and other AAV capsid structure have mapped epitopes targeted for CD8+ T cell recognition (Li et al., 2009; Mays et al., 2013; Sabatino et al., 2005a). Due to the immunogenicity of AAVrh32.33, it was recommended that this serotype be developed as a vaccine vector candidate (Lin et al., 2009). For the vaccine studies in this thesis, AAVrh32.33 vector was utilized as a control.

The immune cell tropism may also play a role in the quality of immune response generated against the transgene. Adenovirus, a vector capable of inducing robust immune responses against its transgene, was shown to infect dendritic cells efficiently and induce maturation of these cells, most likely contributing to efficient presentation to cytotoxic T cells and stimulation of effective immune responses (Adams et al., 2009; Morelli et al., 2000). As well, the hexon protein of adenovirus was shown to have adjuvant-like properties, enhancing the immune responses generated against the

transgene (Kramp, Six, Drake, & Kasel, 1979; Molinier-frenkel et al., 2002). For these reasons, adenovirus has been developed as a vaccine vector against numerous diseases including human immunodeficiency virus, rabies virus, Ebola virus, etc. (Tatsis & Ertl, 2004). It was suggested that AAVs sharing qualities similar to adenovirus may serve as potential vaccine vector candidates. As mentioned previously, T cell epitopes have been mapped on numerous AAVs such as AAVrh32.33, which may contribute to the stimulation of robust T cell responses. As well, there is mounting evidence that certain AAVs can transduce dendritic cells efficiently (Aldrich et al., 2006; Aslanidi et al., 2012; Chiriva-Internati et al., 2003; Y. Liu et al., 2000; Ponnazhagan, Mahendra, Curiel, & Shaw, 2001; Veron et al., 2007; Xin et al., 2006). Other than the transduction of dendritic cells, the transduction of alternative antigen presenting cells has been largely ignored. In this thesis, AAV transduction of both dendritic cells and B cells was achieved, providing a mechanistic means for protective efficacy against influenza and Ebola virus infection in mice.

For the most part, AAVs are generally tolerogenic, as exemplified by the successfulness of AAV vectors in numerous clinical trials. It appears as though AAV is able to avoid inflammatory signals and actively suppress T cell responses (Somanathan, Breous, Bell, & Wilson, 2010). An example of AAV actively suppressing the immune response was shown in a mouse study with adenovirus; adenovirus is known to be highly immunogenic and results in generation of T cell responses against its transgene when injected into a host (Jooss & Chirmule, 2003). When adenovirus vector was administered post-AAV injection, the immune responses were found to be actively suppressed against the adenovirus transgene product (Somanathan et al., 2010). In order to understand the

mechanism behind tolerance induction, AAV8, previously shown to have tolerogenic properties, was extensively studied. The mechanisms behind AAV8 tolerance in the mouse muscle was attributed to the poor transduction of antigen presenting cells (APC), T cell exhaustion, and minimal MHCI upregulation on target cells (Mays et al., 2014). As well, AAV8 was shown to produce aberrant and non-functional T cell responses (Lin, Zhi, Mays, & Wilson, 2007a). The immune responses against AAV1 and AAV8 were compared, showing that both B and T cell responses were generated against AAV1 transgene product, whereas only B cell responses were generated against the AAV8 transgene product (Lu & Song, 2009). Therefore, it was proposed that AAV8 would serve as an excellent gene therapy vector due to its ability to induce tolerance. In this thesis, AAV8 was utilized in hopes that it would serve as a poor vaccine vector candidate. However, the results were quite unexpected, as will be described in the Results and Discussion sections of this thesis. A comparative analysis on the tropism, host immune response, and protective efficacy against lethal challenge using AAV has never been investigated. In this thesis, the role that AAV tropism plays in the protective efficacy against lethal challenge of influenza or Ebola virus will be discussed.

1.9 AAV VECTOR DESIGN

1.9.1 CONCEPTS BEHIND AAV VECTOR DESIGN

AAV are essentially defective viral particles relying heavily upon host machinery and protein from other productive viral infections in order to replicate. As such, AAV by itself is considered safe as it is not associated with any known human illnesses or diseases. The AAV genome is composed of two main genes: *rep* and *cap*, which are flanked by two ITRs. The ITRs are recognized by the Rep proteins, allowing for

cleavage and packaging of the AAV genomes into the pre-formed viral capsid particles. Researchers have realized the potential of AAV as viral vectors and deleted the *cap* and *rep* genes, replacing them with a gene of interest such as beta-galactosidase (β-gal) or green fluorescent protein (GFP). In gene therapy applications, a therapeutic gene to supplement a defective gene is usually inserted into the rAAV particles, whereas for vaccine applications, an immunogenic gene is utilized to stimulate the host immune response to protect against infection. For production of rAAV vectors, the *rep* and *cap* genes are expressed from plasmids provided in *trans*, while the gene of interest is produced in *cis* in relation to the viral vectors. When a gene of interest is flanked by AAV ITRs, this allows for recognition by AAV Rep proteins, thereby allowing packaging of the gene of interest into the AAV capsid particle and resulting in creation of rAAV vectors.

1.9.2 ELEMENTS OF GENE EXPRESSION CASSETTE

As mentioned previously, the gene of interest is flanked by AAV ITRs derived from AAV2. AAV Rep is also derived from AAV2, allowing for recognition of AAV2 ITRs, resulting in cleavage of the terminal resolution site and creation of recombinant single-stranded AAV genomes of either negative or positive polarity. AAV ITRs are responsible for the replication and packaging of recombinant AAV genomes into the vector particles. In the case of self-complementary AAV genomes, the terminal resolution site on one of the ITRs is deleted, preventing the Rep proteins from recognizing and cleaving this site. The resulting genome consists of both positive and negative sense polarity, which can fold upon itself and act as a double-stranded DNA genome. It is theorized that the self-complementary AAV DNA allows for quicker and

more efficient expression of the gene-of-interest due to elimination of the rate-limiting second-strand synthesis step (McCarty, Monahan, & Samulski, 2001). Apart from the ITRs, a promoter/enhancer is included for expression of the gene of interest. The cytomegalovirus (CMV) immediate early promoter/enhancer is a commonly used promoter, shown to be ubiquitous for driving high expression levels of transgene in a wide range of mammalian tissues and cells (Papadakis, Nicklin, Baker, & White, 2004). Downstream of the CMV promoter is the gene of interest, followed by a bovine growth hormone (bGH) polyadenylation (polyA) sequence for stabilization of messenger ribonucleic acid (mRNA) structure (Ribeiro, Monteiro, & Prazeres, 2004). AAV2 ITRs flank both the 5' and 3' end of the rAAV genome.

1.9.3 AAV PLASMID CONSTRUCTION

There are two main plasmids used in the production of AAV particles: *cis* and *trans*. The *cis* plasmid is comprised of the AAV2 ITRs, which flank a promoter, the gene of interest, and bGH polyA signal sequence. This plasmid also contains an origin of replication to allow for amplification of the plasmid in bacterial cells, as well as an ampicillin resistance gene for screening. As for the *trans* plasmid, this construct is responsible for the expression of Rep and Cap proteins. The *trans* plasmid is comprised of *rep* and *cap* genes containing three internal promoters: p5, p19, and p40. The p5 promoter initiates transcription for the large Rep proteins, p19 for the small Rep proteins, and p40 for the Cap proteins. For the helper plasmid, adenoviral genes E2a, E4, and VA are expressed to drive expression of *trans* plasmid genes and replication of rAAV genomes in the *cis* plasmid. All three plasmids contain the ampicillin resistance gene for screening of transformed bacterial cells.

1.9.4 AAV VECTOR PRODUCTION, EXPANSION, AND PURIFICATION FROM HEK293 CELLS

AAV vectors are generally produced in HEK293 or HEK293T cells. Triple transfection can be performed using the *cis*, *trans*, and adenoviral helper plasmid. These plasmids can be transfected using Lipofectamin, but due to the copious amounts of transfecting reagent required for large-scale production of AAV vector, a cheaper alternative is the calcium phosphate transfection method (Chen & Okayama, n.d.). 48-72 hours following transfection, cells are scraped and pelleted. The pellet is resuspended in buffer and the mixture undergoes 3-4 rounds of freeze-thaw cycles to liberate the viral particles from the cells. Following the freeze-thaw, viral particles can be found in the supernatant. Cellular debris is spun down and the supernatant collected for the clarification step. Several AAV vectors exhibit a binding affinity to heparin sulfate proteoglycan, allowing the use of heparin columns to purify the virus (Zolotukhin et al., 1999). If the binding affinity is not known, classical purification using iodixonal or cesium chloride (CsCl) gradients has also been used to purify virus (Choi, Asokan, Haberman, & Samulski, 2007). Chromatography is another option for purification of AAV vector particles, although binding is limited to specific AAV serotypes (Smith, Yang, & Kotin, 2008). All current methods of purifying AAV vectors are lengthy and cumbersome, but one lab has recently established a protocol, which can be completed within a week from start to finish using polyethylene glycol (PEG)/aqueous two-phase partitioning method, resulting in vector with higher transduction efficiency and higher yield to that of traditional methods (Guo et al., 2012).

1.9.5 TITRATION OF AAV VECTORS

Quantitative real-time PCR (qPCR) is a technique generally utilized for detection or quantification of pathogens, quantification of micro ribonucleic acid (microRNA), or quantification of gene expression products (Schmittgen & Livak, 2008). A commonality between all our AAV vectors is the presence of the bGH polyA signal sequence within the recombinant genomes. This sequence was exploited and primers designed to amplify a 70 base pair (bp) sequence from this region. A probe was designed to bind the middle of sequence. The probe is coupled with the fluorophore 6FAM on the 5'-end and the quencher TAMRA on the 3'-end. *Taq* polymerase has 5' – 3' exonuclease activity. When the probe is bound to the amplicon, *Taq* will release the fluorophore as it processes the DNA for amplification. *Taq* cleaves the rest of the probe sequence, and the remaining sequence eventually melts off the amplicon. Essentially, the quencher absorbs energy from fluorophore when they are both bound to the probe. Once liberated, the fluorophore's level of fluorescence increases, while the quencher's decreases, allowing for detection of fluorescence elicited by the fluorophore. The level of fluorescence is exponential as it directly relates to the amount of DNA being amplified, and the amount detected at a set threshold of the assay is directly related to the amount of starting material.

1.9.6 MODIFICATION OF AAV CAPSID

One major constraint associated with rAAV vectors is the possible presence of pre-existing immunity against the vector capsid, thus complicating vector administration or re-administration (Calcedo & Wilson, 2013; Calcedo et al., 2011b). To overcome pre-existing immunity, vector dose could be increased, likely increasing the chances of

toxicity. Another alternative is the use of AAV serotypes, which the human population has not been exposed to. Other methods to overcome pre-existing immunity include modification of the AAV capsid surface, thus preventing recognition by the host immune system. Such modifications include PEGylation of capsid surfaces, random peptide display, amino-acid mutations, or swapping of capsid surface domains (Asokan et al., 2012; Müller et al., 2003; X. Shen, Storm, & Kay, 2007). AAV naturally infects humans and a majority of the population has pre-existing immunity towards several human and NHP AAV serotypes, including AAV2, AAV5, and AAV8 (Calcedo, Vandenberghe, Gao, Lin, & Wilson, 2009). To modify tissue tropism, increase transduction efficiency, and circumvent pre-existing immunity, intelligent design employed shuffling of AAV genomes or the creation of tyrosine-mutants (Grimm et al., 2008; Zhong et al., 2008). However, although tailor-made AAVs have gained considerable popularity over the last few years, intelligent design is limited to the utilization of traits exhibited only by existing AAVs.

1.9.7 AAV AS GENE THERAPY VECTORS

Recently, AAV has been exploited as a gene therapy vector showing excellent safety profile in humans with varying degrees of success. AAV has been used in a number of phase I, II, and III clinical trials to treat inherited or acquired genetic disorders such as Batten's, Alzheimer's, Leber's Congenital Amaurosis (LCA), muscular dystrophy, and hemophilia (Asokan et al., 2012; Daya & Berns, 2008). Because AAV is limited to a packaging size of approximately 5 kb, many labs have come up with novel ways to overcome this disadvantage such as Oligo-Assisted AAV Genome Recombination (OAGR), or the modification of genes to reduce their footprint, yet retain their

functionality, such as the mini-Dystrophin gene (Hirsch, Storici, Li, Choi, & Samulski, 2009; Zhang & Duan, 2012). These vectors are promising as they have been shown to produce therapeutic levels of transgene in injected hosts.

Other than the limited packaging size, some AAV vectors are also susceptible to adverse immune responses, as seen in some human clinical trials. Pre-existing immunity towards several AAV serotypes subsists in the general human population, which was shown to affect several AAV vectors in early clinical trials. In a hemophilia clinical trial involving the use of AAV2, one patient who had a neutralizing antibody titer of 1:2 against AAV2 capsid was able to produce ~11% normal levels of human factor IX (FIX), while another patient who elicited an antibody titer of 1:17 prevented detection of therapeutic human FIX expression (Manno et al., 2006). AAV8, one of the best known AAVs to transduce hepatocellular targets, was used in a separate hemophilia clinical trial with therapeutic levels of human FIX achieved in most recipients; although transient levels of AAV8 CD8+ T cells were observed in the blood of one subject (Nathwani et al., 2011). In these studies, pre-existing humoral immunity and cross-reactive T cell responses may be responsible for ablation of transduced cells. In order to circumvent undesirable responses against AAV vectors, immunosuppression was carried out using glucocorticoids, allowing for maintenance of therapeutic levels of FIX expression. Although adverse immune responses were seen in some patients, prophylaxis in other patients was discontinued due to a lack of evidence of opposing host immune responses.

1.9.8 AAV AS VACCINE VECTORS

AAV is considered an attractive vehicle for gene therapy applications due to its apparent lack of pathogenicity and its safety record. However, very little is known about

the protective efficacy of AAV as a vaccine vector to treat infectious diseases. Previous studies have utilized AAV to vaccinate against disease such as human papilloma virus, simian immunodeficiency virus, human immunodeficiency virus, herpes simplex virus, and swine influenza in animal models (Johnson et al., 2005; D. W. Liu et al., 2000; Manning et al., 1997; Sipo et al., 2011; Xin et al., 2006). In each of these studies, AAV was able to elicit robust and protective immune responses towards its transgene. However, in phase I and II clinical trials for AAV expressing HIV gag, protease, and parts of the reverse transcriptase, the results were somewhat disappointing (Mehendale et al., 2008; Vardas et al., 2010). Since development of a vaccine against HIV has proven somewhat difficult, AAV should not be ruled out as a vaccine vector candidate for other diseases since robust immune responses were still observed for the HIV vaccine, as well as other vaccines utilizing AAV.

Although AAVs such as AAV8 are thought to be tolerogenic, other AAVs are being developed as vaccine vector candidates. As mentioned previously, AAVrh32.33 was shown to induce robust T and B cell responses against its transgene, including β-gal and HIV gag (Lin et al., 2009; Mays et al., 2009). Recently, AAV9 encoding H1N1 HA was employed as an H1N1 vaccine vector, affording 100% protection in challenged mice (Sipo et al., 2011). Though AAV8 is thought to induce tolerance, recent studies employing AAV8 as a vaccine vector against henipaviruses was found to induce robust humoral responses, affording 100% protection against lethal challenge of Nipah virus in guinea pigs (Ploquin et al., 2013).

Another emerging method of AAV vaccinology is the development of AAVs as immunoprophylaxis. The recent identification of broadly neutralizing antibodies has

permitted researchers to encode these sequences into AAV vectors, providing expression of these antibodies when injected into a host. AAVs encoding broadly neutralizing antibodies for HIV or pandemic swine influenza showed efficacy in mice (Balazs et al., 2012; Balazs, Bloom, Hong, Rao, & Baltimore, 2013; Limberis et al., 2013). This technique was described as vectored immunoprophylaxis and may one day prove useful in long-term, broad protection against future strains of influenza or HIV.

Overall, the characterization of AAVs as vaccine vectors has been very limited over the last 15 years, with only a handful of publications being produced. One of the goals in this thesis is the development of porcine-derived AAVs as vaccine vectors. Here, the protective efficacy and immune responses of porcine-derived AAVs are compared to AAV8 and AAVrh3.33. The results found that the protective efficacy of each AAV differed from one another. As well, a relationship was observed between the immune responses elicited, protective efficacy, and tropism of certain AAVs in a lethal Ebola virus challenge. These findings contribute to a better understanding on the role of AAV as vaccine vector candidates.

1.10 THE IMMUNE SYSTEM

1.10.1 INTRODUCTION

The host immune system is composed of two parts: the innate and the adaptive immune system. Essentially, the innate immune response is the first responder to a pathogen and considered non-specific. Components of the innate immune system include barriers such as the skin and mucosal surfaces, as well as the complement system. Another element of the innate immune system are specialized cells called antigen presenting cells (APC), which are capable of engulfing invading pathogens and degrading

their proteins in the lysosome. The antigenic peptides can then be presented to T cells, in effect activating them. Because APCs are capable of activating T cells, they are considered an important link between the innate and adaptive immune system. APCs can activate naïve T cells by presenting peptides via MHC II to the T cell receptor. APCs include cell types such as dendritic cells (DC), B cells, and macrophages. On the surface and found within APCs are pattern recognition receptors, also known as Toll-like receptors. These receptors have evolved to recognize pathogen-associated molecular patterns, which are not normally found within mammalian cells. When Toll-like receptors bind their ligand, they activate a signaling cascade which results in production of cytokines or chemokines, signaling and modulating the responses of other specialized immune cells to the site of infection.

Other cells of the innate immune response include natural killer cells. These cells are similar to T cells in that they can recognize and kill infected cells presenting peptide via MHC I. However, natural killer cells are different from T cells due to their ability to kill without being activated, allowing for a faster response as with viral infection. Natural killer cells kill infected cells via granzyme action. Apart from recognition of MHC I, natural killer cells are also capable of inducing apoptosis in opsonized cells via antibody-dependent cell-mediated cytotoxicity (Clynes, Towers, Presta, & Ravetch, 2000). Natural killer cells express FcγrIII, which can bind the Fc portion of antibodies on opsonized targets.

The adaptive immune system consists of cells highly specific for an antigen. Two branches of the adaptive immune system are T cells and B cells. T and B cells arise from a common progenitor cell in the bone marrow. However, B cells are selected for in the

bone marrow and mature in the spleen, whereas T cells undergo selection and maturation in the thymus. The T cell, B cell paradigm consists of two arms of the immune system, capable in inhibiting each other. When Th1 cells are activated, IFNγ expression can inhibit Th2 responses, whereas when Th2 cells are activated, IL-4 secretion prevents Th1 type responses.

1.10.2 T CELL RESPONSES

T cells consist of two main subtypes: $CD4^+$ T cells (helper cells) and $CD8^+$ T cells. Helper cells can by further divided into subsets including Th1, Th2, Th17, and Tregs. Briefly, T cells must first be activated by APCs before they can respond to a pathogen. Essentially, $CD4^+$ T cells are activated when presented antigen via MHC II on an APC, as well as binding to co-stimulatory molecules CD40 and CD80 via CD40L and CD28, respectively. $CD8^+$ cells are activated when they encounter antigen presented via MHC I and co-stimulatory molecules, and proliferate in the presence of IL-2. T cells can be found circulating in the lymphatic system and blood stream, and are also found in secondary lymphoid tissues such as the spleen and lymph nodes. There, T cells encounter APCs which can present antigen, allowing for activation of the T cell. Generally, when activated, Th1 cells secrete IFNγ, IL-12, and TNFα, Th2 secrete IL-4, IL-5, and IL-13, whereas Treg secrete IL-10 and TGFβ. Th1 cells facilitate cell-mediate immune responses, whereas Th2 promote humoral immune responses. As the name suggests, Tregs are capable of suppressing T cell responses. When $CD8^+$ T cells are activated, they become cytotoxic T lymphocytes capable of recognizing antigen bound to MHC I on infected cells, and killing of these cells via granzyme activity, also referred to as the cell-mediated immune response. Cytotoxic T lymphocytes are also capable of

killing infected cells by binding its Fas ligand to the Fas receptor on the infected cell, inducing apoptosis.

1.10.3 B CELL RESPONSES

B cells originate in the bone marrow and are selected for when bound weakly to self-antigen. When bound strongly (indicative of binding to self-antigen), the B cell will receive signals to undergo apoptosis or anergy. If the B cell passes the selection process, it leaves the bone marrow, but is still considered immature. B cells will mature when they migrate to the spleen, becoming naïve, follicular zone, or marginal zone B cells.

One of the most important aspects of B cell immunology is their ability to secrete antibody in response to a pathogen. Initial antibody secretion from marginal zone B cells consists of IgM. After encounter with its antigen, the marginal zone B cell will convert to an IgM secreting plasma cell. Soon afterwards, the B cell will undergo class switching and affinity maturation, becoming plasma cells capable of secreting IgG antibodies. The secreted antibodies can serve many functions including neutralization, opsonization, or antibody-dependent cell-mediated cytotoxicity.

Other than antibody secretion, B cells have also been shown to serve as APCs. B cells express TLR9 and when activated, are capable of presenting antigen to $CD4^+$ and $CD8^+$ T cells via MHC II, as well as upregulation of co-stimulatory molecules CD40 and CD80 (Hua & Hou, 2013; W. Jiang et al., 2007; Rodríguez-Pinto, 2005). In this manner, the activated T cells showed signs of proliferation when co-cultured with B cells.

Apart from APC function, Breg function has recently been described in the suppression of T cell responses (Kessel et al., 2012). In an experiment, Bregs were purified from human blood and co-cultured with $CD4^+$ T cells. Even in the presence of anti-CD3, anti-CD28 antibody, and IL-2, the proliferative capacity of the $CD4^+$ T cells was severely reduced in the presence of Bregs. In a separate experiment, Tregs were isolated and co-cultured with Bregs. The results showed that Bregs can enhance Treg function by increasing Foxp3 and CTLA-4 expression in the Tregs.

1.11 HIGHLY PATHGENIC AVIAN INFLUENZA VIRUSES (H5N1)

1.11.1 INTRODUCTION

Influenza viruses belong to the family *Orthomyxoviridae*, comprised of five genera: *Influenza A, Influenza B, Influenza C, Thogotovirus, and Isavirus*. Viruses in this family have a negative-sense, single-stranded, segmented RNA genome. Influenza viruses are responsible for highly contagious respiratory diseases, quite often with fatal outcomes. In consideration of this thesis, this section will focus on the highly pathogenic avian influenza viruses (H5N1) belonging to *Influenza A. Influenza A* viruses possess a lipid membrane obtained from the host cell. The envelope is composed of the hemagglutinin (HA), neuraminidase (NA), and M2 proteins. Beneath the envelope are the matrix protein and the ribonucleoprotein complex, making up the core of the virion. This core consists of: viral segmented RNA genome, polymerase proteins polymerase basic 1 (PB1) and polymerase basic 2 (PB2), the nucleoprotein, and polymerase acid (PA).

Symptoms of H5N1 infection include headache, fever, sore throat, cough, sneezing, and body aches. Incubation of H5N1 typically takes between 2 to 5 days

before the onset of disease; however, periods of 8 to 17 days incubation periods have been reported (Beigel et al., 2005). Case fatality occurred in 89% of children less than 15 years of age in Thailand, with the average death occurring between days 9 and 10 after onset of illness (Chotpitayasunondh et al., 2005). Even though avian influenza infects primarily birds and poultry, the realization of the transmission directly into humans have raised the threat level of H5N1. Granting human-to-human transmission of H5N1 has not been confirmed, there still remains the possible threat of H5N1 becoming a pandemic.

1.11.2 EPIDEMEOLOGY

The first strain of the highly pathogenic avian influenza A (H5N1) recognized to infect humans occurred in May 1997 (Ku & Chan, 1999). Soon afterwards, 17 more cases were detected in late 1997 in Honk Kong, with 6 deaths (P. K. S. Chan, 2002). To date, 4 clades (0, 1, 2, and 7) and 3 subclades (2.1, 2.2, 2.3) of H5N1 virus strains have been shown to infect people (Abdel-Ghafar et al., 2008). In Southeast Asia between the years 2003-2005, most H5N1 infections were caused by clade 1 strains. Between 2004-2005, clade 2 H5N1 strains were circulating in poultry and wild bird populations from Africa, Asia, Middle East, and Europe (Webster & Govorkova, 2006). Subclade 2.1 virus strains circulating in poultry caused infection in humans in Indonesia, while subclade 2.2 strains infected humans and birds in Asia, Africa, and Europe (Abdel-Ghafar et al., 2008). Transmission of subclade 2.3 between poultry and humans has been detected in China and Southeast Asian countries (Le et al., 2008). According to the World Health Organization, as of January 2014, there existed 650 cases of H5N1 with 386 deaths in humans

(http://www.who.int/influenza/human_animal_interface/EN_GIP_20140124CumulativeNumberH5N1cases.pdf?ua=1).

1.11.3 IMPACT ON HUMAN HEALTH

Influenza viruses have a very high mutation rate often carried out by genetic drift (accumulation of mutations within a gene) or genetic shift (two or more viruses of different strains infect the same host and recombine to form a new subtype). Mutations can occur by reassortment or rearrangement of viral RNA segments between two different influenza subtypes infecting a common host. The high mutation rate of influenza allows for evasion of the host immune response, as most infected hosts are unlikely to have had a previous encounter with the evolved virus. The highly pathogenic H5N1 avian influenza viruses have been shown to evolve rapidly, leading to outbreaks in poultry. H5N1 viruses bind specifically to sialic acid α2,3 receptors (van Riel et al., 2006). In humans, the upper respiratory contains mainly sialic acid α2,6 receptors, preventing this binding. However, avian influenza was shown to predominantly bind to type II pneumocytes, alveolar macrophages, and nonciliated cuboidal epithelial cells in the lower respiratory tract in humans (van Riel et al., 2006). Although not yet considered pandemic, the high mortality rate (>60%) and high mutation rate of H5N1 is of importance for its socioeconomic implications and clinical threat (Neumann, Chen, Gao, Shu, & Kawaoka, 2010).

1.11.4 IMMUNE RESPONSES TOWARDS INFLUENZA

High replication of H5N1 in pharyngeal tissue is correlated with low T cell counts and high levels of chemokines and cytokines in the peripheral blood of diseased patients (de Jong et al., 2006). As well, elevated levels of proinflammatory cytokines could be

found in human primary alveolar and bronchial epithelial cells, as well as in macrophages from patients infected with H5N1 (M. C. W. Chan et al., 2005; Cheung et al., 2002). In mouse models, H5N1 appears to facilitate inflammation leading to alveolar damage by releasing of oxidized phospholipids, which activates Toll-like receptor 4 and stimulates IL-6 production (Imai et al., 2008). In the H5N1 outbreaks of 1997, elevated levels of IL-6, TNFα, interferon gamma (IFNγ), and soluble IL-2 receptor were reported in infected patients (To et al., 2001). In patients who succumbed to disease, plasma levels of inflammatory mediators (IL-6, IL-8, IL-1β, and monocyte chemoattractant protein 1) were found to be much higher than in survivors (Beigel et al., 2005). Although studies point towards the inflammatory response as contributing to pathogenesis of H5N1 infection, mice deficient in IL-6, TNFα, CCL2 chemokine, or mice treated with corticosteroids still succumbed to disease when infected with H5N1 (Salomon, Hoffmann, & Webster, 2007). Therefore, proinflammatory cytokines contribute to overall pathogenesis of disease symptoms, but other immune responses may also be implicated in the immunopathology of influenza viruses.

1.11.5 CURRENT VACCINE STRATEGIES AND EXPERIMENTAL VACCINES

Due to the high mutation rates and lethality of H5N1 infection, this disease provides a challenging setting for vaccine development. The hemagglutinin (HA) glycoprotein is responsible for receptor binding and facilitating fusion of the virion, and both protective cellular and humoral immune responses can be generated against this antigen (Shantha Kodihalli, Goto, Kobasa, Kawaoka, & Webster, 1999). Although vaccines are developed yearly for seasonal strains of influenza, currently no approved vaccine has been developed against H5N1 in humans. Generally, a good vaccine against

H5N1 should elicit a hemagglutination-inhibition (HAI) titer >40 when injected into a host; this titer is also considered the 'gold-standard' as a correlate of anti-influenza immunity (Defang et al., 2012). The purpose of HAI assays is to test the ability of sera to disrupt binding of the influenza hemagglutinin antigen to the sialic-acids on red blood cells, which represents the ability of the antibodies to prevent attachment of influenza virus to the sialic acid residues on target cells of the respiratory tract. Other than humoral responses, cell-mediated immune responses have also been shown to play a role in clearance of influenza virus. In B cell depleted naïve mice infected with influenza, viral clearance was facilitated by cell-mediated immune responses with only slightly delayed kinetics compared to wild type (Teijaro, Verhoeven, Page, Turner, & Farber, 2010). One method of measuring cell-mediated immunity induction is by enzyme-linked immunospot (ELISPOT) assay of IFN-γ expressing cells (Mbawuike, Zang, & Couch, 2007). However, due to the variable HLA backgrounds and responses in humans, cell-mediated correlates are unlikely to be universal, but would provide a means to detect for the presence of cytotoxic T lymphocytes (Mccullers & Huber, 2012).

Experimental vaccination against H5N1 include whole inactivated virus produced by a Vero cell line, shown to elicit neutralizing antibodies in human clinical trials (Ehrlich et al., 2008). In a separate phase III clinical study, 3.75 μg HA of A/Indonesia/05/2005 was administered with $AS03_A$ adjuvant (10% DL-α-tocopherol-based oil-in-water emulsion), resulting in the generation of HAI titers in human volunteers (Langley et al., 2011). In a phase I clinical trial involving the intradermal administration of low-dose H5N1 vaccine, immune responses similar to that of a regular

dose intramuscular vaccine were observed, providing an alternative, less invasive administration route (S. M. Patel, Atmar, El Sahly, Cate, & Keitel, 2010).

Recently, the development of adenovirus based vaccine vectors against H5N1 has been evaluated with great promise (A. Patel, Tikoo, & Kobinger, 2010). Other experimental methods proposed the genetic modification of chickens expressing a short-hairpin RNA that can control H5N1 infection (Lyall et al., 2011). As well, an AAV vaccine expressing the A/Mexico/4603/2009 (H1N1) HA of H1N1 was shown to provide 100% protection against homologous challenge in mice (Sipo et al., 2011). Recently, neutralizing antibodies preventing the fusion process, which follows binding of HA to its receptor, were described (Ekiert & Wilson, 2012). These antibodies were characterized as having broad spectrum neutralization as they were able to bind to conserved regions of multiple sub types of influenza. Knowing the sequence of these antibodies, researchers have cloned this into AAVs, which can constitutively express the antibody and provide protective efficacy against multiple subtypes of influenza in mice and ferrets (Limberis et al., 2013).

As mentioned previously, one of the criteria for successful influenza vaccines are those which can produce high HAI titers. As well, the correlates of protection for influenza infection point more towards humoral responses. As such, the AAV vectors utilized in this thesis were assessed for their ability to induce humoral responses against the influenza HA transgene. For the experimental purposes of this thesis, AAV vectors encoding the HA gene from the A/Hanoi/30408/2005 H5N1 (H05) influenza A isolate (AAV-H05HA) were employed. For humoral immune responses, the neutralizing antibody and HAI titers were examined, whereas for cell-mediated immunity, the IFN-γ

responses by ELISPOT were investigated. The analysis of challenge studies and the ensuing immune responses from AAV-H05HA vaccinations will help accompany the goals of this paper, linking the protective efficacy of AAV vectors to its tissue tropism.

1.12 ZAIRE EBOLAVIRUS

1.12.1 INTRODUCTION

The *Ebolavirus* genus is a member of the family *Filoviridae* in the order *Mononegavirales*. *Ebolaviruses* are enveloped viruses whose genome is a nonsegmented, negative-sense RNA molecule. To date, five species of *ebolavirus* belong to *Filoviridae*: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SEBOV), *Reston ebolavirus* (REBOV), *Tai Forest ebolavirus* (ICEBOV), and *Bundibugyo ebolavirus* (BEBOV). For the sake of this thesis, the focus of this section will be placed on EBOV. The EBOV virion forms a pleomorphic shape, appearing as elongated filamentous particles up to 14,000 nm with an 80 nm diameter (Beer, Kurth, & Bukreyev, 1999). The virion membrane contains VP24 (causing interferon signalling interference) and glycoproteins (GP1 and GP2) (Reid et al., 2006). Contained within the virion is the genome encoding 7 genes: nucleoprotein, virion protein (VP) 24, VP30, VP35, VP40, glycoprotein, and RNA-dependent RNA polymerase (L) (Beer et al., 1999). The nucleoprotein encapsulates the genome and is associated with VP30, VP35, and L, forming the transcriptase-replicase complex (Mühlberger, Weik, Volchkov, Klenk, & Becker, 1999). VP35 functions as an interferon antagonist, while VP40 facilitates particle formation and forms the matrix protein (Basler et al., 2000; Noda et al., 2002).

1.12.2 EPIDEMEOLOGY

Naturally occurring *Ebolavirus* infections most often originate from the rain forests of sub-Saharan Africa, as well as the Philippines (Peterson, Bauer, & Mills, 2004; Peterson, Lash, Carroll, & Johnson, 2006). However, imported cases of *Ebolavirus* originating from the Philippines or Africa have been reported in both humans and non-human primates in USA and Germany, highlighting the risk of potential outbreaks from imported virus (T W Geisbert & Jahrling, 1990; Siegert, Shu, Slenczka, Peters, & Müller, 1967). Disease occurs when a human comes into contact with an infected animal, and outbreaks result from person-to-person transmission of the virus through direct contact with the body, bodily fluid, clothing, or linens of infected individuals (Baron, McCormick, & Zubeir, 1983; Bausch et al., 2007). The first reported cases of filovirus hemorrhagic fever occurred in Germany and the former Yugoslavia in 1967, with the causative agent determined to be MEBOV (Siegert et al., 1967). Almost ten years later, *Zaire ebolavirus* emerged in 1976 in the former northern Zaire, now Democratic Republic of Congo. At the same time during this outbreak, there also emerged the presence of *Sudan ebolavirus* in southern Sudan. Since then, EBOV has emerged/re-emerged in regions of Republic of Congo, Dominican Republic of Congo, and Gabon, with the latest EBOV outbreaks occurring in 2008-2009 in the province of Kasai Occidental in the Dominican Republic of Congo, and the most current in 2014 in Guinea and neighboring Liberia.

1.12.3 IMPACT ON HUMAN HEALTH

EBOV virulence in humans is variable, with case-fatalities ranging between 60-90%. Symptoms associated with EBOV infection often include fever, weakness, muscle

pain, sore throat, and headaches. Progression to severe disease onset presents symptoms such as vomiting, diarrhea, rash, kidney and liver dysfunction, and both internal and external bleeding. EBOV incubation time ranges between 2 to 14 days, with death occurring between days 6 and 16 (Heinz Feldmann & Geisbert, 2011). As EBOV has one of the highest lethality of the hemorrhagic fever viruses, it is often regarded as the prototypic pathogen of the viral hemorrhagic fever viruses. EBOV is one of the most aggressive viruses known to man and is considered an important public health pathogen and Category A biothreat pathogen due to the high lethality associated with EBOV infection and absence of pre- and post-exposure treatment or vaccine. The increase in frequency of natural EBOV infection, potential for importation, absence of treatments or vaccine, and potential as a bioterrorism weapon warrants the development of treatments and vaccines as a priority for many nations.

1.12.4 HOST IMMUNE RESPONSES TO ZAIRE EBOLAVIRUS

In order to mount an effective immune response against viral infection, early events are crucial to establish a balance between stimulatory and inhibitory signals. Establishment of this balance is carried out by mediators of the innate response such as macrophages and dendritic cells, shown to be important for initiating a link to the adaptive immune response. Some of the primary targets of EBOV have been shown to be macrophages, dendritic cells, and monocytes (Thomas W Geisbert et al., 2003; Ryabchikova, Kolesnikova, & Luchko, 1999). Infection of macrophages, monocytes, and dendritic cells by EBOV appear to facilitate release of pro-inflammatory cytokines including tumour-necrosis factor (TNF) and IFN-γ, resulting in disruption of the endothelium architecture and causing leaky membranes (H Feldmann et al., 1996; Wahl-

jensen, Kurz, Hazelton, & Schnittler, 2005). EBOV has also shown to disrupt primary innate immune responses by active suppression of type I IFNs using its VP35 protein, as well as the interference of IFNα, IFNβ, and IFNγ, to prevent induction of antiviral states in the cellular environment (Bosio et al., 2003; Cárdenas et al., 2006; Reid et al., 2006). NK cells, another component of the innate immune response, were shown to be important for early protection against filoviruses in mice (Warfield et al., 2004). As IFNs are important for activation of NK cells, it was suggested that the EBOV downregulation of IFN indirectly affects the efficacy of NK cells during infection (Mohamadzadeh, Chen, & Schmaljohn, 2007).

For adaptive immune responses, mature dendritic cells are crucial for T cell signaling. Studies have shown that EBOV infection in dendritic cells prevents the transition from the immature to mature antigen-presenting stage, thus preventing proper T cell signaling (Mahanty et al., 2003). Infection of dendritic cells found co-stimulatory molecules (CD40, CD86, and IL-12) to be downregulated, indicating a dysfunctional adaptive response important for T cell activation.

1.12.5 VACCINE STRATEGIES

The gold-standard for recapitulation of EBOV symptoms seen in humans is infection in guinea-pigs and non-human primates; therefore, vaccine development and treatment against EBOV is most reliable in these animal models (Connolly et al., 1999; Xu et al., 1998). Although a mouse adapted strain of EBOV (MA-EBOV) has been developed to cause lethality in mice, the symptoms seen in this model are not faithfully reproduced as those seen in humans (Bray, Davis, Geisbert, Schmaljohn, & Huggins, 1998). However, the development of treatments against EBOV in mice is a good

stepping stone as these animals are relatively cheap compared to guinea pigs and non-human primates.

The immune correlates of protection against EBOV are still unclear and quite controversial, as either T cells or B cells have been implicated in protection against EBOV infection in animal models (Bradfute, Warfield, & Bavari, 2008; Qiu et al., 2012). Nevertheless, many successful experimental vaccines have been developed, which can stimulate both robust T and B cell responses, successfully protecting against EBOV in animal models. One major target of EBOV for vaccine development is the surface glycoprotein (GP), shown to be necessary and sufficient for protection against lethal challenge in non-human primates. Guinea pigs vaccinated with a DNA vaccine encoding the EBOV GP elicited robust antibody and T cell responses, affording long-lasting protection when challenged with lethal virus (Xu et al., 1998). Other vaccine strategies include virus-like particles (VLPs), Venezuelan equine encephalitis virus, adenovirus vectors, and vesicular stomatitis virus; all of which were able to afford complete protection in mice and guinea pigs, while stimulating robust cell-mediated and humoral immune responses (Garbutt et al., 2004; Pushko et al., 2000; Richardson et al., 2009).

In previous studies, passive transfer of antibodies from vaccinated guinea pigs into naïve animals did not confer protection (Nancy J Sullivan et al., 2011; Xu et al., 1998). The researchers hypothesized that T cell responses played more of an important role in protection against EBOV infection. Using these criteria, AAV expressing EBOV GP was utilized to evaluate the potential of the AAV transgene to stimulate T cell responses. The use of AAV as a vaccine vector against EBOV has never been described in the literature. In this thesis, the protective efficacy of AAV vectors expressing EBOV

GP was evaluated against lethal challenge of EBOV in mice. Much like the influenza studies component of this thesis, the immune responses generated against the AAV-EBOVGP vector were evaluated. The protective efficacy and ensuing immune responses elicited by the AAV-EBOVGP vectors were compared with its tissue tropisms.

1.13 OJECTIVES AND HYPOTHESES

1.13.1 SIGNIFICANCE OF RESEARCH

AAV is a non-pathogenic virus, which shows great promise in gene therapy and vaccine applications; however, many AAVs utilized in these studies are predisposed to pre-existing immunity within the general population. Since human and porcine share considerable sequence homology and immune similarities, we hypothesized that AAVs exist in porcine tissues and AAVs isolated from pigs would be able to transduce mammalian and human cells efficiently. Pig heart valves have been used extensively in xenotransplants in human recipients, with an apparent lack of toxicity. As well, cellular components such as the swine leukocyte antigen and human leukocyte antigen share significant homology; antigens presented by swine leukocyte antigen have been shown to stimulate T cells derived from humans. These examples highlight the similarities between human and pig tissues and the reason why AAVs isolated from porcine tissues may one day be used as gene delivery tools to supply a gene-of-interest into human recipients. In these studies, the isolation of novel porcine-derived AAV vectors were not neutralized by human sera and did not generate cross neutralizing antibodies (NAb) towards other published AAV serotypes. Here, the novel porcine-derived AAVs were characterized *in vivo* and their ability to transduce common organ targets for gene therapy and vaccine applications such as the lung, brain, liver, and muscle were assessed. The

main focus of this lab is vaccine vector development and as such, the porcine-derived AAVs were characterized for their potential to protect against some of the most lethal and aggressive diseases known to man, namely H05 and EBOV infections. As the project advanced, it became apparent that each AAV exhibited unique immune responses towards its transgene. Here, the immune response towards the AAV transgene was characterized in order to identify AAV vectors best suited for vaccine applications. It was observed that AAV vectors exhibit pronounced differences between each other in respect to their tropisms and their abilities to protect against lethal challenge, and a link between these two traits may exist. It was hypothesized that AAV vectors showing protective efficacy against lethal challenge may share common tissue targets. Looking more closely at the immune cellular level, it was hypothesized that AAV vectors able to infect APCs such as dendritic cells or B cells would provide for better protective efficacy. These studies are significant because a relationship between tissue tropism and the ensuing protective efficacy and host immune responses has never been examined as in depth as presented in this thesis. Also, a relationship between AAV immune cell transduction and protective efficacy in *in vivo* studies has never before been described in the literature. The ability of AAV in transduction of B cells has mainly been ignored, and in these studies, AAVs equipped to protect well against lethal challenge were also shown to transduce B cells quite efficiently. The results of this phenomenon will be discussed in more detail in the discussion section (Chapters 4.6 and 4.7).

1.13.2 HYPOTHESES

According to the literature, AAV has never before been described in pig tissues. Thus, the original hypothesis for this project postulated that novel AAV DNA can be

isolated from pig tissues. With the close genetic similarities between human and pig genomes, it was also hypothesized that porcine-derived AAVs can efficiently transduce mammalian and human cells:

1) AAV DNA exists within porcine genomic DNA and can be isolated by PCR

2) AAVs isolated from porcine tissues can transduce mammalian and human cells efficiently

Following advancement of this project and the realization that each AAV has its own unique tissue tropism, more hypotheses were generated to address the issue of the differing immune responses observed against the transgene of each AAV vector serotype. These hypotheses were designed to provide a challenge model, which the protective efficacies of each vector could be compared to each other, as well as show a relationship between the tissue tropisms of the different AAV serotypes to their respective protective and immunostimulatory properties:

3) AAVs delivering immunogenic transgene can protect against homologous lethal infection

4) AAVs offering protective efficacy against lethal challenge share common tissue tropisms

As more evidence emerged during work on this thesis, further attention was paid to the tropism of AAV in the hosts' immune cells. It was hypothesized that AAV transduction of immune cells itself is important for dictating the quality of protective immune

responses elicited in a host. Vaccine vectors utilizing adenovirus are shown to be highly efficacious due to the immunogenicity of the hexon protein (adjuvant-like qualities), as well as the ability of adenovirus to infect dendritic cells (Adams et al., 2009; Kramp et al., 1979). For the same reasons, it was hypothesized that AAVs, which can stimulate robust immune responses and infect antigen presenting cells, would make good vaccine vectors.

5) AAVs affording protection against lethal infection can transduce antigen presenting cells such as dendritic cells or B cells

As controls for lethal challenge, AAV8 and AAVrh32.33 were utilized. Initial studies involving AAV8 showed that this vector resulted in aberrant T cell responses and these cells were mainly non-functional (Lin et al., 2007a; Mays et al., 2009). Further investigation into the mechanism of action for AAV8 showed that this vector is capable of inducing T cell tolerance via three factors: poor APC transduction efficiency, T cell exhaustion, and minimal upregulation of MHCI on transduced cells (Mays et al., 2014). For these reasons, AAV8 was suggested to be more suited for gene therapy as it may serve as a poor vaccine vector candidate. Therefore, it was intended that AAV8 be used as a negative control, with the expectation that it would perform poorly in challenge studies. However, quite the opposite was true as will presented in the Results and Discussion sections of this thesis. For the positive control, AAVrh32.33 was used. The capsid of AAVrh32.33 was shown to be highly immunogenic, resulting in complete ablation of transgene expression (Mays et al., 2009). As well, AAVrh32.33 exhibited robust cellular and humoral immune responses when it delivered HIV gag into monkeys (Lin et al., 2009). Furthermore, the structural motifs in the AAVrh32.33 capsid,

specifically in the hypervariable region IV of VP3, were determined to contribute to its antigenicity (Mays et al., 2013). Here, it was predicted that AAV8 would serve as a poor vaccine vector, while AAVrh32.33 would provide protective immune responses; however, the opposite was true for both vectors. A model describing the unexpected results will be presented in the Discussion section. Regardless of the outcome, the results of all the hypotheses were still able to provide a unifying model, showing the relationship between AAV tissue tropism and the protective efficacy elicited by the AAV transgene.

1.13.3 OBJECTIVES

In order to address the hypotheses of this project, the following objectives were performed in no particular order:

1) Design primers based on conserved regions of the AAV genome and isolate AAV DNA using PCR on pig genomic DNA
2) Once AAV DNA has been identified, design primers to isolate the full *cap* gene of unique AAV isolates
3) Produce viable AAV particles (based on the unique porcine-derived AAV sequences) with reporter gene
4) Determine if AAV capsid are not cross-neutralized by antisera against other AAV serotypes
5) Determine if pre-existing immunity exists against porcine-derived AAVs within the general human population
6) Evaluate the transduction efficiency and tropism of porcine-derived AAVs *in vitro* and *in vivo*

7) Evaluate the protective efficacy of porcine-derived AAVs in lethal H05 and EBOV challenge

8) Evaluate immune responses generated against AAV-H05HA and -EBOVGP transgene

9) Evaluate tropism of porcine-derived AAVs in immune cells

CHAPTER II

2.0 MATERIALS AND METHODS

2.1 DISCOVERY OF NOVEL AAV SEQUENCES FROM PORCINE TISSUES BY PCR

2.1.1 ISOLATION OF GENOMIC DNA FROM PORCINE TISSUES

Pig organs from rural farms across Manitoba were collected and kindly provided by the Canadian Inspection Food Agency (CFIA). Organs such as the heart, liver, lung, spleen, and gut were harvested from sickly pigs. Genomic DNA was isolated from the pig organs using QIAamp® DNA Mini Kit as per manufacturer's protocols (QIAGEN). Briefly, approximately 25 mg of tissue was cut into tiny pieces using a scalpel. The cut tissue was placed into a 2 mL screw cap cryotube along with a steel bead and 80 uL phosphate buffered saline (PBS). The tissue was lysed using a Tissue Lyser II (QIAGEN), at a frequency of 30 hertz (Hz) per second for 1 minute. 100 uL ATL buffer was added to the tube and everything was spun down at 13,000 RPM for 1 minute. 20 uL proteinase K was added to the tubes and incubated at 56°C overnight. Following incubation, 200 uL of Buffer AL was added to the tube and the mixture was incubated at 70°C for 10 minutes. 200 uL of ethanol was then added to the sample and mixed, and the whole mixture transferred to QIAamp mini spin columns and centrifuged at 8,000 RPM for 1 minute, and the filtrate discarded. The column was washed with 500 uL of Buffer AW1, centrifuged at 8,000 RPM for 1minute, and filtrate discarded. The column was washed an additional time with 500 uL Buffer AW2 and spun at 13,000 RPM for 3

minutes. DNA was eluted with 200 uL Buffer AE and eluate collected in a 1.5 mL eppendorf tube.

2.1.2 REAGENTS AND PRIMERS FOR PCR

Genomic DNA isolated from pig tissues was screened for the presence of AAV sequences using primers based on conserved regions of the AAV genome. The primer RC+ (5'-GGTAATTCCTCGGGAAATTGGCATT-3') was designed to bind to a conserved region ~1 kb into the *rep* region of the AAV genome (Mori, Wang, Takeuchi, & Kanda, 2004), while SIG- (5'-GGTAATTCCTCGGGAAATTGGCATT-3') was designed to bind to a conserved region ~700 bp into the *cap* gene (G.-P. Gao et al., 2002), giving a PCR product of ~1.7 kb. PCR for screening was carried out using iProof™ High-Fidelity Polymerase (Bio-Rad).

2.1.3 AGAROSE GEL ELECTROPHORESIS AND CLONING INTO TOPO-TA CLONING KITS

PCR products for screening were run on a 1% agarose gel (Life Technologies™) with TAE buffer for 1 hour at 100 volts. 2-Log DNA Ladder was run alongside the PCR products to approximate the size of the DNA fragments (New England Biolabs®). PCR products were visualized with SYBR® Safe DNA Gel Stain (Life Technologies) and products ~1.7 kb in size were excised from the gel using a scalpel, and the DNA purified using QIAquick Gel Extraction Kit as per manufacturer's instructions (QIAGEN). DNA was eluted from QIAquick columns using 50 µl if Buffer EB. In order to proceed with cloning into PCR®2.1 TOPO® TA Cloning Kits, the PCR products were required to have 3'-A overhangs (Life Technologies™). Since iProof® produces products having blunt ends, the PCR screening products were treated with Taq DNA Polymerase to add

these 3'-A overhangs (Life Technologies™). The treated PCR screening products were then cloned into the PCR®2.1 TOPO® TA Cloning Kits as per manufacturer's protocols. Briefly, the cloned PCR screening products were transformed into competent TOP10 cells (Life Technologies™). The cells were plated onto LB agar plates with 50 µg ampicillin and 40 µl of 20 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Empty plasmids contain functional β-galactosidase (lacZ) gene, while successfully cloned vectors have their lacZ disrupted. Therefore, white colonies were picked for screening, whereas blue colonies were disregarded. Picked colonies were grown overnight in 10 mL LB Lennox broth, and the DNA was isolated using QIAprep Spin Miniprep Kit in accordance with manufacturer's instructions (QIAGEN). DNA was eluted from QIAprep columns using 100 µl of Buffer EB. Isolated DNA was sequenced by the DNA Core Facility at the National Microbiology Laboratory, and sequences were submitted online for Basic Local Alignment Search Tool (BLAST) analysis.

2.2 ISOLATION OF FULL LENGTH AAV *CAP* GENE

2.2.1 REAGENTS AND PRIMERS FOR PCR

Once sequences were verified to be unique, primers were designed based on common regions of the AAV isolates within the *rep* region, and used in nested thermal asymmetric interlaced (TAIL) PCR in three successive PCRs, along with an arbitrary degenerate primer. The three specific primers were Nestedcap1+2.7kb, Nestedcap2+2.4KB, and Nestedcap3+2.2kb (5'-TGGAGGACCGAATGTTCAAGTTTG-3', 5'-CGACCGATCGATTGGACCTC-3', and 5'-AAGCGAGTAGTCATGTCGTTTG-3', respectively). The arbitrary degenerate primer was CED- (5'-ACTGAMACGAAT(H/_)AMMCGGTTTAT

TGA-3'). Nested TAIL PCR was carried out using HotStarTaq DNA Polymerase (QIAGEN). The reagents used in the nested TAIL PCR can be found in Table 1.

2.2.2 NESTED-TAIL PCR RUN PARAMETERS

In the primary nested TAIL PCR reaction, low stringency conditions allow for annealing of the arbitrary degenerate primer, with annealing temperatures as low as 30°C. During the secondary reaction, more stringent conditions were used, with annealing temperatures as low as 44°C. For the tertiary reaction, highly stringent conditions were used, with annealing temperatures of Tm-5°C of the specific primer 3. Conditions for nested TAIL PCR can be found in Table 2.

2.2.3 AGAROSE GEL ELECTROPHORESIS

PCR products for the nested TAIL PCR were loaded onto a 1% agarose gel along with 2-log Ladder and run at 100 volts for one hour. Expected DNA bands were visualized with SYBR® Safe DNA Gel Stain (Life Technologies), excised, purified, cloned into PCR®2.1 TOPO® TA vectors, and amplified in a similar fashion to that of the PCR screening products in 2.1.3. Isolated DNA was sequenced by the DNA Core Facility at the National Microbiology Laboratory and sequences submitted online for BLAST analysis.

Table 1: Nested TAIL PCR Reagent List

1° Reaction Reagents	**Volume**
1.5 uM specific primer	1 uL
2 uM degenerate primer	1 uL
buffer 10x	2 uL
100 uM dNTP	1 uL
HotStarTaq (1 unit)	1 uL
Water	13 uL
1 ug DNA	1 uL

2° Reaction Reagents	**Volume**
1.5 uM specific primer 2	1 uL
2 uM degenerate primer	1 uL
buffer 10x	2.5 uL
100 uM dNTP	1 uL
HotStarTaq (1 unit)	1 uL
Water	17.5 uL
1° PCR Product 1:40	1 uL

3° Reaction Reagents	**Volume**
1.5 uM specific primer 3	1 uL
2 uM degenerate primer	1 uL
buffer 10x	5 uL
100 uM dNTP	1 uL
HotStarTaq (1.5 units)	1.5 uL
Water	39.5 uL
2° PCR Product 1:10	1 uL

Table 2: Nested TAIL PCR Run Parameters

1° Reaction

Step	Process	Temp	Time	
1	Denature	94°C	15 min	
2	Melting	94°C	45 sec	
3	Annealing	60.6°C	30 sec	
4	Extension	72°C	90 sec	go to step 2 (5X)
5	Melting	94°C	45 sec	
6	Annealing	30°C	30 sec	
7	Extension	72°C	90 sec	
8	Melting	94°C	45 sec	
9	Annealing	44°C	30 sec	
10	Extension	72°C	90 sec	go to step 8 (10X)
11	Melting	94°C	45 sec	
12	Annealing	60.6°C	30 sec	
13	Extension	72°C	90 sec	go to step 11 (1X)
14	Melting	94°C	45 sec	
15	Annealing	44°C	30 sec	
16	Extension	72°C	90 sec	go to step 11 (12X)

2° Reaction

Step	Process	Temp	Time	
1	Denaturing	94°C	15 min	
2	Melting	94°C	45 sec	
3	Annealing	67°C	30 sec	
4	Extension	72°C	90 sec	go to step 2 (1X)
5	Melting	94°C	45 sec	
6	Annealing	44°C	30 sec	
7	Extension	72°C	90 sec	go to step 2 (10X)

3° Reaction

Step	Process	Temp	Time	
1	Denaturing	94°C	15 min	
2	Melting	94°C	45 sec	
3	Annealing	51°C	30 sec	
4	Extension	72°C	90 sec	go to step 2 (1X)
5	Melting	94°C	45 sec	
6	Annealing	44°C	30 sec	
7	Extension	72°C	90 sec	go to step 2 (10X)
8	Extension	72°C	10 min	

2.3 CLONING OF AAV *CAP* GENES INTO *TRANS* PLASMID

Two main plasmids were used in construction of the rAAV vectors: *cis* and *trans* plasmids. The *trans* plasmid consisted of an AAV2 *rep* sequence followed by the desired *cap* gene. An existing *trans* plasmid called pACK2/5 was kindly provided by Dr. Alberto Aurrichio's lab. The cloning sites utilized in this plasmid were SwaI and NotI. Once isolated, AAV *cap* was cloned into pCR®2.1-TOPO® TA along with restriction sites SwaI on the 5'-end and NotI on the 3'-end and amplified. pACK2/5 and the pCR®2.1-TOPO® TA vectors were cut using SwaI and NotI, and the fragment isolated from the pACK2/5 vector was incubated with calf intestinal alkaline phosphatase (CIP) to remove phosphate groups from the 5'-end, thus preventing self-ligation. The pACK2/5 backbone and pCR®2.1-TOPO® TA insert were ligated together using T4 DNA Ligase (Life Technologies), resulting in the *trans* plasmid capable of expressing AAV2 *rep* proteins and the AAV *cap*-of-choice when transfected into human embryonic kidney (HEK) 293T cells. For helper virus function, pAdΔF6 was used to provide the Adenoviral genes E2a, E4, and VARNA; necessary to drive replication of rAAV particles (Xiao, Li, & Samulski, 1998b).

2.4 CLONING OF EBOVGP AND H05HA TRANSGENES INTO *CIS* PLASMID

For the *cis* plasmid, the vector pAAV2.1CMV-LacZ was utilized to clone the transgene. For A/Hanoi/30408/2005 H5N1 hemagglutinin antigen (H05HA) cloning, the restriction sites employed were NotI on the 5'-end and SacII on the 3'-end of the transgene. For cloning of EBOVGP, the restriction sites NotI on the 5'-end and BglII on the 3'-end were used to clone the transgene into the *cis* plasmid. Completed genes with

restriction sites were produced by gene synthesis with primers designed by the online site Gene2Oligo (http://berry.engin.umich.edu/gene2oligo/). Primers were designed to have melting temperatures around 58°C. Completed transgene was cloned into pCR®2.1-TOPO® TA, transformed into TOP10 cells, and amplified. pAAV2.1CMV-LacZ backbone and the cloned transgenes were digested with either NotI and SacII for H05 cloning, or NotI and BglII for EBOVGP cloning. pCR®2.1-TOPO® TA digested transgene fragment and pAAV2.1CMV-LacZ digest backbone were ligated using T4 DNA Ligase, producing the completed *cis* plasmid.

2.5 PRODUCTION AND PURIFICATION OF AAV VECTORS WITH TRANSGENE

2.5.1 CELL CULTURE OF HEK293T CELLS

HEK293T cells were required for all transfections of AAV vectors. HEK293T cells were grown on 150 mm TC-Treated Culture Plates (Corning®). HEK293T cells were amplified to 90% confluency into 21-150 mm plates and a day before transfection, cells were seeded at a ratio of 1:3 into 50-150 mm plates. The growth media consisted of Delbecco's Modified Eagle's Medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Life Technologies). Cells were trypsinized using 0.25% Trypsin-EDTA (Life Technologies).

2.5.2 TRANSFECTION OF PLASMIDS USING CALPHOS TRANSFECTION KITS

At least one hour before transfection, 50-150 mm plates at a confluency of ~60-80% were given fresh media. Triple transfection was performed to produce rAAV

particles in HEK293T cells using CalPhos™ Mammalian Transfection kits as per manufacturer's protocols (Clontech® Laboratories). The plasmids involved in transfection were the *trans* packaging plasmid (expressing the AAV2 *rep* and AAV *cap*-of-choice), the *cis* transgene plasmid (producing the recombinant AAV genome to be packaged within the *trans* plasmid Cap product), and the helper plasmid pAdΔF6 (contains the Adenoviral genes capable of driving expression of the AAV genes in the *trans* plasmid and replication of the recombinant AAV genomes from the *cis* plasmid).

2.5.3 HARVEST AND CLARIFICATION OF AAV PARTICLES

72 hours following transfection, cells were scraped and supernatant harvested and placed into 500 mL Screw Cap Conical Bottom Centrifuge Tubes (Corning). The mixture of supernatant and cells were spun in a Sorvall Legend RT and the supernatant was discarded. The cell pellet was resuspended in Resuspension Buffer I and freeze-thawed three times by freezing in a dry ice-ethanol bath and thawing in a 37°C water bath. The mixture was subjected to 5000 units of DNase I at 37°C for one hour to remove exogenous DNA (Life Technologies). Afterwards, a clarification step was performed to remove debris. For the clarification step, the mixture was centrifuged at 3000 RPM for 15 minutes, and supernatant collected and transferred to a fresh 50 mL Falcon™ conical centrifuge tube (Fisher Scientific). The clarification step was repeated a second time and transferred to another fresh 50 mL conical tube. A solution of 10% Octyl β-D-glucopyranoside was added to the mixture for solubilisation of membrane proteins (Sigma-Aldrich).

2.5.4 PURIFICATION AND FRACTIONATION OF AAV PARTICLES BY CESIUM CHLORIDE GRADIENTS

Following the clarification step, supernatant was collected and overlayed onto a Cesium Chloride (CsCl) gradient, loaded into an SW32Ti rotor, and spun in an Optima L-90K ultracentrifuge at 25,951 RPM overnight (Beckman Coulter). Fractions were collected and those having a refractive index between 1.3750-1.3660 were pooled together. Refractive indexes were recorded using an Abbe™ benchtop refractometer (Fisher Scientific). The pooled fractions loaded into a Type90Ti rotor spun again in the Optima L-90K ultracentrifuge at 62,119 RPM overnight. Fractions were collected and those reading 1.3750-1.3660 were pooled. The sample was desalted and concentrated using Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore) and washed with Final Formulation Buffer. The sample was spun down and resuspended in ~2 mL of Final Formulation Buffer, then aliquoted and frozen at -80°C.

2.5.5 QUANTITATIVE REAL-TIME PCR TO DETERMINE AAV TITERS

Each AAV vector sample was diluted 1:100, and 10 uL was incubated with 40 units of DNaseI in a total volume of 100 uL at 37°C for 1 hour (Life Technologies). The preparation was boiled at 99°C for 5 minutes, and quenched on ice for at least 10 minutes. For each quantitative RT-PCR reaction, 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), 1 uM primer BGH FW (5'-TCTAGTTGCCAGCCATCTGTTGT-3'), 1 uM primer BGH REV (5'-TGGGAGTGGCACCTTCCA-3'), and 0.2 uM BGH PROBE (6FAM-TCCCCCGTGCCTTCCTTGACC-TAMRA) were used, along with 10 uL of the diluted sample. The PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes,

followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Acquisition occurred during the 40 cycles of 60°C for 1 minute step. Reactions were loaded onto a LightCycler® 480 Multiwell Plate 96 and run on a LightCycler® 480 (Roche). Standard plasmid was prepared by linearization using NotI, then purified using QIAquick PCR purification kit as per manufacturer's instructions (QIAGEN). The concentration of DNA of the linearized plasmid was determined using a ND-1000 Spectrophotometer (NanoDrop Technologies, Inc), and the molar concentration calculated. Since 1M = $6.023x10^{23}$ copy numbers, the number of copies of linearized standard plasmid can be calculated in a given volume. The standard plasmid ladder was diluted from $2.5x10^{6}$ copies/uL to $1x10^{0}$ copies/uL. The following formula was used to determine the concentration of AAV vector: copy number X 10 X dilution factor X 2 X 10^{3}; with the copy number determined from the cycle time, in which the threshold was achieved in both the standard ladder and the sample; 10 for the volume loaded into the well; the dilution factor of 100; 2 to account for 2 strands of AAV genome of opposite polarities being produced; 10^{3} to convert from µl to ml.

2.6 WESTERN BLOT ANALYSIS OF AAV PARTICLE PRODUCTION

Following production of AAV particles via triple transfection of helper plasmid, *trans* plasmid, and *cis* plasmid into HEK293T cells in a 6 well flat bottom plate, cell supernatant was aspirated and 100uL of radioimmunoprecipitation (RIPA) buffer added to the cells. Cells were scrapped and incubated with 5X Sample Protein buffer at 99°C for 10 minutes. The sample was then loaded and run on a 5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) stacking gel and 10% SDS-PAGE resolving gel to separate the proteins, along with MagicMark™ XP Western Protein

Standard and SeeBlue® Plus 2 Pre-stained Protein Standard (Life Technologies). Samples were run at 100 V for 2 hours. Afterwards, samples were transferred to a polyvinylidene fluoride (PVDF) membrane via vertical electrophoresis using a Mini-PROTEAN® Tetra (Bio-Rad) for 1 hour at 350 mA.

The membrane blots were washed with PBS 0.1% Tween 20 and incubated with ADENO-Associated virus VP1, VP2, VP3 mouse antibody (Fitzgerald) diluted at 1:1000 in skim milk for 1 hour at room temperature. The membrane blots were washed again with PBS 0.1% Tween 20 and incubated with the secondary antibody (goat anti-mouse IgG horseradish peroxidase (HRP)) (Fitzgerald) diluted 1:3000 in skim milk for 1 hour. Samples were washed with PBS 0.1% Tween 20 and the protein was incubated with substrate using Amersham ECL Plus™ Western Blotting Detection Reagent (GE Healthcare Life Sciences) as per manufacturer's protocols. Protein was detected using Amersham Hyperfilm ECL (GE Healthcare Life Sciences) and the film developed using an SRX-201A film processor (Konica Minolta).

2.7 ELECTRON MICROSCOPY OF AAV PARTICLES

AAV vectors were produced by triple transfection in HEK293T cells as previously mentioned. Following harvest and purification via CsCl gradients, AAV vector samples were processed by the Monoclonal Antibody Unit, & Bioforensics Assay Development and Diagnostics at the NML for electron microscropy (EM). Briefly, AAV samples were attached to glow discharged carbon coated formvar films on 400 mesh copper grids by adsorption for 1 minute. 2% methylamine tungstate was used as a negative contrast (Nanoprobes). An FEI Tecnai 20 transmission electron microscope at

200 kV and magnification of 200 000 X was used to image the samples, and digital images acquired using an AMT Advantage XR 12 CCD camera (AMT).

2.8 INFECTION OF AAV VECTORS IN CELL LINES

AAV-LacZ vectors were prepared at various dilutions in plain DMEM. Cell lines were seeded onto flat bottom 96 well plates to obtain a confluency of 60-80% the day of infection (Nunc™). Media was removed and replaced with the prepared AAV-LacZ dilutions and incubated at 37°C for one hour. Complete DMEM was then added to the infected cells to give a final concentration of 1% penicillin/streptomycin, 10% FBS. The next morning, the media was removed and replaced with fresh complete media. 48 hours post infection, media was removed and cells were washed 2 times with PBS and fixed with 1% glutaraldehyde in PBS for 10 minutes. After fixing, cells were washed 4 times with PBS and stained with X-gal Staining Solution (36 mM potassium ferricyanide, 36 mM potassium ferrocyanide, 0.1 mM $MgCl_2$ and 1 mg/mL X-gal in PBS).

2.9 NEUTRALIZING ANTIBODY ASSAY AGAINST AAV USING AAV ANTI-SERUM AND POOLED HUMAN IG

AAV capsids were injected IM into the tibialis anterior muscle of mice at a dose of $1x10^{11}$ genome copies (gc)/mouse, and blood was collected via cardiac puncture 28 days post injection. Serum was separated and collected using BD serum separator tubes. Serum was then heated to 56°C for 45 minutes to inactivate complement. Various porcine AAV-LacZ particles were incubated with heat inactivated sera for 1 hour, then transferred unto HEK293T cells (giving ~100 transduced cells per well) in a 96-well format for an additional hour, upon which complete media was added. 48 hours post-infection, cells were fixed using 2% glutaraldehyde in PBS, and incubated with X-gal

Staining Solution at 37°C with 5% CO_2 overnight. Neutralization titer was reported for serum able to neutralize ~50% of transducing particles compared to PBS incubated particles. In a similar fashion, pooled human Igs purchased from CSL Behring was incubated with AAV-LacZ particles, starting at a concentration of 12 mg/mL, with a 2-fold dilution going down the wells. Neutralization was reported when ~50% of transducing particles were neutralized when compared to the non-neutralized control wells.

2.10 INJECTION ROUTES OF AAV FOR *IN VIVO* STUDIES

2.10.1 IM ADMINISTRATION OF AAV VECTORS IN VIVO

AAV vectors administered by the intramuscular route (IM) in BALB/c mice were injected in either the tibialis anterior for characterization of transduction efficiency in the muscle, or in the thigh for vaccine and survival studies. Mice were anesthetized in a chamber with isoflurane. Vectors were administered in a 50 µl volume with a 27G X ½ inch needle (BD™), at doses ranging between $1x10^7$ – $1x10^{11}$ gc per mouse. At the appropriate time-point, mice were sacrificed using isoflurane and cervical dislocation, and the muscle was harvested and processed.

2.10.2 IN INJECTION OF AAV VECTORS IN VIVO

AAV vectors administered intranasally (IN) were used to characterize transduction efficiency of the vectors in the lung. AAV-LacZ vectors were injected using a P200 pipette at a dose of $1x10^{11}$ in a 50 µl volume. Briefly, BALB/c mice were anesthetized in a chamber with isoflurane and given the vector IN using a P200 pipette. 28 days post-injection, the lungs and trachea were harvested.

2.10.3 IVTV INJECTION OF AAV VECTORS IN VIVO

For biodistribution studies, AAV vectors were administered thru the intravenous tail vein route (IVTV). Briefly, the tails of BALB/c mice were submerged in warm water to allow for dilation of blood vessels. AAV vectors were then administered in the lateral tail vein at a dose of $1x10^{11}$ gc in a volume of 100 µl using a 27G x ½ inch needle. Mice were incubated for an applicable amount of time before proceeding with organ harvest.

2.10.4 GENERAL ANIMAL HUSBANDRY

Mice were checked daily for any signs of any disease, distress, or abnormalities. Food and water levels were checked daily and replaced as needed. Bedding was changed at least once a week or as needed. Animal husbandry was undertaken in accordance with the guidelines provided by the Animal Care Committee at the National Microbiology Laboratory.

2.11 *IN VIVO* TRANSDUCTION EFFICIENCIES

2.11.1 ORGAN HARVEST FOR CRYOSECTION

For the IM route, the tibialis anterior muscle was harvested. The muscle was snap frozen in isobutene chilled with liquid nitrogen, then frozen in a dry ice-ethanol bath with Optimal Cutting Temperature (O.C.T.™) compound (Tissue-Tek®). For IN injection, the lung and trachea were harvested. Harvested lungs were inflated with a 1:1 mixture of PBS and O.C.T. before freezing. For IVTV injected mice, the brain and liver were harvested for cryosectioning and processed in a similar fashion to the organs harvested by IM and IN route. All samples were stored at -80°C for at least 24 hours before sectioning.

For cryosectioning, samples were mounted and cut into 10 µm sections at -20°C using a Leica CM 1850 (Leica Biosystems). Sections were mounted onto ColorFrost™ slides and allowed to air dry (Thermo Scientific).

2.11.2 BIODISTRIBUTION AND QUANTITATIVE PCR OF AAV

For biodistribution studies, organs were harvested from mice injected via IVTV. Mice were sacrificed and euthanized using Isoflurane and cervical dislocation. The spleen, pancreas, lung, heart, liver, kidney, small intestine, large intestine, ovary, tibialis anterior muscle, and brain were harvested from injected mice. Approximately 25 mg of each organ was cut and placed into a 2 mL screw cap cryotube along with a steel bead. DNA was extracted and purified using QIAamp® DNA Mini Kit (QIAGEN) in a similar fashion to the pig genomic DNA as described in 2.1.1.. Extracted DNA was then subjected to quantitative RT-PCR to determine AAV gc numbers in a similar fashion to the viral titers in 2.5.5.. Mammalian cells contain an average of 1500 ng of DNA per cell. Since the amount of DNA loaded for each sample was known, the amount of AAV gc per cell can be calculated based on the known amount of molecules in the standard DNA ladder.

2.11.3 HISTOCHEMICAL AND X-GAL STAINING OF MOUSE TISSUES

Sections were air-dried on glass slides and fixed for 10 minutes with a 2% glutaraldehyde-PBS solution (Sigma-Aldrich). The slides were washed 3 times with PBS. The slides were incubated with X-gal staining solution overnight at 37°C and washed 3 times with PBS the following morning. Afterwards, the X-gal stained slides were processed for Eosin staining and dehydration. The slides were immersed in eosin for 2 minutes and rinsed with water (Sigma-Aldrich). Following eosin staining, the slides

were dehydrated in 70%, 90%, and 2x100% ethanol baths for 2 minutes in each bath. The slides were immersed in xylene (Sigma-Aldrich) to remove residual alcohol, and coverslips were mounted using Permount™ (VWR). The coverslips were allowed to adhere overnight before scanning. Slides were scanned using a Zeiss Mirax Midi scanner, and pictures were recorded using 3D Histech Pannoramic Viewer.

2.11.4 BETA-GAL ASSAY FOR IM INJECTED AAV-LACZ AT VARIOUS TIME-POINTS

For beta-gal assays, 10 - 10 μm muscle sections from various locations of the muscle were collected for each different time-point. Sections were washed with PBS to remove O.C.T. and spun down in a 2 mL cryotube. Samples were processed in a 96 well flat-bottom plate using Genlantis β-gal assay kit as per manufacturer instructions to determine the amount of β-gal expression per sample and presented as β-gal milliunits based on the standard curve for known amounts of β-gal. Absorbance was recorded at 570 nm using a plate reader.

2.12 IMMUNOLOGICAL AND CHALLENGE STUDIES FOR AAV-H05HA OR AAV-EBOVGP VACCINATED MICE

2.12.1 IM INJECTION OF VECTORS

For vaccination studies, mice were anesthetized with isoflurane, then injected in the left thigh with AAV-H05HA or AAV-EBOVGP vector, at doses ranging from $1x10^8$ – $2.5x10^{10}$ gc/mouse. 50 μl of each vector was injected into mice using BD 1 ml syringes and 27 G X ½ inch needles.

2.12.2 TRIAL BLEEDS FOR SERUM COLLECTION

Mice were trial bled from the saphenous vein. Briefly, the leg fur was shaved over the saphenous vein and vaseline used to lubricate the area. The vein was punctured using a 27 G X ½ inch needle, and ~100 µl of blood collected using BD Microtainer® tube with serum separator (BD). The tubes were centrifuged at 1300 g for 15 minutes to allow for sufficient separation of serum from blood. The serum was transferred to 1.5 ml Eppendorf tubes and heat inactivated at 56°C for 45 minutes, then stored at -80°C.

2.12.3 ISOLATION OF SPLENOCYTES FROM MOUSE SPLEEN

Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. The surgical area was sterilized with 70% ethanol and a small incision was made on the left side of the mouse and the spleen extracted. For studies involving dendritic cells, the spleen was treated with 500 µg Collagenase D (Roche) in PBS containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ for 1 hour at 37°C. The spleen was mashed through a 40 µm Cell Strainer (BD Falcon) to free the splenocytes. The cell suspension was spun down at 1500 g for 5 minutes and the supernatant was discarded. The pellet was washed with PBS, then resuspended in complete media (Roswell Park Memorial Institute – 1640 media (RPMI-1640) supplemented with 10% FBS and 1% penicillin/streptomycin) (Life Technologies). Splenocytes were counted using a hemocytometer and resuspended in complete RPMI at a concentration of $1x10^7$ cells/ml.

2.12.4 FLOW CYTOMETRY PROTOCOL

Cells processed for flow cytometry were treated with Ammonium-Chloride-Potassium (ACK) Lysing Buffer (Life Technologies) in order to lyse the red blood cells. Splenocytes were cultured in a 96-well round bottom plate at a concentration of $1x10^6$

cells/well and stimulated with antigen or peptide pools for 5 hours. Cells were washed twice with PBS. Antibodies for extracellular staining were diluted in PBS and cells were stained for 30 minutes in the dark. The cells were washed twice more with PBS and the membranes were permeabilized using a Cytofix/Cytoperm™ kit (BD) as per manufacturer's instructions. Following permeabilization, cells were washed twice with Perm/Wash™ (BD) and intracellularly stained for 30 minutes using antibodies diluted in Perm/Wash™ (BD). Cells were fixed using 1% paraformaldehyde in PBS. For flow cytometry, cells were run through an LSR II (BD) and data was analyzed with FACSDiva™ (BD) or FlowJo (Tree Star Inc).

2.12.5 ANIMAL HUSBANDRY, CONTAINMENT LEVELS AND SCORING CRITERIA FOR EUTHANASIA

Upon arrival, mice were acclimatized for at least 7 days before manipulation. Mice were injected after acclimatization and incubated for up to 28 days post-injection in the Animal Care facility at the National Microbiology Laboratory in a biosafety level 2 (BSL2) setting. Mice were checked daily for food, water, and signs of disease or distress. For challenge studies, mice were transferred to a biosafety level 4 (BSL4) laboratory setting at the National Microbiology Laboratory. Mice were housed 5 mice per cage in isolator cages and supplied with high-efficiency particulate adsorption (HEPA) filtered air. Post-challenge, mice were weighed and scored daily based on disease symptoms such as ruffled fur, decreased activity, increased weight loss or neurological signs of disease. Animals were scored from 0-3, with 0 having no symptoms, 1 for ruffled fur, slow activity, and loss of body condition, 2 for labored breathing and hunched posture, and 3 being death. When mice received a score of 2 or more, they were considered for

euthanasia and were anesthetized with isoflurane and sacrificed via cervical dislocation. As well, if the mouse lost more than 20% of its initial body weight, it was subject to euthanasia. Mouse cages were checked daily for food and water, and bedding was changed at least once a week or as needed.

2.12.6 STUDIES FOR IN VIVO INJECTED AAV-H05HA VECTORS

2.12.6.1 H05 CHALLENGE TIME-LINE AND DOSE-RESPONSE

The strain of influenza used for lethal challenge was A/Hanoi/30408/2005 H5N1 (H05). Mice were vaccinated with AAV vectors expressing H05HA or PBS as a control, with vector doses ranging between $1x10^8 – 2.5x10^{10}$ gc/mouse. 25 days post-vaccination, mice were trial-bled then transferred from BSL2 to BSL4. Mice were challenged with H05 at a dose of $100LD_{50}$ in 50 µl per mouse via the intranasal route 28 days post vaccination. Mice were monitored and scored for signs of disease or distress for up to 28 days post-challenge.

2.12.6.2 ELISPOT ANALYSIS OF SPLENOCYTES FROM AAV-H05HA INJECTED MICE

Splenocytes obtained from 2.12.3 were used for ELISPOT analysis. Peptide libraries consisted of 15 amino acid sequences with 10 amino acid overlaps spanning the entire H05 HA protein, resulting in 27 peptide pools used to stimulate the splenocytes (Mimitopes). The peptides were resuspended at a concentration of 100 µg/µl in dimethyl sulfoxide (DMSO) and stored at -80°C. Mouse IFN-γ ELISPOT Kit (BD) was used for the ELISPOT assay. Purified anti-mouse IFN-γ antibody was diluted in PBS 1:200 and 100 µl of solution per well was used for coating the plate overnight at 4°C. The

following morning, the plate was blocked with RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin for at least 3 hours at room temperature. Spleen was collected from vaccinated or naïve mice and splenocytes isolated as described in 2.12.3. $5x10^5$ splenocytes and 2.5 µg/ml of peptide pool were added to a well and incubated for 16 hours at 37°C with 5% CO_2. Negative controls consisted of peptide pool-free media, whereas positive controls were stimulated with 1 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 0.2 µg/ml Ionomycin. Afterwards, plates were washed 2x with water and 3x with Wash Buffer I (PBS containing 0.05% Tween-20). Biotinylated anti-mouse IFN-γ was diluted 1:250 in PBS containing 10% FBS and added onto the wells for 2 hours at room temperature. Following incubation, the plates were washed 3x with Wash Buffer I. Streptavidin-HRP diluted 1:100 in PBS containing 10% FBS was added to the wells and incubated for 1 hour at room temperature. The wells were washed 4 times with Wash Buffer I, followed by 2 washes with PBS. 3-Amino-9-Ethylcarbazole (AEC) was diluted in 0.1M Acetate solution from the AEC Substrate Reagent kit (BD) (Final Substrate Solution) and added to the wells to develop spots. Approximately 15 minutes after addition of substrate, spot development was stopped by washing the wells with water. The plate was allowed to air-dry overnight and the spots were counted using an AID ELISPOT Reader (Cell Technology). Spots produced from all 27 pools were added together and reported as spot forming units (SFU) per million cells. Any spots produced from the unstimulated controls were considered background and subtracted from the simulated samples.

2.12.6.3 HAI ASSAYS FOR MOUSE SERUM FROM AAV-H05HA VACCINATED MICE

Serum from mice vaccinated with AAV-H05HA or PBS injected was harvested 25 days post-injection as described in 2.2.12. Serum was treated with receptor destroying enzyme II (RDE) (Seiken) at 1:3 and incubated at 37°C overnight. The following day, the reaction was stopped by incubating at 56°C for 45 minutes. RDE-treated mouse serum was then used in the HAI assay by performing 2-fold serial dilutions beginning at 1:10. Briefly, 50 µl of diluted treated sera were added to each well of a 96 well V-bottom plate (Corning) and four hemagglutinating doses (AD) of H05 virus were added to each well and incubated at room temperature for 1 hour. Following incubation, 50 µl of 1% horse red blood cells were added to each well and incubated at room temperature for up to 1.5 hours. The reciprocal of the highest dilution of red blood cell agglutination not observed was used to report the HAI titer.

2.12.6.4 H05 NEUTRALIZING ANTIBODY ASSAY

Mice were vaccinated with different serotypes of AAV-H05HA and serum was harvested via trial bleed 25 days post vaccination. Serum was treated in a similar fashion as described in 2.12.6.3. Following RDE treatment protocol, samples were diluted two-fold following an initial 1:10 dilution in 96-well round bottom plate. 100 plaque forming units (PFU) of H05 virus was added to each well and incubated at 37°C for 60 minutes. The virus/serum mixture was added onto subconfluent Madin-Darby canine kidney (MDCK) cells in a 96-well flat bottom plate and incubated at 37°C with 5% CO_2 for 48 hours. Media consisted of MEM supplemented with 0.3% BSA, 1% penicillin/streptomycin, and 3.0 µg/ml Tosyl phenylalanyl chloromethyl ketone (TPCK)

trypsin. The highest dilution that cytopathic effect (CPE) was not observed was reported as the reciprocal of the neutralizing antibody titer.

2.12.7 STUDIES FOR IN VIVO INJECTED AAV-EBOVGP VECTORS

2.12.7.1 EBOV CHALLENGE STUDY TIME-LINE

Similar to the H05 challenge studies in 2.12.6.1, mice vaccinated with AAV-EBOVGP at a dose of $2.5x10^{10}$ were incubated in BSL2 conditions. 25 days post-vaccination, animals were trial-bled and transferred to BSL4. 28 days post-vaccination, the mice were challenged with $1000LD_{50}$ of mouse adapted EBOV (MA-EBOV) in 2x 100 µl intraperitoneal injections per mouse. The EBOV strain used for challenge was the Mayinga strain of mouse adapted *Zaire ebolavirus*. The mice were scored and monitored for up to 28 days post-challenge as per the criteria found in 2.12.5.

2.12.7.2 ELISPOT ANALYSIS OF SPLENOCYTES FROM AAV-EBOVGP INJECTED MICE

Protocols for the analysis of splenocytes from AAV-EBOVGP injected mice by ELISPOT were the same as those found in 2.12.6.2, with the only difference being the peptide pools; peptide sequences 15 amino acid long with 10 amino acid overlaps spanning the entire EBOV GP were used (Mimitopes). Splenocytes from mice were isolated as described in 2.12.3. Peptides were resuspended at a concentration of 28 µg/µl in DMSO and stored at -80°C. Peptides were pooled together to form 4 peptide pools. Briefly, ELISPOT plates were coated with purified anti-mouse IFN-γ antibody diluted in PBS at 1:200. The next morning, the plate was blocked with RPMI supplemented with 10% FBS and 1% penicillin/streptomycin for at least 3 hours. Each of the 4 peptide

pools were added at a concentration of 2 µg/ml to a well containing $5x10^5$ splenocytes to stimulate them overnight. The negative control consisted of peptide-free media and the positive control had media containing 1 ng/ml PMA and 0.2 µg/ml Ionomycin. Following incubation, the plate was washed 2x with deionized water and 3x with Wash Buffer I. The detection antibody (biotinylated anti-mouse IFN-γ) was diluted 1:250 in PBS containing 10% FBS and added onto the wells for 2 hours at room temperature. The plates were then washed 3x with Wash Buffer I. The enzyme conjugate (Streptavidin-HRP) was diluted 1:100 in PBS containing 10% FBS and added to the wells and incubated for 1 hour at room temperature. The wells were washed 4x with Wash Buffer I, followed by 2 washes with PBS. AEC Substrate Reagent kit (BD) was used to develop spots. Approximately 15 minutes after addition of substrate, spot development was stopped by washing the wells with water. The plate was allowed to air-dry overnight and the spots were counted using an AID ELISPOT Reader (Cell Technology). Spots produced from all 4 pools were added together and reported as spot forming units per million cells. The number of spots formed from negative controls was subtracted from spots from each peptide pool.

2.12.7.3 FLOW CYTOMETRY OF SPLENOCYTES FROM AAV-EBOVGP INJECTED MICE

Splenocytes isolated from AAV-EBOVGP or PBS injected mice were processed for flow cytometry as described in 2.12.4. Briefly, cells were stimulated for 5 hours at 37°C with 5% CO_2 with each of the 4 peptide pools as described in 2.12.7.2. After stimulation, cells were processed for extracellular staining with the following antibodies: CD4-Pac Blue, CD8-PerCPCy5.5, and CD107a-Alexa Fluor 488. For intracellular

staining, the antibodies used were: IFNγ-PE, IL2-APC, and TNFα-PE-Cy7. Stained cells were run on an LSRII Flow Cytometer (BD) and results reported were based on the percentage of the responsive $CD4^+$ and $CD8^+$ T cell populations expressing the following combinations: TNFα+IFNγ-IL2-, TNFα+IFNγ+IL2-, TNFα-IFNγ+IL2-, TNFα-IFNγ+IL2+, or TNFα-IFNγ-IL2+.

2.12.7.4 ELISAS FOR TOTAL IgGs GENERATED AGAINST AAV-EBOVGP

For ELISAs, 96-well high protein binding ELISA plates (Corning) were coated with 30 ng of purified EBOV GP per well and incubated overnight at 4°C. The following morning, the wells were blocked with PBS containing 5% milk powder and 0.2% Tween-20 (Dilution Buffer) for 90 minutes at 37°C. Serum was heat inactivated at 56°C for 45 minutes and diluted 1:100 in Dilution Buffer. The plate was washed 3 times with PBS containing 0.1% Tween-20 (Wash Buffer), and diluted serum added to the wells and incubated at 37°C for 1 hour. During incubation, goat anti-mouse IgG-conjugated HRP antibody (Cedarlane) was diluted 1:3000 in Dilution Buffer. After incubation, the plate was washed 3 times with Wash Buffer, and the diluted secondary antibody added to the wells and incubated for 1 hour at 37°C. The plate was then washed 3 times with Wash Buffer and ABST (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulphonic acid]-diammonium salt) and peroxidase substrate (Cedarlane) were added to each well and incubated at 37°C for at least 30 minutes to visualize antibody binding. Absorbance was read at OD_{405} on a plate reader and the results were considered positive if they were > 3 standard deviations from that of the negative serum.

2.12.7.5 NEUTRALIZING ANTIBODY ASSAY

Sera harvested from AAV-EBOVGP or PBS vaccinated mice was heat inactivated at 56°C for 45 minutes. Heat inactivated sera was serially diluted two fold beginning with 1:20 in DMEM containing 2% FBS. The diluted sera was incubated at 37°C for 90 minutes with ~100 infecting particles of EBOV expressing enhanced green fluorescent protein (EGFP) per well. The mixture was transferred onto 60-80% confluent VeroE6 cells in 96-well flat bottom plates and incubated at 37°C for 1 hour. Following incubation, complete DMEM supplemented with 10% FBS and 1% penicillin/streptomycin was added to the wells. The plates were incubated at 37°C for 48 hours and the cells were fixed and removed from BSL4 with an overnight incubation in formalin, followed by replacement of media with fresh formalin. The plates were read using an AID ELISPOT Reader on the GFP channel, and the reciprocal of the highest dilution, in which sera prevented >50% of GFP positive cells, were recorded as the neutralizing antibody titer.

2.13 TRANDUCTION EFFICIENCY OF AAV-scGFP IN MOUSE SPLENOCYTES

2.13.1 ISOLATION OF MOUSE SPLENOCYTES AND ANTIBODY PANEL

Mouse splenocytes were used to evaluate transduction efficiency of AAVs expressing self-complementary (sc) GFP vectors in immune cells. Mice were injected via IVTV with $1x10^{11}$ gc of AAV-scGFP vectors in 150 µl PBS. 48 hours post-injection, the mice were sacrificed by isoflurane and cervical dislocation and the spleens harvested. Spleens were treated with Collagenase D for 1 hour at 37°C to liberate dendritic cells. Following Collagenase D treatment, spleens were processed for FACS analysis using the

methods described in 2.12.3 and 2.12.4. After isolation and purification of splenocytes, extracellular staining was performed using the following antibody panel: Vivid-Pac Blue, CD19-BV650, CD11c-PerCPCy5.5, CD49b-APC, CD8-APC-Cy7, CD11b-BV711, CD4-Alexa Fluor 700, CD3-PE-Cy7, and CD115-BV605. Stained cells were fixed over-night with 1% paraformaldehyde (PFA) in PBS and the samples were run on an LSRII for flow cytometric analysis. The readout of the analysis reported the percentage of each immune type population expressing GFP, denoting AAV transduction in these cells types. Samples were considered positive if GFP expression was at least three times that of the PBS control.

2.14 STATISTICAL ANALYSIS

For statistical analysis, data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test, or two-way ANOVA followed by Bonferroni post tests, when appropriate. For survival curves, Log-rank (Mantel-Cox) test was performed. Samples were considered significantly different if $p<0.05$. Means were reported with standard error of the mean (SEM).

CHAPTER III

3.0 RESULTS

3.1 PCR SCREENING AND PHYLOGENETIC ANALYSIS OF NEW AAV ISOLATES FROM PORCINE TISSUES

In order to address the hypothesis that 'AAVs exist within porcine genomes and can be isolated by PCR', genomic DNA was isolated from pig organs such as the gut, lung, liver, spleen, and heart, and screened for the presence of AAV sequences by using primers specific for conserved regions of the AAV genome. These primers were designed to isolate a region spanning approximately 800 base pairs (bp) of the 3'-end of the AAV *rep* gene and 900 bp of the 5'-end of the AAV *cap* gene, for a total screening product of ~1.7 kilobases (kb) (Figure 1).

The AAV sequences were cloned into PCR®2.1 TOPO® vector and amplified. Following amplification and sequencing, the sequences were submitted online to GenBank and BLAST analysis confirmed that a pool of over 25 unique porcine AAV isolates were generated using this technique. The *rep* of AAV is fairly conserved among all AAVs, whereas the *cap* is more variable and responsible for determining the tissue tropism and antigenicity of AAV vectors. Keeping in mind that the *cap* is important for vector attributes, an alignment was performed using the *cap* portion of the new porcine-derived AAV isolates, along with the corresponding region of the *cap* gene of other published AAVs (Figure 2). The porcine-derived *cap* isolates are denoted as AAVpo*, where * is the sequential number, in which the porcine AAVs were isolated.

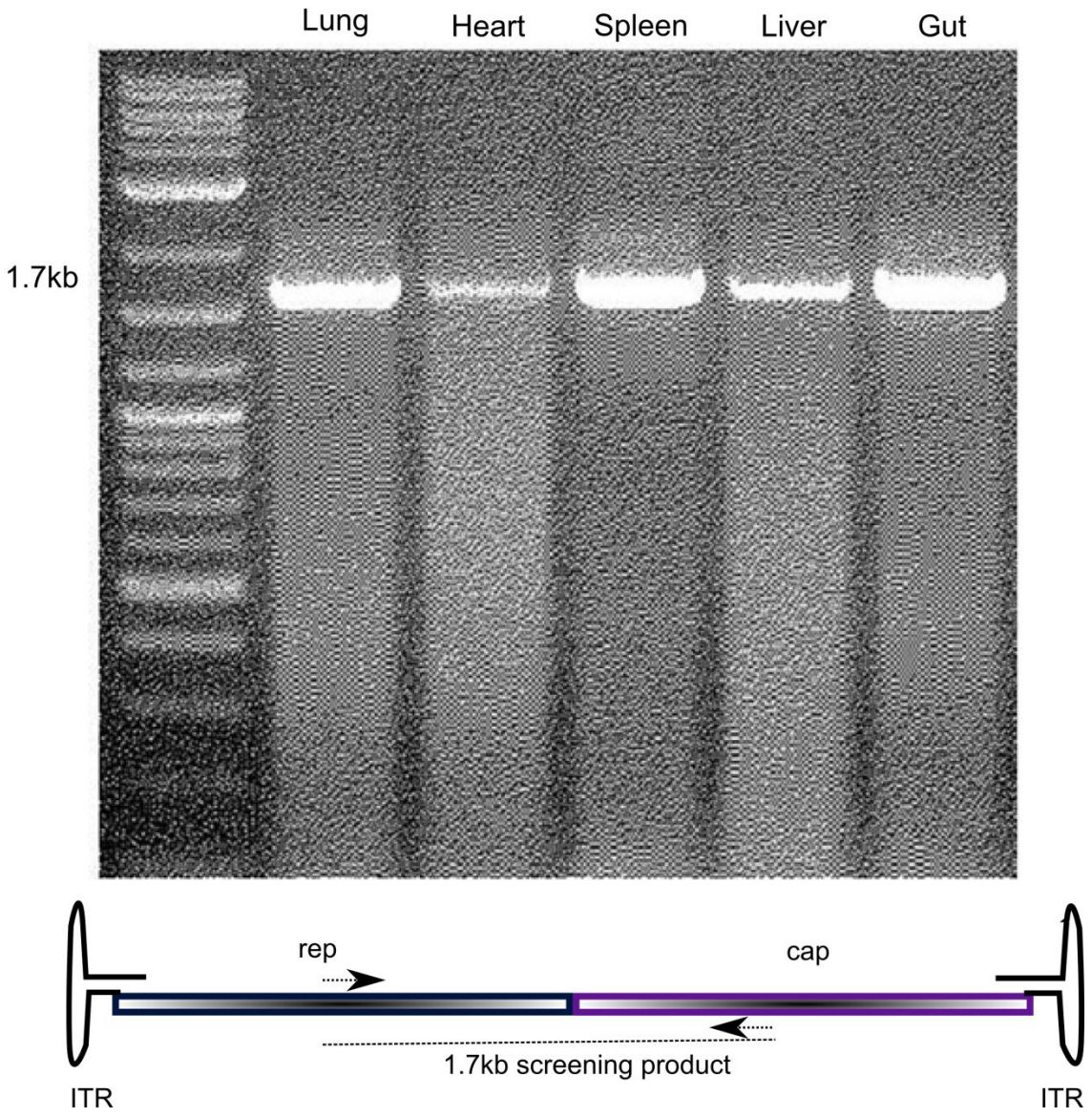

Figure 1 AAV DNA can be isolated from the genomic DNA of various pig organs. Genomic DNA was isolated from pig organs including the lung, heart, spleen, liver, and gut. PCR was performed on pig genomic DNA using the primers RC+ and SIG- to isolate unique AAV sequences ~1.7 kb in length. The isolated AAV DNA spans the 3' end of the *rep* and the 5' end of the *cap* gene.

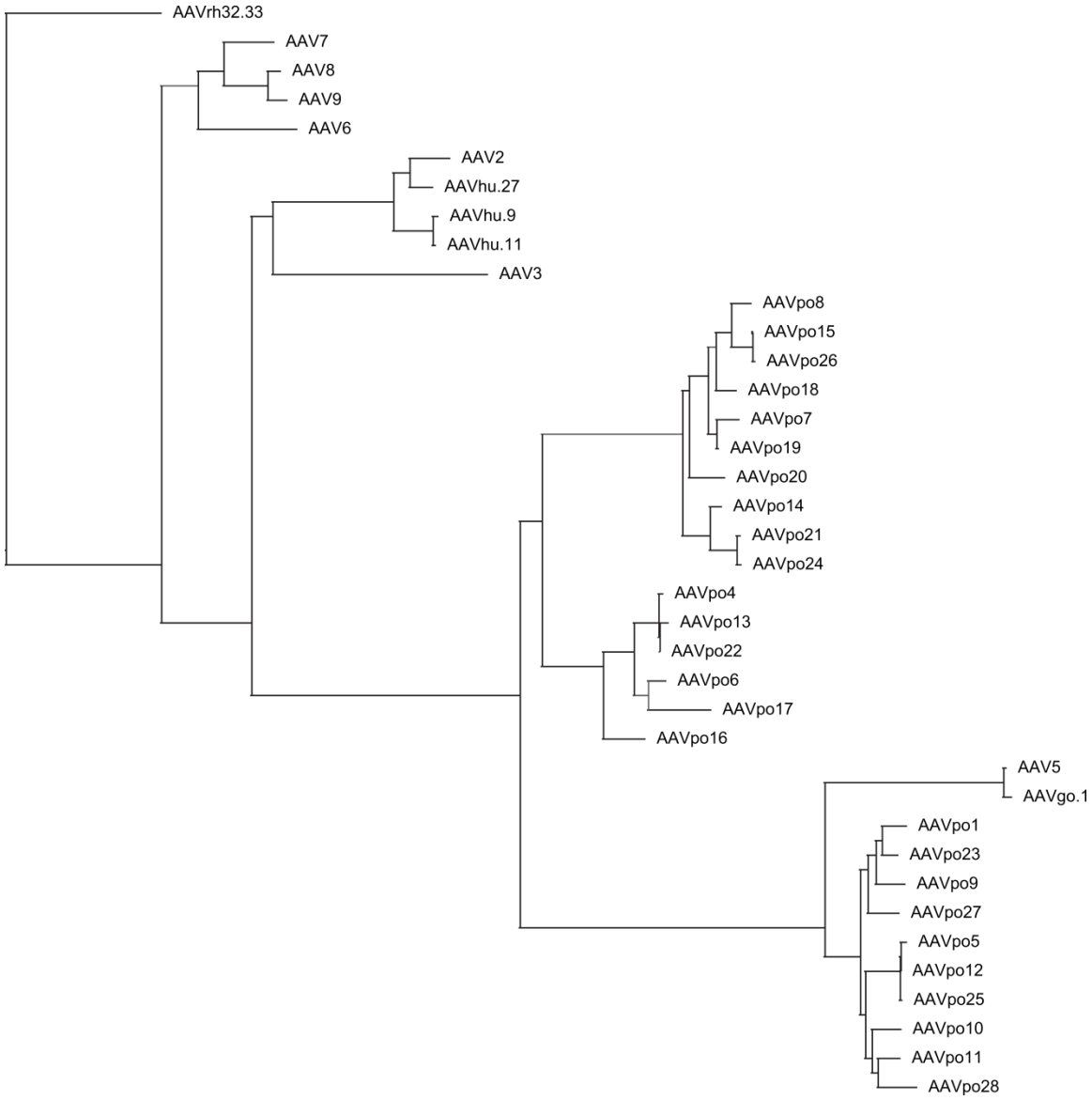

Figure 2 Porcine-derived AAVs are highly divergent from published AAV sequences. Partial *cap* sequences corresponding with approximately the first 900 bp of the *cap* gene of porcine-derived AAVs was aligned with the same region of published AAVs. Alignments reveal that porcine-derived AAVs (denoted by AAVpo*, where * is the sequential order, in which the porcine AAVs were isolated) are unique and form three new clades within the AAV species. Alignments were performed using Clustal W method in DNASTAR® MegAlign™. Phylogenetic trees were generated using TreeView and manipulated with Inkscape.

3.2 ISOLATION AND PHYLOGENETIC ANALYSIS OF NOVEL FULL-LENGTH AAV CAP GENES

In order to isolate the full-length *cap* genes of novel porcine-derived AAVs, nested thermal asymmetric interlaced (TAIL) PCR was utilized. For this PCR, two specific primers (Nestedcap1+2.7kb and Nestedcap2+2.4kb), along with an arbitrary degenerate primer (CED-), were used in two consecutive PCRs. From the alignment of the porcine-derived AAVs, primers were designed based on conserved regions shared between all the porcine-derived AAVs, allowing for isolation of DNA 2.7 kb and 2.4 kb in length for the primary and secondary PCR reactions, respectively (Figure 3).

Using this method, the complete *cap* genes were produced for 6 of the novel porcine AAVs thereafter denoted AAVpo1, -po4, -po5, -po6, -po7, and -po8. The completed *cap* genes were then cloned into an expression vector along with AAV2 *rep*. Furthermore, although only partial sequence of the 3'-end of *cap* for AAVpo2 was obtained, this isolate shared 95.2% homology with AAVpo6. It was conceptualized that the high homology between the two AAVs would allow for production of viable rAAV hybrid particles. By this logic, the first 255 base pairs (bp) of AAVpo6 *cap* was used to complete the missing beginning portion of the AAVpo2 *cap*, resulting in the creation of a hybrid capsid of both AAVs and production of viable particles named AAVpo2.1. From these results, the first hypothesis that 'AAV exists within porcine genomic DNA' was answered by successful isolation of the full-length *cap* genes of AAVs from pig tissues.

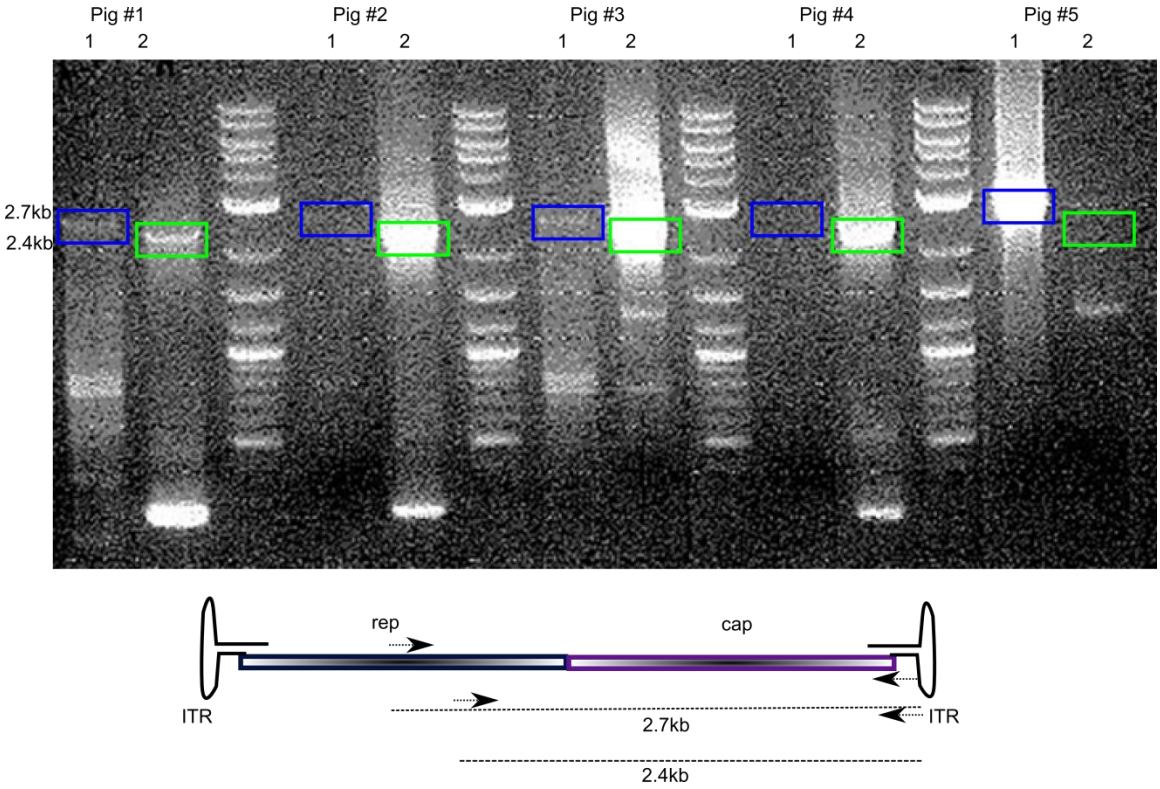

Figure 3 Full-length AAV *cap* can be isolated by nested TAIL PCR. Primers for specific conserved regions within the *rep* gene of porcine-derived AAVs and an arbitrary degenerate primer were utilized in a Nested TAIL PCR to isolate DNA ~2.7 kb in length for the primary reaction (as indicated by blue boxes), and 2.4 kb in length for the secondary reaction (as indicated by green boxes).

Following isolation of the *cap* gene, a phylogenetic analysis was performed with the *cap* of other published AAVs to determine whether or not porcine-derived AAV *cap* genes were indeed unique sequences. In fact, the results showed that porcine-derived AAVs were divergent from published AAV sequences. Results indicate that AAVpo2.1, -po4, and –po6 nucleotide sequences are most closely related to AAV2 with 80.6%, 79.8%, and 80.6% similarity, respectively. In contrast AAVpo1 and AAVpo5 were closer to AAV5 with 84.8% and 85.4% sequence homology, respectively (Table 3, Figure 4). The closest relatives to AAVpo7 were AAV2 and AAVpo2.1 with 77.3 and 90.1% homology, respectively; AAVpo8 was found to be highly divergent from known published human and NHP AAVs, as well as porcine-derived AAVs, with its nearest relatives being AAV8 and AAVpo2.1 at 68.7 and 75% homology, respectively.

Since AAV2 has been extensively studied and AAV1 was the first AAV described, an alignment of protein sequences from the porcine-derived AAVs and AAV1 and AAV2 capsid sequences was performed using the Clustal W method of MegAlign software. Here, the alignment was used to determine whether or not VP1, VP2, and VP3 were being translated at the same relative positions to that of other published AAVs. According to the alignment, porcine-derived AAV translation of VP1 and VP3 begins with methionine, while VP2 beings with threonine, similar to that of AAV1 and AAV2. The alignment revealed that VP1, VP2, and VP3 for the porcine-derived AAVs were being translated at the same relative positions to AAV1 and AAV2. The predicted lengths of VP1 are: 717 aa for AAVpo1, 727 aa for AAVpo2.1, 728 aa for AAVpo4, 717 aa for AAVpo5, 727 aa for AAVpo6, 726 aa for AAVpo7 and 729 aa for AAVpo8, compared to 737 aa and 736 aa for AAV1 and AAV2, respectively (Figure 5).

Table 3: Percent similarity of porcine-derived AAVs with published AAV sequences

	AAV po1	AAV po2.1	AAV po4	AAV po5	AAV po6	AAV po7	AAV po8
AAVpo1	-	-	-	-	-	-	-
AAVpo2.1	67.5	-	-	-	-	-	-
AAVpo4	68.5	90.5	-	-	-	-	-
AAVpo5	89.8	67.8	68.7	-	-	-	-
AAVpo6	67.5	92.4	90.1	67.9	-	-	-
AAVpo7	66.9	90.1	86.9	66.6	87.6	-	-
AAVpo8	66.5	75	74.4	65.7	74	78.8	-
AAV1	64.8	77.1	77.3	63.2	76.9	74.5	67.7
AAV2	64.5	80.6	79.8	63.9	80.6	77.3	68.6
AAV3	63.5	76.3	76.1	63.5	76.2	75.1	68.1
AAV4	61.1	65.3	66.3	60.9	65.8	64.2	63.4
AAV5	84.8	66.9	67.6	85.4	66.9	66.5	65.4
AAV6	64.7	77.2	77.3	63.1	76.8	74.5	68
AAV7	64.8	77.2	77.3	64.8	77.2	75.3	69
AAV8	65.7	77.1	77.5	65.6	77.9	75.3	68.7
AAV9	64.9	75.8	76.6	64.3	76.4	74.2	66.8
AAV rh32.33	60.2	64.1	64	59.7	64.5	63.3	62.6

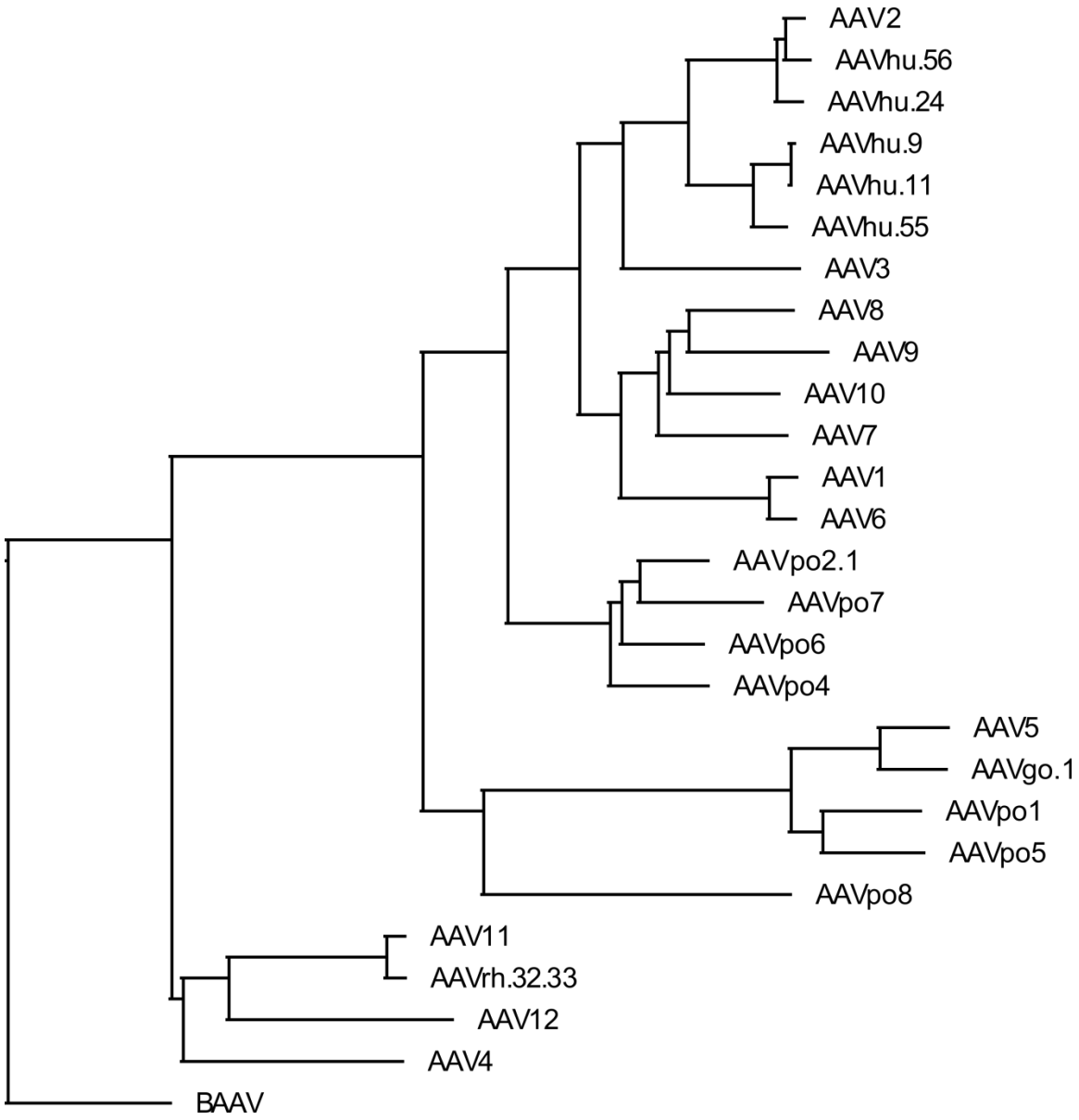

Figure 4 Porcine-derived full-length *cap* is highly divergent from published AAV sequences. The *cap* of porcine-derived AAVpo* (where * indicates the sequential order, in which the porcine AAVs were identified) were aligned with known published AAV *caps*. Alignments were performed using the Clustal W Method in MegAlign of the DNASTAR Lasergene 9 software package. The phylogenetic tree was visualized using TreeView and manipulated with Inkscape.

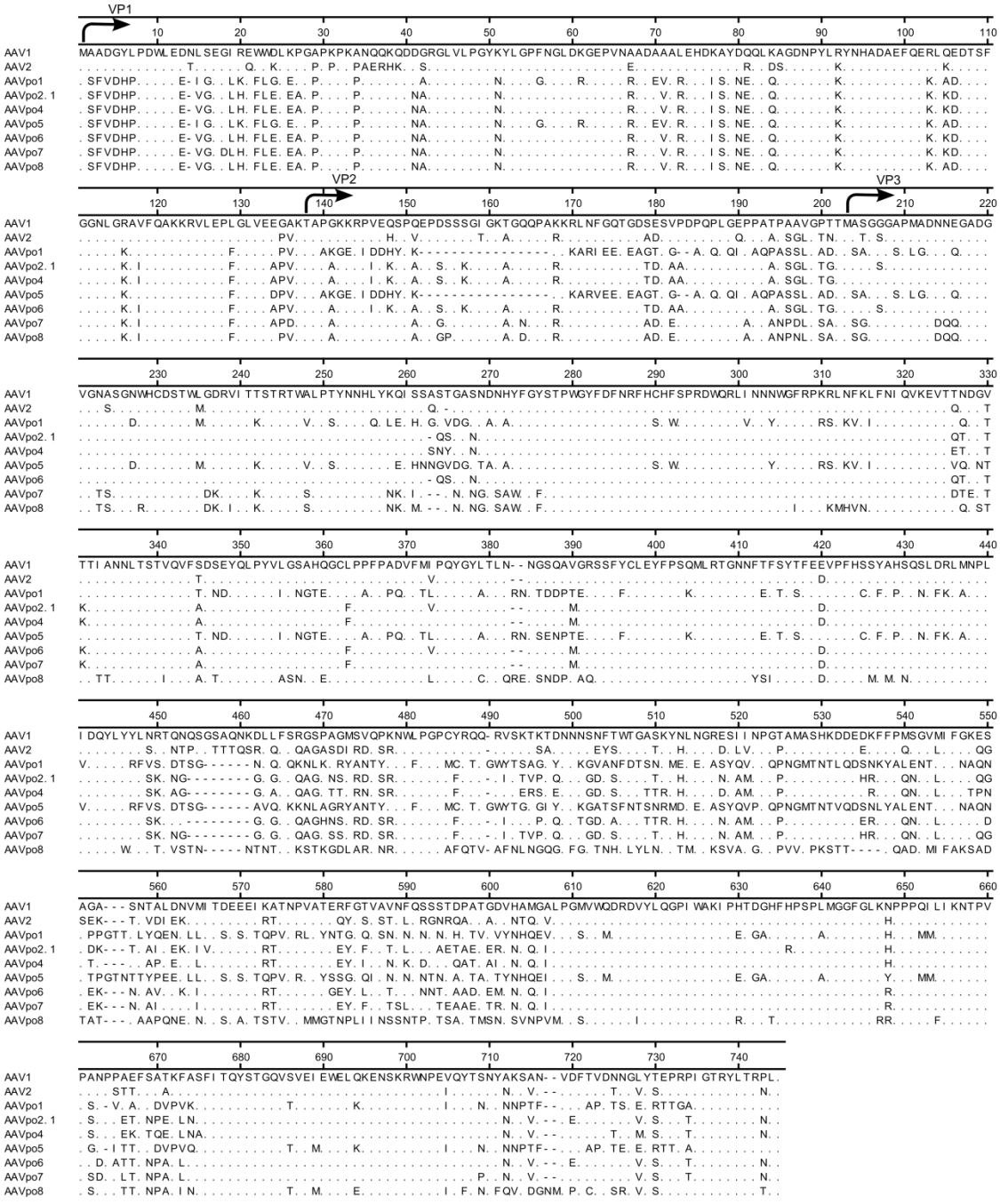

Figure 5 Porcine-derived AAV Cap amino acid sequences are highly divergent from AAV1 and AAV2 cap sequences. The amino acid sequences of porcine-derived AAVs were aligned using the Clustal W method of MegAlign software with AAV1 and AAV2. The start positions for VP1, VP2, and VP3 are indicated by curved arrows. VP1 and VP3 begin with an M, whereas VP2 begins with a T. Conserved residues are indicated by dots, whereas absent amino acids are indicated by dashes.

3.3 PRODUCTION AND VISUALIZATION OF RECOMBINANT PORCINE AAV VECTORS EXPRESSING LACZ, H05HA, OR EBOVGP

Before answering the second hypothesis that 'AAVs derived from porcine tissues can efficiently transduce mammalian and human cells', it was important to verify that the *cap* sequences resulted in formation of capsid proteins and the production of viable particles. Production of porcine recombinant AAV (rAAV) vectors expressing LacZ, H05HA or EBOVGP was achieved via triple transfection of helper, packaging, and transgene plasmids using a calcium phosphate transfection kit. Briefly, *trans*-packaging plasmid consisting of porcine-derived AAV *cap* and AAV2 *rep*, *cis*- plasmid containing lacZ, H05HA, or EBOVGP flanked by AAV2 ITRs, and the helper plasmid pAdΔF6, were transfected into HEK293T cells producing chimeric rAAV particles. Cesium chloride gradient sedimentation has been used for over 40 years to purify viruses and this proven technique was utilized in the purification of rAAV vectors in this thesis (Aaslestad & Hoffman, 1968). Cesium chloride gradients allows for the separation of empty and fully packaged viral particles from cellular proteins and debris based on their buoyant densities. Since AAV banding patterns are difficult to visualize in CsCl gradients, fractions are collected and their refractive indexes measured. Complete AAV viral particles with genomes typically band in fractions with refractive indexes between 1.375-1.3660. With the aid of a refractometer, each fraction was read and refractive index determined. Fractions reading between 1.375-1.3660 were pooled together and purified. AAV particles are generally sized at 3.9 MegaDaltons; therefore, a 100 KiloDalton filter was used to purify the viral particles in the final purification step (Sonntag et al., 2010).

To confirm viral particle production, VP1, VP2, and VP3 capsid protein expression was examined by Western blot analysis for AAVpo1, -po2.1, -po4, -po5, -po6, and AAV5 and -8 as controls from transfected cellular pellet (Figure 6). Although *cap* gene was isolated for AAVpo7 and –po8, proteins from these AAVs were not successfully visualized by Western blot. Using commercial monoclonal antibodies for AAV VP1, VP2, and VP3, Western blot analysis revealed that VP1, VP2, and VP3 were being produced for AAVpo1, -po2.1, -po4, -po5, and –po6, with sizes of approximately 87, 72, and 62 KiloDaltons, respectively.

Positive identification of proteins via Western blot analysis does not necessarily indicate particle formation; therefore, electron microscopy (EM) was employed to visualize particle formation. Each AAV serotype was subjected to EM to confirm that capsids were being formed. From these results, it was found that transfection of AAV *cap* DNA for AAVpo1, -po4, -po5, and –po6 resulted in the formation of icosahedral capsids, approximately 26nm in diameter (Figure 3.3.2). Empty capsid particles are signified by icosahedral particles with a donut-like appearance on the inside, whereas fully packaged virions appear as solid icosahedral particles. From EM analysis of the porcine-derived AAV viral preparations, >90% of virions appeared to be intact, signifying vectors carrying genomes.

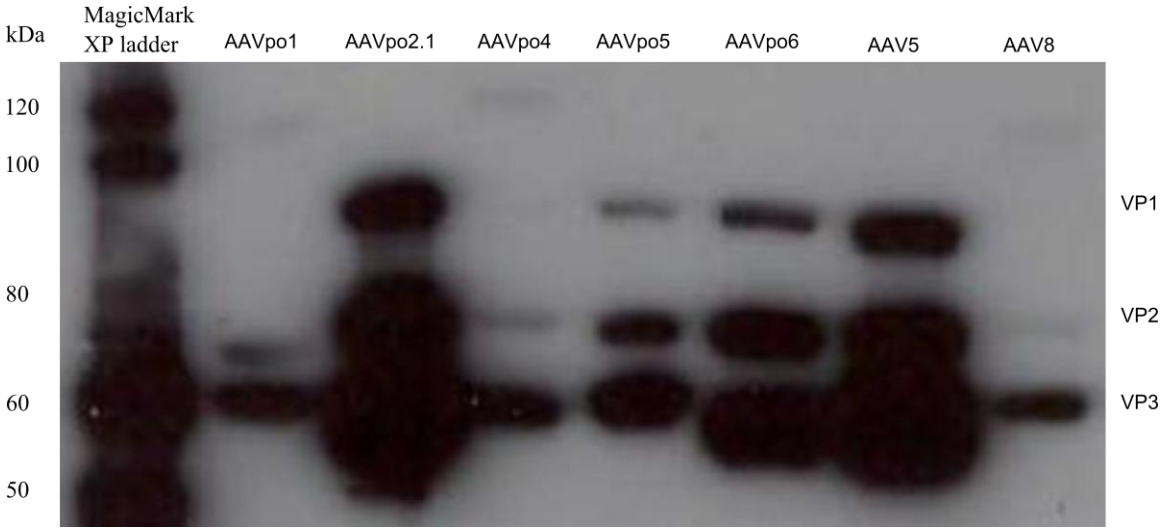

Figure 6 Cap of porcine-derived AAVs form VP1, VP2, and VP3 proteins. *Trans* plasmid containing DNA for *cap* of AAVpo1, -po2.1, -po4, -po5, and –po6 were transfected into HEK293T cells. Cell lysate was harvested 48 hours post-transfection and Western blot analysis performed to visualize AAV proteins using polyclonal antibodies against AAV VP1, VP2, and VP3 at ~87, 72, and 62 kDa.

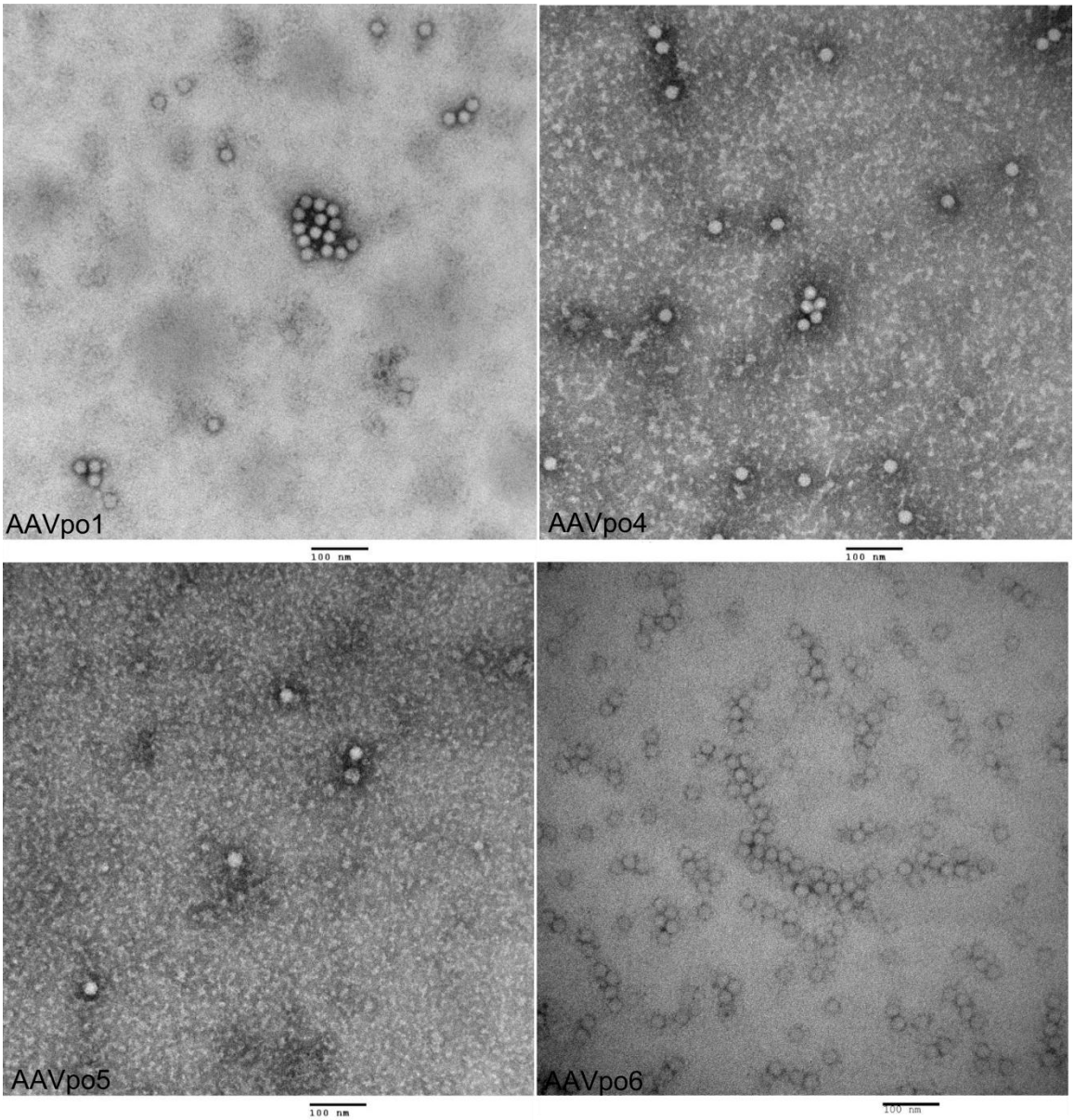

Figure 7 Porcine-derived AAVs form icosahedral particles. Porcine-derived AAVs were transfected into HEK293T cells and harvested and purified 72 hours post-transfection. The viral preparations were processed for EM, revealing isosahedral capsids of ~26 nm in diameter being formed for AAVpo1, -po4, -po5, and –po6 at x100,000 magnification.

3.4 IN VITRO TESTING OF AAV-LACZ VECTORS IN CELL LINES

Following verification of the production of complete AAV vectors, the vectors were tested for transduction efficiency *in vitro* in a number of cell lines (Table 4). Initial testing indicated that AAVpo1, -po4, and –po5 transduced a number of different cell lines originating from different species including human, monkey, dog, pig, and mouse at different efficiencies when infected at the same multiplicity of infection. Cell types included liver, lung, kidney, retina, fibroblast, and macrophage cells. AAV5 was used as a comparative control and was found to transduce a majority of these cell lines at a greater efficiency, with the exception of VR1BL, in which all the porcine-derived AAVs tested performed much better than AAV5. AAVpo2.1 and AAVpo6 were only tested in HEK293 cells and both were found to transduce this cell type efficiently. The goal of this experiment was to determine if porcine-derived AAVs can transduce human cell lines and it was found that porcine-derived AAVs did indeed transduce human cells quite efficiently along with cell lines derived from other species, although their efficiencies may not have been as high as AAV5 for some cell types.

Table 4: Transduction Efficiency of AAV Vectors in Various Cell Lines

		Vector					
Cell Lines	Source	AAVpo1	AAVpo4	AAVpo5	AAV5	AAVpo2.1	AAVpo6
Hep G2	human liver	1.27E+03	4.88E+02	5.38E+01	5.60E+02	N/A	N/A
A549	human lung	2.30E+00	3.16E+00	3.87E+00	9.67E+00	N/A	N/A
COS	monkey kidney	3.37E+03	2.98E+03	5.70E+01	1.11E+04	N/A	N/A
MDCK	canine kidney	2.42E+02	1.81E+01	7.63E+00	5.89E+01	N/A	N/A
VR1BL	porcine retina	5.30E+04	6.39E+04	7.63E+03	8.11E+02	N/A	N/A
NIH3T3	mouse fibroblast	1.27E+01	4.18E+01	1.28E+01	9.89E+01	N/A	N/A
RAW 264.7	mouse macrophage	3.33E+01	2.70E+00	1.40E+01	6.56E+00	N/A	N/A
Vero E6	monkey kidney	3.94E+02	6.32E+02	1.88E+03	9.44E+02	N/A	N/A
ARPE 19	human retina	1.44E+01	4.56E+01	3.66E+00	2.67E+03	N/A	N/A
HEK293	human kidney	3.20E+01	9.30E+01	5.20E+01	1.90E+02	4.20E+01	2.00E+03

Values represent the mean number of transduced cells at a multiplicity of infection (MOI) of 250 gc of AAV vector per cell (*n*= 3 wells). N/A = not analyzed

3.5 BIODISTRIBUTION OF AAV-LACZ VECTORS

The second part of the second hypothesis was to evaluate whether or not porcine-derived AAVs can transduce mammalian cells *in vivo.* For this experiment, biodistribution data for AAV5 and AAV8 were already established and these vectors were shown to transduce cells efficiently when injected systemically. Therefore, AAV5 and AAV8 were utilized as controls for biodistribution experiments in this thesis. Each AAV vector expressing LacZ or PBS was administered through intravenous tail vein (IVTV) injection at a dose of $1.0x10^{11}$ gc per mouse in a 100 µl solution (n= 3). Mice were sacrificed 28 days post-injection to allow for sufficient gene expression and tissues were harvested. The tissues processed for DNA extraction were the spleen, pancreas, heart, lung, liver, kidney, small intestine, large intestine, tibialis anterior muscle, ovary, and brain. When delivered systemically, both AAVpo1 and -po2.1 performed relatively poorly in all major organs except the muscle. AAVpo2.1 specifically targeted the heart and kidneys as higher titers were obtained from this particular organ compared to its relative levels in other sampled tissues (Figure 8). For AAVpo4 and –po6, transduction efficiencies were very similar to each other, as well as AAV8. High titers were obtained in every organ sampled, especially in the liver where an average of 61.9, 21.0, and 52.4 genome copies (gc)/cell were measured for AAVpo4, AAVpo6, and AAV8, respectively (Figure 8). More interestingly, the presence of AAVpo4 and AAVpo6 gc were observed in the mouse brain at mean titers of 0.188 gc/cell for AAVpo4 and 0.147 gc/cell for AAVpo6, in contrast to AAVpo2.1 and –po5, which only produced 0.013 and 0.002 gc/cell, respectively. The presence of AAV gc for AAVpo4 and –po6 raised the question whether or not these vectors have the ability to pass through the blood-brain barrier and

transduce cells in the brain, leading to further investigation into the transduction efficiency of these vectors in the mouse brain. AAVpo5 was shown to perform very poorly in the biodistribution studies, with levels similar to the negative control and a low mean gc/cell across the board for all the major organs tested. Samples were considered positive for the presence of AAV DNA if genome copies were at least three times that of the PBS negative control.

3.6 TRANSDUCTION EFFICIENCY IN THE MUSCLE AT VARIOUS TIME-POINTS

Expanding upon the second hypothesis, the ability of porcine-derived AAVs to transduce certain cell types when injected in a specific location was evaluated. Many genetic therapies and vaccinations deliver AAV vectors directly into the muscle of a host; therefore, the ability of porcine-derived AAVs to transduce mouse muscle fibers was assessed. AAV1 and AAV5 were utilized as controls because of their high transduction efficiency in the mouse muscle (Louboutin, Wang, & Wilson, 2005). Each porcine-derived AAV vector was administered into the tibialis anterior muscle of BALB/c mice (n= 3) in a 50 µl volume at a titer of $1x10^{11}$ gc, and the muscle was harvested at days 7, 14, 21, and 28 post-injection. Histochemistry and X-gal staining was performed to reveal transduction efficiency at each time point. For levels of X-gal expression from the various porcine-derived AAVs, the rankings from the fastest to slowest are as follows: AAV1>AAVpo6>AAVpo5>AAVpo4>AAVpo1/AAV5>AAVpo2.1 (Figure 9). However, when peak expression was examined, the rankings from highest to lowest expression are as follows: AAV1/AAVpo4>AAVpo6>AAVpo5>AAVpo2.1 (AAVpo1 and AAV5 were not included in this comparison as samples were not taken at the later

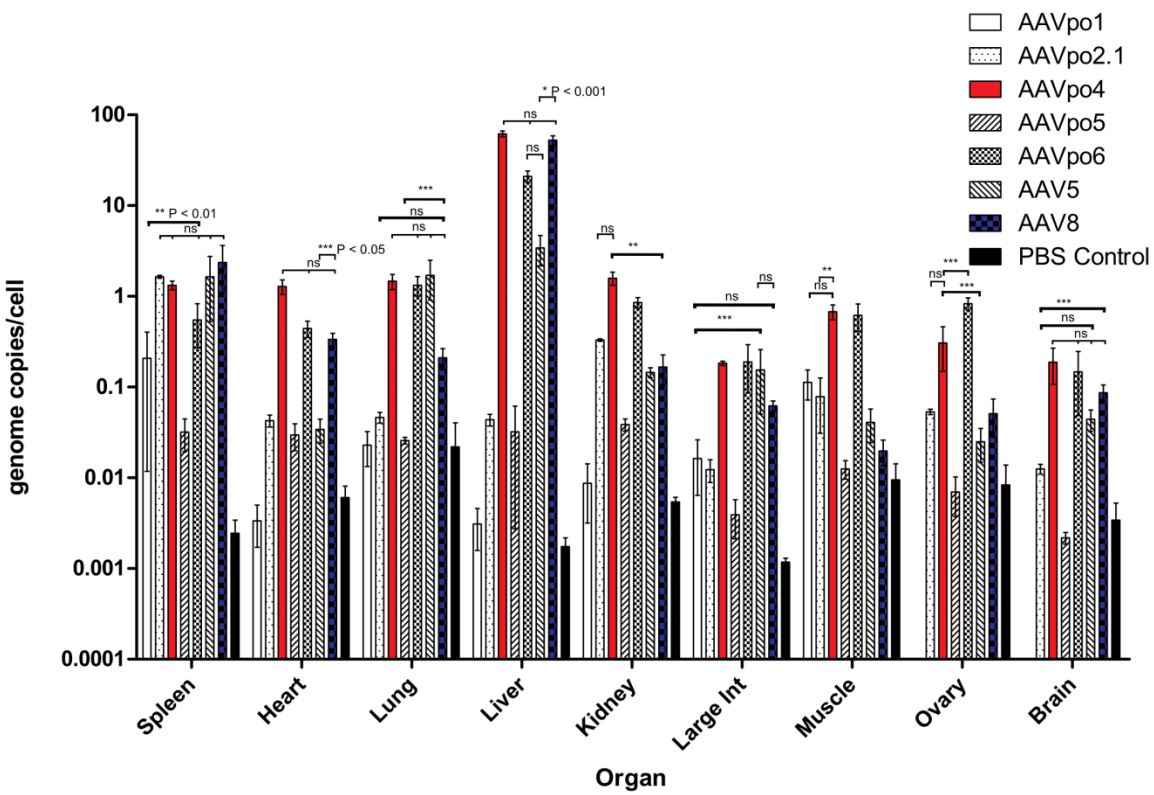

Figure 8 Certain porcine-derived AAVs can transduce most mouse organs efficiently compared to 'gold-standard' AAVs. Mice were injected via IVTV with $1x10^{11}$ gc/mouse of AAV-LacZ vector. 28 days post-injection, the organs were harvested and processed for DNA extraction. qPCR was performed on all organs and the amount of AAV vector genome copies/cell was reported. *n*= 3 mice, bars represent the mean±SEM. ns= not statistically significant

time-points). Beta-gal expression was quantified using a Beta-gal Assay kit from Genlantis (Figure 10). Peak expression levels of Beta-gal were achieved and maintained up to 28 days post injection by AAVpo6 on day 14 similar to that of AAV1, with mean expression levels of 7.77±.994 and 9.75±.037 beta-gal milliunits, respectively. Expression from the AAVpo4 vector did not reach similar mean levels until day 28 (8.61± beta-gal milliunits). Beta-gal expression was not measurable for AAVpo2.1 using our methods up until day 14, although minimal expression was observed on stained sections by day 7. Peak beta-gal expression for AAVpo2.1 was at day 21 with 0.707±.066 beta-gal milliunits. AAVpo5 appeared to have consistent mean beta-gal expression throughout all time-points beginning with 1.62±.092 beta-gal milliunits at day 7 and 2.54±.186 beta-gal milliunits by day 28; albeit peak levels were relatively low compared to more efficient AAV vectors (AAVpo4, -po6, and AAV1). AAVpo6 was the most efficient porcine-derived AAV at transducing the muscle, and had expression levels similar to that of AAV1 at all time-points.

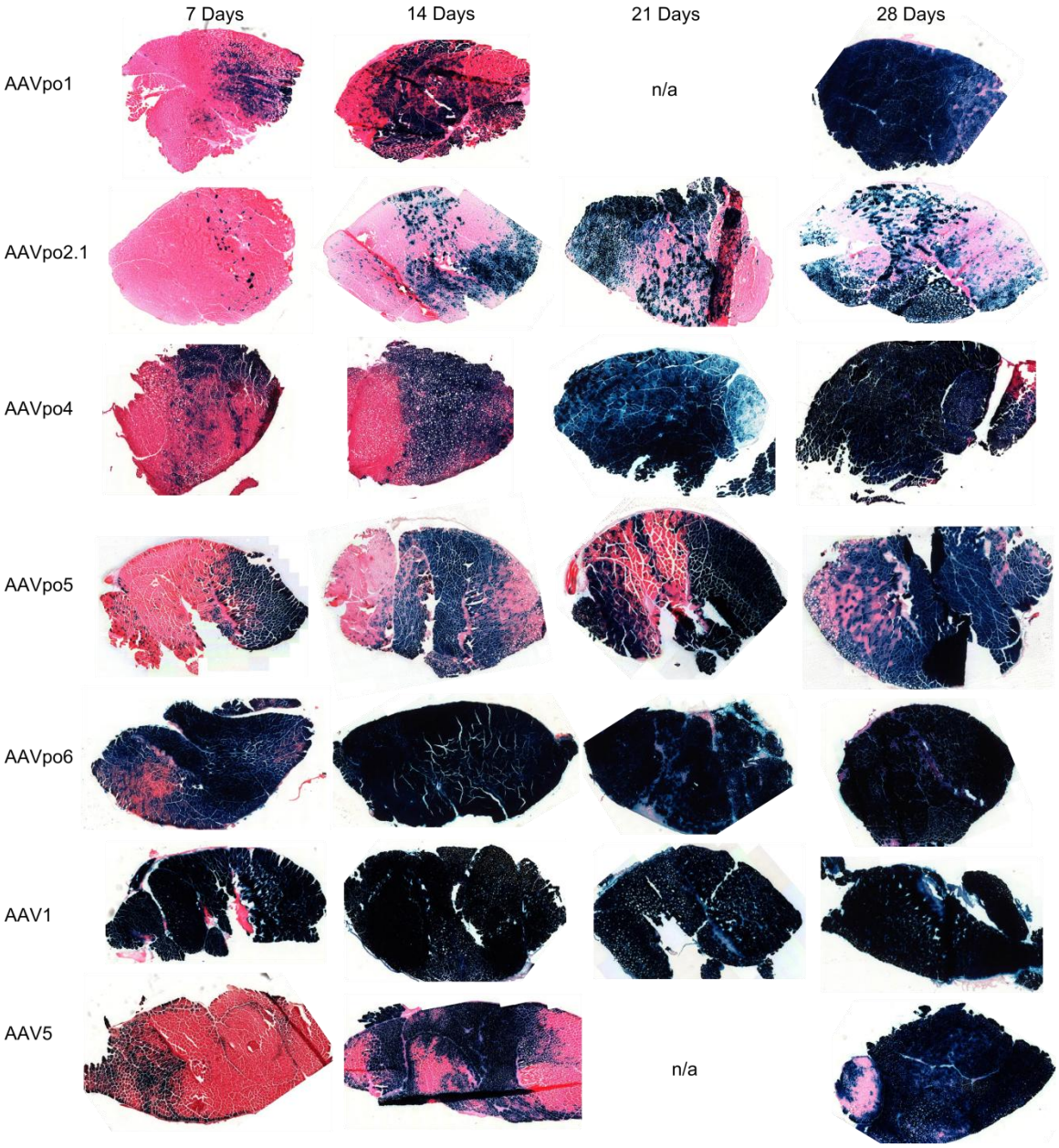

Figure 9 Porcine-derived AAVs have robust transgene expression in the mouse muscle when administered via IM injection. AAV-LacZ was administered at a dose of $1x10^{11}$ gc per mouse via IVTV. Muscles were harvested at various time-points and frozen with O.C.T., cryosectioned at 10 µm, and stained with eosin and X-gal. Blue cells are indicative of AAV transduced cells. Pictures are representative of *n*= 5 mice.

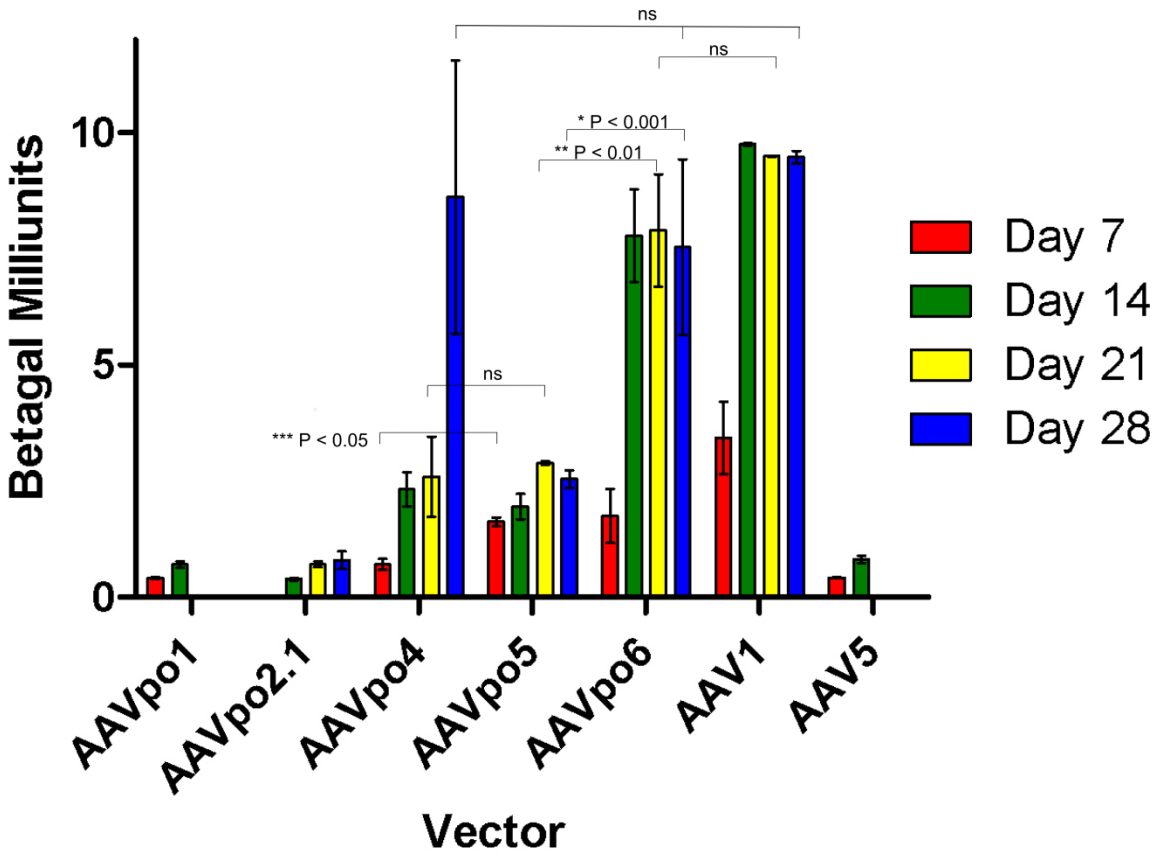

Figure 10 AAVpo4 and –po6-LacZ vectors produce high levels of β-gal expression equivalent to AAV1-LacZ in mouse muscle. Mice were injected with AAV-LacZ vectors and muscle was harvested at different time-points. 10 sections at 10 µm thick were collected from various areas throughout the muscle and a beta-gal assay was performed using the Genlantis Betagal Kit. *Values indicate mean beta-gal milliunits per sample. *n*= 3 mice. Values reported are the mean±SEM. Absorbance was recorded at 595 nm.

3.7 TRANSDUCTION EFFICIENCY IN THE LUNG

To assess the ability of porcine-derived AAVs for potential of utilization for therapies in the lung, AAV vectors were administered through the intranasal route in Balb/c mice. Porcine-derived AAVs were injected intranasally (IN) into mice at a dose of $1x10^{11}$ gc per mouse and lungs were harvested 28 days post injection. For better separation of tissue, lungs were inflated with a mixture of PBS and O.C.T., then frozen in O.C.T. and cut using a cryostat, followed by histochemistry and X-gal staining on each section. From these results, AAVpo1, -po4, and –po5 performed poorly in the lungs, with only AAVpo6 transducing these tissues. When delivered through the intranasal route, AAVpo6 transduced both airway epithelia and pneumocytes, making AAVpo6 a potential candidate for transduction of lung tissues (Figure 11). As a control, AAV5 was utilized as it is considered one of the most efficient AAVs for transduction of lung tissue (Zabner et al., 2000).

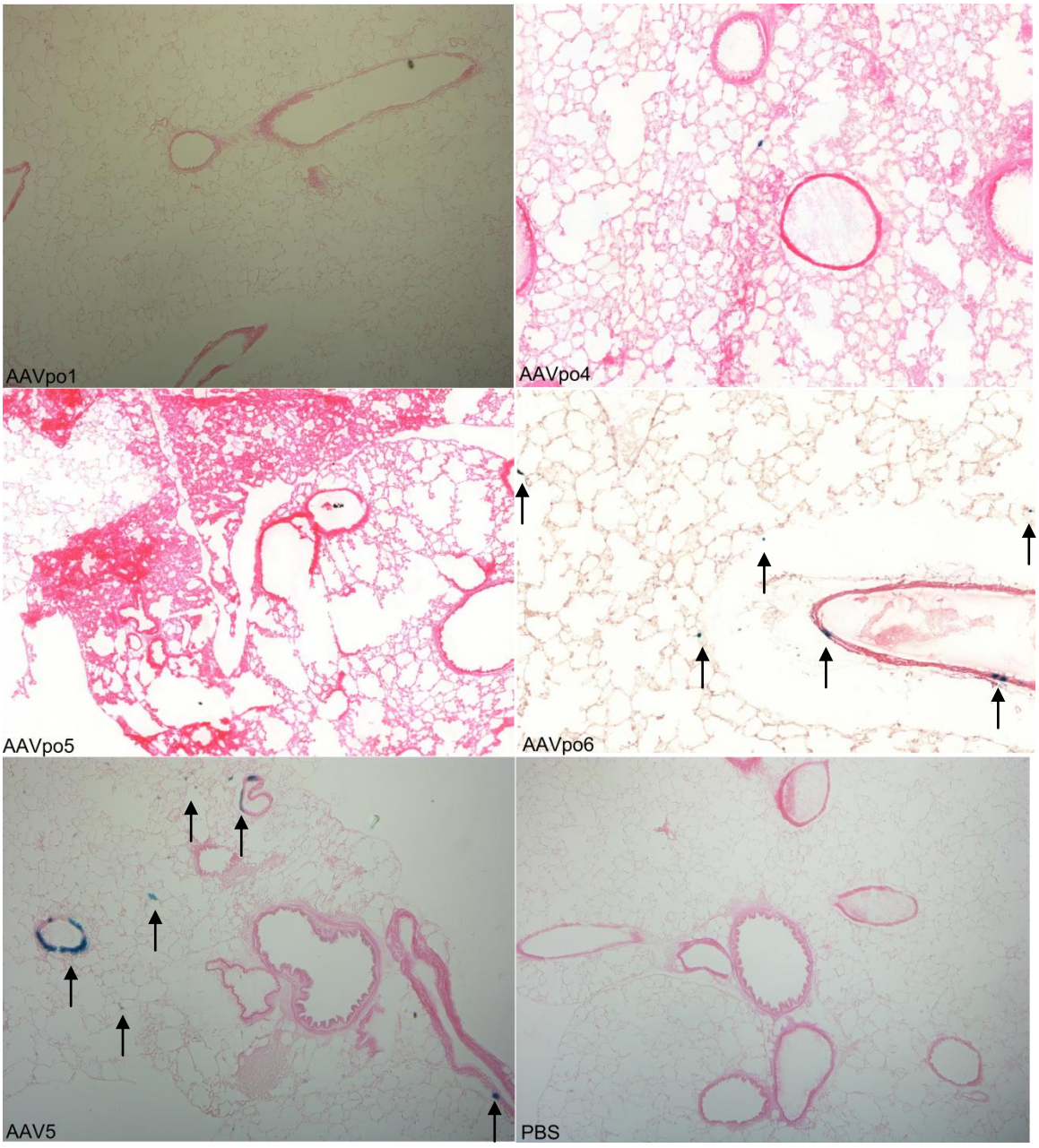

Figure 11 AAVpo6-LacZ transduces cells in the lung parenchyma. Mice were injected intranasally with $1x10^{11}$ gc of AAV-LacZ vector and lungs were harvested 28 d.p.i. The lungs were frozen and cut using a cryostat, then stained with X-gal and eosin. Arrows indicate blue cells successfully transduced by AAV-LacZ vector.

3.8 TRANSDUCTION EFFICIENCY IN THE LIVER

Many genetic diseases affecting the liver such as human FIX and α1-antitrypsin deficiency have been treated with gene therapy vectors AAV2 and AAV8 with promising results. However, for other genetic diseases requiring targeting to specific organs other than the liver, detargeting of the liver may be beneficial for vectors required for systemic transduction. For example: AAVs specifically targeting muscle cells and not liver may result in minimizing hepatocellular damage. In order to evaluate the performance of porcine-derived AAVs in liver tissue, each AAV vector was delivered systemically through IVTV in BALB/c mice (*n*= 3 mice) and the liver was harvested 28 days post-injection. Liver sections from IVTV injected mice were embedded in O.C.T. and histochemistry and X-gal staining performed to visualize β-gal activity of transduced cells (Figure 12). Results from histochemistry and X-gal staining on liver samples from AAVpo4 and –po6 injected mice corroborated with the qPCR biodistribution data presented previously. AAV8, a 'gold-standard' AAV vector for transduction of hepatocytes, was used as a comparison to evaluate the performance of the porcine-derived AAVs. Agreeing with previous studies, AAV8 was shown to have high gc present in the liver. Interestingly, it was found that the average number of gc/cell present in the liver for AAV8 injected mice (52.4) was very similar to that of animals injected with the AAVpo4 vector (61.9). There was anecdotal evidence of lack of hepatic transduction from mice injected with AAVpo1, AAVpo2.1, or AAVpo5.

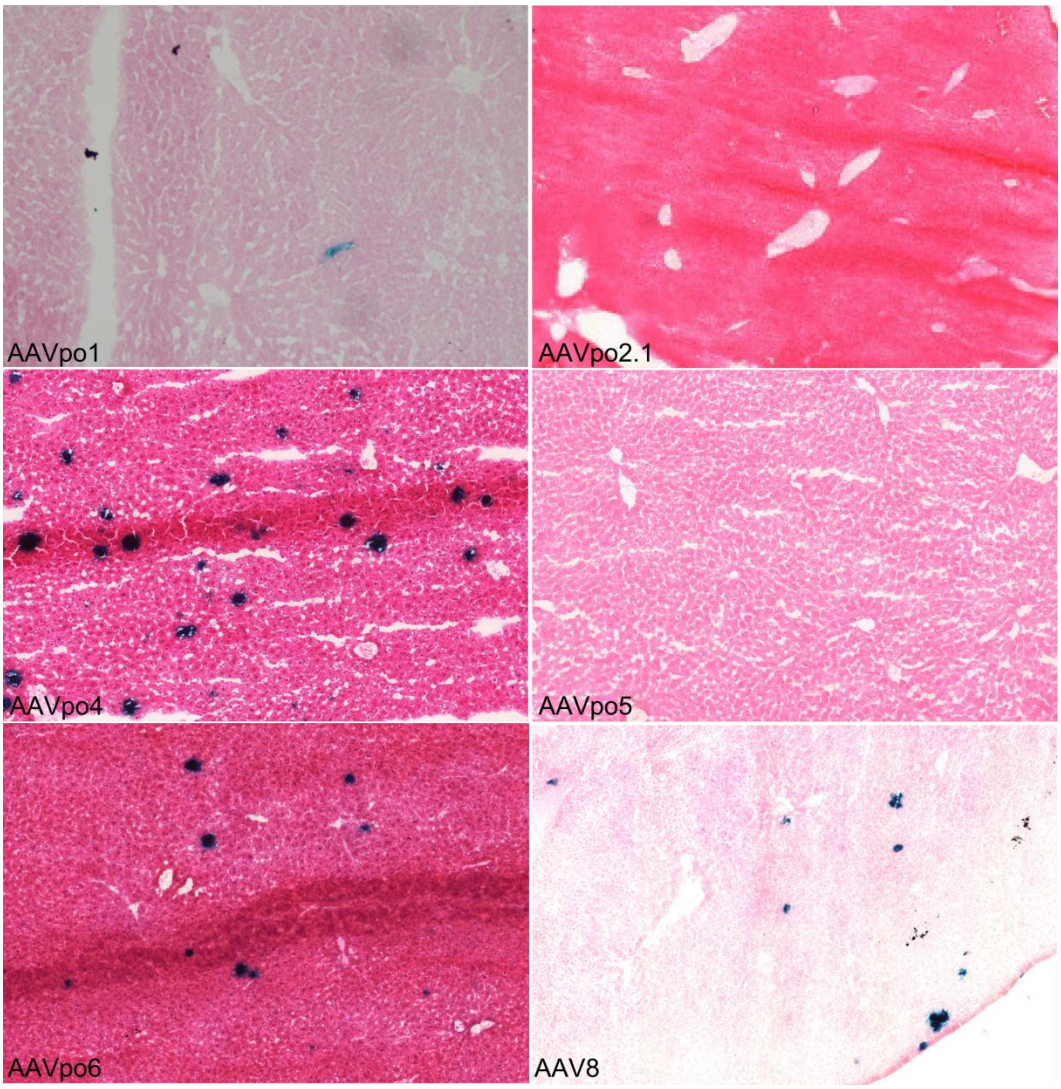

Figure 12 AAVpo4 and –po6-LacZ show robust transgene expression in the mouse liver when delivered systemically. Mice were injected intravenously via tail-vein with $1x10^{11}$ gc of AAV-LacZ vector, and livers were harvested 28 d.p.i. The liver was frozen in O.C.T. and cut using a cryostat. X-gal staining was performed to reveal successfully transduced cells as indicated in blue.

3.9 TRANSDUCTION EFFICIENCY IN BRAIN

Diseases such as Alzheimer's and Batten's disease primarily affect the brain, with many favorable results from clinical trials utilizing AAV2 in gene therapies to combat these ailments. From biodistribution studies presented in this thesis, of interest to note was the presence of rAAV genomes in the brain following AAVpo4 and –po6 IVTV injection, indicating that these AAV vectors may be able to traverse the blood-brain barrier and transduce cells in the brain. Following up on these results, mice were injected with AAV-LacZ vectors by IVTV and the different sections of the brain harvested 28 days post-injection. Brain samples from injected mice indicate that transduction by AAVpo4 and -po6 can be observed in widespread areas of the brain including the forebrain, midbrain, and hindbrain (Figure 13). These results are evidence that AAVpo4 and -po6 can traverse the blood-brain barrier, corroborating with results obtained from our biodistribution efforts.

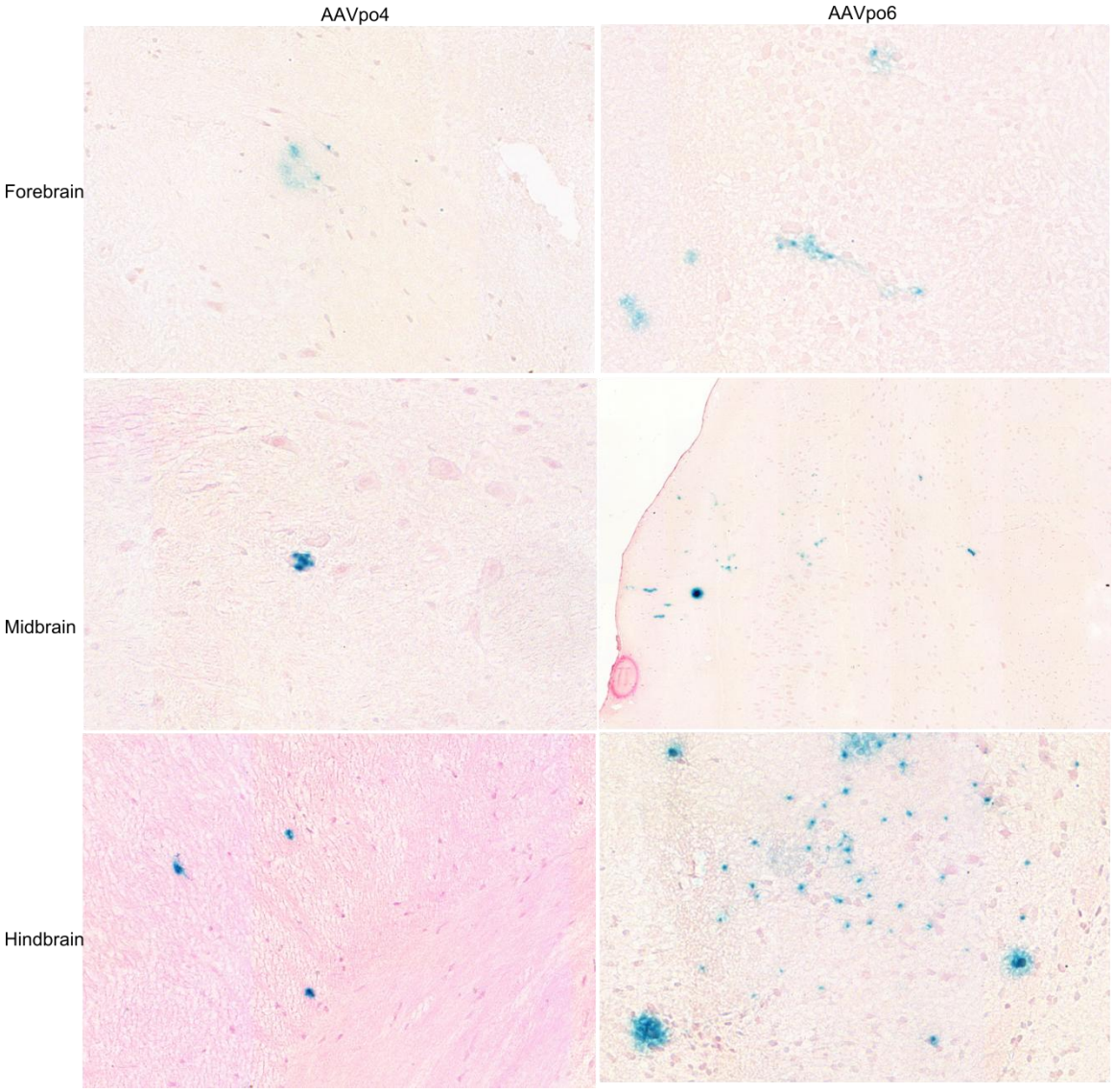

Figure 13 AAVpo4 and –po6-LacZ show transgene expression in the mouse brain when delivered systemically. Mice were injected IVTV with AAV-LacZ vector at a dose of $1x10^{11}$gc and forebrain, midbrain, and hindbrain harvested 28 d.p.i. Tissue was frozen in O.C.T. and sectioned at 10 µm using a cryostat. Sections were eosin and X-gal stained to reveal transduced cells as indicated by blue stains.

3.10 PRE-EXISTING IMMUNITY AND CROSS-REACTIVITY OF AAV ANTI-SERUM WITH AAV VECTOR

Pre-existing immunity towards current AAV vectors is a major drawback for their use in the clinic; novel AAV should have low seroprevalence in the human population to improve their efficacy and reduce toxicity from increased vector dose. AAVs have been shown to be cross-neutralized by anti-sera generated towards others, making it crucial to find AAVs not neutralized by pre-existing immunity or not cross-reactive with other serotypes (Boutin et al., 2010b). Therefore, it was assessed whether neutralizing antibodies against the novel porcine AAVs were detectable in the human population. Neutralization assays were performed using pooled human IgGs from 10,000 to 60,000 donors obtained from CLS Behring, and incubated with each porcine-derived AAV. Briefly, each AAV vector was incubated with pooled human IgGs antisera generated against different AAV serotypes at various dilutions for one hour, followed by incubation of the mixture on HEK293T cells for an additional hour. After infection, the cells were stained with X-gal to look for evidence of transduction. Interestingly, pooled human IgGs failed to neutralize the porcine-derived AAVs, with the exception of AAVpo6 where a relatively high concentration (1.5mg/mL) of pooled human IgGs neutralized 50% of transducing AAVpo6-LacZ particles (Table 5). In contrast, circulating antibodies towards AAV5 was previously reported and it was found that AAV5 was neutralized by a much lower concentration of pooled human IgGs compared to AAVpo6 (0.1875 mg/mL and 1.5 mg/ml, respectively) (Bello et al., 2009).

Historically, AAV vector injection elicits humoral responses to the vector, rendering subsequent use of the same vector in the same individual challenging due to

risk of neutralization by pre-existing immunity. As mentioned previously, there also exists pre-existing immunity towards a number of human and NHP AAVs in the human population, thus making it crucial to identify AAV vectors not cross-neutralized against circulating antibodies. Therefore, it was investigated whether the newly isolated porcine AAVs induce cross-neutralizing antibodies against each other and against other published AAV vectors. To assess this, each porcine-derived AAV was injected IM into naïve mice and serum harvested 28 days post injection. Sera from injected mice were incubated with different AAV vectors for neutralizing antibody assays for 1 hour. Sera from mice vaccinated with AAV vectors failed to neutralize AAVs of other serotypes, showing that each porcine-derived AAV is a distinct serotype. An exception was AAVpo6, which was neutralized by AAVpo2.1 antisera at a 40 fold dilution, in contrast to its AAVpo6 antisera neutralizing it at a 640 fold dilution. As mentioned previously, AAVpo2.1 was constructed using partial sequence of AAVpo6, which may explain why AAVpo6 was partially neutralized by antisera against AAVpo2.1. AAV1, -5, -6, and -8 expressing LacZ were not neutralized by any of the anti-sera produced against porcine-derived AAVs.

Table 5: Neutralizing Antibody Assay of Porcine-Derived AAVs with Mouse antisera against different AAV Serotypes or Pooled Human IgGs.

	Vectors								
Anti-sera	**AAVpo1**	**AAVpo2.1**	**AAVpo4**	**AAVpo5**	**AAVpo6**	**AAV5**	**AAV1**	**AAV6**	**AAV8**
AAVpo1	1:1280	<1:20	<1:20	<1:20	<1:20	<1:20	<1:20	<1:20	<1:20
AAVpo2.1	<1:20	1:320	<1:20	<1:20	1:40	<1:20	<1:20	<1:20	<1:20
AAVpo4	<1:20	<1:20	1:640	<1:20	<1:20	<1:20	<1:20	<1:20	<1:20
AAVpo5	<1:20	<1:20	<1:20	1:40	<1:20	<1:20	<1:20	<1:20	<1:20
AAVpo6	<1:20	<1:20	<1:20	<1:20	1:640	<1:20	<1:20	<1:20	<1:20
AAV5	<1:20	<1:20	<1:20	<1:20	<1:20	1:5120	n/a	n/a	n/a
pooled human Ig (mg/mL)	>12	>12	>12	>12	1.5	0.1875	n/a	n/a	n/a

Values indicate the highest mean neutralizing titer where >50% of transducing particles were neutralized by serum, *n*= 3 mice.

3.11 PROTECTIVE EFFICACY OF AAV VECTORS EXPRESSING H05HA *IN VIVO*

3.11.1 DOSE-RESPONSE OF AAV-H05HA VACCINATED MICE CHALLENGED WITH H05

In order to test the protective efficacy of rAAV vectors in a lethal challenge model, mice were vaccinated against avian influenza H5N1 using AAV encoding the H05HA gene and challenge with $100LD_{50}$ of virus. In a lethal challenge of avian influenza H5N1, DNA vaccines expressing HA afforded 100% protection against challenge, although the mice still showed signs of disease (S Kodihalli et al., 1999). In order to establish a working vaccination dose, a dose-response experiment was performed at doses of $1x10^8$, $1x10^9$, and $2.5x10^{10}$ gc (Figure 14). Mice were injected with the different doses of AAV-H05HA or PBS (*n*= 10 mice) and challenged 28 days post vaccination with $100LD_{50}$ of H05. All AAV-H05HA vectors at a dose $1x10^8$ gc resulted in similar protection between 20-40%, with AAVpo6 having the worst protection at 20% and AAVpo4 and AAVrh32.33 having the best protection at 40%. Increasing the dose one log to $1x10^9$ gc provided a different scenario with AAV8 and AAVrh32.33 conferring the best protection at 90% and 100%, respectively. All the porcine-derived AAVs performed poorly at this dose, affording protection between 50-70% (For AAVpo1 and AAVrh32.33, $p<0.05$ following Mantel-Cox test). However, going up to $2.5x10^{10}$ gc resulted in 100% protection between all the AAV serotypes, with the exception of AAVpo5, which only elicited 90% survival ($p=0.3171$). The mice were weighed daily and signs of disease noted. Looking at the maximum average weight loss, it was apparent that mice vaccinated at the lowest dose resulted in a higher maximum average

weight loss, lower survival, and more severe clinical signs of disease including ruffled fur, hunched posture, and lethargy (Table 6). Vaccination at the highest dose resulted in absence of clinical signs of disease and little to no weight loss, with the exception of the one mouse from the AAVpo5 group, which succumbed to disease (Figure 15). The PBS injected group experienced severe weight loss >20% up to the time of death or when criteria for euthanasia was met. One-way ANOVA of data and Tukey's Multiple comparison Test revealed that there was a significant difference of weight between AAVpo5 vs AAVrh32.33 and AAVpo6 vs AAV8; $p<0.05$. There was no significant difference between all other AAV groups; $p>0.05$.

3.11.2 HUMORAL IMMUNE RESPONSES TO AAV-H05HA VACCINATED MICE

HA, the target of neutralizing antibodies, was shown to be important for protection against influenza (Hobson, Curry, Beare, & Ward-Gardner, 1972). As such, the quality of humoral immune responses generated towards the H05HA transgene was evaluated via neutralizing antibody assays and hemagglutination-inhibition antibody assays.

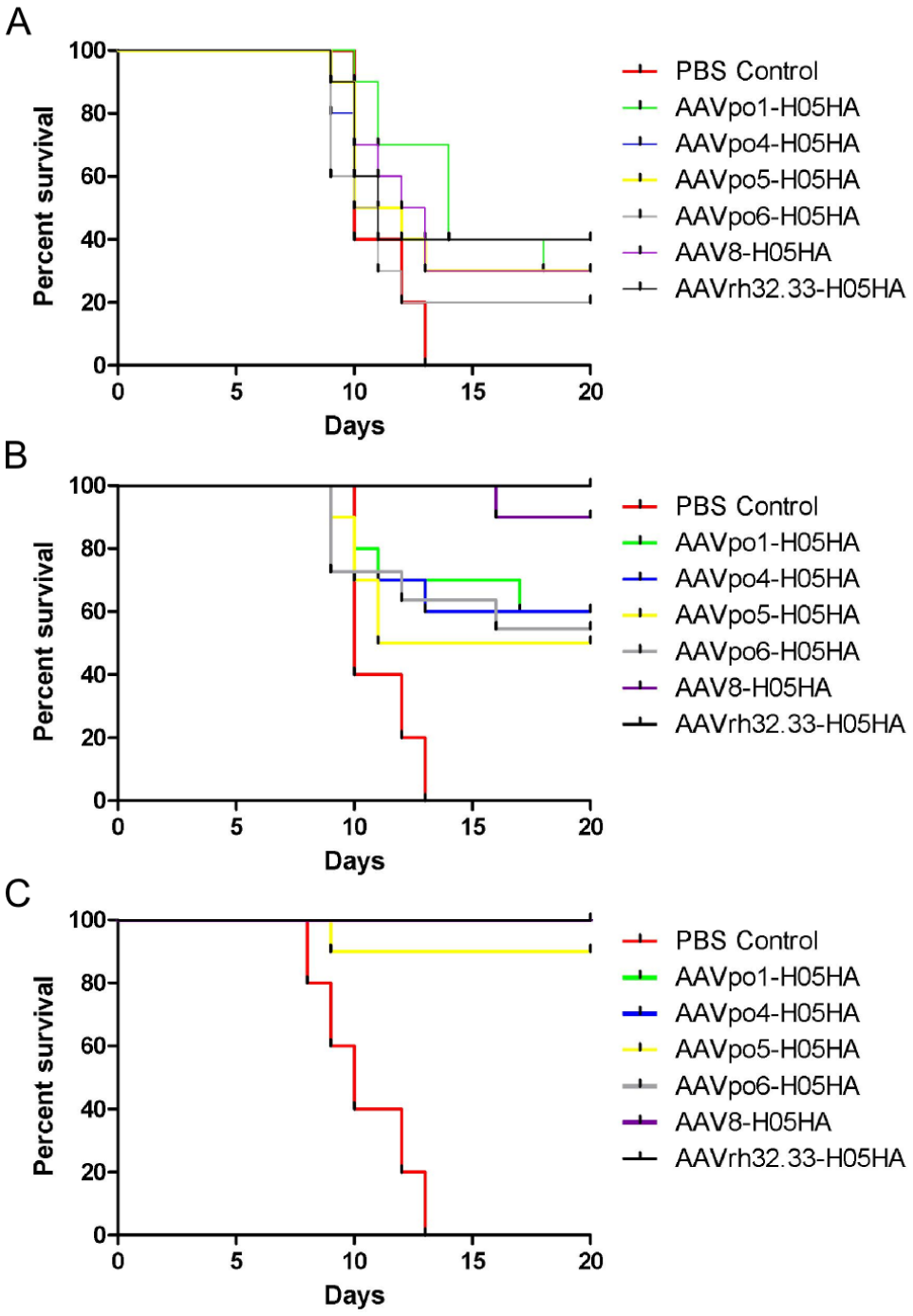

Figure 14 AAV-H05HA vectors protect against lethal challenge of H05 in mice. Mice were injected with different serotypes of AAV expressing H05HA at various doses and challenged with $100LD_{50}$ of H05 28 day post-vaccination. A) Mice vaccinated at a dose of $1x10^8$ gc, B) mice vaccinated at a dose of $1x10^9$ gc, and C) mice vaccinated with $2.5x10^{10}$ gc. The number of surviving mice were recorded daily and expressed as a percentage. *n*= 10 mice.

Table 6 Maximum Average Weight Loss and Survival of AAV-H05HA Vaccinated Mice at Various Doses and Challenged with 100LD_{50} of H05

Vaccine	Dose	Maximum Average Weight Loss (%)	Survival (%)	Clinical Symptoms
PBS Control	$1x10^8$	>20	0	Severe
AAVpo1-H05HA	$1x10^8$	>20	30	Severe
AAVpo4-H05HA	$1x10^8$	>20	40	Severe
AAVpo5-H05HA	$1x10^8$	>20	30	Mild-Severe
AAVpo6-H05HA	$1x10^8$	>20	20	Severe
AAV8-H05HA	$1x10^8$	>20	30	Mild-Severe
AAV.rh.32.33-H05HA	$1x10^8$	20	40	Mild-Severe
AAVpo1-H05HA	$1x10^9$	14	70	None-Mild
AAVpo4-H05HA	$1x10^9$	12	60	None-Mild
AAVpo5-H05HA	$1x10^9$	17	50	Mild
AAVpo6-H05HA	$1x10^9$	7	60	None-Mild
AAV8-H05HA	$1x10^9$	4	90	None-Mild
AAV.rh.32.33-H05HA	$1x10^9$	5	100	None-Mild
AAVpo1-H05HA	$2.5x10^{10}$	2	100	None
AAVpo4-H05HA	$2.5x10^{10}$	2	100	None
AAVpo5-H05HA	$2.5x10^{10}$	1	90	None-one severe
AAVpo6-H05HA	$2.5x10^{10}$	1	100	None
AAV8-H05HA	$2.5x10^{10}$	4	100	None
AAV.rh.32.33-H05HA	$2.5x10^{10}$	2	100	None

n= 10 mice

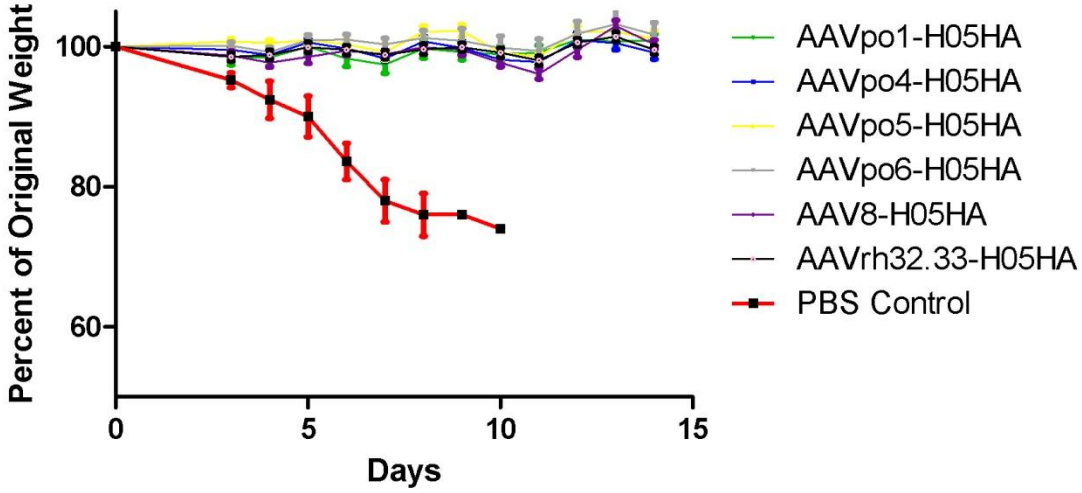

Figure 15 AAV-H05HA vaccinated mice do not show weight loss or signs of disease when challenged with H05. Mice were vaccinated with $2.5x10^{10}$ gc of AAV-H05HA vectors and challenged 28 days post vaccination with $100LD_{50}$ of H05. The weight was recorded daily and reported as percent of original weight. Each time-point is represented by mean weight. Error bars = SEM, *n*= 10 mice.

Sera from AAV-H05HA vaccinated mice were harvested 25 days post vaccination and treated with receptor destroying enzyme (RDE) overnight. The following day, sera were inactivated at 56°C for 45 minutes. Treated sera were incubated with 100 PFU of H05 per sample for one hour, and the mixture added onto subconfluent MDCK cells. 48 hours post-infection, the highest dilution that did not result in CPE was recorded as the reciprocal of the neutralizing antibody titer. All AAVs were able to produce modest levels of neutralizing antibody titers, although some members from all AAV groups elicited no neutralizing antibody titers against H05 (Figure 16). Mean neutralizing antibody titers for AAV samples ranged between 3 and 33.33 and one-way analysis of variance (ANOVA) revealed that the means were not significantly different ($p=0.139$).

Sera for HAI assay were treated in a similar fashion to that of the neutralizing antibody assay. Briefly, sera were treated with RDE at 37°C overnight and inactivated at 56°C the following morning. Afterwards, sera were diluted 2-fold beginning at a 1:10 dilution and incubated with 4 agglutinating doses (AD) of H05 for 1 hour. 100 µl of 1% horse red blood cells was added to each well and observed for up to 1.5 hours for signs of hemagglutination-inhibition. The highest dilution that resulted in inhibition of hemagglutination, was reported as the reciprocal of the dilution. The mean HAI titers ranged between 104 and 184, with AAVpo1 producing the lowest mean titer and AAVpo4 producing the highest mean titer (Figure 17). One-way ANOVA revealed that the difference between the means were not statistically significant ($p=0.1137$).

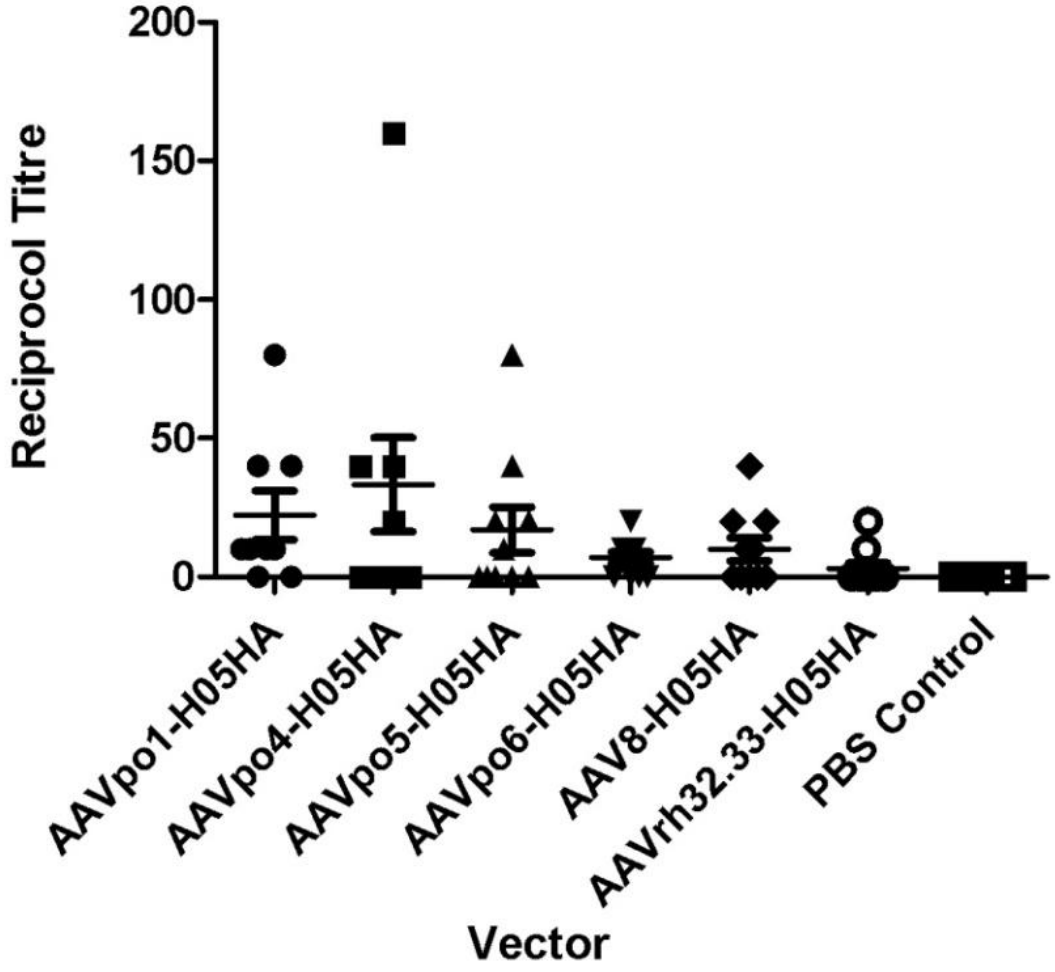

Figure 16 AAV-H05HA vectors elicit similar neutralizing antibody levels in mice. Mice were vaccinated with AAV-H05HA vectors and the serum harvested 25 days post-vaccination. The serum was tested for neutralizing ability by incubating with H05 virus and infecting on MDCK cells. The reported values are the reciprocal of the titer that cytopathic effect was not observed in cells. *n*= 10 mice.

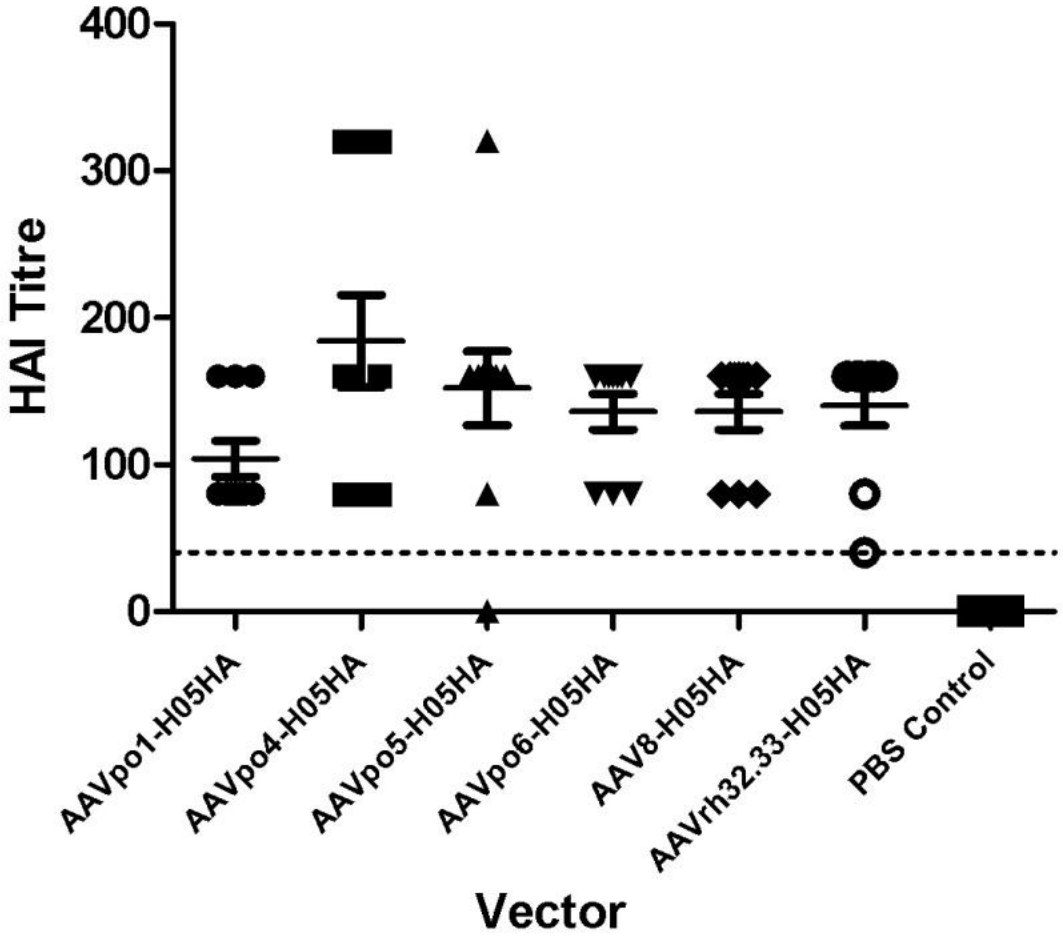

Figure 17 AAV-H05HA vectors elicit similar HAI titers in mice. Sera was harvested from AAV-H05HA vaccinated mice and treated overnight with receptor destroying enzyme. Treated serum was incubated with 4 agglutinating doses of H05 and 1% horse red blood cells were added. The reported titer is the reciprocal of the highest dilution preventing hemagglutination. The dotted line represents an HAI titer of 40, which is considered protective in humans. *n*= 10 mice.

3.11.3 CELL-MEDIATED IMMUNE RESPONSES TO AAV-H05HA VACCINATED MICE

Following examination of humoral responses, the cellular immune responses were next assessed. IFNγ is a cytokine promoting Th1 responses and can activate macrophages. Therefore, the secretion of IFNγ from T cells was assayed. Mice were vaccinated with $2.5x10^{10}$ gc of AAV-H05HA (*n*= 3 mice). At days 10, 20, 30, and 40, the spleen was harvested from vaccinated mice and the splenocytes extracted. Enzyme-linked immunospot (ELISPOT) plates were coated overnight with anti-mouse IFNγ capture antibody and $5x10^5$ splenocytes were added to the wells the next day, with each well containing a peptide pool from 27 different peptide pools spanning the entire HA region. The next day, the cells were washed from the plates and biotinylated secondary antibody and streptavidin enzyme were used to reveal secreted IFNγ from cells when substrate was added. The readout indicated spot forming units (SFU) per million cells. From these results, it was found that most AAVs had very low IFNγ secretion by day 10, with means ranging between 0 and 262 SFU/million cells (Figure 18). There was no significant difference at day 10 between AAVrh32.33 and all other AAVs ($p>0.05$). However, at the same time-point, AAV8 produced levels of 872 SFU/million cells, relatively larger than what was seen with AAVrh32.33 ($p<0.001$ following two-way ANOVA and Bonferroni post test). Most AAVs reached their peaks by day 20, with gradual decreases seen by day 40. AAVrh32.33 had the highest peak IFNγ responses of 6119 SFU/million cells on day 20 compared to all other AAVs tested. At all time-points other than day 10, IFNγ expression levels from AAVrh32.33 was significantly different

than all the other AAVs tested (two-way ANOVA followed by Bonferroni post test, $p<0.001$). Overall, AAVpo5 resulted in the poorest IFNγ secretion, whereas AAVrh32.33 produced the highest levels of IFNγ secretion out of all the AAVs tested.

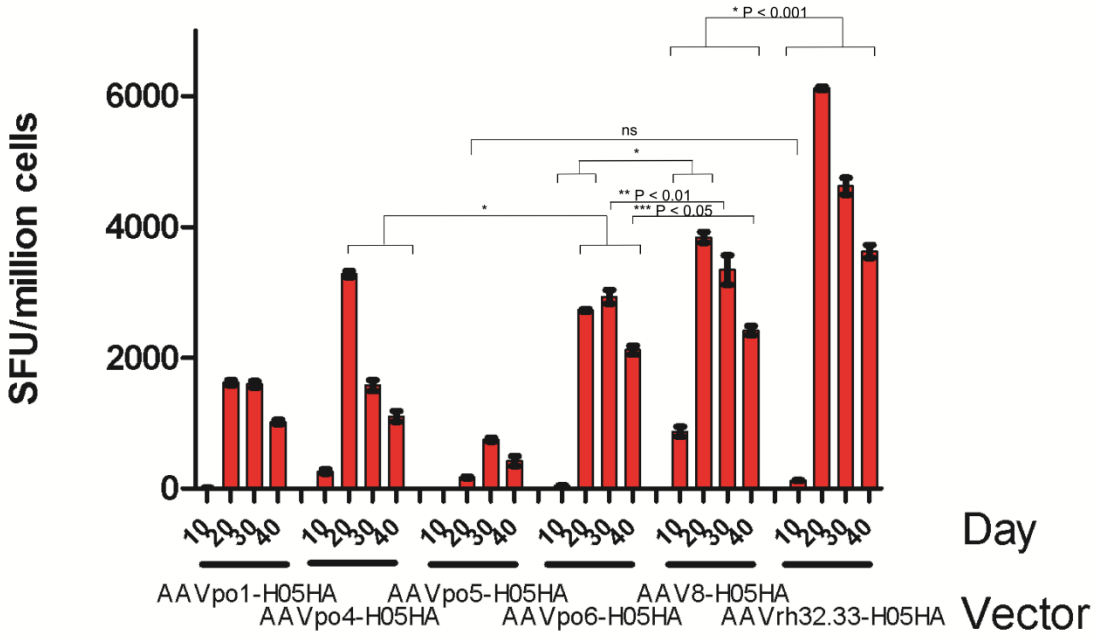

Figure 18 AAV-H05HA vectors elicit IFNγ responses from splenocytes isolated from vaccinated mice. Mice were injected with AAV-H05HA vectors and spleen was harvested at different time-points and processed for ELISPOT analysis. The splenocytes were stimulated overnight with 27 different peptide pools spanning the H05 HA protein. IFNγ responses were revealed the next day, pooled together, and reported as spot forming units per $1x10^6$ cells. *n*= 3 mice.

3.12 PROTECTIVE EFFICACY OF AAV VECTORS EXPRESSING EBOV GP *IN VIVO*

3.12.1 MA-EBOV CHALLENGE IN MICE VACCINATED WITH 2.5X10^{10} GC OF AAV-EBOVGP VECTORS

In order to test the protective efficacy of AAV vectored vaccines against some of the most aggressive viruses known to man, EBOV was chosen for animal studies as the mortality rate can reach up to 90% (Beer et al., 1999). Mice were vaccinated with 2.5x10^{10} gc of AAV-EBOVGP or PBS (*n*= 10 mice) and challenged 28 days post-vaccination with 1000LD$_{50}$ of mouse-adapted EBOV (MA-EBOV). The mice were monitored for signs of disease or distress for a period of up to 20 days and sacrificed if they met criteria for euthanasia or reached the end-point of the experiment. Results showed that AAVpo4 and AAV8 provided the best protective efficacy, affording 90% survival for each group (Figure 19). Compared to AAVpo4 and AAV8, AAVpo1 protected second best with 50% survival (p=0.0706), whereas AAVpo5 protected third best with 20% survival (p=0.0016). For PBS controls, 40% died on day 6, while the remaining 60% succumbed to disease by day 7. An interesting observation was that mice from the AAVpo5, AAVpo6, and AAVrh32.33 vaccinated groups began dying two days before the PBS controls did (day 4). By the end of the experiment, there were 0% survivors for the AAVpo6 and AAVrh32.33 vaccinated groups, as the remaining mice died around the same time as the PBS controls, on days 7 and 8, respectively.

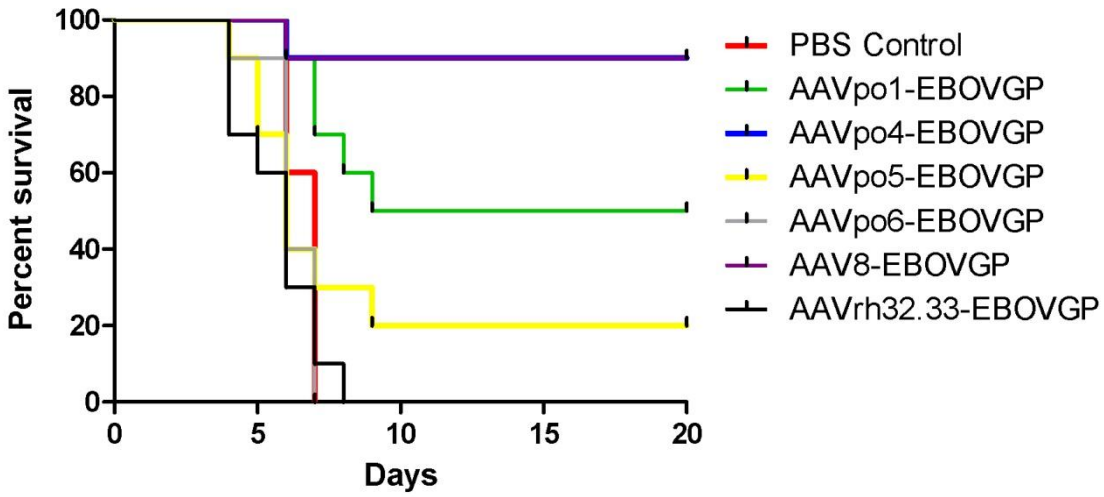

Figure 19 Some AAV-EBOVGP vectors protect well against lethal challenge of EBOV in mice, while others do not. Mice were vaccinated with $2.5x10^{10}$ gc of AAV-EBOVGP vectors. 28 days post-vaccination, mice were challenged with $1000LD_{50}$ of mouse-adapted EBOV. Mice were monitored for up to 20 days post challenge and percent survivors reported. *n*= 10 mice

The weights of infected mice were recorded daily and reported as a percentage of the original (Figure 20). A one-way ANOVA followed by Tukey's Multiple Comparison Test revealed statistical significance in weight loss between the following groups: AAVpo1 vs AAVrh32.33, AAVpo4 vs AAVpo6, AAVpo4 vs AAVrh32.33, and AAVpo6 vs AAVrh32.33, where $p<0.05$. Regarding the percent weight loss graphs for AAV-EBOVGP vaccinated mice, the AAVpo4 and AAV8 vaccinated groups maintained their weights throughout the entire experiment with no apparent changes in weight. AAVpo1 and AAVpo5 began showing weight loss by day 4 with their lowest weights observed on days 6 and 8, respectively. >20% weight loss was observed in groups injected with AAVpo6, AAVrh32.33, and PBS. Those animals having lost >20% of their body weight were euthanized as per protocol.

3.12.2 HUMORAL IMMUNE RESPONSES TO AAV-EBOVGP VACCINATED MICE

To assess the humoral responses elicited by AAV-EBOVGP vectors, neutralizing antibody assays and total IgG enzyme-linked immunosorbent assays (ELISA) were performed.

Mice were vaccinated with PBS or $2.5x10^{10}$ gc of AAV-EBOVGP vectors and serum was harvested 25 days post vaccination. The serum was heat inactivated for 45 minutes at 56°C and diluted two fold starting at a 1:10 dilution. Diluted serum was incubated with 100 focus forming units of EBOV-EGFP for 3-4 hours and the mixture was added onto subconfluent Vero E6 cells. 48 hours post-infection, the cells were fixed with formalin and transported from BSL4 to BSL2. GFP positive cells were recorded using a fluorescent plate reader. The highest dilution that resulted in inhibition of >50%

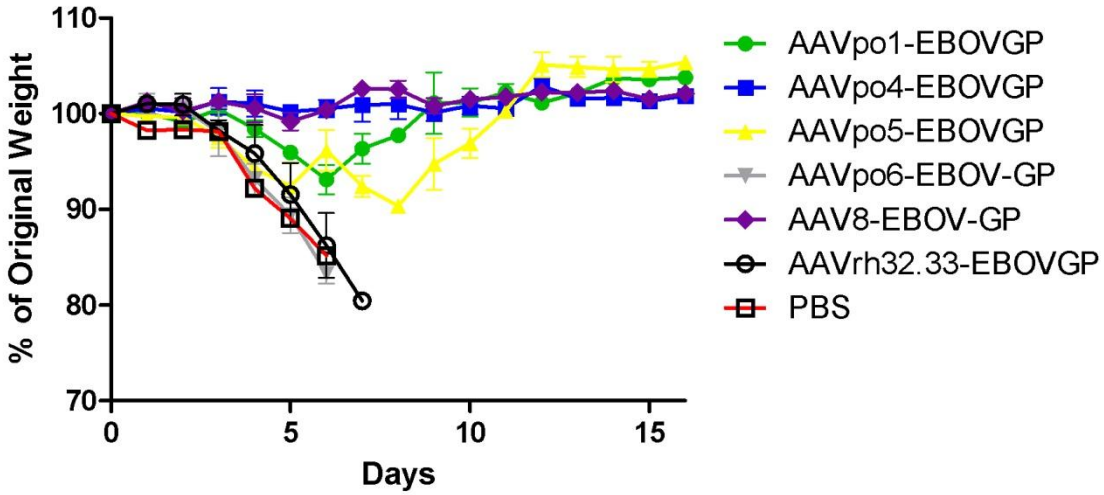

Figure 20 AAVpo4 and -8-EBOVGP vaccinated surviving mice do not experience disease symptoms or weight loss when challenged with lethal dose of MA-EBOV. Vaccinated mice were challenged with 1000LD_{50} of mouse adapted EBOV. The average weight was recorded daily and reported as a percentage of the original starting weight. *n*= 10 mice.

Since neutralizing antibody assays were not very informative on the humoral responses elicited by AAV-EBOVGP vectors, another approach was to analyze total IgGs by ELISA (Figure 22). Serum was harvested from mice vaccinated with AAV-EBOVGP or PBS and processed in the same manner as the neutralizing antibody assay described in the previous section. Purified EBOV GP was added to 96-well microplates with high protein binding affinity and incubated overnight. The plates were washed and serum added to the wells for one hour. Secondary anti-mouse antibody with HRP was added to the wells and incubated for an additional hour. Substrate was added to the wells to visualize total IgG ELISA titers. The absorbance of the wells was recorded at 405 nm using a plate reader. Statistical analysis by two-way ANOVA followed by Bonferroni posttests found that AAVpo4, AAVpo6, and AAV8 had similar kinetics for total IgG ELISA at all time-points ($p>0.05$), reaching their maximum absorbance threshold by day 20. The lowest mean absorbance recorded at day 10 for all AAVs was similar ($p>0.05$). At day 20, AAVpo1 and AAVpo5 had significantly different total IgG expression vs AAVpo4, AAVpo6, and AAV8 ($p<0.001$). Maximum detection threshold of absorbance by total IgG ELISA was reached by AAV-EBOVGP vectors between days 20 and 40, with mean maximum absorbance threshold ranging between 1.85 and 1.97. Threshold was reached and maintained for all AAV-EBOVGP vaccinated groups, except for AAVpo5, which reached its maximum threshold at day 40, with levels declining by day 50. The mean absorbance of total IgG ELISA for AAVpo5 was significantly different from AAVpo4, AAVpo6, and AAV8 at days 20, 30, and 50, where $p<0.001$, $p<0.05$ and $p<0.01$ for the time-points, respectively.

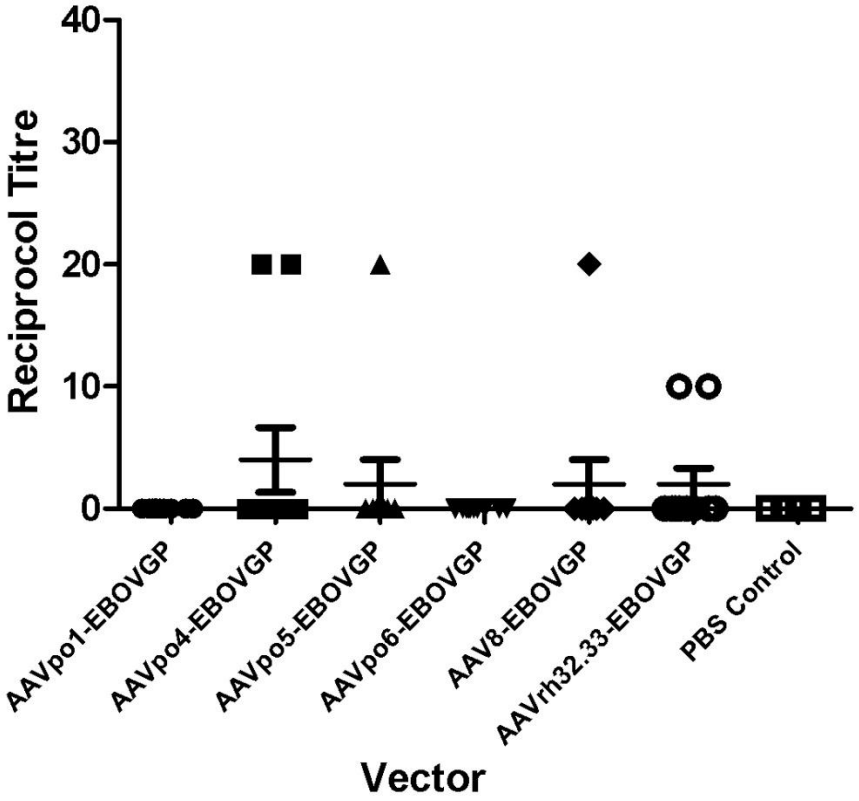

Figure 21 AAV-EBOVGP vaccinated mice produce little or no neutralizing antibodies against EBOV-EGFP. Sera from AAV-EBOVGP vaccinated mice was harvested 25 days post-injection and incubated with EBOV expressing EGFP. Serum and virus were transferred unto VERO E6 cells and incubated for 48 hours. The titer reported is the reciprocal of the highest dilution able to neutralize >50% of transducing EBOV-EGFP particles. *n*= 10 mice

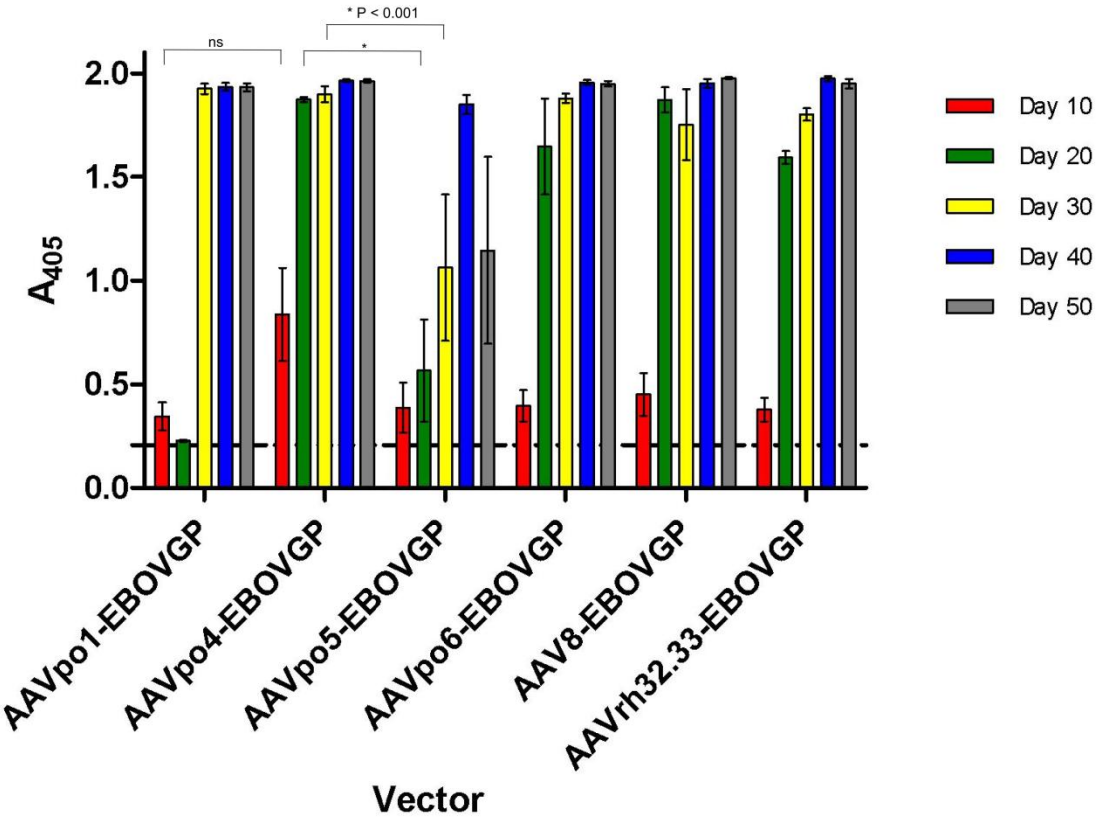

Figure 22 AAV-EBOVGP vaccinated mice produce varying total IgG levels at various time-points. Sera from mice vaccinated with AAV-EBOVGP were harvested at various time-points and assessed for the presence of IgGs by ELISA. Absorbance of samples was recorded at 405 nm. The dashed line represents the absorbance of PBS control samples. *n*= 3 mice.

3.12.3 CELL-MEDIATED IMMUNE RESPONSES TO AAV-EBOVGP VACCINATED MICE

Following characterization of humoral immune responses against AAV-EBOVGP vectors, the cell-mediated immune responses were analyzed via IFNγ ELISPOT analysis and flow cytometry.

For ELISPOT analysis, mice were vaccinated with $2.5x10^{10}$ gc of AAV-EBOVGP vectors and the spleen harvested 28 days post vaccination (*n*= 3 mice). A day before processing spleens for ELISPOT analysis, ELISPOT plates were coated with anti-mouse IFNγ capture antibody and incubated at 4°C overnight. The next day, splenocytes were extracted from spleens and added to ELISPOT plates at a concentration $5x10^5$ cells/well. A peptide pool from four different peptide pools spanning the entire EBOV GP were added to a well and incubated overnight with the cells. The following day, the cells were washed from the plates and biotinylated anti-mouse IFNγ antibody added to the wells. An hour later, streptavidin-HRP was added to the wells and AEC substrate used to visualize capture of IFNγ. Spots were counted using a plate reader and reported as SFU/million cells. Results indicated that AAVpo4 produced higher levels of IFNγ at days 20 and 40 compared to AAVpo1, AAVpo5, AAVpo6, AAV8, and AAVrh32.33 ($p<0.01$) (Figure 23). AAVpo4 had a mean peak IFNγ expression at day 20 (767 SFU/million cells), and these levels declined drastically by day 30 (251 SFU/million cells). AAVpo1, AAVpo5, and AAV8 had mean peak expression by day 30 (268, 211, and 413 SFU/million cells, respectively), with levels declining by day 40 (92, 96, and 102 SFU/million cells, respectively). Overall, AAVpo4 and AAV8 provided the highest mean peak IFNγ expression (767 and 413 SFU/million cells, respectively), albeit these

levels were achieved at different time-points. Mean peak IFNγ expression for AAV8 at day 30 was significantly different from all other AAVs at this time-point ($p<0.001$). AAVrh32.33 provided the lowest levels of IFNγ with mean expression of 20, 9, and 10 SFU/million cells for days 20, 30, and 40, respectively.

Another analysis performed to evaluate cell-mediate immune responses was flow cytometry (Figure 24). Briefly, mice were vaccinated with AAV-EBOVGP vectors and the spleens harvested 28 days post vaccination (n= 3 mice). The splenocytes were extracted and pooled together with members from the same group. The splenocytes were stimulated overnight with a peptide pool from four different peptide pools consisted of 15mers with 10 aa overlaps, spanning the entire EBOV GP. The following day, the cells were stained extracellularly with CD4-PacBlue and CD8-PerCPCy5.5. Cells were treated with Cytofix/Cytoperm™ (BD) and permwash, and stained intracellulary with IFNγ-PE, TNFα-PE-Cy7, and IL2-APC. The samples were subjected to flow cytometry and the readouts recorded as the percent populations of CD4+ or CD8+ T cells responding to peptide pool stimulation. Each individual cytokine or combinations of cytokines upregulated as a result of peptide pool stimulation are displayed in Figure 24. Essentially, those AAVs causing poor protection against EBOV challenge had CD4+ T cells secreting IL-2 upon peptide stimulation (AAVpo5, AAVpo6 and AAVrh32.33). As well, IL-2 production was observed in these groups for CD8+ T cells, with the exception of AAVpo6, which only saw TNFα production. For the group affording protection in 50-90% of vaccinated mice, CD4+ T cells were absent of IL-2 responses (AAVpo1, AAVpo4, and AAV8). Also seen in this group was CD8+ T cells responses mainly limited to TNFα and IFNγ responses, although a very small population of stimulated

CD8+ T cells from the AAVpo1 vaccinated group did show a combinatorial response of IFNγ and IL-2 (<4%), and IL-2 alone (<10%); whereas CD8+ T cells responses for AAVpo4 and AAV8 were devoid of IL-2 responses. One caveat for the AAV8 CD4+ T cell response is that these cells, for the most part, were non-functional, as a difference of cytokine expression between unstimulated and peptide-stimulated cells was not observed.

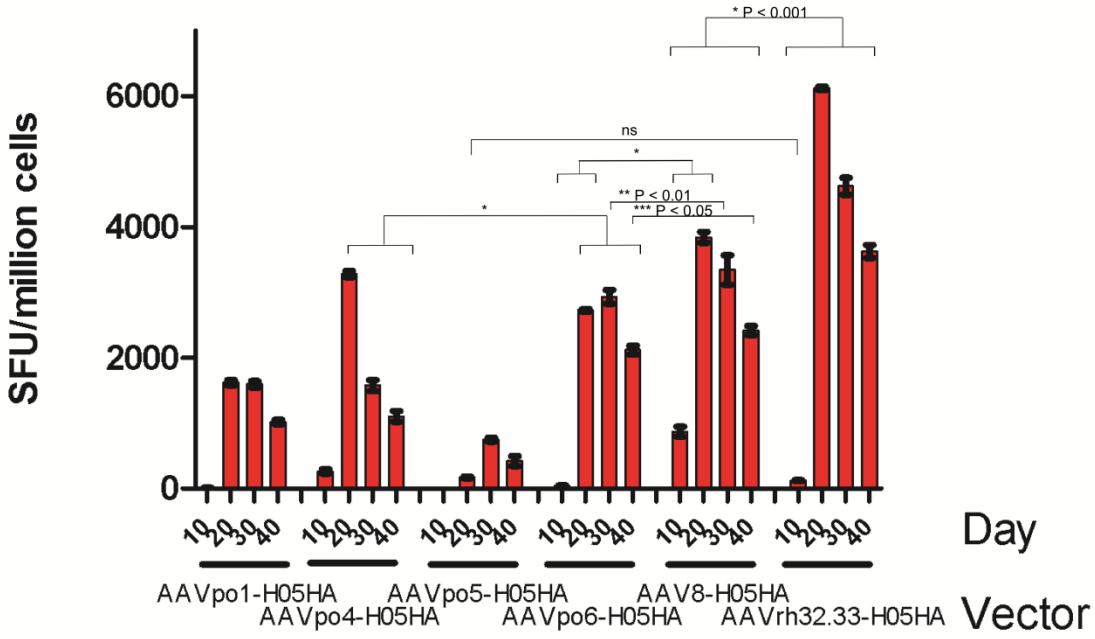

Figure 23 AAVpo4 and -8-EBOVGP produce higher IFNγ responses in vaccinated mice compared to other AAV vectors. Mice were vaccinated with $2.5x10^{10}$ gc of AAV-EBOVGP vectors and spleens were harvested at various time-points. Splenocytes were processed for ELISPOT analysis and stimulated with a peptide pool from four different peptide pools spanning the entire EBOV GP. Spots were visualized with substrate. The spots counted from each peptide pool were recorded and the background from unstimulated cells subtracted. The additive of the means±SEM for each peptide pool is displayed as bars on the graph for each time-point. *n*= 3 mice.

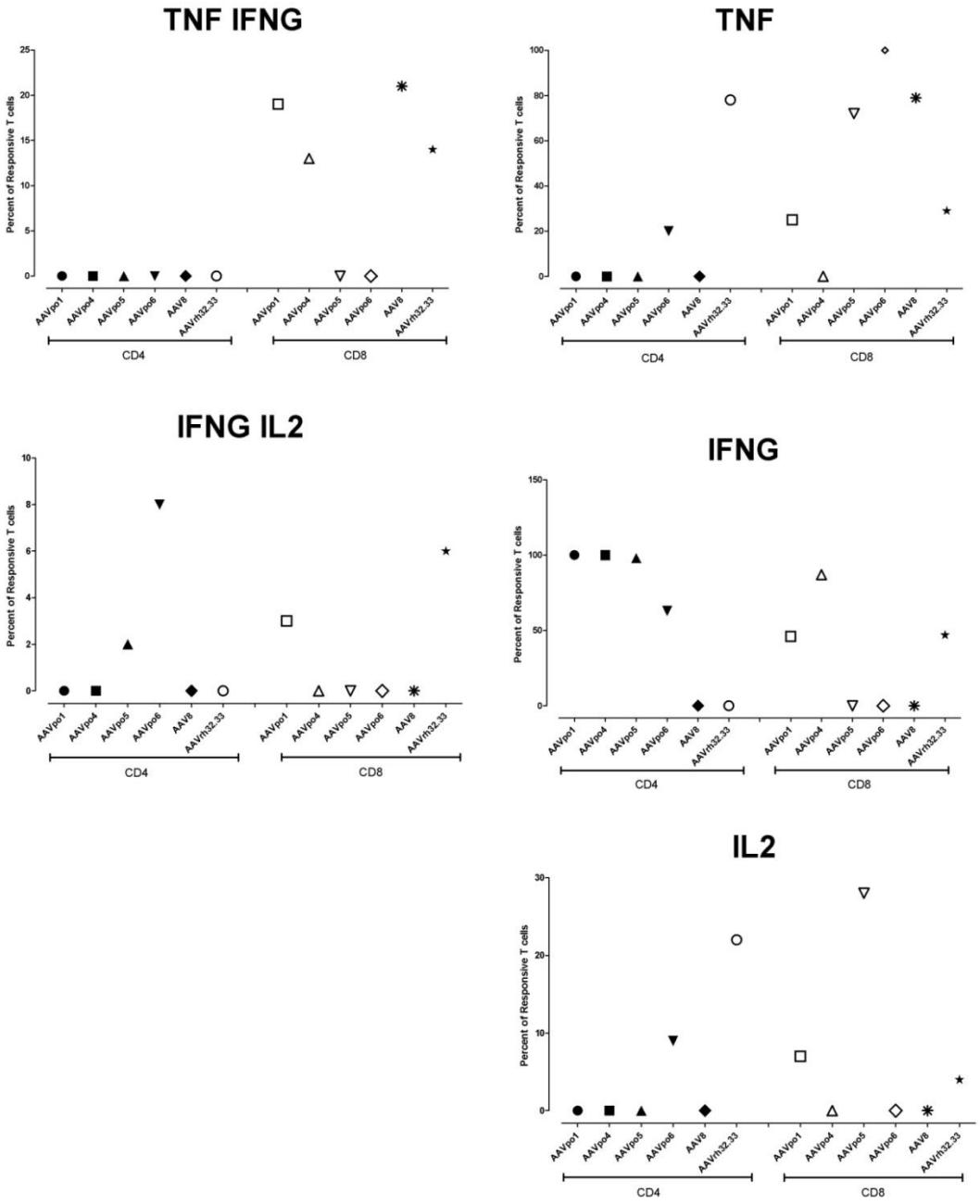

Figure 24 AAVpo4 and -8-EBOVGP vectors do not elicit IL-2 responses. Mice were vaccinated with AAV-EBOVGP vectors and spleens harvested 28 days post vaccination. The splenocytes were extracted and stimulated with a peptide pool from four different peptide pools spanning the entire EBOV GP region. The cells were stained with CD4-PacBlue, CD8-PerCPCy5.5, IFNγ-PE, TNFα-PE-Cy7, and IL2-APC. Flow cytometry was performed on the samples and the readout recorded as the proportions of CD4+ or CD8+ T cells responding to peptide stimulation via IFNγ, IL-2, or TNFα responses, or different combinations of all three cytokines. *n*= 3 mice.

3.13 EVALUATION OF AAV TROPISM IN IMMUNE CELLS

Following the assessment of AAV vectors in tissue tropism and protective efficacy against lethal challenge, the immune cell tropisms of AAVs were examined. AAV vectors carrying self-complementary (sc) GFP were injected IVTV in BALB/c mice. 48 hours post-injection, the spleen was harvested and subjected to treatment with Collagenase D to liberate DCs. The spleen was mashed with a plunger on a mesh screen and filtered through a .45 μm filter. The splenocytes were stained extracellularly with: Vivid-Pac Blue (live/dead staining), CD19-BV650 (B cell), CD11c-PerCPCy5.5 (DC), CD49b-APC (NK cell), CD8-APC-Cy7 (cytotoxic T cell), CD11b-BV711 (macrophage), CD4-Alex 700 (helper T cell), CD3-PE-Cy7 (pan T cell), and CD115-BV605 (monocyte/macrophage). Following staining, the cells were fixed overnight with 1% PFA in PBS and flow cytometry performed using an LSRII. The readout of the analysis was the percentage of the different immune cell types expressing GFP following AAV transduction. Samples were considered positive if GFP expression was three times that of the PBS control. From these results, it was found that AAVs showing poor protection against lethal challenge (AAVpo1 and AAVpo5) did not efficiently transduce immune cells, while those AAVs eliciting the best protective efficacy (AAVpo4 and AAV8) transduced a number of immune cells types quite effectively (Figure 25). Qualitatively, AAVpo4 and AAV8 transduced B cells and DCs most efficiently, with NK cells having modest GFP expression. Transduction of the overall population of splenocytes shows that many cells express GFP following AAVpo4 and AAV8 administration in mice. AAVpo1 and AAVpo5 did not transduce any of these cells as efficiently, as the

percentages were only slightly higher than the PBS control. None of the AAV-scGFP vectors transduced CD4+ or CD8+ cells effectively.

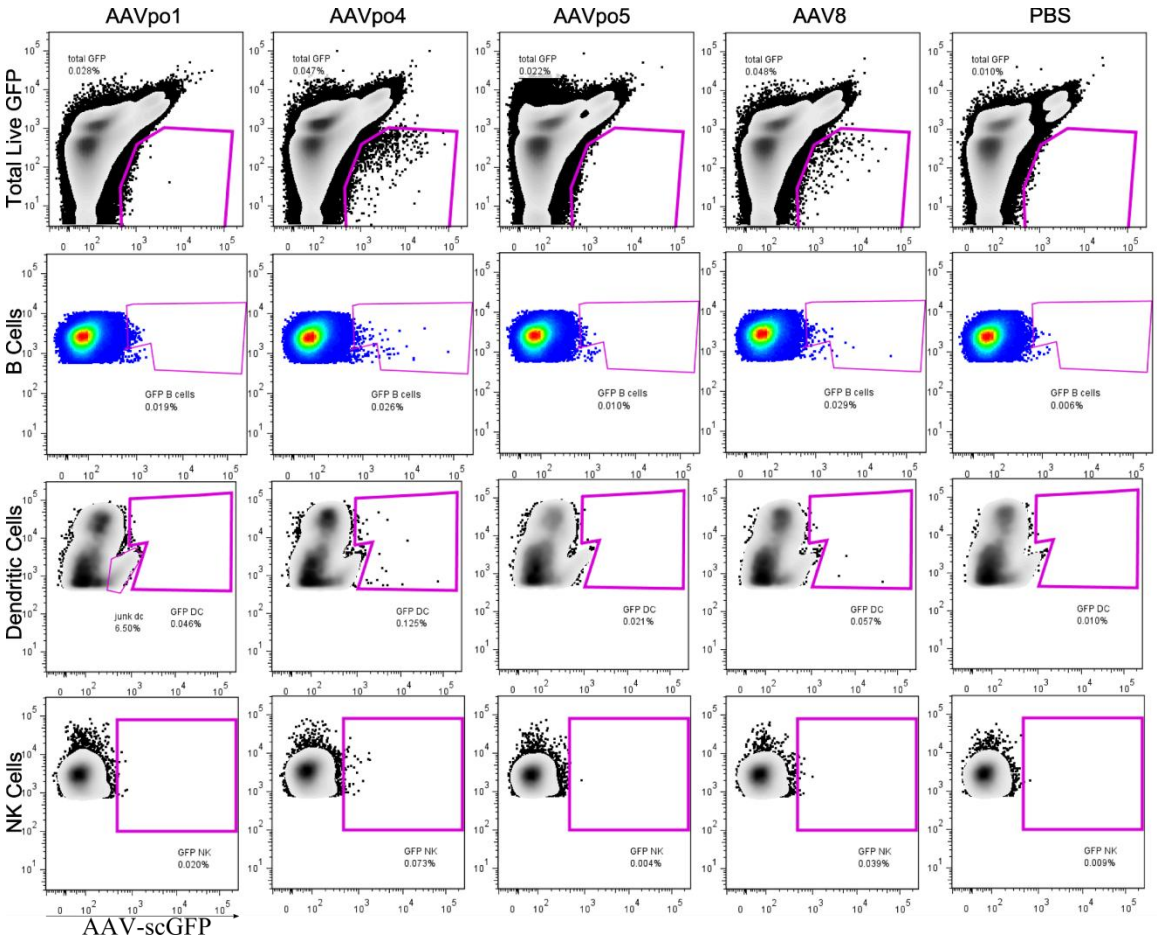

Figure 25 AAVpo4 and -8-scGFP vectors show robust transgene expression in total splenocytes from mice, including APCs such as DC and B cells. Mice were injected with $1x10^{11}$ gc of AAV-scGFP vectors via IVTV and the spleens harvested 48 hours post-injection. The spleens were subjected to Collagenase D treatment and staining performed using CD19-BV650, CD11c-PerCPCy5.5, CD49b-APC, CD8-APC-Cy7, CD11b-BV711, CD4-Alexa 700, CD3-PE-Cy7, and CD115-BV605. AAV-scGFP vectors were assessed for their ability to transduce immune cells by GFP expression via flow cytometry. Red boxes indicate the gating for GFP^+ cells. *n*= 3 mice.

CHAPTER IV

4.0 DISCUSSION

4.1 IDENTIFICATION AND GENERATION OF NOVEL AAV FROM PORCINE TISSUES

AAVs are considered safe and do not result in any known illness, disease, or tumorigenesis when delivered into a host. As well, AAVs have been shown to efficiently deliver genes into a host and have specific tissue tropisms. As such, AAVs have great potential as modalities for safe and tolerable gene therapy or vaccine applications. AAV was first isolated in the 1960s and described as a contaminant in human adenovirus stocks (Atchison et al., 1965). From the 1960's to early 21st century, only 12 serotypes of human and non-human primate AAVs were described (Atchison et al., 1965; Bantel-Schaal & zur Hausen, 1984b; G.-P. Gao et al., 2002; Mori et al., 2004). However, with the utilization of different isolation techniques, hundreds of new isolates of AAV have been identified from numerous species including cows, birds, snakes, rats, mice, etc. (Arbetman et al., 2005; Clarke, McFerran, McKillop, & Curran, 1979; Coria & Lehmkuhl, 1978; Farkas, 2004; G. Gao et al., 2004a; Lochrie et al., 2006; Myrup, Mohanty, & Hetrick, 1976; Olson et al., 2004; Yates, el-Mishad, McCormick, & Trentin, 1973). Of the newly identified AAVs being developed for gene therapy treatments, the majority were isolated from human and non-human primates. Recent research has shown that pre-existing immunity is present against these vectors, and that T cells generated against some human serotypes are cross-reactive against those of non-human primates (G. Gao et al., 2009; Jooss & Chirmule, 2003; Madsen, Cantwell, O'Brien, Johnson, & Mahon, 2009; Sabatino et al., 2005b; Zaiss & Muruve, 2008). For the purpose of AAV

as vectors for gene therapy or vaccine applications, it is crucial to identify AAVs of low seroprevalence in the human population, or non-cross-reactive with other AAVs. Therefore, the initial goal of this thesis was to identify new AAV serotypes humans have not been exposed to, yet that are able to transduce human cells efficiently.

Pigs and humans share considerable sequence homology as exemplified by xenotransplantation utilizing pig heart valves in human recipients (Hermida-Prieto et al., 2007; Herring, Cunningham, Whittam, Fernández-Suárez, & Langford, 2001; Luciani, Viscardi, Cresce, Faggian, & Mazzucco, 2007). Pig heart valves or stents have been used successfully to treat humans with valvular heart disease. Of course, immune-suppression is administered during these procedures, but a lack of toxicity suggests that pig tissues are highly compatible to that of humans. As well, swine leukocyte antigen (SLA) and human leukocyte antigen (HLA) are highly homologous; human T cells have been shown to recognize SLA antigens of antigen presenting cells from the xenograft, described as "direct xenorecognition" (Chardon, Renard, Gaillard, & Vaiman, 2000; Shishido, Naziruddin, Howard, & Mohanakumar, 1997; Varela, 2003). Other examples of cross-species transmission can be seen with many other viruses including influenza, Nipah, and EBOV, all of which are able to cross the species barrier and infect both pigs and humans (Kobinger et al., 2011; Kulkarni, Tosh, Venkatesh, & Senthil Kumar, 2013; Thacker & Janke, 2008). For these reasons, this thesis hypothesizes that AAVs isolated from pig tissues would be able to transduce human cells efficiently.

The first hypothesis states that 'AAV DNA exists within porcine genomic DNA and can be isolated by PCR'. Following the objectives stated in 1.13.2, PCR was utilized to "fish" for new AAV serotypes from pig genomic DNA (Figure 1). Although AAV can

differ in nucleotide identity anywhere from 60 to 99%, conserved regions exist within the *rep* and *cap* genes of the AAV genome can be exploited for identification of novel AAVs. Using these methods, several new AAVs from pig tissues were isolated using primers designed to bind to the conserved regions. During the process, numerous pig tissues were screened including the heart, lung, liver, spleen, and gut. Of the novel porcine-derived AAVs, AAVpo1 was the first identified and found in virtually all organs sampled in numerous pigs (Bello et al., 2009). However, full *cap* sequences for AAVpo2.1, AAVpo4, AAVpo5, and AAVpo6 were found from the gut of only one pig sample (Puppo et al., 2013). Screening of this sample also resulted in identification of 28 unique porcine-derived AAV sequences (Figure 2). To represent AAV natural infection, various NHP organs were previously screened for the presence of AAV (G. Gao et al., 2003). Results from this experiment found that AAV genomic recombination was observed to a high degree, similar to that of RNA viruses. As well, mutations occurred mainly on hyper variable regions located mainly on the capsid surface, which may explain the unique tissue tropism and binding to various receptors. High mutation rates in parvoviruses and other single-stranded DNA viruses have been previously described, and during an active AAV infection, these mutations may be taking place. Genetic diversity during an active AAV infection may help drive its fitness and specific tissue tropism (Duffy, Shackelton, & Holmes, 2008). Therefore, the later porcine-derived AAVs may have been isolated during a productive AAV infection, explaining why numerous AAV isolates with unique genetic sequences were observed. Once BLAST analysis was performed on the novel pig AAV sequences and these sequences aligned with published ones, it was determined that these sequences were indeed unique and

formed 3 new clades within the AAV family (Figure 4). More than 20 new isolates of AAV were identified and focus on isolation of the full *cap* gene was placed on those AAVs belonging to new clades. The isolation of the full *cap* was achieved by performing nested TAIL PCR (Figure 3). Essentially, two rounds of PCR of low and high stringent conditions utilizing two specific successive primers are performed one after the other, using the PCR product from the previous PCR. The two successive primers were designed based on conserved regions of the 5' end outside of *cap*. For the 3' end, a degenerate primer was utilized, since the 3' end of *cap* can vary between AAV serotypes. Using these methods, several new full-length AAV *cap* sequences were successfully isolated and named AAVpo1, AAVpo2.1, AAVpo4, AAVpo5, and AAVpo6.

Although the main goal was to isolate AAV *cap* genes from pig genomic DNA, isolation of the full *rep* gene and ITRs was also attempted. Much like the arbitrary degenerate primer designed to bind to the 3' end of the *cap* gene, an arbitrary degenerate primer was designed to bind to the 5' end of the *rep* gene. However, attempts were unsuccessful as isolation of any of the *rep* or ITRs from the pig genomic DNA proved ineffective. Previous isolation of full AAV genomic DNA was performed on intact AAV particles present as contaminants in adenoviral preparations, or on rescued AAVs from infected cell lines. In this thesis, PCR was performed on genomic DNA, in which case it was assumed that the AAV genome has already integrated and no progeny virus is present. In this case, the isolation of the ITRs and *rep* would be difficult, due in part by the complexity of tertiary and quaternary structures formed by the genomic DNA and ITRs, as well as the high GC content of the ITRs.

4.2 CLONING OF TRANSGENE INTO CIS PLASMID AND GENERATION OF RECOMBINANT AAV VECTORS

Following the isolation of porcine-derived AAV *cap*, it was important to determine that intact AAV particles were being produced. Porcine-derived AAV *cap* from each isolate was cloned into an expression vector (in place of AAV5 *cap*) providing the Rep and Cap in *trans* during a transfection. An AAV2 *rep* is located just upstream of the AAV5 *cap.* Provided in *cis* is the gene-of-interest, which is cloned into a transgene plasmid and flanked by AAV2 ITRs. Even though AAV2 has only 62.9% sequence homology to that of AAV5, the Rep proteins of AAV2 are able to recognize the AAV2 ITRs flanking the gene-of-interest and sequester the recombinant genomes to the AAV5 capsid particles, allowing for packaging of the genomes into these vectors. Here, it was hypothesized that AAV2 *rep* would be able sequester recombinant porcine-derived AAV genomes to the porcine-derived capsid particles. HEK293T cells were transfected with the *cis* and *trans* plasmids, along with a helper plasmid, providing helper virus functions to drive replication and production of recombinant AAV particles. Indeed, viable porcine-derived AAVs particles were produced when the AAV2 Rep and ITRs were utilized, showing that porcine-derived Cap is compatible with AAV2 Rep and AAV2 ITRs (Figure 6, Figure 7).

4.3 PROTECTIVE EFFICACY OF PORCINE AAVS AND IMMUNE RESPONSES AGAINST INFLUENZA AND EBOLA

One of the hypotheses of this thesis states that ‘AAVs delivering immunogenic transgene can protect against homologous lethal infection’. In order to provide a baseline for the overall goal of this thesis (unifying the transduction efficiencies of

porcine-derived AAVs and the connection to its protective efficacy), two aggressive disease models were utilized: avian influenza H5N1 and Ebola virus infection. The initial goal was to select disease models requiring two different arms of the immune response for prevention of disease: avian influenza H5N1 is thought to require a humoral response for protection, whereas Ebola virus infection is thought to require more of a cellular-immune response. However, as outlined in recent emerging studies, the exact opposite may hold true for EBOV infection, where a B cell response is thought to be important for protection (Wong et al., 2012a). With this in mind, the evaluation of AAV as vaccine vectors was not to elicit any specific type of immune response from the porcine-derived AAV vectors, but to understand whether or not these vectors can protect against some of the deadliest and most aggressive viruses known to man. In conjunction with the challenge studies, the humoral and cellular immune responses elicited by the transgene were investigated. As controls for the challenge and cellular assays, AAV8 was used due to its distinct property of inducing non-functional T cell responses against the transgene (Lin, Zhi, Mays, & Wilson, 2007b; Mays et al., 2009). An additional control was AAVrh32.33, which is highly immunogenic and produces robust immune responses (Lin et al., 2009). Therefore, it was proposed that AAVrh32.33 would serve as an applicable vaccine vector candidate for its immunogenic properties, whereas AAV8 would perform poorly. However, this was not the case, as both vectors protected equally as well against H05 challenge. One caveat is that for EBOV challenge, AAVrh32.33 unexpectedly conferred 0% protection, whereas AAV8 protected 90% of mice; the causes behind this anomaly will be discussed in more detail later in this discussion.

The exploitation of AAV as vaccine vectors has been very limited, with only a handful of publications since 1997. Early studies of the immune responses towards transgene of AAV vectors have shown that immune tolerance can be achieved against the transgene in mice (Brockstedt et al., 1999). As such, researchers directed their focus on developing AAV as a gene therapy vector rather than a vaccine platform. The neglect AAV has received for vaccine vector development is apparent as most studies have shown that AAV is capable of eliciting protective immune responses in animals and prevent infection when challenged, yet very few AAV vaccine studies exist (Johnson et al., 2005). In this thesis, challenge studies with influenza found that all AAVs administered at the highest dose protected 100% of the challenged mice equally as well, with the exception of AAVpo5, which only protected up to 90% of injected mice (Figure 14). At the lowest vaccination dose, none of the AAVs protected well; whereas moving up a log, only AAV8 and AAVrh32.33 conferred the best protection. Recent publications have shown that vector dosing and administration route can have an impact on the immune responses against the transgene and the AAV capsid itself (Bharadwaj et al., 2010; Brockstedt et al., 1999; Kelly, Zhuo, Bharadwaj, & Chao, 2009; Mingozzi et al., 2009). At lower doses, not enough antigen is present, resulting in lack of recognition of the transgene by the hosts' immune system. At intermediate doses, immunity develops as there is enough antigen to stimulate an immune response. At higher doses, tolerance develops as too much antigen can result in T cell exhaustion (Mays et al., 2014). From these results, a three zone immune mechanism dependent on AAV dose has been proposed by Kelly *et al*: ignorance, immunity, and tolerance. The influenza disease model used in this thesis met criteria for two of the three zones; lower dosing with AAV-

H05HA vectors may have been ignored by the host immune system, whereas intermediate dosing resulted in immune activation and recognition of the transgene as foreign. However, criteria for the third zone have not been tested. According to the hypothesis suggested by Kelly *et al*, the third zone would result in tolerance of the transgene at higher doses. If this hypothesis were to be tested with AAV-H05 vectors, decreased survival may be seen with challenged animals as the immune system would essentially develop tolerance against the H05HA antigen. Regardless, the goal for the dose-response experiment was to identify the dose immunity could be established against the transgene; the consequence of a higher vaccine dose has yet to be determined.

For the H05 challenge study, it was predicted that AAV8 would not protect well in this disease model due to its inability to produce functional CD4+ T cell responses (Lin et al., 2007a). As mentioned previously, AAV8 did, in fact, confer protection in vaccinated mice, which may have been due to vector dosing or antigenicity of the transgene. Most transgenes utilized in clinical trials have been developed to replace defective genes for gene therapy purposes, thereby allowing the host to recognize the expressed transgene product as self-antigens. An antigen the host has not been previously exposed to is recognized as non-self and can stimulate the adaptive immune response. For the vaccine studies presented in this thesis, the antigens chosen are considered highly antigenic, and may provide another clue as to why protection was seen with the AAV8 vector. Cellular assays were performed and results found that AAV8 was indeed able to elicit IFNγ responses from stimulated splenocytes. However, although the splenocytes secreted IFNγ, it is not necessarily indicative of functional T cell responses. Even more surprisingly, IFNγ responses for AAV8 vectors appeared at a higher intensity

much sooner than the porcine-derived AAVs or AAVrh32.33. In the literature, AAV8 is one of the most efficient vectors for uncoating and delivery of genetic material to the nucleus, translating to faster expression of the gene-of interest (Thomas, Storm, Huang, & Kay, 2004). This finding may explain why IFNγ responses for AAV8 were much higher and sooner than those of other vectors. Overall, splenocytes from AAVpo5 vaccination had the lowest IFNγ production upon peptide stimulation, which may explain why AAVpo5 had lower survival compared to the other serotypes. AAVrh32.33 has been shown to induce robust T cell responses and as expected, vaccination with AAVrh32.33-H05HA resulted in the highest peak expression of IFNγ at day 20.

Analysis of the humoral response generated against AAV-H05HA vectors revealed that all AAVs were able to confer neutralizing antibody responses, as well as hemagglutination-inhibition responses (Figure 16, Figure 17). Levels were very similar between the different AAV serotypes for both humoral assays. As mentioned in the previous paragraph, an intermediate dose and route of administration can dictate the immune response generated against the transgene. AAVs expressing human factor IX (FIX) via IM in mice or canine were found to elicit inhibitory antibodies, whereas the same vectors injected via intrahepatic portal vein resulted in noninhibitory antibodies (Herzog et al., 1997; Snyder et al., 1999); the generation of neutralizing antibody against H05HA transgene in this thesis is consistent with the findings as seen with human FIX in animal models. From the AAV-H05HA studies, no significant difference was observed between the AAV vectors when survival and humoral immune responses were examined (except for cellular immune responses, where a difference in the kinetics of IFNγ expression was observed). Therefore, investigation of the protective efficacy of AAV

vectors against a much more aggressive viral infection was undertaken in order to establish a difference in protective efficacies. Here, a comparison can be made between the different AAVs when administered at an intermediate dose, permitting immunity to develop against the gene-of-interest.

For the second disease model presented in this thesis, EBOV infections in mice were employed. EBOV is one of the most aggressive viruses known to man and causes more than 90% fatality in infected humans. The immune correlates of protection are not clearly understood for EBOV infection in mice. However, recent emerging evidence suggests that the humoral response is important for protection against infection (Wong et al., 2012b). Therefore, it was decided to examine both humoral and cell-mediated immune responses generated when mice were vaccinated with various AAV-EBOVGP vectors. As well, determining the AAVs displaying the best or worst protective efficacies would provide a parameter upon which a comparison can be drawn; the protective efficacies of each AAV can then be compared to their corresponding transduction efficiencies. As predicted, differences in protective efficacy between the different AAV serotypes were observed. AAVpo4 and AAV8 had the best efficacy with 90% protection, whereas surprisingly, AAVpo6 and AAVrh32.33 resulted in 0% survivors and had mice dying two days before the PBS control mice did (Figure 19). From these results, it was obvious that the AAVs could either be detrimental or beneficial in disease prevention. Here, it was hypothesized that AAV tropism may dictate the quality of immune responses elicited, which may have on impact on protective efficacy.

When examining the immune responses elicited by the AAV-EBOVGP vectors against the EBOV glycoprotein, neutralizing antibody analysis produced very little to no

titers. The same phenomenon can be seen in other studies, where neutralizing antibodies do not seem to play a significant role in protection, but total IgG content did (Wong et al., 2012b). Therefore, overall IgG production was examined using ELISA. Here, AAVs reached a maximum detection threshold between days 20 and 30, and these levels were maintained up to day 50 (Figure 22). However, AAVpo5 did not reach this maximum threshold until day 40, and even then, levels began decreasing by day 50. Examination of total IgG levels saw that groups that survived displayed high levels of total IgG (AAVpo4 and AAV8). However, one caveat to note is that groups that started dying before the controls had high IgG levels as well (AAVpo6 and AAVrh32.33). Although total IgGs are important for protection against EBOV infection, the quality of these antibodies may be equally as significant. For example, the subtypes of IgG being produced such as IgG1, IgG2, IgG3, or IgG4, may be important for generation of a high quality antibody response. As well, the epitopes that the antibodies target may be important for their protective efficacy. Another caveat was the detection threshold of the total IgG in the assays; the maximum absorbance allowed was ~2.0 at 405nm. In order to have a better understanding of the levels of antibodies produced by vaccinated mice, dilution series will have to be performed to achieve end-point total IgG titers until the maximum absorbance threshold is no longer attained.

Other than humoral responses, cellular immune responses against AAV-EBOVGP vaccinated mice were also evaluated at different time-points. The kinetics of pooled IFNγ responses in ELISPOT analysis found that AAVs affording protection had responses which peaked between day 20 and 30 (Figure 23). AAVpo6 and AAVrh32.33, which showed no protection in EBOV challenge studies, had relatively lower IFNγ

production at all time-points. AAVs affording the best protective efficacy (AAVpo4 and AAV8) offered the highest levels of IFNγ production. Those AAVs which offered moderate or minimal protection had similar IFNγ expression profiles to one another, with levels significantly lower than those seen with AAVpo4 and AAV8. Overall, AAV vaccination seems to point at IFNγ as being important for vaccine protection against EBOV in mice. Looking at the cytokine expression profiles of CD4+ and CD8+ cells from AAV-EBOVGP vaccinated mice, the general consensus for AAVs bestowing poor protection seems to point at CD4+ or CD8+ cells producing IL-2 (Figure 24). IL-2 is a signaling molecule important for proliferation and differentiation of T cells. Perhaps T cell responses are not as important for protection against EBOV as previously thought, and may in fact direct the immune response in a detrimental manner, contributing to disease progression. The subject manner for the immune correlates of protection for EBOV infection is quite controversial, yet data presented in this thesis appear to point towards a humoral response, rather than cell-mediated response for protection.

The pathology of EBOV infections has been described as a "cytokine storm", in which unbiased and uncontrolled release of cytokines allows for dysregulation of membrane integrity and the immune system, contributing to the symptoms often observed during lethal infection. In this thesis, the absence of IL-2 responses in animals vaccinated with AAVpo4-EBOVGP and AAV8-EBOVGP suggests that a constraint may be placed on T cells, and that these cells are not proliferating when in the presence of AAVpo4 or AAV8. Therefore, B cell responses may be as important for protection since these responses were observed and signaling molecules for T cell proliferation were absent. As well, AAVpo4 and AAV8 resulted in TNFα production, a pyrogen capable of inducing

antiviral properties such as fever, which may also contribute to protection (Figure 24). Although T cell signaling was measured in response to stimulation via EBOV GP peptides, the question about whether or not these T cells are truly functional or cytotoxic still remains. To elucidate this, future experiments for proliferation assays involving carboxyfluorescein succinimidyl ester (CFSE) or cytotoxic T cell assays can be performed. The results obtained from these studies were quite interesting, but an in-depth analysis was not the overall goal of this thesis; it is merely a basis to compare the protective efficacy and immune responses elicited by the different AAVs to their overall biodistribution and tissue tropism. Therefore, the following sections will discuss how AAV tissue tropism relates to protective efficacy.

4.4 *IN VITRO* AND *IN VIVO* TRANSDUCTION EFFICIENCIES OF PORCINE AAVS

The second hypothesis states 'AAVs isolated from porcine tissues can transduce mammalian and human cells efficiently'. It has been previously established that AAVs have specific tissue tropisms and can transduce both immortalized cell lines and animal tissues. However, transduction *in vitro* does not necessarily translate to transduction efficiency *in vivo*. Recent studies with AAV8 found that transduction of different mouse primary cell types including fibroblasts, skeletal muscle progenitor, keratinocytes, as well as immortalized cell lines, were very low (Ellis et al., 2013). However, when injected *in vivo*, AAV8 transduced a number of organs quite effectively including the liver. When injected intrahepatically, AAV8 performance was unquestionable as it displayed unrestricted transduction of hepatocytes in mice (Nakai et al., 2005; L. Wang et al., 2010). With this in mind, for *in vitro* studies, AAV8 transduced HepG2 at a very low

efficiently, even though this cell line is derived from hepatocytes. Intuitively, AAV8 should be able to transduce HepG2 efficiently since it is one of the most effective vectors for transducing hepatocytes *in vivo*. In this example, it is evident that *in vitro* transduction efficiency does not translate into *in vivo* performance. Therefore, minimal importance will be placed on the discussion of *in vitro* transduction of porcine-derived AAVs, as information obtained from these experiments was only a proof-of-concept that porcine-derived AAVs can infect cells derived from other species. Briefly, porcine-derived AAVs were able to transduce a number of different cell lines from different sources including human, non-human primates, and canines, showing that porcine-derived AAVs are capable of crossing the species barrier (Table 4). It was hypothesized that the similarities between human and pig tissues would allow for porcine-derived AAVs to transduce cells obtained from human sources. Indeed, this was observed in cellular sources from both humans and non-human primates.

The kinetics and intensity of gene-of-interest expression may be important for the quality of immune response generated against the transgene. Here, the time it takes for transgene expression and the levels expressed may dictate the immune response elicited. Because vaccination took place in the muscle, it was important to compare the kinetics of expression in the muscle and quantify this expression. To evaluate this, mice were injected intramuscularly with AAV-LacZ vectors and expression was measured both qualitatively and quantitatively at various time-points (Figure 9, Figure 10). AAV1 was shown to have comparative transduction efficiency in the muscle to that of AAV8 (Louboutin et al., 2005). Therefore, AAV1 was utilized in place of AAV8 due to the lack of availability of AAV8-LacZ vector. When compared to the porcine-derived AAVs, it

was observed that the kinetics and intensity of expression did not seem to play a role in protective efficacy. For example, LacZ expression for AAV1 vector peaked and was maintained by day 14, similar to that of AAVpo6. On the other hand, LacZ expression for AAVpo4 did not reach similar levels until day 28. Recall that AAVpo4 and AAV8 protected the best against EBOV challenge; from the kinetic studies for LacZ expression, the intensity of early transgene expression does not seem to play a role with immunity. If the intensity of expression were important, some level of protection should have been seen with the AAVpo6 vector. However, AAVpo6 actually resulted in mice dying days before the PBS control, suggesting that AAVpo6 may be having some sort of detrimental effect on the host's immune response. AAV is generally thought to be tolerogenic; perhaps AAVpo6 induced tolerance towards the EBOV GP transgene, which would explain why the mice succumbed to disease much sooner than the controls did. Although tolerance was not examined in this thesis, these results warrants further investigation into the role of AAV tolerance induction during vaccination. As for AAVrh32.33, recent evidence has shown immune responses generated towards the capsid results in abolishment of transgene expression (Mays et al., 2009). As well, the EBOV GP was shown to contain an immunosuppressive domain capable of decreasing cell surface expression of major histocompatibility complex class I molecules (Nancy J Sullivan et al., 2005; Volchkov, Blinov, & Netesov, 1992). This may provide a clue as to why AAVrh32.33 did not perform well in vaccine studies against EBOV; perhaps the immunosuppressive motifs of the EBOV GP, in conjunction with the antigenicity of the AAVrh32.33 capsid, directed the host immune response against the capsid itself, masking the signature of the EBOV GP. Taking into account that IFNγ responses of

AAVrh32.33-EBOVGP were minimal when examined by ELISPOT analysis, these results corroborate the outcome of AAVrh32.33-EBOV challenge in mice. The same may be true for AAVpo6-EBOVGP, where IFNγ ELISPOT responses were minimal compared to that of the other porcine-derived AAVs and AAV8 (Figure 23).

Looking at the overall biodistribution, AAVs with higher protective efficacy shared similar transduction profiles; efficient transduction was observed in almost all the major organs sampled (Figure 8). Those AAVs affording meager protective efficacy performed poorly in biodistribution studies (with the exception of AAVpo6). This suggests that AAVpo4 and AAV8 (which protected well in challenge studies) may share similar receptors, and that these receptors may be important for initiating protective immune responses. AAV8 binds to the laminin receptor, which is widely expressed on human tissues, and may explain the broad tropism of this vector (Akache et al., 2006). Even though AAVpo6 performed poorly in protective efficacy against EBOV infection in mice, it did share similar transduction profiles to AAVpo4 and AAV8 in biodistribution studies. AAV9, another AAV also displaying broad tropism, binds to terminal *N*-linked galactose as its primary receptor (Bell, Gurda, Van Vliet, Agbandje-McKenna, & Wilson, 2012; S. Shen, Bryant, Brown, Randell, & Asokan, 2011). Although purely speculative, it could be envisioned that AAVpo4 and AAV8 may share a common receptor such as laminin, whereas AAVpo6 may utilize a different receptor such as galactose. Unlike other AAVs shown to exploit the binding of a combination of receptors such as laminin and galactose (for AAV9), AAV8 was shown to bind solely to laminin, irrespective of the presence or absence of other receptors.

Receptor binding was not investigated in this thesis, but the characterization of the receptor binding of porcine-derived AAVs may shed more light on their tissue tropism and ability to cross the species barrier. Some questions to be asked are: do the receptors that porcine-derived AAVs bind to exist on other species such as human or non-human primates? As well, does binding of AAV to specific receptors play an important role in resulting immune responses? As mentioned previously, the pig SLA shares considerable sequence homology to the human HLA, and antigens bound by the pig SLA were able to present antigens to T cells from the opposite species (Shishido et al., 1997). Other examples of shared receptors are sialic acid α(2,6), which can be found in both humans and pigs (Nelli et al., 2010). Studies with AAV1 and AAV6 have shown that these AAVs can bind to both sialic acid α(2,3) and –α(2,6), suggesting that these AAVs can cross the species barrier (Wu, Miller, et al., 2006). As will be discussed later, a parallel can be drawn where AAVs showing higher protective efficacy are able to transduce specific types of immune cells, suggesting they may share similar receptors.

To further complement the tropism studies, transduction of porcine-derived AAVs in organs including the lung, liver, and brain were studied. Transduction of porcine-derived AAVs in the lung did not show a correspondence between transduction in this organ to protective efficacy (Figure 11). Overall, only AAVpo6 was able to transduce lung tissue efficiently, while all other porcine-derived AAVs did not fare as well. AAVpo1, AAVpo4, and AAVpo5 showed only anecdotal transduction in the lungs. A relationship between AAV transduction efficiency in the lung and protective efficacy could not be established.

Looking at transduction in the liver, AAVs providing good protection expressed their transgene in the liver (Figure 12). However, AAVpo6, which did not provide protection against EBOV challenge, also showed high transduction efficiency in the liver. Going back to the theme of receptor binding, perhaps AAVpo4 and AAV8 share a common receptor found on the liver, whereas AAVpo6 is able to bind to a different receptor also present on the hepatocellular surface. Both laminin and galactose receptors can be found on liver cells, which could allow for transduction of AAVs utilizing different receptors on a common target tissue (Kempka & Kolb-Bachofen, 1985; Thepparit & Smith, 2004). This would explain why we see AAVpo4, AAVpo6, and AAV8 in hepatocytes, but only the presence of AAVpo6 in lung tissue.

From biodistribution data, the presence of AAV genome copies in the brain was observed (Figure 8). Genome copies do not necessarily reveal transduction in a tissue, warranting further investigation into the ability of porcine-derived AAVs to infect cells in the brain. It is important to note that viral infection in the brain is rare due to the ability of the blood-brain barrier to prevent translocation of viral particles from the blood stream into the brain. The blood-brain barrier forms junctions tight enough to prevent transport of very small molecules, including antibodies, making treatment of cerebral infection quite difficult (Abbott, Rönnbäck, & Hansson, 2006). Recently, AAV9 was observed to cross the blood-brain barrier and infect cells in the brain (Foust et al., 2010). It is thought that retrograde transport can help facilitate translocation of AAV and infect cells away from the site of initial infection (Salegio et al., 2013). AAV8 was shown to undergo retrograde transport and infect the spinal cord and dorsal root ganglion following administration via IM injection (Zheng, 2010). AAVpo4 and AAVpo6 were investigated

for their ability to infect cells in the brain and indeed, transduction was observed following IVTV injection (Figure 13). This suggests that AAVpo4 and AAVpo6 are able to cross the blood-brain barrier and that these AAVs may also undergo retrograde transportation or transcytosis. This property may play an important role as will be discussed in the unifying model of the tropism and protective efficacy of AAV vectors.

Examining the broad tissue tropism of AAV and its relation to protective efficacies did not provide much insight into a mechanism of how tropism affected immunity. The goal of these experiments was to determine if there was a link between tropism in specific tissues and protective efficacy, and it appears that AAVs performing well against lethal challenge did share common tissue tropisms, but AAVs which offered no protection at all during EBOV infection also shared similar tropisms. A proposed mechanism where AAVpo6 is thought to skew the immune response will be discussed near the end of this chapter. Therefore, it was proposed that tropism of AAV in immune cells itself, rather than general tissue tropism, may provide insight into protective efficacy elicited by certain AAV serotypes.

4.5 TRANSDUCTION EFFICIENCY OF AAV-SCGFP VECTORS IN MOUSE IMMUNE CELLS

It was hypothesized that broad tropism may play a role with immunity and protective efficacy during AAV administration, but no conclusive results were obtained. Therefore, the tropism of AAV in immune cell types was closely examined, as tropism of certain immune cells such as antigen presenting cells may dictate the type of immune response elicited. Recent vaccination strategies utilizing adenoviral vectors have shown great promise (Tatsis & Ertl, 2004). The hexon protein of adenovirus can act as a potent

adjuvant, making adenoviral vectors effective stimulators of the cellular immune response (Molinier-frenkel et al., 2002). As well, adenovirus has the ability to infect and induce maturation of dendritic cells, allowing for effective priming of the adaptive immune response (Morelli et al., 2000). Furthermore, adenoviral vectors carrying the EBOVGP afforded complete protection in non-human primates (N J Sullivan, Sanchez, Rollin, Yang, & Nabel, 2000). Perhaps the immunostimulatory properties present in adenovirus may also be observed in certain AAV serotypes. Therefore, it was hypothesized that 'AAVs affording protection against lethal infection can transduce antigen presenting cells such as dendritic cells or B cells'. This experiment was carried out by injecting mice via IVTV with AAV vectors carrying the self-complementary DNA for GFP. Self-complementary AAV DNA allows for faster and more efficient expression of the transgene, providing a tool to identify transduced immune cells during early infection (McCarty et al., 2001). From the results, none of the AAV vectors were able to transduce helper T cells or cytotoxic T cells, effectively. However, the results from the transduction of other immune cells were stunning and did confirm the infection of antigen presenting cell hypothesis; AAVs having higher protective efficacy against EBOV challenge were able to infect dendritic cells and B cells, whereas AAVs having low protective efficacy did not. As well, transduction of total population of immune cells was much higher for the AAVs which protected well versus the poor protectors. Here, an association does exist for tropism and protective efficacy in the context of the immune system, where AAVs affording the highest protection against lethal EBOV challenge (AAVpo4 and AAV8) were able to transduce B cells and dendritic cells (Figure 25). AAVpo1 and AAVpo5 did not transduce any of these cell types nearly as efficiently as

AAVpo4 and AAV8. AAV transduction of APCs appears to play a role with immunity and may explain why differences were observed in protective efficacy between the various AAV serotypes.

4.6 LINK BETWEEN TISSUE TROPISM, TRANSDUCTION EFFICIENCY, AND IMMUNE RESPONSES TO AAV VECTORS

The overall goal of this thesis was to compare the tissue tropisms of porcine-derived AAVs to their protective efficacies against lethal challenge models. AAVs providing good protection showed broad tropism and able to transduce most of the major organs when distributed systemically. One caveat is that an AAV which offered no protection and resulted in death of mice significantly sooner than the controls in EBOV infection models, also showed similar tropism. As well, irrespective of their protective efficacies, all AAVs transduced muscle fibers quite effectively, showing that the intensity and kinetics of transgene expression in the muscle does not seem to play a role on host immunity when AAVs are administered at $1x10^{11}$ gc. Although the kinetics of transgene expression varied between the different serotypes, a parallel could not be established with the protective efficacies. It was mentioned previously that receptor binding may play an important role in defining tissues AAVs can transduce. The tropism of AAVs in immune cells found that those with higher protective efficacy actually transduced antigen presenting cells quite effectively. Perhaps the receptors/co-receptors present on the transduced immune cells can also be found on the transduced cells in the biodistribution studies.

Very little is known about the transduction efficiency of AAV in dendritic cells and much controversy has arisen in questioning the effectiveness of AAV in these cells

(Aslanidi et al., 2012; Chiriva-Internati et al., 2003; Y. Liu et al., 2000). Investigations of AAV transduction efficiency in dendritic cells were performed mainly *ex vivo* or *in vitro*, utilizing monocyte-derived dendritic cells or purified dendritic cells, never actually investigating the actual efficiency of AAVs *in vivo* in immune cells. As mentioned previously, *in vitro* transduction initiated by AAV does not necessarily translate to *in vivo* transduction. Bell *et al* noted that for AAV9 receptor binding, β-galactose linkages were important (Bell et al., 2011, 2012). In their studies, they found that administration of neuraminidase into mice cleaved sialic acid residues, resulting in exposure of β-galactose, facilitating increased binding of AAV9. Here, *in vivo* transduction relies heavily upon exogenous factors for efficiency. These results provide an explanation on the discordance between *in vitro* and *in vivo* transduction for AAV vectors; factors important for transduction *in vivo* may be absent in *in vitro* environments. In this thesis, a natural environment for AAV infection was provided via *in vivo* transduction in mice, providing a better representation for the tropism of AAV in a natural immune environment. Theoretically speaking, transduction of dendritic or B cells by AAV may provide assistance with activation of the immune system, and may explain why protective efficacy afforded by AAVpo4 and AAV8 against lethal EBOV challenge was much higher than the other AAV serotypes. Contrary to therein, it was believed that AAV8 would actually perform poorly in challenge experiments as previous studies have shown that AAV8 administration in mice results in non-functional T cell responses and poor transduction of antigen-presenting cells (Lin et al., 2007a; Mays et al., 2014). As mentioned previously, discordance exists between *in vitro* and *in vivo* transduction and most experiments involving AAV transduction of dendritic cells were facilitated *in vitro*

or *ex vivo*. However, the findings in this thesis do corroborate with the production of non-functional T cells following AAV8 vaccination, as results indicate that CD4+ T cells were mainly unresponsive when stimulated with EBOV GP peptides. Therefore, it is counter-intuitive to believe that AAV8 would serve as an effective vaccine vector candidate as tolerance may be achieved when utilizing this vector. In order to explain the conflicting results with the AAV8 vaccine vector, closer attention should be placed on the disease model utilized. All of the AAV vectors tested were able to effectively protect against influenza infection in mice. When tested in the EBOV infection model, AAVs were found to have contradictory protective efficacies. It was hypothesized that with the influenza model, all the AAVs utilized were able to elicit effective, protective, classical immune responses. For example, the AAVs transduced and expressed their transgene in the muscle fibers. In turn, antigen presenting cells were able to recognize the foreign protein on the cell surface, resulting in engulfment of transduced cells. The protein is processed for presentation by the antigen presenting cells and presented to CD4+ or CD8+ T cells, resulting in activation of the adaptive immune response. As well, the CD4+ T cells are able to stimulate B cells, allowing for production of antibodies. This, of course, is an over-simplified version of what is going on during a classical immune response. In the EBOV model, these same responses may not be sufficient enough to protect against disease. During natural EBOV infection, an overabundance of cytokine expression is observed and dysregulation of immune responses and uncontrolled inflammation occurs. The same can also be seen in Lassa virus infection, where the host's cellular immune response seems to contribute to disease progression (Flatz et al., 2010). In this case, the key to protection may actually be the downregulation of

inflammatory immune responses. In the EBOV studies, AAV8 and AAVpo4 vaccination may induce T cell tolerance, thereby skewing the immune response to the humoral arm. AAVpo4 and AAV8 were shown to transduce B cells, perhaps increasing the quality of antibodies required for protection against EBOV infection, as well as dictating what type of immune response will be elicited. Because all AAVs protected equally as well during influenza challenge, it cannot be ruled out that these vectors may one day serve as vaccine vector candidates. The EBOV studies can be considered an anomaly where B cells can be considered important for protection, whereas an overactive T cell response can be detrimental.

It was mentioned that skewing of the immune response may be occurring. The capsid of AAVrh32.33 was deemed highly immunogenic and its structural determinants responsible for enhanced T cell activation have been mapped (Mays et al., 2013). The combination of the immunogenicity of AAVrh32.33 capsid and immunosuppressive properties of EBOV GP may influence the immune response to act against AAV, resulting in tolerance of the EBOV GP. The observation of minimal IFNγ responses in AAVpo6 and AAVrh32.33 vaccinated mice fits very nicely in this model, where a destructive tolerance is achieved and EBOV GP mainly ignored, allowing the disease to run rampant and cause disease symptoms and death much sooner than control animals. This anomaly can be detrimental with these vectors, as mice in these groups started dying days before the control mice did.

4.7 AAV TRANSDUCTION OF B CELLS

Aside from the observation of protective AAVs transduction in dendritic cells, transduction of B cells was also detected. This is quite significant as the potential of

AAV transduction in B cells has never been closely looked at or described in the literature. As well, there are no previous publications discussing the ability of AAV to transduce B cells. In the literature, B cells acting as antigen-presenting cells are capable of inducing peripheral T cell tolerance (Raimondi, Zanoni, Citterio, Ricciardi-Castagnoli, & Granucci, 2006; Tsitoura, Yeung, DeKruyff, & Umetsu, 2002). Recall that AAVpo4 and AAV8 both transduce B cells; examination of the quality of cellular immune responses found cytokine production limited to IFNγ and TNFα for these vectors, as well as a lack of IL-2 production, which corroborates with data from other labs displaying the ability of AAV8 to produce non-functional T cell responses. Recently, correlates of protection for EBOV infection pointed more towards a humoral immune response, and the use of AAVpo4 and AAV8 may be conducive for protective efficacy in this model. However, transduction of B cells by AAV alone theoretically cannot activate B cells. AAV was previously shown to activate TLR9 (Faust et al., 2013). As well, other studies have shown that TL9 stimulation in B cells will drive naïve B cells to attain antigen presenting function (W. Jiang et al., 2007). Here, AAV can theoretically stimulate B cell activation if it can transduce these cells, independent of T cell activation.

In this thesis, the five hypotheses outlined in 1.13.2 were addressed. The main goal was to isolate new serotypes of AAV and show a relationship of their transduction efficiencies to protective efficacy, and by doing so, it was shown that: 1) AAV exists in porcine tissues and can be isolated by PCR, 2) porcine-derived AAVs can transduce mammalian cells efficiently, 3) porcine-derived AAVs can protect against some of the most aggressive and lethal viral infections known, 4) AAVs protective against lethal challenge have broad tissue tropism, and 5) AAVs able to transduce APCs (B cells and

DC) afforded the best protection against lethal challenge in mice. Each hypothesis was successfully proven. From these results, 3 possible scenarios arise from this data: 1) All AAVs protective against lethal influenza challenge stimulate classical immune responses, 2) AAV transduction of dendritic and B cells may show enhanced B cell responses, and 3) AAV transduction of DC or B cells may induce peripheral T cell tolerance, while producing quality, protective humoral responses. Those AAVs, which could not efficiently transduce B cells, showed very little or no protection against lethal EBOV challenge in mice. AAVs are known to stimulate robust humoral responses, but perhaps this is not enough to protect against EBOV. Therefore, direct B cell transduction by AAV may be important for driving the production of specific antibodies not easily attained by classical immune responses. As mentioned previously, emerging studies are beginning to show the importance of B cell responses in protection against EBOV infection. With influenza, it is not quite clear, but a T cell response was thought to be important for protection. In the influenza model, equal protection between all the AAV serotypes was observed. In this situation, it can be assumed that all AAVs stimulated protective B cell responses as high HAI titers were attained. In a study with lethal challenge of Nipah in guinea pigs, researchers decided to utilize AAV8 over AAVrh32.33 for vaccination purpose due to its ability to produce more robust humoral responses over the latter (Ploquin et al., 2013). Here, 100% protection was obtained, highlighting the importance of humoral immune responses over cell-mediated (Ploquin et al., 2013). T cell responses very much play an important role in immunity, but perhaps more importance should be placed on vaccine vectors capable of eliciting quality humoral immune responses in unique disease models such as EBOV or Lassa infection. The

results from this thesis warrant further investigation of the role of AAV infection in B cells and its implication on the host immune response.

4.7 LIMITATIONS

Initially, the goal of this thesis was to isolate novel AAV *cap* genes from pig genomic DNA. AAV *cap* genes were successfully isolated; however, the rest of the AAV genome was not. Attempts were made to isolate the rest of the AAV genome including the ITRs and *rep* gene, but with no success. One method attempted to isolate the full AAV genome was inverse PCR. Essentially, fragmented genomes are circularized and inverse primers utilized. An alternative method would be to transfect the AAV DNA into cells and infect the cells with Adenovirus, allowing for production of wtAAV. It would have been informative to compare the homology between porcine-derived AAV *rep* genes and those derived from human and NHPs, and if the same functions are retained. Also, it would be interesting to see if porcine-derived AAV ITRs share similar homology to published AAV sequences or between themselves. AAV2 ITRs are known to integrate into specific sites within the human genome, and it would be informative to see if porcine-derived AAVs can do the same in pigs or in humans.

Another limitation was the inability to produce viable particles from AAVpo7 or –po8 DNA. Perhaps these two vectors cannot form functional particles due to their inability to assemble properly. A Western blot was performed and viral capsid proteins were not detected for these vectors, which could mean one of two things: either the capsid proteins are not being expressed or they are not recognized by the monoclonal antibodies. As AAVpo7 and –po8 are highly divergent from other porcine-derived

AAVs, it would have been interesting to characterize these two vectors. This matter will have to be revisited to determine the absence of vector production.

For tropism studies, organ sections were examined qualitatively for transgene expression. Even though the number of transduced cells can be enumerated in this matter and the average number of AAV genome copies present per diploid genome determined, this does not inform us of the actual levels of transgene being produced. The only *in vivo* experiment involving measurement of transgene expression took place following the IM administration of AAV-LacZ in the mouse muscle, with the kinetics of β-gal expression. It would have been valuable to see the levels of transgene expression in lung, liver, or brain following AAV-LacZ administration. Beta-gal levels can be measured using a beta-gal ELISA kit, beta-gal expression assay, or imaging software such as ImageJ, which can visually quantify the number of transduced cells in an image.

In challenge studies, some experiments were repeated only once due to lack of available vector. AAV-H05HA challenge experiments were performed three times. However, the AAV-EBOVGP challenge study was only performed once. It would have been informative to repeat challenge studies to determine if the same outcomes could be replicated.

For delivery of H05HA or EBOVGP to the mouse muscle, immunohistochemistry would have been informative to deduce the outcome of transgene expression. A publication utilizing AAV2-HA vectors showed dramatic decrease of HA expression by day 26, and complete abolishment by day 60 (Zhu et al., 2009). It would be

advantageous to see if the same results could be replicated with porcine-derived AAVs, and if the same results could also be seen with AAV-EBOVGP vectors.

For characterization of total IgG responses in AAV-EBOVGP vaccinated mice, ELISA was utilized to measure the levels from undiluted serum. However, the end-point titer of total IgG was not determined. As most AAVs reached the peak detection threshold for total IgG levels, it would have been more informative if the end-point titers were determined. Essentially, the serum is serially diluted and the titer, in which the detection threshold is not reached, would be the end-point titer. Again, due to the lack of vector and serum, the end-point titers were not determined. However, from the assays we performed, we were still able to show the kinetics of total IgG expression. As well, it would have been useful if more time-points were added to the kinetics study for both total IgG ELISA and IFNγ ELISPOT assays.

For the characterization of immune responses via flow cytometry, the only responses examined were IL-2, TNFα, and IFNγ for $CD4^+$ and $CD8^+$ T cells. Nearing the end of the work in this thesis, it was determined that B cells and DC may play an important role in dictating the host immune response elicited against the transgene. Due to a lack of time, these responses were not characterized. If provided with the proper resources and reagents, other markers for B cell and DC assays could be employed, including IL-4, IL-10, CD80, MHCII, and CD40. Other aspects of B cell infection could look at the activation status of infected B cells. Are infected cells activated to become APCs or Bregs? Can infected B cells proliferate? Also, what is the functional status of T cells derived from vaccinated mice? Are these cells cytotoxic, or non-functional? These

questions will have to be revisited in order to determine the consequences of AAV transduction of B cells and DC.

Another aspect of immunity not examined was the immunogenicity of the AAV capsids themselves. Previous studies have shown that the AAVrh32.33 capsid is highly immunogenic, resulting in the host immune response mounting an effective immune response against transduced cells and abolishment of transgene expression. It would be interesting to see if the capsid proteins of porcine-derived AAVs are immunogenic themselves, much like AAVrh32.33. The immunogenicity of the AAV capsid could also be a determining factor in the type of immune responses elicited against the transgene. However, due to the high variability between the AAV capsids and the fact that immunogenicity against capsid proteins is poorly characterized, peptide pools spanning the entire AAV capsid would have to be generated for each porcine-derived AAV capsid. It would be of importance to determine the immunogenicity of each AAV capsid, but due to time and budget constraints, this aspect of AAV immunity was not explored.

Another limitation was the animal models used for viral infections. The strain of EBOV used to infect mice was mouse adapted and disease outcome in this model does not faithfully recapitulate the disease symptoms as seen in humans. A better animal model would have been the guinea pig of non-human primate. However, due to the costs associated with larger animal models, it was decided that AAV-EBOVGP vaccination studies would be limited to mouse models.

4.8 FUTURE PROSPECTS

During the characterization of porcine-derived AAVs in mice, each AAV displayed its own unique tissue tropism, which can be exploited to benefit future therapies in man. AAVpo1 was shown to specifically target muscle, while detargeting most major organs when delivered systemically; AAVpo1 may one day be useful for treatment of diseases such as muscular dystrophy. Vectors such as AAVpo5 performed very poorly in during biodistribution studies; however, when injected IM, AAVpo5 showed efficient transgene expression in the muscle. This suggests that AAVpo5 cannot cross the endothelial barrier, reducing the risk of it transducing other bystander organs if it were injected IM for therapeutic or vaccine purposes. AAVpo4 and AAVpo6 transduced all the organs we sampled when injected IVTV, and even transduced cells in the brain. Here, these vectors can be developed to treat ailments affecting the brain such as Alzheimer's or Batten's disease. As well, AAVpo4 and AAVpo6 showed high transduction levels in the liver, sharing similar efficiencies with the gold-standard AAV8. These vectors could be used in hepatocytes to treat factor IX or alpha-1 antitrypsin deficiencies. AAVpo6 showed high transduction levels in the lung, making it a potential candidate for treatment of lung disorders such as cystic fibrosis. In all, the porcine-derived AAV vectors should be considered as future gene therapy vectors as they contend with gold-standard AAVs and pre-existing immunity was not observed for most within the general human population.

Here, the significant finding that AAV can infect B cells was also reported. It would contribute greatly to the field if the consequence of this phenomenon is elucidated. In this thesis, a link between the tissue tropisms to protective efficacies was established.

However, the true outcome of the infection of cell types such as B cells or dendritic cells was not examined. The question remains: does AAV infection of B cells induce peripheral T cell tolerance? AAV8 was shown to induce T cell tolerance via poor APC transduction, T cell exhaustion, and minimal MHCI upregulation. The findings in this thesis provide evidence for another mechanism to add to the list: transduction of B cells induces peripheral T cell tolerance. This warrants further investigation to determine if AAV can indeed induce tolerance by transduction of B cells. AAV8 was previously shown to induce Treg activation in the liver (Dobrzynski et al., 2006; Martino et al., 2009). However, the mechanism of this Treg activation is still unknown. Perhaps the AAV transduction of B cells presented in this thesis provide a clue as to how Treg activation and tolerance is achieved during AAV gene transfer. To add to this, what subsets of B cell are being transduced, and what is the quality of antibodies being produced by AAVpo4 and AAV8 transduced B cells? All AAVs were able to induce antibody responses when delivering EBOV GP into a host, but characterization of these antibodies was not performed. It could be quite informative if the subclasses and binding sites of the antibodies are identified.

As suggested, AAV transduction of B cells may be able to elicit a humoral response, resulting in production of quality antibodies. Perhaps these antibodies are targeted to epitopes not normally seen with classical immune responses. One way to test this would be heterologous challenge with influenza. If AAVs can produce quality antibodies targeting conserved regions of the influenza HA, theoretically, these antibodies should be able to protect against divergent strains of influenza sharing this common region. This was seen with the identification of the broadly neutralizing F16

antibody, which afforded protection against influenza strains from both H5N1 and H1N1 groups (Limberis et al., 2013).

Overall, the findings in this thesis have opened new doors in the AAV field. Here, new AAV serotypes from pig tissues have been provided and the sequences shared online, with the potential as modalities for gene therapy and vaccine applications. As well, a relation between AAV tissue tropism and its protective efficacy was suggested, in which AAVs that have broad tissue tropism had high protective efficacy against lethal challenge in mice. In addition, new mechanisms for AAV induction of protective responses have been proposed, with regards to aspects of the immune system never before considered as having an influence on B cell activation or T cell tolerance following AAV transduction.

CHAPTER V

5.0 BIBLIOGRAPHY

Aaslestad, H. G., & Hoffman, E. J. (1968). Fractionation of Eastern Fractionation of Eastern Equine Encephalitis Virus by Density Gradient Centrifugation in CsCl.

Abbott, N. J., Rönnbäck, L., & Hansson, E. (2006). Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews. Neuroscience*, *7*(1), 41–53. doi:10.1038/nrn1824

Abdel-Ghafar, A.-N., Chotpitayasunondh, T., Gao, Z., Hayden, F. G., Nguyen, D. H., de Jong, M. D., … Uyeki, T. M. (2008). Update on avian influenza A (H5N1) virus infection in humans. *The New England Journal of Medicine*, *358*(3), 261–73. doi:10.1056/NEJMra0707279

Adams, W. C., Bond, E., Havenga, M. J. E., Holterman, L., Goudsmit, J., Karlsson Hedestam, G. B., … Loré, K. (2009). Adenovirus serotype 5 infects human dendritic cells via a coxsackievirus-adenovirus receptor-independent receptor pathway mediated by lactoferrin and DC-SIGN. *The Journal of General Virology*, *90*(Pt 7), 1600–10. doi:10.1099/vir.0.008342-0

Agorastos, T., Chrisafi, S., Lambropoulos, A. F., Mikos, T., Constandinides, T. C., Schlehofer, J. R., … Bontis, J. N. (2008). Adeno-associated virus infection and cervical neoplasia: is there a protective role against human papillomavirus-related carcinogenesis? *European Journal of Cancer Prevention : The Official Journal of the European Cancer Prevention Organisation (ECP)*, *17*(4), 364–8. doi:10.1097/CEJ.0b013e3282b6fd2e

Akache, B., Grimm, D., Pandey, K., Yant, S. R., Xu, H., & Kay, M. a. (2006). The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *Journal of Virology*, *80*(19), 9831–6. doi:10.1128/JVI.00878-06

Alazard-Dany, N., Nicolas, A., Ploquin, A., Strasser, R., Greco, A., Epstein, A. L., … Salvetti, A. (2009). Definition of herpes simplex virus type 1 helper activities for adeno-associated virus early replication events. *PLoS Pathogens*, *5*(3), e1000340. doi:10.1371/journal.ppat.1000340

Aldrich, W. a, Ren, C., White, a F., Zhou, S.-Z., Kumar, S., Jenkins, C. B., … Ponnazhagan, S. (2006). Enhanced transduction of mouse bone marrow-derived dendritic cells by repetitive infection with self-complementary adeno-associated virus 6 combined with immunostimulatory ligands. *Gene Therapy*, *13*(1), 29–39. doi:10.1038/sj.gt.3302601

Arbetman, A. E., Lochrie, M., Zhou, S., Wellman, J., Scallan, C., Doroudchi, M. M., … Colosi, P. (2005). Novel Caprine Adeno-Associated Virus (AAV) Capsid (AAV-Go . 1) Is Closely Related to the Primate AAV-5 and Has Unique Tropism and Neutralization Properties, *79*(24), 15238–15245. doi:10.1128/JVI.79.24.15238

Aschauer, D. F., Kreuz, S., & Rumpel, S. (2013). Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PloS One*, *8*(9), e76310. doi:10.1371/journal.pone.0076310

Aslanidi, G. V, Rivers, A. E., Ortiz, L., Govindasamy, L., Ling, C., Jayandharan, G. R., … Srivastava, A. (2012). High-efficiency transduction of human monocyte-derived dendritic cells by capsid-modified recombinant AAV2 vectors. *Vaccine*, *30*(26), 3908–17. doi:10.1016/j.vaccine.2012.03.079

Asokan, A., Hamra, J. B., Govindasamy, L., Agbandje-McKenna, M., & Samulski, R. J. (2006). Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. *Journal of Virology*, *80*(18), 8961–9. doi:10.1128/JVI.00843-06

Asokan, A., Schaffer, D. V, & Samulski, R. J. (2012). The AAV vector toolkit: poised at the clinical crossroads. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *20*(4), 699–708. doi:10.1038/mt.2011.287

Asuri, P., Bartel, M. a, Vazin, T., Jang, J.-H., Wong, T. B., & Schaffer, D. V. (2012). Directed evolution of adeno-associated virus for enhanced gene delivery and gene targeting in human pluripotent stem cells. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *20*(2), 329–38. doi:10.1038/mt.2011.255

Atchison, R. W., Casto, B. C., & Hammon, W. M. (1965). Adenoviru-associated defective virus particles. *Science (New York, N.Y.)*, *149*(3685), 754–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14325163

Balazs, A. B., Bloom, J. D., Hong, C. M., Rao, D. S., & Baltimore, D. (2013). Broad protection against influenza infection by vectored immunoprophylaxis in mice. *Nature Biotechnology*, *31*(7), 647–52. doi:10.1038/nbt.2618

Balazs, A. B., Chen, J., Hong, C. M., Rao, D. S., Yang, L., & Baltimore, D. (2012). Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature*, *481*(7379), 81–4. doi:10.1038/nature10660

Bantel-Schaal, U., & zur Hausen, H. (1984a). Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology*, *134*(1), 52–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6324476

Bantel-Schaal, U., & zur Hausen, H. (1984b). Characterization of the DNA of a defective human parvovirus isolated from a genital site. *Virology*, *134*(1), 52–63.

Baron, R. C., McCormick, J. B., & Zubeir, O. A. (1983). Ebola virus disease in southern Sudan: hospital dissemination and intrafamilial spread. *Bulletin of the World Health Organization*, *61*(6), 997–1003. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2536233&tool=pmcentrez&rendertype=abstract

Bartlett, J. S., Wilcher, R., & Samulski, R. J. (2000). Infectious Entry Pathway of Adeno-Associated Virus and Adeno-Associated Virus Vectors Infectious Entry Pathway of Adeno-Associated Virus and Adeno-Associated Virus Vectors, *74*(6). doi:10.1128/JVI.74.6.2777-2785.2000.Updated

Basler, C. F., Wang, X., Mühlberger, E., Volchkov, V., Paragas, J., Klenk, H. D., … Palese, P. (2000). The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(22), 12289–94. doi:10.1073/pnas.220398297

Bausch, D. G., Towner, J. S., Dowell, S. F., Kaducu, F., Lukwiya, M., Sanchez, A., … Rollin, P. E. (2007). Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. *The Journal of Infectious Diseases*, *196 Suppl* (Suppl 2), S142–7. doi:10.1086/520545

Bearer, E. L., Breakefield, X. O., Schuback, D., Reese, T. S., & LaVail, J. H. (2000). Retrograde axonal transport of herpes simplex virus: evidence for a single mechanism and a role for tegument. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(14), 8146–50. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=16684&tool=pmcentrez&rendertype=abstract

Beer, B., Kurth, R., & Bukreyev, A. (1999). Characteristics of Filoviridae: Marburg and Ebola viruses. *Die Naturwissenschaften*, *86*(1), 8–17. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10024977

Beigel, J. H., Farrar, J., Han, A. M., Hayden, F. G., Hyer, R., de Jong, M. D., … Yuen, K.-Y. (2005). Avian influenza A (H5N1) infection in humans. *The New England Journal of Medicine*, *353*(13), 1374–85. doi:10.1056/NEJMra052211

Bell, C. L., Gurda, B. L., Van Vliet, K., Agbandje-McKenna, M., & Wilson, J. M. (2012). Identification of the galactose binding domain of the adeno-associated virus serotype 9 capsid. *Journal of Virology*, *86*(13), 7326–33. doi:10.1128/JVI.00448-12

Bell, C. L., Vandenberghe, L. H., Bell, P., Limberis, M. P., Gao, G., Vliet, K. Van, … Wilson, J. M. (2011). The AAV9 receptor and its modification to improve in vivo lung gene transfer in mice, *121*(6). doi:10.1172/JCI57367DS1

Bello, a, Tran, K., Chand, a, Doria, M., Allocca, M., Hildinger, M., … Kobinger, G. P. (2009). Isolation and evaluation of novel adeno-associated virus sequences from porcine tissues. *Gene Therapy*, *16*(11), 1320–8. doi:10.1038/gt.2009.82

Berns, K. I., & Giraud, C. (1996). Biology of adeno-associated virus. *Current Topics in Microbiology and Immunology*, *218*, 1–23. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8794242

Bharadwaj, A. S., Kelly, M., Kim, D., & Chao, H. (2010). Induction of immune tolerance to FIX by intramuscular AAV gene transfer is independent of the activation status of dendritic cells. *Blood*, *115*(3), 500–9. doi:10.1182/blood-2009-08-239509

Bleker, S., Sonntag, F., & Kleinschmidt, A. (2005). Mutational Analysis of Narrow Pores at the Fivefold Symmetry Axes of Adeno-Associated Virus Type 2 Capsids Reveals a Dual Role in Genome Packaging and Activation of Phospholipase A2 Activity, *79*(4), 2528–2540. doi:10.1128/JVI.79.4.2528

Bosio, C. M., Aman, M. J., Grogan, C., Hogan, R., Ruthel, G., Negley, D., … Schmaljohn, A. (2003). Ebola and Marburg viruses replicate in monocyte-derived dendritic cells without inducing the production of cytokines and full maturation. *The Journal of Infectious Diseases*, *188*(11), 1630–8. doi:10.1086/379199

Bossis, I., & Chiorini, J. A. (2003). Cloning of an Avian Adeno-Associated Virus (AAAV) and Generation of Recombinant AAAV Particles, *77*(12), 6799–6810. doi:10.1128/JVI.77.12.6799

Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M. F., & Masurier, C. (2010a). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Human Gene Therapy*, *21*(6), 704–12. doi:10.1089/hum.2009.182

Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M. F., & Masurier, C. (2010b). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Human Gene Therapy*, *21*(6), 704–12. doi:10.1089/hum.2009.182

Bradfute, S. B., Warfield, K. L., & Bavari, S. (2008). Functional CD8+ T cell responses in lethal Ebola virus infection. *Journal of Immunology (Baltimore, Md. : 1950)*, *180*(6), 4058–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18322215

Brantly, M. L., Chulay, J. D., Wang, L., Mueller, C., Humphries, M., Spencer, L. T., … Flotte, T. R. (2009). Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proceedings of the National*

Academy of Sciences of the United States of America, *106*(38), 16363–8. doi:10.1073/pnas.0904514106

Bray, M., Davis, K., Geisbert, T., Schmaljohn, C., & Huggins, J. (1998). A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. *The Journal of Infectious Diseases*, *178*(3), 651–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9728532

Brockstedt, D. G., Podsakoff, G. M., Fong, L., Kurtzman, G., Mueller-Ruchholtz, W., & Engleman, E. G. (1999). Induction of immunity to antigens expressed by recombinant adeno-associated virus depends on the route of administration. *Clinical Immunology (Orlando, Fla.)*, *92*(1), 67–75. doi:10.1006/clim.1999.4724

Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., … Wilson, J. M. (2011a). Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clinical and Vaccine Immunology : CVI*, *18*(9), 1586–8. doi:10.1128/CVI.05107-11

Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., … Wilson, J. M. (2011b). Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clinical and Vaccine Immunology : CVI*, *18*(9), 1586–8. doi:10.1128/CVI.05107-11

Calcedo, R., Vandenberghe, L. H., Gao, G., Lin, J., & Wilson, J. M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *The Journal of Infectious Diseases*, *199*(3), 381–90. doi:10.1086/595830

Calcedo, R., & Wilson, J. M. (2013). Humoral Immune Response to AAV. *Frontiers in Immunology*, *4*(October), 341. doi:10.3389/fimmu.2013.00341

Cárdenas, W. B., Loo, Y.-M., Gale, M., Hartman, A. L., Kimberlin, C. R., Martínez-Sobrido, L., … Basler, C. F. (2006). Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *Journal of Virology*, *80*(11), 5168–78. doi:10.1128/JVI.02199-05

Casto, B. C., Atchison, R. W., & Hammon, W. M. (1967). Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. *Virology*, *32*(1), 52–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4290509

Chan, M. C. W., Cheung, C. Y., Chui, W. H., Tsao, S. W., Nicholls, J. M., Chan, Y. O., … Peiris, J. S. M. (2005). Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. *Respiratory Research*, *6*, 135. doi:10.1186/1465-9921-6-135

Chan, P. K. S. (2002). Outbreak of avian influenza A(H5N1) virus infection in Hong Kong in 1997. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *34 Suppl 2*, S58–64. doi:10.1086/338820

Chang, L., Shi, Y., & Shenk, T. (1989). an adenovirus E1A-inducible element and a binding site for the major late transcription Adeno-Associated Virus P5 Promoter Contains an Adenovirus ElA- Inducible Element and a Binding Site for the Major Late Transcription Factor.

Chardon, P., Renard, C., Gaillard, C. R., & Vaiman, M. (2000). The porcine major histocompatibility complex and related paralogous regions: a review. *Genetics, Selection, Evolution : GSE*, *32*(2), 109–28. doi:10.1051/gse:2000101

Chen, C. A., & Okayama, H. (n.d.). Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *BioTechniques*, *6*(7), 632–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3273409

Cheung, C. Y., Poon, L. L. M., Lau, A. S., Luk, W., Lau, Y. L., Shortridge, K. F., … Peiris, J. S. M. (2002). Mechanisms of disease Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses : a mechanism for the unusual severity of human disease ? GLOSSARY, *360*, 1831–1837.

Chiriva-Internati, M., Liu, Y., Weidanz, J. a, Grizzi, F., You, H., Zhou, W., … Hermonat, P. L. (2003). Testing recombinant adeno-associated virus-gene loading of dendritic cells for generating potent cytotoxic T lymphocytes against a prototype self-antigen, multiple myeloma HM1.24. *Blood*, *102*(9), 3100–7. doi:10.1182/blood-2002-11-3580

Choi, V. W., Asokan, A., Haberman, R. a, & Samulski, R. J. (2007). Production of recombinant adeno-associated viral vectors for in vitro and in vivo use. *Current Protocols in Molecular Biology / Edited by Frederick M. Ausubel ... [et Al.]*, *Chapter 16*, Unit 16.25. doi:10.1002/0471142727.mb1625s78

Chotpitayasunondh, T., Ungchusak, K., Chunsuthiwat, S., Srisan, P., Suwan, P., Kanjanawasri, S., … Chokephaibulkit, K. (2005). Human Disease from Influenza A, *11*(2), 201–209.

Clarke, J. K., McFerran, J. B., McKillop, E. R., & Curran, W. L. (1979). Isolation of an adeno associated virus from sheep. Brief report. *Archives of Virology*, *60*(2), 171–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/226038

Clynes, R. a, Towers, T. L., Presta, L. G., & Ravetch, J. V. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine*, *6*(4), 443–6. doi:10.1038/74704

Connolly, B. M., Steele, K. E., Davis, K. J., Geisbert, T. W., Kell, W. M., Jaax, N. K., & Jahrling, P. B. (1999). Pathogenesis of experimental Ebola virus infection in guinea pigs. *The Journal of Infectious Diseases*, *179 Suppl* , S203–17. doi:10.1086/514305

Coria, M. F., & Lehmkuhl, H. D. (1978). Isolation and identification of a bovine adenovirus type 3 with an adenovirus-associated virus. *American Journal of Veterinary Research*, *39*(12), 1904–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/219733

Dalkara, D., Byrne, L. C., Klimczak, R. R., Visel, M., Yin, L., Merigan, W. H., … Schaffer, D. V. (2013). In vivo-directed evolution of a new adeno-associated virus for therapeutic outer retinal gene delivery from the vitreous. *Science Translational Medicine*, *5*(189), 189ra76. doi:10.1126/scitranslmed.3005708

Daya, S., & Berns, K. I. (2008). Gene therapy using adeno-associated virus vectors. *Clinical Microbiology Reviews*, *21*(4), 583–93. doi:10.1128/CMR.00008-08

De Jong, M. D., Simmons, C. P., Thanh, T. T., Hien, V. M., Smith, G. J. D., Chau, T. N. B., … Farrar, J. (2006). Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nature Medicine*, *12*(10), 1203–7. doi:10.1038/nm1477

Defang, G. N., Martin, N. J., Burgess, T. H., Millar, E. V, Pecenka, L. a, Danko, J. R., … Luke, T. C. (2012). Comparative analysis of hemagglutination inhibition titers generated using temporally matched serum and plasma samples. *PloS One*, *7*(12), e48229. doi:10.1371/journal.pone.0048229

Di Pasquale, G., Davidson, B. L., Stein, C. S., Martins, I., Scudiero, D., Monks, A., & Chiorini, J. a. (2003). Identification of PDGFR as a receptor for AAV-5 transduction. *Nature Medicine*, *9*(10), 1306–12. doi:10.1038/nm929

DiPrimio, N., Asokan, A., Govindasamy, L., Agbandje-McKenna, M., & Samulski, R. J. (2008). Surface loop dynamics in adeno-associated virus capsid assembly. *Journal of Virology*, *82*(11), 5178–89. doi:10.1128/JVI.02721-07

Dobrzynski, E., Fitzgerald, J. C., Cao, O., Mingozzi, F., Wang, L., & Herzog, R. W. (2006). Prevention of cytotoxic T lymphocyte responses to factor IX-expressing hepatocytes by gene transfer-induced regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(12), 4592–7. doi:10.1073/pnas.0508685103

Duffy, S., Shackelton, L. a, & Holmes, E. C. (2008). Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews. Genetics*, *9*(4), 267–76. doi:10.1038/nrg2323

Ehrlich, H. J., Müller, M., Oh, H. M. L., Tambyah, P. a, Joukhadar, C., Montomoli, E., … Barrett, P. N. (2008). A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *The New England Journal of Medicine*, *358*(24), 2573–84. doi:10.1056/NEJMoa073121

Ekiert, D. C., & Wilson, I. A. (2012). Broadly neutralizing antibodies against influenza virus and prospects for universal therapies. *Current Opinion in Virology*, *2*(2), 134–41. doi:10.1016/j.coviro.2012.02.005

Ellis, B. L., Hirsch, M. L., Barker, J. C., Connelly, J. P., Steininger, R. J., & Porteus, M. H. (2013). A survey of ex vivo/in vitro transduction efficiency of mammalian primary cells and cell lines with Nine natural adeno-associated virus (AAV1-9) and one engineered adeno-associated virus serotype. *Virology Journal*, *10*(1), 74. doi:10.1186/1743-422X-10-74

Farkas, S. L. (2004). A parvovirus isolated from royal python (Python regius) is a member of the genus Dependovirus. *Journal of General Virology*, *85*(3), 555–561. doi:10.1099/vir.0.19616-0

Farr, G. A., Zhang, L., & Tattersall, P. (2005). Parvoviral virions deploy a capsid-tethered lipolytic enzyme to breach the endosomal membrane during cell entry. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(47), 17148–53. doi:10.1073/pnas.0508477102

Faust, S. M., Bell, P., Cutler, B. J., Ashley, S. N., Zhu, Y., Rabinowitz, J. E., & Wilson, J. M. (2013). CpG-depleted adeno-associated virus vectors evade immune detection, *123*(7), 1–8. doi:10.1172/JCI68205.2994

Feldmann, H., Bugany, H., Mahner, F., Klenk, H. D., Drenckhahn, D., & Schnittler, H. J. (1996). Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. *Journal of Virology*, *70*(4), 2208–14. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=190060&tool=pmcentrez&rendertype=abstract

Feldmann, H., & Geisbert, T. W. (2011). Ebola haemorrhagic fever. *Lancet*, *377*(9768), 849–62. doi:10.1016/S0140-6736(10)60667-8

Fisher, K. J., Gao, G. P., Weitzman, M. D., DeMatteo, R., Burda, J. F., & Wilson, J. M. (1996). Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *Journal of Virology*, *70*(1), 520–32. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=189840&tool=pmcentrez&rendertype=abstract

Flatz, L., Rieger, T., Merkler, D., Bergthaler, A., Regen, T., Schedensack, M., … Pinschewer, D. D. (2010). T cell-dependence of Lassa fever pathogenesis. *PLoS Pathogens*, *6*(3), e1000836. doi:10.1371/journal.ppat.1000836

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Curtis, M., & Kaspar, B. K. (2010). NIH Public Access, *27*(1), 59–65. doi:10.1038/nbt.1515.Intravascular

Gao, G., Alvira, M. R., Somanathan, S., Lu, Y., Vandenberghe, L. H., Rux, J. J., … Wilson, J. M. (2003). Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(10), 6081–6. doi:10.1073/pnas.0937739100

Gao, G., Vandenberghe, L. H., Alvira, M. R., Lu, Y., Calcedo, R., Zhou, X., … Wilson, J. M. (2004a). Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues. doi:10.1128/JVI.78.12.6381

Gao, G., Vandenberghe, L. H., Alvira, M. R., Lu, Y., Calcedo, R., Zhou, X., … Wilson, J. M. (2004b). Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues. doi:10.1128/JVI.78.12.6381

Gao, G., Wang, Q., Calcedo, R., Mays, L., Bell, P., Wang, L., … Wilson, J. M. (2009). Adeno-associated virus-mediated gene transfer to non-human primate liver can elicit destructive transgene-specific T cell responses. *Human Gene Therapy*, *20*(9), 930–42. doi:10.1089/hum.2009.060

Gao, G.-P., Alvira, M. R., Wang, L., Calcedo, R., Johnston, J., & Wilson, J. M. (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(18), 11854–9. doi:10.1073/pnas.182412299

Garbutt, M., Liebscher, R., Wahl-jensen, V., Jones, S., Wagner, R., Volchkov, V., … Stro, U. (2004). Properties of Replication-Competent Vesicular Stomatitis Virus Vectors Expressing Glycoproteins of Filoviruses and Arenaviruses, *78*(10), 5458–5465. doi:10.1128/JVI.78.10.5458

Geisbert, T. W., Hensley, L. E., Larsen, T., Young, H. a, Reed, D. S., Geisbert, J. B., … Davis, K. J. (2003). Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. *The American Journal of Pathology*, *163*(6), 2347–70. doi:10.1016/S0002-9440(10)63591-2

Geisbert, T. W., & Jahrling, P. B. (1990). Use of immunoelectron microscopy to show Ebola virus during the 1989 United States epizootic. *Journal of Clinical Pathology*, *43*(10), 813–6. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=502829&tool=pmcentrez&rendertype=abstract

Geoffroy, M., Epstein, A. L., Moullier, P., Salvetti, A., Toublanc, E., & Bernard, C. (2004). Herpes Simplex Virus Type 1 ICP0 Protein Mediates Activation of Adeno-Associated Virus Type 2 rep Gene Expression from a Latent Integrated Form Herpes Simplex Virus Type 1 ICP0 Protein Mediates Activation of Adeno-Associated Virus Type 2 rep Gene Expressi. doi:10.1128/JVI.78.20.10977

Georg-Fries, B., Biederlack, S., Wolf, J., & zur Hausen, H. (1984). Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology*, *134*(1), 64–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6200995

Grieger, J. C., Johnson, J. S., Gurda-Whitaker, B., Agbandje-McKenna, M., & Samulski, R. J. (2007). Surface-exposed adeno-associated virus Vp1-NLS capsid fusion protein rescues infectivity of noninfectious wild-type Vp2/Vp3 and Vp3-only capsids but not that of fivefold pore mutant virions. *Journal of Virology*, *81*(15), 7833–43. doi:10.1128/JVI.00580-07

Grieger, J. C., Snowdy, S., & Samulski, R. J. (2006). Separate basic region motifs within the adeno-associated virus capsid proteins are essential for infectivity and assembly. *Journal of Virology*, *80*(11), 5199–210. doi:10.1128/JVI.02723-05

Grimm, D., Lee, J. S., Wang, L., Desai, T., Akache, B., Storm, T. a, & Kay, M. a. (2008). In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. *Journal of Virology*, *82*(12), 5887–911. doi:10.1128/JVI.00254-08

Guo, P., El-Gohary, Y., Prasadan, K., Shiota, C., Xiao, X., Wiersch, J., … Gittes, G. K. (2012). Rapid and simplified purification of recombinant adeno-associated virus. *Journal of Virological Methods*, *183*(2), 139–46. doi:10.1016/j.jviromet.2012.04.004

Haberman, R. P., Mccown, T. J., Samulski, J., & Cown, T. J. M. C. (2000). Novel Transcriptional Regulatory Signals in the Adeno-Associated Virus Terminal Repeat A / D Junction Element Novel Transcriptional Regulatory Signals in the Adeno-Associated Virus Terminal Repeat A / D Junction Element. doi:10.1128/JVI.74.18.8732-8739.2000.Updated

Halbert, C. L., Miller, A. D., McNamara, S., Emerson, J., Gibson, R. L., Ramsey, B., & Aitken, M. L. (2006). Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors. *Human Gene Therapy*, *17*(4), 440–7. doi:10.1089/hum.2006.17.440

Hermida-Prieto, M., Domenech, N., Moscoso, I., Diaz, T., Ishii, J., Salomon, D. R., & Mañez, R. (2007). Lack of cross-species transmission of porcine endogenous retrovirus (PERV) to transplant recipients and abattoir workers in contact with pigs. *Transplantation*, *84*(4), 548–50. doi:10.1097/01.tp.0000275203.91841.23

Herring, C., Cunningham, D. A., Whittam, A. J., Fernández-Suárez, X. M., & Langford, G. A. (2001). Monitoring xenotransplant recipients for infection by PERV. *Clinical Biochemistry*, *34*(1), 23–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11239511

Herzog, R. W., Hagstrom, J. N., Kung, S. H., Tai, S. J., Wilson, J. M., Fisher, K. J., & High, K. A. (1997). Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(11), 5804–9. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=20861&tool=pmcentrez&rendertype=abstract

Hirsch, M. L., Storici, F., Li, C., Choi, V. W., & Samulski, R. J. (2009). AAV recombineering with single strand oligonucleotides. *PloS One*, *4*(11), e7705. doi:10.1371/journal.pone.0007705

Hobson, D., Curry, R. L., Beare, a S., & Ward-Gardner, A. (1972). The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *The Journal of Hygiene*, *70*(4), 767–77. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2130285&tool=pmcentrez&rendertype=abstract

Hölscher, C., Kleinschmidt, J. A., Bürkle, A., & Bu, A. (1995). High-level expression of adeno-associated virus (AAV) Rep78 or Rep68 protein is sufficient for infectious-particle formation by a rep-negative AAV mutant . High-Level Expression of Adeno-Associated Virus (AAV) Rep78 or Rep68 Protein Is Sufficient for .

Hoque, M., Ishizu, K., Matsumoto, a, Han, S. I., Arisaka, F., Takayama, M., … Handa, H. (1999). Nuclear transport of the major capsid protein is essential for adeno-associated virus capsid formation. *Journal of Virology*, *73*(9), 7912–5. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=104328&tool=pmcentrez&rendertype=abstract

Hua, Z., & Hou, B. (2013). TLR signaling in B-cell development and activation. *Cellular & Molecular Immunology*, *10*(2), 103–6. doi:10.1038/cmi.2012.61

Hüser, D., Gogol-Döring, A., Lutter, T., Weger, S., Winter, K., Hammer, E.-M., … Heilbronn, R. (2010). Integration preferences of wildtype AAV-2 for consensus rep-

binding sites at numerous loci in the human genome. *PLoS Pathogens*, *6*(7), e1000985. doi:10.1371/journal.ppat.1000985

Imai, Y., Kuba, K., Neely, G. G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., … Penninger, J. M. (2008). Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*, *133*(2), 235–49. doi:10.1016/j.cell.2008.02.043

Inagaki, K., Fuess, S., Storm, T. a, Gibson, G. a, Mctiernan, C. F., Kay, M. a, & Nakai, H. (2006). Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *14*(1), 45–53. doi:10.1016/j.ymthe.2006.03.014

Jay, F. T., Laughlin, C. a, & Carter, B. J. (1981). Eukaryotic translational control: adeno-associated virus protein synthesis is affected by a mutation in the adenovirus DNA-binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, *78*(5), 2927–31. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=319472&tool=pmcentrez&rendertype=abstract

Jiang, H., Pierce, G. F., Ozelo, M. C., de Paula, E. V, Vargas, J. a, Smith, P., … Arruda, V. R. (2006). Evidence of multiyear factor IX expression by AAV-mediated gene transfer to skeletal muscle in an individual with severe hemophilia B. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *14*(3), 452–5. doi:10.1016/j.ymthe.2006.05.004

Jiang, W., Lederman, M. M., Harding, C. V, Rodriguez, B., Mohner, R. J., & Sieg, S. F. (2007). TLR9 stimulation drives naïve B cells to proliferate and to attain enhanced antigen presenting function. *European Journal of Immunology*, *37*(8), 2205–13. doi:10.1002/eji.200636984

Johnson, P. R., Schnepp, B. C., Connell, M. J., Rohne, D., Robinson, S., Krivulka, G. R., … Clark, K. R. (2005). Novel Adeno-Associated Virus Vector Vaccine Restricts Replication of Simian Immunodeficiency Virus in Macaques, *79*(2), 955–965. doi:10.1128/JVI.79.2.955

Jooss, K., & Chirmule, N. (2003). Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy. *Gene Therapy*, *10*(11), 955–63. doi:10.1038/sj.gt.3302037

Kaludov, N., Brown, K. E., Walters, R. W., Zabner, J., & Chiorini, J. A. (2001). Adeno-Associated Virus Serotype 4 (AAV4) and AAV5 Both Require Sialic Acid Binding for Hemagglutination and Efficient Transduction but Differ in Sialic Acid Linkage Specificity, *75*(15), 6884–6893. doi:10.1128/JVI.75.15.6884

Kashiwakura, Y., Tamayose, K., Iwabuchi, K., Hirai, Y., Shimada, T., Matsumoto, K., … Oshimi, K. (2005). Hepatocyte Growth Factor Receptor Is a Coreceptor for Adeno-Associated Virus Type 2 Infection, *79*(1), 609–614. doi:10.1128/JVI.79.1.609

Kelkar, S., De, B. P., Gao, G., Wilson, J. M., Crystal, R. G., & Leopold, P. L. (2006). A common mechanism for cytoplasmic dynein-dependent microtubule binding shared among adeno-associated virus and adenovirus serotypes. *Journal of Virology*, *80*(15), 7781–5. doi:10.1128/JVI.00481-06

Kelly, M. E., Zhuo, J., Bharadwaj, A. S., & Chao, H. (2009). Induction of immune tolerance to FIX following muscular AAV gene transfer is AAV-dose/FIX-level dependent. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *17*(5), 857–63. doi:10.1038/mt.2009.25

Kempka, G., & Kolb-Bachofen, V. (1985). Galactose-specific receptors on liver cells. I. Hepatocyte and liver macrophage receptors differ in their membrane anchorage. *Biochimica et Biophysica Acta*, *847*(1), 108–14. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2996612

Kessel, A., Haj, T., Peri, R., Snir, A., Melamed, D., Sabo, E., & Toubi, E. (2012). Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmunity Reviews*, *11*(9), 670–7. doi:10.1016/j.autrev.2011.11.018

Kobinger, G. P., Leung, A., Neufeld, J., Richardson, J. S., Falzarano, D., Smith, G., … Weingartl, H. M. (2011). Replication, pathogenicity, shedding, and transmission of Zaire ebolavirus in pigs. *The Journal of Infectious Diseases*, *204*(2), 200–8. doi:10.1093/infdis/jir077

Kodihalli, S., Goto, H., Kobasa, D. L., Kawaoka, Y., & Webster, R. G. (1999). DNA Vaccine Encoding Hemagglutinin Provides Protective Immunity against H5N1 Influenza Virus Infection in Mice DNA Vaccine Encoding Hemagglutinin Provides Protective Immunity against H5N1 Influenza Virus Infection in Mice.

Kodihalli, S., Goto, H., Kobasa, D. L., Krauss, S., Kawaoka, Y., & Webster, R. G. (1999). DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. *Journal of Virology*, *73*(3), 2094–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=104453&tool=pmcentrez&rendertype=abstract

Kotin, R. M., Linden, R. M., & Berns, K. I. (1992). Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *The EMBO Journal*, *11*(13), 5071–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=556985&tool=pmcentrez&rendertype=abstract

Kramp, W. J., Six, H. R., Drake, S., & Kasel, J. a. (1979). Liposomal enhancement of the immunogenicity of adenovirus type 5 hexon and fiber vaccines. *Infection and Immunity*, *25*(2), 771–3. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=414511&tool=pmcentrez&rendertype=abstract

Ku, A. S., & Chan, L. T. (1999). The first case of H5N1 avian influenza infection in a human with complications of adult respiratory distress syndrome and Reye's syndrome. *Journal of Paediatrics and Child Health*, *35*(2), 207–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10365363

Kulkarni, D. D., Tosh, C., Venkatesh, G., & Senthil Kumar, D. (2013). Nipah virus infection: current scenario. *Indian Journal of Virology : An Official Organ of Indian Virological Society*, *24*(3), 398–408. doi:10.1007/s13337-013-0171-y

Kurzeder, C., Koppold, B., Sauer, G., Pabst, S., Kreienberg, R., & Deissler, H. (2007). CD9 promotes adeno-associated virus type 2 infection of mammary carcinoma cells with low cell surface expression of heparan sulphate proteoglycans. *International Journal of Molecular Medicine*, *19*(2), 325–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17203208

Kyöstiö, S. R., Wonderling, R. S., & Owens, R. a. (1995). Negative regulation of the adeno-associated virus (AAV) P5 promoter involves both the P5 rep binding site and the consensus ATP-binding motif of the AAV Rep68 protein. *Journal of Virology*, *69*(11), 6787–96. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=189590&tool=pmcentrez&rendertype=abstract

Labow, M. a, Hermonat, P. L., & Berns, K. I. (1986). Positive and negative autoregulation of the adeno-associated virus type 2 genome. *Journal of Virology*, *60*(1), 251–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=253923&tool=pmcentrez&rendertype=abstract

Langley, J. M., Risi, G., Caldwell, M., Gilderman, L., Berwald, B., Fogarty, C., … Fries, L. (2011). Dose-sparing H5N1 A/Indonesia/05/2005 pre-pandemic influenza vaccine in adults and elderly adults: a phase III, placebo-controlled, randomized study. *The Journal of Infectious Diseases*, *203*(12), 1729–38. doi:10.1093/infdis/jir172

Le, M. T. Q., Wertheim, H. F. L., Nguyen, H. D., Taylor, W., Hoang, P. V. M., Vuong, C. D., … Horby, P. (2008). Influenza A H5N1 clade 2.3.4 virus with a different antiviral susceptibility profile replaced clade 1 virus in humans in northern Vietnam. *PloS One*, *3*(10), e3339. doi:10.1371/journal.pone.0003339

Li, C., Goudy, K., Hirsch, M., Asokan, A., Fan, Y., Alexander, J., … Samulski, R. J. (2009). Cellular immune response to cryptic epitopes during therapeutic gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(26), 10770–4. doi:10.1073/pnas.0902269106

Limberis, M. P., Adam, V. S., Wong, G., Gren, J., Kobasa, D., Ross, T. M., … Wilson, J. M. (2013). Intranasal antibody gene transfer in mice and ferrets elicits broad protection against pandemic influenza. *Science Translational Medicine*, *5*(187), 187ra72. doi:10.1126/scitranslmed.3006299

Limberis, M. P., Vandenberghe, L. H., Zhang, L., Pickles, R. J., & Wilson, J. M. (2009). Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *17*(2), 294–301. doi:10.1038/mt.2008.261

Lin, J., Calcedo, R., Vandenberghe, L. H., Bell, P., Somanathan, S., & Wilson, J. M. (2009). A new genetic vaccine platform based on an adeno-associated virus isolated from a rhesus macaque. *Journal of Virology*, *83*(24), 12738–50. doi:10.1128/JVI.01441-09

Lin, J., Zhi, Y., Mays, L., & Wilson, J. M. (2007a). Vaccines based on novel adeno-associated virus vectors elicit aberrant CD8+ T-cell responses in mice. *Journal of Virology*, *81*(21), 11840–9. doi:10.1128/JVI.01253-07

Lin, J., Zhi, Y., Mays, L., & Wilson, J. M. (2007b). Vaccines based on novel adeno-associated virus vectors elicit aberrant CD8+ T-cell responses in mice. *Journal of Virology*, *81*(21), 11840–9. doi:10.1128/JVI.01253-07

Linden, R. M., Winocour, E., & Berns, K. I. (1996). The recombination signals for adeno-associated virus site-specific integration. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(15), 7966–72. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=38858&tool=pmcentrez&rendertype=abstract

Liu, D. W., Tsao, Y. P., Kung, J. T., Ding, Y. a, Sytwu, H. K., Xiao, X., & Chen, S. L. (2000). Recombinant adeno-associated virus expressing human papillomavirus type 16 E7 peptide DNA fused with heat shock protein DNA as a potential vaccine for cervical cancer. *Journal of Virology*, *74*(6), 2888–94. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=111780&tool=pmcentrez&rendertype=abstract

Liu, Y., Santin, A. D., Mane, M., Chiriva-Internati, M., Parham, G. P., Ravaggi, A., & Hermonat, P. L. (2000). Transduction and utility of the granulocyte-macrophage colony-stimulating factor gene into monocytes and dendritic cells by adeno-associated virus. *Journal of Interferon & Cytokine Research : The Official Journal*

of the International Society for Interferon and Cytokine Research, *20*(1), 21–30. doi:10.1089/107999000312702

Lochrie, M. a, Tatsuno, G. P., Arbetman, A. E., Jones, K., Pater, C., Smith, P. H., … Colosi, P. (2006). Adeno-associated virus (AAV) capsid genes isolated from rat and mouse liver genomic DNA define two new AAV species distantly related to AAV-5. *Virology*, *353*(1), 68–82. doi:10.1016/j.virol.2006.05.023

Louboutin, J.-P., Wang, L., & Wilson, J. M. (2005). Gene transfer into skeletal muscle using novel AAV serotypes. *The Journal of Gene Medicine*, *7*(4), 442–51. doi:10.1002/jgm.686

Lu, Y., & Song, S. (2009). Distinct immune responses to transgene products from rAAV1 and rAAV8 vectors. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(40), 17158–62. doi:10.1073/pnas.0909520106

Luciani, G. B., Viscardi, F., Cresce, G. D., Faggian, G., & Mazzucco, A. (2007). Seven-year performance of the Edwards Prima Plus stentless valve with the intact non-coronary sinus technique. *Journal of Cardiac Surgery*, *23*(3), 221–6. doi:10.1111/j.1540-8191.2008.00592.x

Lyall, J., Irvine, R. M., Sherman, A., McKinley, T. J., Núñez, A., Purdie, A., … Tiley, L. (2011). Suppression of avian influenza transmission in genetically modified chickens. *Science (New York, N.Y.)*, *331*(6014), 223–6. doi:10.1126/science.1198020

Madsen, D., Cantwell, E. R., O'Brien, T., Johnson, P. a, & Mahon, B. P. (2009). Adeno-associated virus serotype 2 induces cell-mediated immune responses directed against multiple epitopes of the capsid protein VP1. *The Journal of General Virology*, *90*(Pt 11), 2622–33. doi:10.1099/vir.0.014175-0

Mahanty, S., Hutchinson, K., Agarwal, S., McRae, M., Rollin, P. E., & Pulendran, B. (2003). Cutting edge: impairment of dendritic cells and adaptive immunity by Ebola and Lassa viruses. *Journal of Immunology (Baltimore, Md. : 1950)*, *170*(6), 2797–801. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12626527

Manning, W. C., Paliard, X., Zhou, S., Pat Bland, M., Lee, a Y., Hong, K., … Dwarki, V. (1997). Genetic immunization with adeno-associated virus vectors expressing herpes simplex virus type 2 glycoproteins B and D. *Journal of Virology*, *71*(10), 7960–2. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=192154&tool=pmcentrez&rendertype=abstract

Manno, C. S., Pierce, G. F., Arruda, V. R., Glader, B., Ragni, M., Rasko, J. J., … Kay, M. a. (2006). Successful transduction of liver in hemophilia by AAV-Factor IX and

limitations imposed by the host immune response. *Nature Medicine*, *12*(3), 342–7. doi:10.1038/nm1358

Martino, A. T., Nayak, S., Hoffman, B. E., Cooper, M., Liao, G., Markusic, D. M., … Herzog, R. W. (2009). Tolerance induction to cytoplasmic beta-galactosidase by hepatic AAV gene transfer: implications for antigen presentation and immunotoxicity. *PloS One*, *4*(8), e6376. doi:10.1371/journal.pone.0006376

Mayor, H. D., Drake, S., Stahmann, J., & Mumford, D. M. (1976). Antibodies to adeno-associated satellite virus and herpes simplex in sera from cancer patients and normal adults. *American Journal of Obstetrics and Gynecology*, *126*(1), 100–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/183503

Mays, L. E., Vandenberghe, L. H., Xiao, R., Bell, P., Nam, H.-J., Agbandje-McKenna, M., & Wilson, J. M. (2009). Adeno-associated virus capsid structure drives CD4-dependent CD8+ T cell response to vector encoded proteins. *Journal of Immunology (Baltimore, Md. : 1950)*, *182*(10), 6051–60. doi:10.4049/jimmunol.0803965

Mays, L. E., Wang, L., Lin, J., Bell, P., Crawford, A., Wherry, E. J., & Wilson, J. M. (2014). AAV8 Induces Tolerance in Murine Muscle as a Result of Poor APC Transduction, T Cell Exhaustion, and Minimal MHCI Upregulation on Target Cells. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *22*(1), 28–41. doi:10.1038/mt.2013.134

Mays, L. E., Wang, L., Tenney, R., Bell, P., Nam, H.-J., Lin, J., … Wilson, J. M. (2013). Mapping the structural determinants responsible for enhanced T cell activation to the immunogenic adeno-associated virus capsid from isolate rhesus 32.33. *Journal of Virology*, *87*(17), 9473–85. doi:10.1128/JVI.00596-13

Mbawuike, I., Zang, Y., & Couch, R. B. (2007). Humoral and cell-mediated immune responses of humans to inactivated influenza vaccine with or without QS21 adjuvant. *Vaccine*, *25*(17), 3263–9. doi:10.1016/j.vaccine.2007.01.073

McCarty, D. M., Monahan, P. E., & Samulski, R. J. (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Therapy*, *8*(16), 1248–54. doi:10.1038/sj.gt.3301514

Mccullers, J. A., & Huber, V. C. (2012). and its complications © 2012 Landes Bioscience . Do not distribute . © 2012 Landes Bioscience . Do not distribute ., *8*(1), 34–44.

Mclaughlin, S. K., Collis, P., & Hermonat, P. L. (1988). vectors : analysis of proviral structures . Adeno-Associated Virus General Transduction Vectors : Analysis of Proviral Structures, *62*(6).

Mehendale, S., van Lunzen, J., Clumeck, N., Rockstroh, J., Vets, E., Johnson, P. R., … Heald, A. E. (2008). A phase 1 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 subtype C adeno-associated virus vaccine. *AIDS Research and Human Retroviruses*, *24*(6), 873–80. doi:10.1089/aid.2008.0292

Mingozzi, F., & High, K. a. (2013). Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood*. doi:10.1182/blood-2013-01-306647

Mingozzi, F., Maus, M. V, Hui, D. J., Sabatino, D. E., Murphy, S. L., Rasko, J. E. J., … High, K. a. (2007). CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nature Medicine*, *13*(4), 419–22. doi:10.1038/nm1549

Mingozzi, F., Meulenberg, J. J., Hui, D. J., Basner-Tschakarjan, E., Hasbrouck, N. C., Edmonson, S. a, … High, K. a. (2009). AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. *Blood*, *114*(10), 2077–86. doi:10.1182/blood-2008-07-167510

Mohamadzadeh, M., Chen, L., & Schmaljohn, A. L. (2007). How Ebola and Marburg viruses battle the immune system. *Nature Reviews. Immunology*, *7*(7), 556–67. doi:10.1038/nri2098

Molinier-frenkel, V., Lengagne, R., Gaden, F., Hong, S., Choppin, J., Gahery-ségard, H., … Gahery-se, H. (2002). Adenovirus Hexon Protein Is a Potent Adjuvant for Activation of a Cellular Immune Response Adenovirus Hexon Protein Is a Potent Adjuvant for Activation of a Cellular Immune Response. doi:10.1128/JVI.76.1.127

Morelli, A. E., Larregina, A. T., Raymond, W., Zahorchak, A. F., Plowey, J. M., Logar, A. J., … Ganster, R. W. (2000). Recombinant Adenovirus Induces Maturation of Dendritic Cells via an NF- κ B-Dependent Pathway Recombinant Adenovirus Induces Maturation of Dendritic Cells via an NF- B-Dependent Pathway. doi:10.1128/JVI.74.20.9617-9628.2000.Updated

Mori, S., Wang, L., Takeuchi, T., & Kanda, T. (2004). Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology*, *330*(2), 375–83. doi:10.1016/j.virol.2004.10.012

Mouw, M. B., & Pintel, D. J. (2000). Adeno-associated virus RNAs appear in a temporal order and their splicing is stimulated during coinfection with adenovirus. *Journal of Virology*, *74*(21), 9878–88. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=102024&tool=pmcentrez&rendertype=abstract

Mühlberger, E., Weik, M., Volchkov, V. E., Klenk, H. D., & Becker, S. (1999). Comparison of the transcription and replication strategies of marburg virus and Ebola virus by using artificial replication systems. *Journal of Virology*, *73*(3), 2333–42. Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=104478&tool=pmcentrez&rendertype=abstract

Müller, O. J., Kaul, F., Weitzman, M. D., Pasqualini, R., Arap, W., Kleinschmidt, J. a, & Trepel, M. (2003). Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nature Biotechnology*, *21*(9), 1040–6. doi:10.1038/nbt856

Myrup, A. C., Mohanty, S. B., & Hetrick, F. M. (1976). Isolation and characterization of adeno-associated viruses from bovine adenovirus types 1 and 2. *American Journal of Veterinary Research*, *37*(8), 907–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/986122

Nakai, H., Fuess, S., Storm, T. A., Muramatsu, S., Nara, Y., & Kay, M. A. (2005). Unrestricted Hepatocyte Transduction with Adeno-Associated Virus Serotype 8 Vectors in Mice, *79*(1), 214–224. doi:10.1128/JVI.79.1.214

Nash, K., Chen, W., & Muzyczka, N. (2008). Complete in vitro reconstitution of adeno-associated virus DNA replication requires the minichromosome maintenance complex proteins. *Journal of Virology*, *82*(3), 1458–64. doi:10.1128/JVI.01968-07

Nathwani, A. C., Tuddenham, E. G. D., Rangarajan, S., Rosales, C., McIntosh, J., Linch, D. C., … Davidoff, A. M. (2011). Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *The New England Journal of Medicine*, *365*(25), 2357–65. doi:10.1056/NEJMoa1108046

Nayak, R., & Pintel, D. J. (2007). Adeno-associated viruses can induce phosphorylation of eIF2alpha via PKR activation, which can be overcome by helper adenovirus type 5 virus-associated RNA. *Journal of Virology*, *81*(21), 11908–16. doi:10.1128/JVI.01132-07

Nelli, R. K., Kuchipudi, S. V, White, G. A., Perez, B. B., Dunham, S. P., & Chang, K.-C. (2010). Comparative distribution of human and avian type sialic acid influenza receptors in the pig. *BMC Veterinary Research*, *6*, 4. doi:10.1186/1746-6148-6-4

Neumann, G., Chen, H., Gao, G. F., Shu, Y., & Kawaoka, Y. (2010). H5N1 influenza viruses: outbreaks and biological properties. *Cell Research*, *20*(1), 51–61. doi:10.1038/cr.2009.124

Noda, T., Sagara, H., Suzuki, E., Takada, A., Kida, H., & Kawaoka, Y. (2002). Ebola Virus VP40 Drives the Formation of Virus-Like Filamentous Particles Along with GP, *76*(10), 4855–4865. doi:10.1128/JVI.76.10.4855

Olson, E. J., Haskell, S. R. R., Frank, R. K., Lehmkuhl, H. D., Hobbs, L. a., Warg, J. V., … Wunschmann, a. (2004). Isolation of an Adenovirus and an Adeno-Associated

Virus from Goat Kids with Enteritis. *Journal of Veterinary Diagnostic Investigation*, *16*(5), 461–464. doi:10.1177/104063870401600518

Papadakis, E. D., Nicklin, S. a, Baker, a H., & White, S. J. (2004). Promoters and control elements: designing expression cassettes for gene therapy. *Current Gene Therapy*, *4*(1), 89–113. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15032617

Patel, A., Tikoo, S., & Kobinger, G. (2010). A porcine adenovirus with low human seroprevalence is a promising alternative vaccine vector to human adenovirus 5 in an H5N1 virus disease model. *PloS One*, *5*(12), e15301. doi:10.1371/journal.pone.0015301

Patel, S. M., Atmar, R. L., El Sahly, H. M., Cate, T. R., & Keitel, W. a. (2010). A phase I evaluation of inactivated influenza A/H5N1 vaccine administered by the intradermal or the intramuscular route. *Vaccine*, *28*(17), 3025–9. doi:10.1016/j.vaccine.2009.10.152

Pereira, D. J., McCarty, D. M., & Muzyczka, N. (1997). The adeno-associated virus (AAV) Rep protein acts as both a repressor and an activator to regulate AAV transcription during a productive infection. *Journal of Virology*, *71*(2), 1079–88. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=191159&tool=pmcentrez&rendertype=abstract

Peterson, a T., Bauer, J. T., & Mills, J. N. (2004). Ecologic and geographic distribution of filovirus disease. *Emerging Infectious Diseases*, *10*(1), 40–7. doi:10.3201/eid1001.030125

Peterson, a T., Lash, R. R., Carroll, D. S., & Johnson, K. M. (2006). Geographic potential for outbreaks of Marburg hemorrhagic fever. *The American Journal of Tropical Medicine and Hygiene*, *75*(1), 9–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16837700

Ploquin, A., Szécsi, J., Mathieu, C., Guillaume, V., Barateau, V., Ong, K. C., … Salvetti, A. (2013). Protection against henipavirus infection by use of recombinant adeno-associated virus-vector vaccines. *The Journal of Infectious Diseases*, *207*(3), 469–78. doi:10.1093/infdis/jis699

Ponnazhagan, S., Mahendra, G., Curiel, D. T., & Shaw, D. R. (2001). Adeno-associated virus type 2-mediated transduction of human monocyte-derived dendritic cells: implications for ex vivo immunotherapy. *Journal of Virology*, *75*(19), 9493–501. doi:10.1128/JVI.75.19.9493-9501.2001

Puppo, A., Bello, A., Manfredi, A., Cesi, G., Marrocco, E., Della Corte, M., … Auricchio, A. (2013). Recombinant vectors based on porcine adeno-associated viral

serotypes transduce the murine and pig retina. *PloS One*, *8*(3), e59025. doi:10.1371/journal.pone.0059025

Pushko, P., Bray, M., Ludwig, G. V, Parker, M., Schmaljohn, a, Sanchez, a, … Smith, J. F. (2000). Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. *Vaccine*, *19*(1), 142–53. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10924796

Qiu, X., Audet, J., Wong, G., Pillet, S., Bello, A., Cabral, T., … Kobinger, G. P. (2012). Successful treatment of ebola virus-infected cynomolgus macaques with monoclonal antibodies. *Science Translational Medicine*, *4*(138), 138ra81. doi:10.1126/scitranslmed.3003876

Raimondi, G., Zanoni, I., Citterio, S., Ricciardi-Castagnoli, P., & Granucci, F. (2006). Induction of peripheral T cell tolerance by antigen-presenting B cells. II. Chronic antigen presentation overrules antigen-presenting B cell activation. *Journal of Immunology (Baltimore, Md. : 1950)*, *176*(7), 4021–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16547237

Rapti, K., Louis-Jeune, V., Kohlbrenner, E., Ishikawa, K., Ladage, D., Zolotukhin, S., … Weber, T. (2012). Neutralizing antibodies against AAV serotypes 1, 2, 6, and 9 in sera of commonly used animal models. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *20*(1), 73–83. doi:10.1038/mt.2011.177

Redemann, B. E., Mendelson, E., & Carter, B. J. (1989). Adeno-associated virus rep protein synthesis during productive infection. *Journal of Virology*, *63*(2), 873–82. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=247761&tool=pmcentrez&rendertype=abstract

Reid, S. P., Leung, L. W., Hartman, A. L., Martinez, O., Shaw, M. L., Carbonnelle, C., … Basler, C. F. (2006). Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. *Journal of Virology*, *80*(11), 5156–67. doi:10.1128/JVI.02349-05

Ribeiro, S. C., Monteiro, G. a, & Prazeres, D. M. F. (2004). The role of polyadenylation signal secondary structures on the resistance of plasmid vectors to nucleases. *The Journal of Gene Medicine*, *6*(5), 565–73. doi:10.1002/jgm.536

Richardson, J. S., Yao, M. K., Tran, K. N., Croyle, M. a, Strong, J. E., Feldmann, H., & Kobinger, G. P. (2009). Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine. *PloS One*, *4*(4), e5308. doi:10.1371/journal.pone.0005308

Rodríguez-Pinto, D. (2005). B cells as antigen presenting cells. *Cellular Immunology*, *238*(2), 67–75. doi:10.1016/j.cellimm.2006.02.005

Rohde, V., Erles, K., Sattler, H. P., Derouet, H., Wullich, B., & Schlehofer, J. R. (1999). Detection of adeno-associated virus in human semen: does viral infection play a role in the pathogenesis of male infertility? *Fertility and Sterility*, *72*(5), 814–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10560983

Ryabchikova, E. I., Kolesnikova, L. V, & Luchko, S. V. (1999). An analysis of features of pathogenesis in two animal models of Ebola virus infection. *The Journal of Infectious Diseases*, *179 Suppl* (Suppl 1), S199–202. doi:10.1086/514293

Sabatino, D. E., Mingozzi, F., Hui, D. J., Chen, H., Colosi, P., Ertl, H. C. J., & High, K. a. (2005a). Identification of mouse AAV capsid-specific CD8+ T cell epitopes. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *12*(6), 1023–33. doi:10.1016/j.ymthe.2005.09.009

Sabatino, D. E., Mingozzi, F., Hui, D. J., Chen, H., Colosi, P., Ertl, H. C. J., & High, K. a. (2005b). Identification of mouse AAV capsid-specific CD8+ T cell epitopes. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *12*(6), 1023–33. doi:10.1016/j.ymthe.2005.09.009

Salegio, E. A., Samaranch, L., Kells, A. P., Mittermeyer, G., San Sebastian, W., Zhou, S., … Bankiewicz, K. S. (2013). Axonal transport of adeno-associated viral vectors is serotype-dependent. *Gene Therapy*, *20*(3), 348–52. doi:10.1038/gt.2012.27

Salomon, R., Hoffmann, E., & Webster, R. G. (2007). Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(30), 12479–81. doi:10.1073/pnas.0705289104

Schmidt, M., Katano, H., Bossis, I., & Chiorini, J. A. (2004). Cloning and Characterization of a Bovine Adeno-Associated Virus, *78*(12), 6509–6516. doi:10.1128/JVI.78.12.6509

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101–1108. doi:10.1038/nprot.2008.73

Seiler, M. P., Miller, A. D., Zabner, J., & Halbert, C. L. (2006). Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. *Human Gene Therapy*, *17*(1), 10–9. doi:10.1089/hum.2006.17.10

Shen, S., Bryant, K. D., Brown, S. M., Randell, S. H., & Asokan, A. (2011). Terminal N-linked galactose is the primary receptor for adeno-associated virus 9. *The Journal of Biological Chemistry*, *286*(15), 13532–40. doi:10.1074/jbc.M110.210922

Shen, X., Storm, T., & Kay, M. a. (2007). Characterization of the relationship of AAV capsid domain swapping to liver transduction efficiency. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *15*(11), 1955–62. doi:10.1038/sj.mt.6300293

Shishido, S., Naziruddin, B., Howard, T., & Mohanakumar, T. (1997). Recognition of porcine major histocompatibility complex class I antigens by human CD8+ cytolytic T cell clones. *Transplantation*, *64*(2), 340–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9256198

Siegert, R., Shu, H. L., Slenczka, W., Peters, D., & Müller, G. (1967). [On the etiology of an unknown human infection originating from monkeys]. *Deutsche Medizinische Wochenschrift (1946)*, *92*(51), 2341–3. doi:10.1055/s-0028-1106144

Siegl, G., Bates, R. C., Berns, K. I., Carter, B. J., Kelly, D. C., Kurstak, E., & Tattersall, P. (1985). Characteristics and taxonomy of Parvoviridae. *Intervirology*, *23*(2), 61–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3980186

Sipo, I., Knauf, M., Fechner, H., Poller, W., Planz, O., Kurth, R., & Norley, S. (2011). Vaccine protection against lethal homologous and heterologous challenge using recombinant AAV vectors expressing codon-optimized genes from pandemic swine origin influenza virus (SOIV). *Vaccine*, *29*(8), 1690–9. doi:10.1016/j.vaccine.2010.12.037

Slanina, H., Weger, S., Stow, N. D., Kuhrs, A., & Heilbronn, R. (2006). Role of the herpes simplex virus helicase-primase complex during adeno-associated virus DNA replication. *Journal of Virology*, *80*(11), 5241–50. doi:10.1128/JVI.02718-05

Smith, R. H., & Kotin, R. M. (2000). An Adeno-Associated Virus (AAV) Initiator Protein , Rep78 , Catalyzes the Cleavage and Ligation of Single-Stranded AAV ori DNA An Adeno-Associated Virus (AAV) Initiator Protein , Rep78 , Catalyzes the Cleavage and Ligation of Single-Stranded AAV ori D, *74*(7). doi:10.1128/JVI.74.7.3122-3129.2000.Updated

Smith, R. H., Yang, L., & Kotin, R. M. (2008). Chromatography-based purification of adeno-associated virus. *Methods in Molecular Biology (Clifton, N.J.)*, *434*, 37–54. doi:10.1007/978-1-60327-248-3_4

Snyder, R. O., Miao, C., Meuse, L., Tubb, J., Donahue, B. A., Lin, H. F., … Kay, M. A. (1999). Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors. *Nature Medicine*, *5*(1), 64–70. doi:10.1038/4751

Somanathan, S., Breous, E., Bell, P., & Wilson, J. M. (2010). AAV vectors avoid inflammatory signals necessary to render transduced hepatocyte targets for destructive T cells. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *18*(5), 977–82. doi:10.1038/mt.2010.40

Sonntag, F., Schmidt, K., & Kleinschmidt, J. A. (2010). A viral assembly factor promotes AAV2 capsid formation in the nucleolus. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(22), 10220–5. doi:10.1073/pnas.1001673107

Sullivan, N. J., Hensley, L., Asiedu, C., Geisbert, T. W., Stanley, D., Johnson, J., … Nabel, G. J. (2011). CD8+ cellular immunity mediates rAd5 vaccine protection against Ebola virus infection of non-human primates. *Nature Medicine*, *17*(9), 1128–31. doi:10.1038/nm.2447

Sullivan, N. J., Peterson, M., Yang, Z., Kong, W., Duckers, H., Nabel, E., & Nabel, G. J. (2005). Ebola Virus Glycoprotein Toxicity Is Mediated by a Dynamin-Dependent Protein-Trafficking Pathway, *79*(1), 547–553. doi:10.1128/JVI.79.1.547

Sullivan, N. J., Sanchez, a, Rollin, P. E., Yang, Z. Y., & Nabel, G. J. (2000). Development of a preventive vaccine for Ebola virus infection in primates. *Nature*, *408*(6812), 605–9. doi:10.1038/35046108

Summerford, C., Bartlett, J. S., & Samulski, R. J. (1999). AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nature Medicine*, *5*(1), 78–82. doi:10.1038/4768

Summerford, C., & Samulski, R. J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *Journal of Virology*, *72*(2), 1438–45. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=124624&tool=pmcentrez&rendertype=abstract

Tatsis, N., & Ertl, H. C. J. (2004). Adenoviruses as vaccine vectors. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *10*(4), 616–29. doi:10.1016/j.ymthe.2004.07.013

Teijaro, J. R., Verhoeven, D., Page, C. a, Turner, D., & Farber, D. L. (2010). Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. *Journal of Virology*, *84*(18), 9217–26. doi:10.1128/JVI.01069-10

Thacker, E., & Janke, B. (2008). Swine influenza virus: zoonotic potential and vaccination strategies for the control of avian and swine influenzas. *The Journal of Infectious Diseases*, *197 Suppl*, S19–24. doi:10.1086/524988

Thepparit, C., & Smith, D. R. (2004). Serotype-Specific Entry of Dengue Virus into Liver Cells : Identification of the 37-Kilodalton / 67-Kilodalton High-Affinity Laminin Receptor as a Dengue Virus Serotype 1 Receptor, *78*(22), 12647–12656. doi:10.1128/JVI.78.22.12647

Thomas, C. E., Storm, T. A., Huang, Z., & Kay, M. A. (2004). Rapid Uncoating of Vector Genomes Is the Key to Efficient Liver Transduction with Pseudotyped Adeno-Associated Virus Vectors, *78*(6), 3110–3122. doi:10.1128/JVI.78.6.3110

To, K. F., Chan, P. K., Chan, K. F., Lee, W. K., Lam, W. Y., Wong, K. F., … Cheng, a F. (2001). Pathology of fatal human infection associated with avian influenza A H5N1 virus. *Journal of Medical Virology*, *63*(3), 242–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11170064

Tsitoura, D. C., Yeung, V. P., DeKruyff, R. H., & Umetsu, D. T. (2002). Critical role of B cells in the development of T cell tolerance to aeroallergens. *International Immunology*, *14*(6), 659–67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12039917

Van Riel, D., Munster, V. J., de Wit, E., Rimmelzwaan, G. F., Fouchier, R. A. M., Osterhaus, A. D. M. E., & Kuiken, T. (2006). H5N1 Virus Attachment to Lower Respiratory Tract. *Science (New York, N.Y.)*, *312*(5772), 399. doi:10.1126/science.1125548

Vardas, E., Kaleebu, P., Bekker, L.-G., Hoosen, A., Chomba, E., Johnson, P. R., … Schmidt, C. (2010). A phase 2 study to evaluate the safety and immunogenicity of a recombinant HIV type 1 vaccine based on adeno-associated virus. *AIDS Research and Human Retroviruses*, *26*(8), 933–42. doi:10.1089/aid.2009.0242

Varela, I. D. (2003). Cross-Reactivity between Swine Leukocyte Antigen and Human Anti-HLA-Specific Antibodies in Sensitized Patients Awaiting Renal Transplantation. *Journal of the American Society of Nephrology*, *14*(10), 2677–2683. doi:10.1097/01.ASN.0000088723.07259.CF

Veron, P., Allo, V., Rivière, C., Bernard, J., Douar, A.-M., & Masurier, C. (2007). Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2. *Journal of Virology*, *81*(10), 5385–94. doi:10.1128/JVI.02516-06

Vihinen-Ranta, M., Yuan, W., & Parrish, C. R. (2000). Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. *Journal of Virology*, *74*(10), 4853–9. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=112008&tool=pmcentrez&rendertype=abstract

Volchkov, V. E., Blinov, V. M., & Netesov, S. V. (1992). The envelope glycoprotein of Ebola virus contains an immunosuppressive-like domain similar to oncogenic retroviruses. *FEBS Letters*, *305*(3), 181–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1299611

Wahl-jensen, V., Kurz, S. K., Hazelton, P. R., & Schnittler, H. (2005). Role of Ebola Virus Secreted Glycoproteins and Virus-Like Particles in Activation of Human Macrophages, *79*(4), 2413–2419. doi:10.1128/JVI.79.4.2413

Walz, C. M., Anisi, T. R., Schlehofer, J. R., Gissmann, L., Schneider, a, & Müller, M. (1998). Detection of infectious adeno-associated virus particles in human cervical biopsies. *Virology*, *247*(1), 97–105. doi:10.1006/viro.1998.9226

Wang, L., Wang, H., Bell, P., McCarter, R. J., He, J., Calcedo, R., … Wilson, J. M. (2010). Systematic evaluation of AAV vectors for liver directed gene transfer in murine models. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *18*(1), 118–25. doi:10.1038/mt.2009.246

Wang, X. S., Ponnazhagan, S., & Srivastava, a. (1995). Rescue and replication signals of the adeno-associated virus 2 genome. *Journal of Molecular Biology*, *250*(5), 573–80. doi:10.1006/jmbi.1995.0398

Ward, P., Falkenberg, M., Elias, P. E. R., Weitzman, M., & Linden, R. M. (2001). Rep-Dependent Initiation of Adeno-Associated Virus Type 2 DNA Replication by a Herpes Simplex Virus Type 1 Replication Complex in a Reconstituted System, *75*(21), 10250–10258. doi:10.1128/JVI.75.21.10250

Warfield, K. L., Perkins, J. G., Swenson, D. L., Deal, E. M., Bosio, C. M., Aman, M. J., … Bavari, S. (2004). Role of natural killer cells in innate protection against lethal ebola virus infection. *The Journal of Experimental Medicine*, *200*(2), 169–79. doi:10.1084/jem.20032141

Webster, R. G., & Govorkova, E. A. (2006). H5N1 influenza--continuing evolution and spread. *The New England Journal of Medicine*, *355*(21), 2174–7. doi:10.1056/NEJMp068205

Weindler, F. W., & Heilbronn, R. (1991). A subset of herpes simplex virus replication genes provides helper functions for productive adeno-associated virus replication. *Journal of Virology*, *65*(5), 2476–83. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=240602&tool=pmcentrez&rendertype=abstract

Weitzman, M. D., & Linden, R. M. (2011). Adeno-Associated Virus, *807*. doi:10.1007/978-1-61779-370-7

Wong, G., Richardson, J. S., Pillet, S., Patel, A., Qiu, X., Alimonti, J., … Kobinger, G. P. (2012a). Immune parameters correlate with protection against ebola virus infection in rodents and non-human primates. *Science Translational Medicine*, *4*(158), 158ra146. doi:10.1126/scitranslmed.3004582

Wong, G., Richardson, J. S., Pillet, S., Patel, A., Qiu, X., Alimonti, J., … Kobinger, G. P. (2012b). Immune parameters correlate with protection against ebola virus infection in rodents and non-human primates. *Science Translational Medicine*, *4*(158), 158ra146. doi:10.1126/scitranslmed.3004582

Wu, Z., Asokan, A., & Samulski, R. J. (2006). Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Molecular Therapy : The Journal of the American Society of Gene Therapy*, *14*(3), 316–27. doi:10.1016/j.ymthe.2006.05.009

Wu, Z., Miller, E., Agbandje-McKenna, M., & Samulski, R. J. (2006). Alpha2,3 and alpha2,6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *Journal of Virology*, *80*(18), 9093–103. doi:10.1128/JVI.00895-06

Xiao, X., Li, J., & Samulski, R. J. (1998a). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *Journal of Virology*, *72*(3), 2224–32. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=109519&tool=pmcentrez&rendertype=abstract

Xiao, X., Li, J., & Samulski, R. J. (1998b). Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *Journal of Virology*, *72*(3), 2224–32. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=109519&tool=pmcentrez&rendertype=abstract

Xie, Q., Bu, W., Bhatia, S., Hare, J., Somasundaram, T., Azzi, A., & Chapman, M. S. (2002). The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(16), 10405–10. doi:10.1073/pnas.162250899

Xie, Q., Somasundaram, T., Bhatia, S., Bu, W., & Chapman, M. S. (2003a). Structure determination of adeno-associated virus 2: three complete virus particles per asymmetric unit. *Acta Crystallographica. Section D, Biological Crystallography*, *59*(Pt 6), 959–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12777756

Xie, Q., Somasundaram, T., Bhatia, S., Bu, W., & Chapman, M. S. (2003b). Structure determination of adeno-associated virus 2: three complete virus particles per asymmetric unit. *Acta Crystallographica. Section D, Biological Crystallography*, *59*(Pt 6), 959–70.

Xin, K.-Q., Mizukami, H., Urabe, M., Toda, Y., Shinoda, K., Yoshida, A., … Okuda, K. (2006). Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. *Journal of Virology*, *80*(24), 11899–910. doi:10.1128/JVI.00890-06

Xu, L., Sanchez, A., Yang, Z., Zaki, S. R., Nabel, E. G., Nichol, S. T., & Nabel, G. J. (1998). Immunization for Ebola virus infection. *Nature Medicine*, *4*(1), 37–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9427604

Yalkinoglu, A. O., Heilbronn, R., Bürkle, A., Schlehofer, J. R., & zur Hausen, H. (1988). DNA amplification of adeno-associated virus as a response to cellular genotoxic stress. *Cancer Research*, *48*(11), 3123–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2835153

Yates, V. J., el-Mishad, a M., McCormick, K. J., & Trentin, J. J. (1973). Isolation and characterization of an Avian adenovirus-associated virus. *Infection and Immunity*, *7*(6), 973–80. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=422790&tool=pmcentrez&rendertype=abstract

Zabner, J., Seiler, M., Walters, R., Kotin, R. M., Fulgeras, W., Davidson, B. L., & Chiorini, J. a. (2000). Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *Journal of Virology*, *74*(8), 3852–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=111893&tool=pmcentrez&rendertype=abstract

Zaiss, a K., & Muruve, D. a. (2008). Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. *Gene Therapy*, *15*(11), 808–16. doi:10.1038/gt.2008.54

Zhang, Y., & Duan, D. (2012). Novel mini-dystrophin gene dual adeno-associated virus vectors restore neuronal nitric oxide synthase expression at the sarcolemma. *Human Gene Therapy*, *23*(1), 98–103. doi:10.1089/hum.2011.131

Zheng, H. (2010). Efficient Retrograde Transport of Adeno-Associated Virus Type 8 to Spinal Cord and Dorsal Root Ganglion, *97*(January), 87–97.

Zhong, L., Li, B., Jayandharan, G., Mah, C. S., Govindasamy, L., Agbandje-McKenna, M., … Srivastava, A. (2008). Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology*, *381*(2), 194–202. doi:10.1016/j.virol.2008.08.027

Zhu, J., Huang, X., & Yang, Y. (2009). The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *The Journal of Clinical Investigation*, *119*(8), 2388–98. doi:10.1172/JCI37607

Zolotukhin, S., Byrne, B. J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., … Muzyczka, N. (1999). Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy*, *6*(6), 973–85. doi:10.1038/sj.gt.3300938

CHAPTER VI

6.0 AUTHOUR'S LIST OF PUBLICATIONS

6.1 PUBLICATIONS (REFEREED ARTICLES)

Bello A, Chand A, Soule G, Auricchio A, and Kobinger G. 2014. Novel Adeno-associated Viruses From Pig Tissues Efficiently Transduce Most Major Organs in Mice. *In submission*.

Qiu X, Audet J, Wong G, Fernando L, **Bello A**, Pillet S, Alimonti J, and Kobinger G. 2013. Sustained protection following treatment of infected non-human primates with ZMAb. Scientific Reports, **3**:3365. DOI:10.1038/srep03365.

***Puppo A**, ***Bello A**, Manfredi A, Cesi G, Marrocco E, Corte MD, Rossi S, Giunti M, Bacci ML, Simonelli F, Surace EM, Kobinger G, and Auricchio A. 2013. Recombinant Vectors Based on Porcine Adeno-Associated Viral Serotypes Transduce the Murine and Pig Retina. PLOS One, DOI: 10.1371/journal.pone.0059025.

Qiu X, Wong G, Fernando L, Audet J, **Bello A**, Strong J, Alimonti J, and Kobinger G. 2013. mAbs and Ad-Vectored IFN-α Therapy Rescue Ebola-Infected Non-human Primates When Administered After the Detection of Viremia and Symptoms. Science Translational Medicine, DOI: 10.1126/scitranslmed.3006605.

Richardson JS, Pillet S, **Bello A**, and Kobinger G. 2013. Airway Delivery of Adenovirus-Based Ebola Virus Vaccine Bypasses Existing Immunity to Homologous Adenovirus in Non-human Primates. Journal of Virology, **87**:3668-77. doi: 10.1128/JVI.02864-12.

Yokose U, Hachiya A, Sriwiriyanont P, Fujimura T, Visscher MO, Kitzmiller WJ, **Bello A**, Tsuboi R, Kitahara T, Kobinger G, and Takema Y. 2012. The Endogenous Protease Inhibitor TIMP-1 Mediated Protection and Recovery From Cutaneous Photodamage. Journal of Investigative Dermatology, **132**:2800-9. doi: 10.1038/jid.2012.204.

Qiu X, Audet J, Wong G, Pillet S, **Bello A**, Cabral T, Strong J, Plummer F, Corbett C, Alimonti J, and Kobinger G. 2012. Successful Treatment of Ebola Virus-Infected Cynomolgous Macaques with Monoclonal Antibodies. Science Translational Medicine, **138**:138ra81. doi: 10.1126/scitranslmed.3003876

Lin F, Shen X, Kichaev G, Mendoza JM, Yang M, Armendi P, Yan J, Kobinger G, **Bello A**, Khan A, Broderick K, and Sardesai N. 2012. Optimization of Electroporation-Enhanced Intradermal Delivery of DNA Vaccine Using a Minimally Invasive Surface Device. 2012. Human Gene Therapy, **23**:157-168. DOI:10.1089/hgtb.2011.209.

Shen X, Soderholm J, Lin F, Kobinger G, **Bello A**, Gregg D, Broderick K, and Sardesai N. Influenza A Vaccines Using Linear Expression Cassettes Delivered *Via* Electroporation Afford Full Protection Against Challenge in a Mouse Model. 2012. Vaccine, **30**:6946-6954.

Ao Z, Wang X, **Bello A**, Jayappa K, Yu Z, Fowke K, He X, Li J, Kobinger G, and Yao X. 2011. Characterization of anti-HIV activity mediated by R88-APOBEC3G mutant fusion proteins in $CD4^+$ T cells, peripheral blood mononuclear cells, and macrophages. Human Gene Therapy **22:**1225-1237.

Kasamatsu S, Hachiya A, Fujimura T, Sriwiriyanont P, Haketa K, Visscher M, Kitzmiller W, **Bello A**, Kitahara T, Kobinger G and Takema Y. 2011. Essential role of microfibrillar-associated protein 4 in human cutaneous homeostasis and in its photoprotection. Scientific Reports **1**, 164. DOI:10.1038/srep00164.

Sriwiriyanont P, Hachiya A, Pickens W, Moriwaki S, Kitahara T, Visscher M, Kitzmiller W, **Bello A**, Takema Y, and Kobinger G. 2011. Effects of IGF-Binding Protein 5 in Dysregulating the Shape of Human Hair. Journal of Investigative Dermatology, **131**:320-328. DOI:10.1038/jid.2010.309.

Bello A, Tran K, Chand A, Doria M, Allocca M, Hildinger M, Beniac D, Kranendonk C, Auricchio A, and Kobinger G. 2009. Isolation and evaluation of novel adeno-associated virus sequences from porcine tissues. Gene Therapy **16:**1320-1328 doi:10.1038/gt.2009.82.

Sriwiriyanont P, Hachiya, A., Pickens, W.L., Moriwaki, S., Ohuchi, A., Kitahara, T., Takema, Y., Kitzmiller, W.J., Visscher, M.O., **Bello, A.,** Tsuboi, R., and Kobinger, G.P. 2009. Lentiviral vector-mediated gene transfer to human hair follicles. Journal of Investigative Dermatology **129:**2296-2299.

6.2 CONFERENCE ABSTRACTS

Bello A, Chand A, and Kobinger G. 2013. Evaluation of Porcine AAVs as Vaccine Vectors Against Avian Flu and Zaire Ebolavirus Infections. Poster presented at 1) the Canadian Society for Immunology, Whistler, BC, Canada.

Bello A, Chand A, Auricchio, A, and Kobinger G. 2012. Characterization of Porcine Adeno-associated viruses Belonging to Unique Clades. Poster presented at: 2) American Society for Gene and Cell Therapy 15th Annual Meeting, Philadelphia, Pennsylvania, USA, 3) 5th Prairie Infectious Immunology Network, Moose Jaw, Saskatchewan, Canada.

Bello A, Chand A, and Kobinger G. 2011. Novel adeno-associated virus from pig tissues as vaccine vectors against avian influenza infection. Poster presented at: 4) Canadian Society for Immunology 2011, Lake Louise, Alberta, Canada and 5) 4th Prairie Infectious Immunology Network 2011, Asessippi Provincial Park, Manitoba, Canada.

Bello A, Chand A, Doria M, Tran K, Allocca M, Auricchio A, and Kobinger G. 2010. Efficient transduction of mouse liver and other organs following intravenous administration of a recombinant porcine AAV. Poster presented at: 6) American Society of Gene Therapy 13th Annual Meeting 2010, Washington, DC, USA and 7) Association de therapie genique du Quebec 2010, Grenville-sur-la-Rouge, Quebec.

Bello A, Chand A, Tran K, Doria M, Allocca M, Auricchio A, and Kobinger G. 2009. Characterisation of a Unique Adeno-Associated Virus Forming a New Clade Within the Dependovirus Genus. Poster presented at: 8) American Society of Gene Therapy 12th Annual Meeting, San Diego, California, USA and 9) Canadian Student Health Research Forum 2009, Winnipeg, Manitoba, Canada .

Bello A, Tran K, Feldmann H, Auricchio A, Doria M, and Kobinger G. 2008. Isolation of Novel Adeno-Associated Viruses from Porcine Tissues. Poster presented at: 10) American Society of Gene Therapy 11th Annual Meeting, Boston, Massachusetts, USA, 11) 4th Canadian Gene Therapy and Vaccines Symposium, Montreal, Quebec, Canada, 12) 2008 Public Health Agency of Canada Research Forum, Ottawa, Ontario, Canada and 13) Canadian Student Health Research Forum 2008, Winnipeg, Manitoba, Canada.

Bello, A., Tran, K., Klassen, M., Grey, M., Feldmann, H., and Kobinger, G.P. 2007. Isolation of Novel Adeno-Associated Viruses from Porcine Tissues. Poster presented at: 14) 2007 Public Health Agency of Canada Research Forum, Winnipeg, Manitoba, Canada and 15) Canadian Student Health Research Forum 2008, Winnipeg, Manitoba, Canada.

6.3 ORAL PRESENTATIONS AT NATIONAL AND INTERNATIONAL CONFERENCES

Bello A, Chand A, and Kobinger G. 2012. Evaluation of Porcine AAVs as Vaccine Vectors Against Avian Flu and Zaire Ebolavirus Infections. Presented at the 15th Annual American Society for Gene and Cell Therapy in Philadelphia, Pennsylvania, USA.

Bello A, Chand A, and Kobinger K. 2011. Novel Adeno-associated Virus From Pig Tissues as Vaccine Vectors Against Avian Influenza Infection. Presented at the 4th Prarie Infectious Immunology Network, Asessippi Provincial Park, Manitoba, Canada.

Bello A, Chand A, Tran K, Auricchio A, and Kobinger G. 2010. The Isolation and Performance of Novel Adeno-associated Virus from Porcine Tissues. Presented at the 5th Canadian Symposium on Gene Therapy and Vaccines, Grenville-sur-la-Rouge, Quebec, Canada.

6.4 PATENTS

Isolation, development and use of novel porcine adeno-associated viruses.

Inventors: **Alexander Bello,** Markus Hildinger, Michael Gray, Matthew Klassen, Heinz Feldmann, and Gary P. Kobinger.

US Provisional Patent Application No.: 60/969,733

This patent describes the new AAV sequences which I was able to isolate for my project, specifically AAVpo1, -po2, and –po3