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Uncovering the Mechanisms Underlying the Immunogenicity of Adenovirus Vaccine Vectors

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UNCOVERING THE MECHANISMS UNDERLYING THE IMMUNOGENICITY OF ADENOVIRUS VACCINE VECTORS

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LIST OF ABREVIATIONS

α	alpha
Å	atomic
Ad	adenovirus
Ad5	human adenovirus class C serotype 5 (hAd-5C)
Ad28	human adenovirus class D serotype 28 (hAd-28D)
AdV	adenovirus vectors
AdV-5	AdV based on Ad5 genome
AdV-28	AdV based on Ad28 genome
APC	Antigen presenting cell
β	beta
CAR	Coxsackievirus-adenovirus receptor
CD	Cluster of differntiation
^{o}C	degrees Celsius
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
ds	Double stranded
DSG-2	Desmoglein-2
EDTA	ethlenediaminetertaacetic acid
FCS	fetal calf serum
GFP	green fluorescent protein
HEK	human embryonic kidney
hpi	hours post infection
hpt	hours post transduction
HRP	horse radish peroxidase

IFA	immunofluorescence assay
IFN	Interferon
IRF	Interferon regulator factor
ISRE	Interferon stimulated response element
Ig	immunoglobulin
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
IP-10	gamma interferon-induced protein 10
JNK	Jun N-terminal kinase
kDa	kilo Dalton
KD	knockdown
KO	knockout
luc	luciferase
M	Molar
MHC	Major histocompatibility complex
µg	microgram
µl	microliter
µM	micromolar
MFI	Mean florescent intensity
ml	milliliter
mM	millimolar
min	minutes
MOI	multiplicity of infection
nM	nanomolar
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cells

PCR	polymerase chain reaction
PI3 kinase	Phosphoinositide 3-kinase
PRR	Pattern recognition receptors
PS	Phosphatidylserine
r	recombinant
RNA	ribonucleit acid
SEM	standard error of the mean
SD	standard deviation
SDS	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLR	Toll-like receptor
TNF	tumor necrosis factor
VA-RNA	Viral associated RNA
VKD	Vitamin K-dependent
VKOR	Vitamin K epoxide reductase
VSV	vesicular stomatitis virus
w/v	weight per unit volume
WT	wild-type

ABSTRACT

Vaccination is historically the most effective tool for preventing infectious disease but current vaccine strategies fail to generate robust immunity to major infectious diseases such as HIV and malaria. Therefore, newer vaccine approaches are needed. Vaccines generated from viral, adenovirus based, vectors (AdVs) have proven highly immunogenic in multiple disease models. However, the clinical use of many AdVs is limited by the presence of pre-existing antibodies in human populations, which prevent expression of antigenic genes during immunization with AdVs based on common adenovirus (Ad) serotypes, such as HAdV-5C. Immunization with rare serotype based AdVs, such as HAdV-28D, are not affected by pre-existing immunity; however, these rare AdVs stimulate high levels of type I interferon (IFN), which suppress antigenic gene expression and hence, preclude antigen-specific immunity. We suggest that there is a way to prevent these rare AdVs from stimulating IFN. This would allow for the generation of Ads that can bypass neutralization from preexisting antibodies but also elicit appropriate immune responses that are sufficient for durable immunization.

We sought to identify and characterize serotype-specific properties of AdVs that contribute to the ability of these vectors to induce potent vaccine immunity. As high levels of type I IFN are known to negatively impact the ability of rare serotype-vectors to express antigenic genes and stimulate antigen-specific immune responses, we focused our studies on determining how different AdV serotypes stimulate unique innate immune responses that vary in type I IFN production. We found that AdV serotypes differentially interact with a serum protein,

Gas6, that is known to negatively regulate innate immunity during viral infection. Our studies show that adenoviruses interact with Gas6 in manner that is mediated by interactions between the Gas6 Gla domain and the HAdV-5C fiber protein shaft domain. Further, we demonstrate that Gas6 reduces the IFN response stimulated by HAdV-5C and enhances HAdV-5C encoded transgene expression. Our studies suggest that Gas6-fiber interactions contribute to AdV immunogenicity. We reason that rare-serotype based AdVs engineered to include Gas6 binding motifs will more effectively deliver DNA-based vaccines.

CHAPTER I

INTRODUCTION

Vaccines

Vaccination is the most successful medical invention for preventing infectious disease since the late eighteenth century when Edward Jenner first developed a vaccine against smallpox (Andre et al., 2003; Nabel et al., 2013). Vaccines significantly contribute to public health by reducing the morbidity and mortality of infectious diseases and limit spread through the population. Widespread vaccination efforts can even lead to the global eradication of a disease as demonstrated by the eradication of smallpox in 1980 (Hinman et al., 1999). Current vaccine initiatives are estimated to save over 3 million lives a year (Andre et al., 2003). While current vaccines against diseases such as polio, measles, mumps, rubella, yellow fever, and hepatitis A and B have successfully lowered the global burden of these diseases other infectious diseases such as HIV, tuberculosis, and malaria remain refractory towards current vaccine approaches. These diseases claim the lives of over 4 million people per year (Nabel et al., 2013). Therefore, new strategies must be developed to safely and effectively combat these unresponsive diseases.

Effective vaccination strategies work by inducing pathogen-specific immune responses that provide long-lasting protection against disease. The majority of vaccines currently in circulation involve immunization with a killed or live-attenuated version of the disease-causing pathogen. While these strategies have been successful, some safety issues are associated with their use. Incomplete inactivation of killed pathogens or reversion of live-attenuated pathogens

virulent strains have the potential to inadvertently result in disease. However, these events are extremely rare and usually the result of manufacturer error (Andre et al., 2003). Alternatively, subunit vaccines, that only contain a fraction of the disease-causing pathogen, remove these risk factors (Plotkin et al., 2013). However, subunit vaccines are less immunogenic and often require other components to help or "adjuvant" the generation of protective immunity (Coffman et al., 2010). Newer vaccine strategies focus on eliminating safety risks while at the same time generating robust immunity to specific pathogens.

Originally vaccines were designed with little insight into the molecular mechanisms required to generate protective immunity. Further, the failure of current vaccine strategies to demonstrate protection against refractory diseases (e.g. HIV, tuberculosis, and Malaria) are due to the inability to elicit appropriate immune responses specific to these pathogens. Advances in molecular biology have increased our knowledge of microbial pathogenesis and allowed for the protective mechanisms of innate and adaptive immunity to begin to be dissected. Newer approaches focus on the rational development of vaccines, able to elicit desirable immune responses that correlate with protection. This has led to the development of newer adjuvants capable of eliciting adaptive immunity, which is more robust and also more tailored to a protective immune phenotype (Coffman et al., 2010). Different diseases require specialized immune responses to mediate protection in vaccine models. However, the scope of this dissertation will mainly focus on the protective correlate of cellular immunity; specifically, the generation of antigen specific $CD8^+$ T cells. I will further discuss the importance of this cell population and how they are generated in subsequent sections.

Advances in DNA technology have allowed for the development of gene based therapies

and vaccines. Genetic vaccines allow for genes that encode a protein for which an immune response can be elicited against, antigenic genes, to be delivered directly to cells. While the previously described vaccination techniques mainly stimulate immune responses that are mediated by antibodies, humoral immunity, genetic vaccines can stimulate both humoral and cellular immune responses. This is specifically advantageous for generating immunity to intracellular pathogens that can often evade humoral immune responses alone. Gene delivery systems have varied over the past few decades. However, adenovirus vectors (AdV) have long stood out as an excellent platform for gene delivery as they exhibit a wide cellular tropism, are easily genetically modified, and can be mass-produced at low cost. AdVs are specifically promising in vaccine studies due to inherent viral properties that allow for targeted cell delivery and induction of innate immune pathways that adjuvant immunity against the antigenic genes they encode (Hartman et al., 2008). AdVs have long been studied in vaccine initiatives but our incomplete understanding of how adenoviruses interact with the host immune system has limited their use clinically.

Adenovirus

Adenoviruses (Ads) are non-enveloped, double-stranded DNA viruses with at least 65 known human serotypes, classified into 7 classes (A-G) (Teigler et al., 2012). Human adenoviruses were initially isolated over 60 years ago by Hilleman and Rowe (Hilleman and Werner, 1954; Rowe and Huebner, 1956) and are associated with acute respiratory, gastrointestinal, and ocular infections (Nemerow et al., 2009). While infection is usually self-limiting in healthy individuals who easily overcome infection, elderly or immunocompromised individuals can experience sever disease and complications following infection (Nemerow et al.,

2009). Adenoviruses have been well studied as a model system for uncovering mechanisms of molecular and cell biology. Our understanding of adenovirus biology, while incomplete, has led to the development of viral vectors for gene delivery and vaccine use. The studies outlined in this dissertation compare the use of two distinct adenoviruses, human adenovirus class C serotype 5 (Ad5) and human adenovirus class D serotype 28 (Ad28) as vaccine vectors.

Adenoviruses possess a linear double stranded DNA genome ranging from 30-38kb, and encoding 30-40 viral genes. The viral genome is packaged into a non-enveloped viral particle that is ~90 nm in diameter constructed of 11 structural proteins that form an icosahedron (Nemerow et al., 2009). The external capsid structure is composed of 3 major capsid proteins: hexon, penton base and fiber. 240 copies of hexon trimers make up the majority of the capsid with, pentamers, of penton base anchoring, trimers of fiber protein. 12 fiber trimers project from each fivefold capsid vertex. Minor capsid proteins (pIIa, pVI, pVIII and pIX) help to stabilize the viral capsid. Protein VI, present inside the viral capsid, is released during cell entry following endosomal acidification, and is responsible for mediating endosomal rupture (Nemerow et al., 2009; Wiethoff et al., 2005). The remaining proteins inside the capsid, terminal protein, Mu, pVII, and pV, are all associated with the viral DNA (Figure 1). Additionally, Ad capsids contain several copies of a viral cysteine protease that are incorporated into the viral particle following their processing of the viral capsid precursor proteins during assembly (Nemerow et al., 2009).

Cell Entry.

Adenovirus cell entry and resulting infection occurs via interaction between adenovirus and primary cellular receptors. This is followed by secondary receptor interactions with cellular integrins that allow for virus internalization. Clatherin-mediated endocytosis is thought to be the

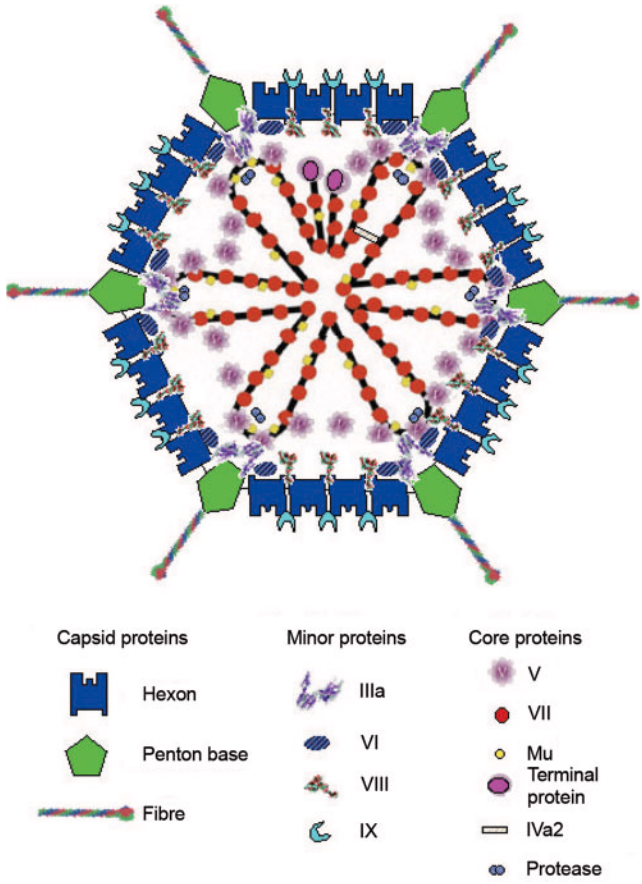


Figure 1. Structure of Adenovirus Capsid
The adenovirus capsid is made up of 7 capsid proteins. Hexon, penton base and fiber proteins make up the majority of the external capsid. Protein IIIa, protein VI, protein VIII and L3-23K protease reside in the interior of the capsid. Within the capsid, Terminal protein, Protein Mu, Protein VII, and Protein V, are associated with the viral DNA. Figure taken from (Russell, 2009).

primary route of Ad entry for most serotypes (Zhang et al., 2014; Zhang and Bergelson, 2005). However, class B Ads have also been shown to alternatively undergo cellular internalization through, clathrin-independent, micropinocytosis (Amstutz et al., 2008; Kalin et al., 2010). Both cellular attachment and internalization route is dependent on capsid components that vary between adenovirus serotype. Receptor binding domains present in the fiber knob dictate primary receptor usage. Most Adenoviruses fibers, including Ad5, utilize the coxsackievirus-

adenovirus receptor (CAR) (Bergelson et al., 1997) with the exception of class B Ads, which utilize CD46 (Wu et al., 2004), and class D Ads which utilize sialic acid (Arnberg et al., 2000; Burmeister et al., 2004). Despite being part of the class D subgroup, the primary receptor for Ad28 remains undefined (Kahl et al., 2010). Additionally, some Ad serotypes have been shown to alternatively interact with desmoglein-2 (DSG-2) (Wang et al., 2011), CD80/86 (Marttila et al., 2005; Short et al., 2004) or heparin sulfate (Dechecchi et al., 2001). A full description of Ad subgroup and serotype-specific receptor binding is depicted in (Table 1). As receptor engagement is the first critical step towards Ad entry, the identification of Ad receptors and attachment factors that drive viral internalization is of the upmost importance.

Intracellular Trafficking and Genome Delivery.

Following cellular binding and virion internalization, Ad particles traffic through the endosomal compartment. There interactions with cellular factors and endosomal acidification results in capsid disassembly and endosomal escape (Figure 2). Primary receptor usage not only facilitates initial cellular attachment and internalization but also dictates the route of intracellular trafficking. CAR-binding adenovirus serotypes, such as Ad5, rapidly escape the endosome but when the fiber protein is swapped with fiber proteins from class B serotypes, that bind alternative primary receptors, virions are retained longer in the endosomal pathway (Miyazawa et al., 2001; Miyazawa et al., 1999; Shayakhmetov et al., 2003). Additionally, the presence of an RGD domain within the penton base protein has been shown to mediate binding to cellular αβ-integrins. Secondary integrin interactions can aid in cellular attachment as well as endosomal escape. Integrin engagement activates signaling cascades involving PI3 kinases (Li et al., 1998b) and Rho GTPases (Li et al., 1998a), which leads to cytoskeleton rearrangement and

Adenovirus Class	Serotype
A	12[a], 18, 31[a]
B1	3[b,d,f], 7[f], 16[b], 21[b], 50[b]
B2	11[b,f], 14[b,f], 34, 35[b]
C	1, 2[a,e], 5[b,e], 6
D	8[c], 9, 10, 13, 15[a], 17, 19p[a], 19a[c], 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37[b,c], 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 51
E	4[a]
F	40, 41[a]

Table 1. Adenovirus receptor binding
Those viruses for which specific receptors have been identified are denoted with footnotes. Receptor use was demonstrated by attachment or infection with these viruses being dependent on expression of the indicated receptor. Adapted from (Zhang and Bergelson 2005).
[a] CAR
[b] CD46
[c] Sialic acid
[d] CD80/86
[e] Heparan sulfate
[f] DSG-2

internalization. Binding to αvβ5 integins has also been shown to promote endosomal membrane permeabilization and therefore escape (Wickham et al., 1994). Partially disassembled Ad capsids, that have escaped the endosomal compartment, associate with microtubules and translocate to the nuclear pore where they deliver their viral DNA genome to the nucleus (Suomalainen et al., 1999). These, temporal, events of Ad entry are depicted below (Figure 2).

Gene Expression.

After DNA delivery, viral gene expression of early transcripts (E1A, E1B, E2, E3, and E4) begins. This is followed by transcription of intermediate transcripts (IX, IVa2, L4, and E2 late). Finally, expression of a late transcript occurs which through alternative splicing generates five distinct mRNAs (L1-L5). All of these transcripts are transcribed by host RNA polymerase II (Morris et al., 2010). Alternatively, Ad serotypes encode one or more non-coding virus-associated RNAs (VA RNAs) (Vachon and Conn, 2016). VA RNA expression occurs early following nuclear delivery and is mediated by cellular RNA polymerase III (Vachon and Conn, 2016). Expression of early adenovirus gene products, including E1A, are required for transcriptional activation of downstream viral gene expression and viral DNA replication (Berk, 1986; Frisch and Mymryk, 2002; Morris et al., 2010).

Adenoviruses whose genomes are deleted of E1 are rendered replication defective and cannot propagate following transduction despite having intact expression of early viral gene transcripts (Rauschhuber et al., 2012). These, recombinant replication defective adenovirus vectors, (AdVs) are desirable gene delivery vehicles for many reasons. First, AdVs have a large coding capacity of 5.2kb-36kb, that dependents on the AdV generation which have variable E1 and E2/E3/E4/ gene deletions (Lee et al., 2017; Rauschhuber et al., 2012). Additionally, AdVs have the ability to effectively transduce a broad range of host cells due to their wide cellular tropism and they can be mass produced at low cost. However, despite being rendered replication-defective, AdVs still trigger immune responses *in vivo* (Rauschhuber et al., 2012). The proinflammatory immune responses induced by AdVs have been detrimental in many gene therapy applications clinically (Buckley, 2002; Giacca and Zacchigna, 2012; Lee et al., 2017;

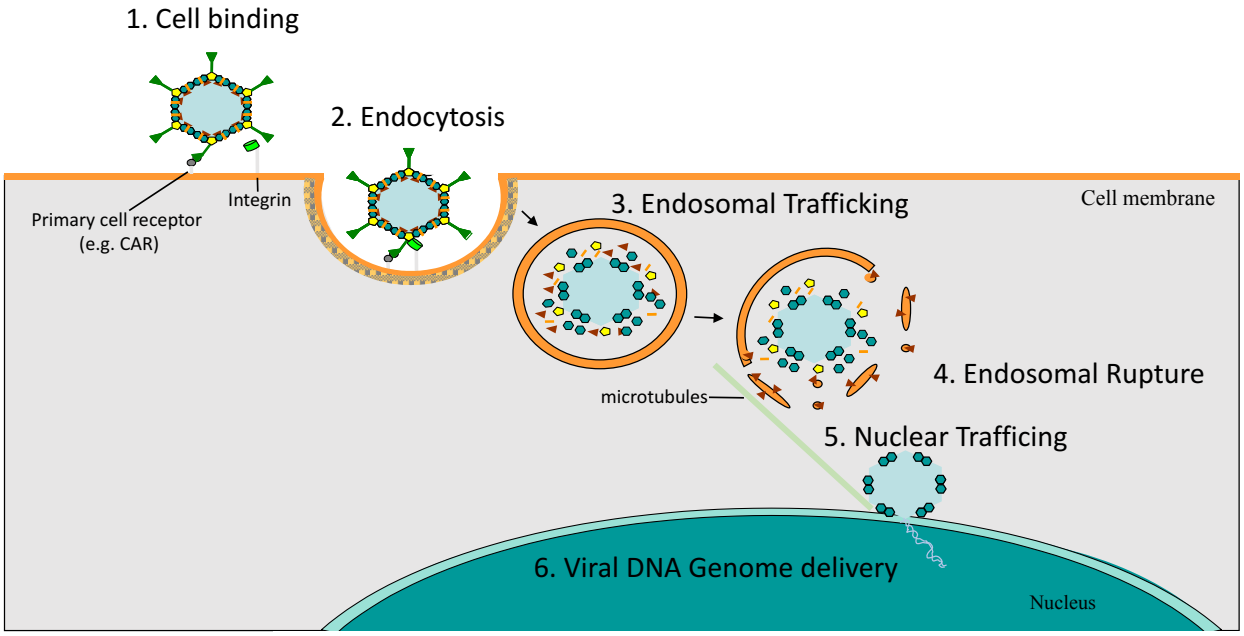


Figure 2. Events of Ad Entry
1) Ad binds cells via interactions with primary cellular receptors and secondary integrin interactions. **2**) Receptor engagement results in internalization of the viral particle. **3)** Ad particles traffic through the endosomal compartment where receptor interactions and endosomal acidification trigger capsid disassembly. **4)** Ad protein VI released from inside the viral capsid, facilitates endosomal rupture. **5)** Partially disassembled capsids traffic along microtubules to reach the nuclear pore complex. **6)** Once docked at the nuclear pore Ad delivers its dsDNA genome to the nucleus where host proteins begin to transcribe viral genes.

Raper et al., 2003; Yang et al., 1996; Yang et al., 1994). However, these proinflammatory responses, while undesirable in gene therapy applications, are advantageous for generating immunity to vaccine antigens. This makes AdVs an ideal vaccine vehicle. I will discuss how AdVs stimulate host immunity and how this stimulation is advantageous in vaccine applications in the following sections.

Innate Immune Response To Adenovirus

Innate immunity is the first line of host defense against invading pathogens, including viral infection. Recognition of pathogen-associated molecular patterns (PAMPs) by cellular

pattern recognition receptors (PRRs) leads to signal transduction events that result in the production of antiviral cytokines and chemokines. These signaling molecules act as secondary messengers to stimulate downstream response pathways and immune cell recruitment in order to limit infection. Numerous studies have highlighted the importance of innate immune responses to adenoviral vaccine vectors for stimulating a robust immune response to vector expressed vaccine antigens (Acsadi et al., 1998; Johnson et al., 2012; Kahl et al., 2010; Teigler et al., 2012; Zhu et al., 2007).

Adenoviruses elicit a potent innate immune response during infection due to the activation of multiple PRRs that occurs during cellular entry and nuclear DNA genome delivery. This response is primarily characterized by the release of cytokines: TNFα, IL-1, IL-6 and type I interferons (IFN) IFNα and IFNβ. Innate immune stimulation by Ad has been attributed to early events of virus entry including, virus-receptor interactions (Tamanini et al., 2006), rupture of endosomal membranes (Barlan et al., 2011a; Barlan et al., 2011b; McGuire et al., 2011; Smith et al., 2011) , recognition of viral DNA (Nociari et al., 2009), and early expression of small non-coding viral associated RNAs (VA-RNA) (Yamaguchi et al., 2010). These events vary significantly between adenovirus serotypes which differ in tropism, receptor usage, intercellular trafficking, and encode 1 or 2 distinct VA-RNA. These differences result in complex immune profiles that are generated during Ad infection. I continue to discuss these events of immune stimulation and how they vary between Ad serotype below.

Differences in Ad proteins are first detected by the innate immune system at the cell surface where the capsid proteins induce a cellular stress response that activates Jun N-terminal kinase (JNK) priming downstream responses for activation of interferon regulatory factor 3

(IRF3) (Nociari et al., 2009) (Figure 3). This activation is believed to be mediated by interaction between the Ad fiber protein and cellular receptor CAR (Tamanini et al., 2006). Others suggest that RGD motifs in the penton base and their interaction with αvβ3 integrins contribute to these initial events of cellular activation (Liu et al., 2003; Shayakhmetov et al., 2003). However, IRF3 phosphorylation and subsequent expression of type I IFNs requires further capsid disassembly (Smith et al., 2011) (Figure 3.) Therefore, innate immune activation is mainly induced by PAMPs and danger signals associated with capsid disassembly and endosomal rupture.

After virions are internalized, they traffic through the endosome. There endosomal acidification triggers capsid disassembly and endosomal rupture. These are the key triggers that activate immunity. In the acidified endosome TLR9 is activated by unmethylated CpG sequences in viral DNA and endosomal rupture releases cellular cathepsins, that act as a danger signal, inducing activation of the NLRP3 inflammasome (Barlan et al., 2011a; Barlan et al., 2011b; Cerullo et al., 2007; McGuire et al., 2011; Zhu et al., 2007) (Figure 4). Class C adenoviruses, such as Ad5, rapidly escape the acidified endosome due to the metastable properties of their capsids and events of CAR-mediated trafficking. This is demonstrated by an Ad2 mutant, temperature sensitive mutant 1 (ts1), that has a hyperstable capsid and is unable to uncoat and remains retained within the endosomal compartment (Silvestry et al., 2009). Alternatively, serotypes that utilize CD46 receptors are retained longer in the endosomal compartment where they can continue to activate TLR9. Additionally, late rupture of these endolysosomes releases an increased concentration of cathepsins that can further stimulate immune signaling pathways (Teigler et al., 2014). In the cytosol, viral DNA sensors DAI and cGas recognize viral DNA PAMPs prior to nuclear entry (Lam et al., 2014; Takaoka et al., 2007) (Figure 4). Early

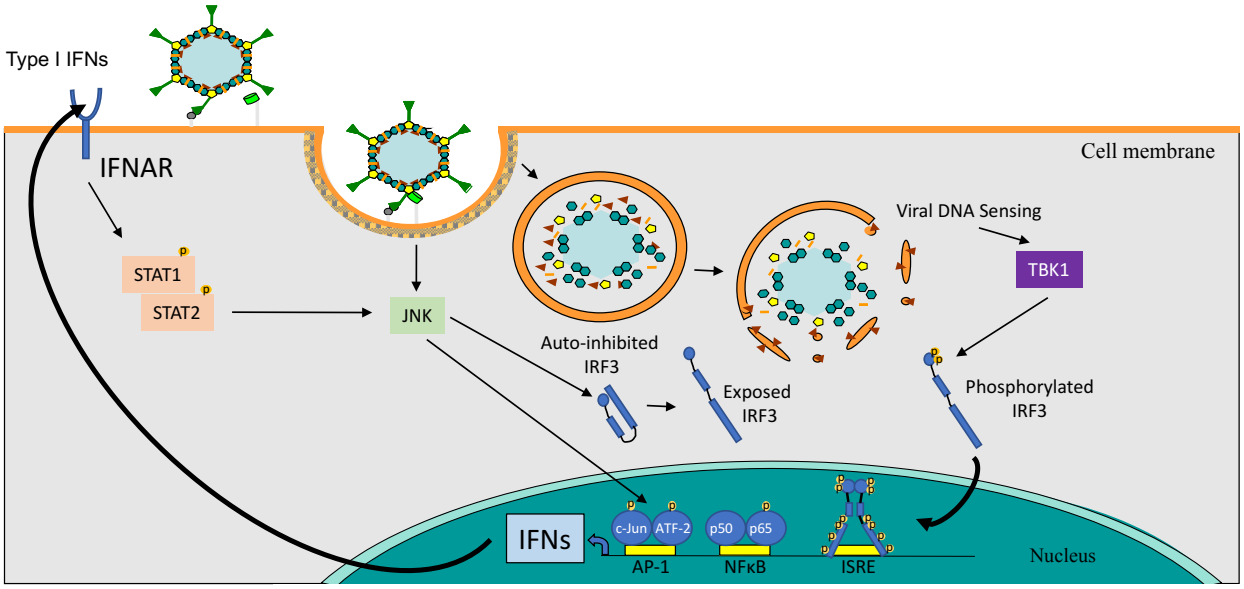


Figure 3. Innate immune signaling begins with receptor engagement
During Ad cell entry interaction with cellular receptors can prime activation of IRF3. CAR binding activates JNK signaling cascades that result in a conformational change in IRF3. IRF3 is self-associated in an inactive form at resting state. Following JNK activation IRF3 exposes its C' terminus. Further activation, phosphorylation, of IRF3 is required for IRF3 to translocate to the nucleus and promote expression of genes encoding proinflammaory cytokines and type I IFN. Endosomal rupture releases Ad PAMP that stimulate PRRs. These PRRs activate TBK1 which phosphorylates the exposed form of IRF3 allowing for the downstream functions listed above. Figure adapted from (Nociari et al., 2009).

Early expressed VA-RNAs are recognized by the viral RNA sensor, RIG-I, (Minamitani et al., 2011; Weber et al., 2013) (Figure 4). All these innate immune pathways are stimulated in the absence of viral replication, as highlighted by studies using replication defective AdVs (Rauschhuber et al., 2012). Therefore, I decided to focus my work on understanding how these initial events of immune activation may vary between serotypes. These differences likely impact the differences that are observed in AdV vaccine utility.

The use of recombinant AdVs has allowed for the innate immune pathways stimulated by

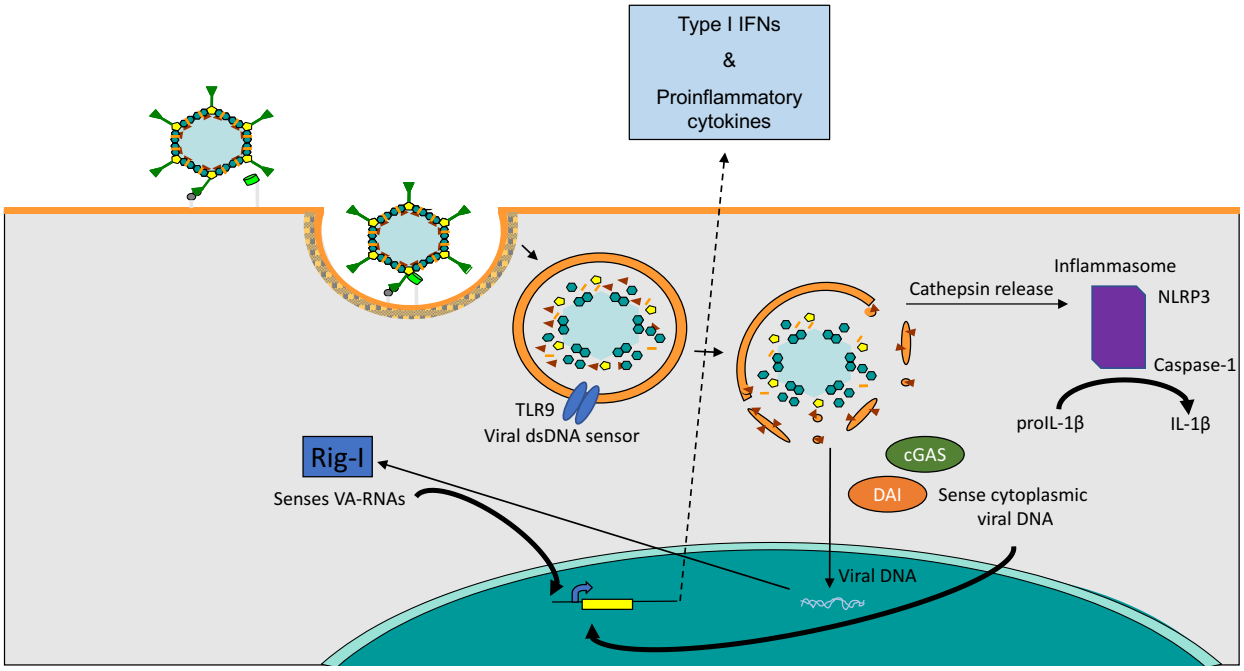


Figure 4. PRR activation in the endosome and following endosomal escape
Following the initial disassembly of Ad capsids, viral dsDNA actives TLR9 in the endosome. Endosomal rupture exposes viral dsDNA to the cytoplasm where DNA sensors, cGas and DAI, are triggered. Cathepsins released from the ruptured endosomes are a danger signal that activates the NLRP3 inflammasome. Following Ad genome deliver to the nucleus, VA-RNAs are produced that further stimulate a viral RNA sensing pathway in the cytoplasm, RIG-I. Together the activation of these PRRs accumulates in the release of proinflammatory cytokines and type I IFNs.

different Ad serotypes to begin to be dissected. AdVs generated from class B and D serotypes Ad35, Ad26 and Ad48 stimulate higher levels of innate cytokines, IL-1RA, IL-6, IFN, and IP-10, compared to Ad5 both in vivo and in vitro (Teigler et al., 2012; Teigler et al., 2014). Capsid-specific interactions were shown to be partially responsible for discrepancies in the magnitude of type I IFN levels elicited between serotypes as demonstrated by capsid modified chimeric AdVs (Teigler et al., 2012). Both hexon and fiber chimeric AdVs induce an altered magnitude of innate cytokine production compared to parent vectors (Teigler et al., 2012). However, I focus my continued discussion on how differences in the fiber protein of Ad serotypes have been shown to

impact innate immunity.

Fiber-receptor interactions alone are implicated in the innate cytokine induction by AdVs. For example, CD46-receptor binding is associated with stimulating an increased type I IFN response. CD46-receptor blocking antibodies reduce the magnitude of the type I IFN levels elicited during *in vitro* Ad35 or Ad26 infection of human peripheral blood mononuclear cells (PBMCs) (Teigler et al., 2012). Additionally, fiber chimeric Ad5 based AdVs (AdV-5) that have fiber proteins from Ad37, Ad16, or Ad35 induce an increased type I IFN response from PMBCs compared to Ad5 (Iacobelli-Martinez and Nemerow, 2007). This discrepancy is believed to be due, in part, to CD46-binding allowing greater infection of immune cells, including plasmacytoid dendritic cells (pDCs), the main producer of IFNα. Additionally, CD46-mediated endosomal trafficking increases Ad dsDNA activation of TLR9 compared to CAR-mediated Ad entry, even at comparable infection levels in Hela cells (Iacobelli-Martinez and Nemerow, 2007). Ad5 fiber chimeric viruses expressing the fiber protein of Ad16 (Ad5F16) have also been shown to more potently activate the NLRP3 inflammasome due to increased retention in cathepsin B containing endosomes prior to endosomal rupture compared Ad5 (Barlan et al., 2011a). CAR-binding Ad5 rapidly escapes the endosomal compartment and traffics to the nucleus. Alternatively, CD46-binding Ad35 and Ad26 accumulate in the late endosome following infection resulting in increased anti-viral cytokine responses: proinflammatory cytokine (IL-1β, TNFα, and IL-1RA) and IFN (IFNα and IFNγ) secretion (Teigler et al., 2014). Therefore, differences in primary receptor usage, specifically CD46 binding, could explain the differences seen in AdV induced immunity.

CD46 is ubiquitously expressed complement regulatory protein that cleaves and

inactivates complement components C3b and C4b (Seya et al., 1990). Several microbial pathogens including: measles virus (Dorig et al., 1993), herpesvirus 6 (Santoro et al., 1999), and *Neisseria sp*. Bacterium (Kallstrom et al., 1997), directly bind CD46 mediating immunosuppressive effects that aid in infection. CD46-utilizing Ads (Ad16, Ad35, and Ad37) have alternatively demonstrated the ability to inhibit secretion of proinflammatory cytokines (IL-12, IL-1α, IL-1β, IL-1Ra, and IL-6) from PBMCs stimulated with IFNγ while CAR-utilizing Ads (Ad5 and Ad2) had no effect (Iacobelli-Martinez et al., 2005). Reports on general proinflammatory cytokine profiles following infection with CD46-utilizing Ads vary based on experiment conditions and serotype but, both class B and D Ads are consistently shown to inhibit IL-12 production (Iacobelli-Martinez et al., 2005; Kahl et al., 2010). While, IL-12 inhibition seems to be a conserved feature across class B and D Ad serotypes not all of these serotypes have been reported to utilize CD46 as a cellular receptor. Class D serotype 28 AdVs (AdV-28) do not require CAR or CD46 for cellular entry and currently utilize an unidentified primary cellular receptor (Kahl et al., 2010). Therefore, CD46 interaction alone cannot explain the discrepancies in AdV induced immunity between serotypes.

AdV-28 has been shown to elicit superior immunogenicity in vaccine studies compared to other class B and D AdVs (Kahl et al., 2010). Therefore, I chose to focus my studies on understanding the immune response to AdV-28. AdV-28 has been shown to suppress proinflammatory cytokine production and elicit high levels of type I IFN much like Ad35 (Kahl et al., 2010). Conflicting data muddles our understanding of the magnitude of proinflammatory cytokine production stimulated by class B and D Ads as compared to class C Ads. Therefore, I focus my studies on understanding the discrepancies in type I IFN production between class D,

Ad28, compared to class C, Ad5. Type I IFN production is thought to be a consistent feature of innate immune stimulation following Ad infection. Additionally, type I IFNs are a hallmark cytokine induced by AdVs and known to play an important role in generating vaccine immunity to AdV encoded antigens. I continue to discuss the importance of type I IFNs below.

Type I IFNs are an important class of cytokines released in response to viral infection. Once released, these IFNs elicit scores of antiviral effects within infected as well as surrounding cells to disrupt multiple stages of the virus life cycle including entry, early gene expression, genomic replication, viral assembly, and egress (Haller et al., 2006; Lam et al., 2014; Zhu et al., 2007). Many viruses have evolved mechanisms to reduce interferon production and evade interferon-inducible antiviral proteins (Hendrickx et al., 2014). Understanding the influence of IFNs on the virus life cycle as well as how viruses suppress IFN responses is critical for the design of targeted antiviral therapies and engineering novel vaccines.

Type I IFN is produced following AdV transduction primarily by dendritic cells (DCs) and macrophage. Plasmacytoid dendritic cells (pDCs) produce high levels of IFNα in response to Ad infection via TLR9 activation while other conventional DC subtypes and macrophage produce IFNβ via TLR-independent signaling cascades (Zhu et al., 2007). Type I IFN production is critical for inducing adaptive immune responses to AdVs as type I IFN receptor deficient mice exhibit reduced antigen-specific T and B cell responses following AdV-5 transduction (Zhu et al., 2007). However, excessive IFN induction by class B and D AdVs can lead to anti-viral immune responses that limit generation of antigen-specific adaptive immune cells (Johnson et al., 2012; Teigler et al., 2012; Zhu et al., 2007). Specifically, IFN has been shown to either promote (Kolumam et al., 2005; Marrack et al., 1999; Thompson et al., 2006) or suppress

(Marshall et al., 2011; Terawaki et al., 2011; Tough et al., 1999) the proliferation and survival of antigen-specific $CD8^+$ T cells depending on its temporal induction and level of production. High levels of type I IFN have been shown to down-regulate gene expression from viral promoters (Acsadi et al., 1998; Papadakis et al., 2004) and enhance activation of Natural killer (NK) cell mediated immune clearance of infected cells (Johnson et al., 2014), both of which inhibit the generation and maintenance of antigen-specific $CD8^+$ T cells (Finn et al., 2009). While type I IFN induction by different AdV serotypes is known to result in variable T cell immunogenicity, little is known about mechanisms by which AdV serotypes differentially induce IFN responses. The studies described in this dissertation aim to identify the serotype-specific mechanisms responsible for these discrepancies in IFN induction magnitude.

These early initial immune responses are required for creating an anti-viral state and recruiting additional immune cells which prevents further infection; inhibiting subsequent viral spread and eliminating virus-containing cells. Innate immune-mediated events are critical for initially limiting disease and for stimulating adaptive immunity. The adaptive immune responses described in the next section, are necessary for providing pathogen-specific immune responses that lead to long-term immune protection. I discuss the adaptive immune responses stimulated by Ad in the following section.

Adaptive Immune Response To Adenovirus

Following Ad exposure and innate immune host responses, cytokines and chemokines prime and recruit adaptive immune cells to the site of infection. Concurrently, Ad-transduced cells present endogenous antigen peptides in the context of MHC class I molecules (MHCI) on their surface. Antigen presenting cells (APCs), typically tissue resident macrophage or dendritic

cells, can also phagocytose apoptotic or necrotic cells and cross-present cell-associated exogenous antigens on MHCI or capture, process, and present secreted antigens on MHC class II molecules (MHCII). The innate immune environment shapes how APC encounter, process, and present antigens. PRR stimulation and production of cytokine and chemokines dictate which APC subtypes are recruited to the site of infection. Once APCs encounter antigen, cytokines further stimulate APC maturation processes to upregulate surface co-stimulator molecules, adhesion molecules, and chemokine receptors that allow for their migration to secondary draining lymph nodes (Alvarez et al., 2008) (Figure 5).

In the lymph node, APCs stimulate the generation of antigen-specific T cells and antigen-specific B cells that are geared toward recognizing specific viral antigens. APCs present viral antigens in the context MHCII, where naïve $CD4^+$ T cell recognize presented antigens via T cell receptor (TCR) interactions (Figure 5). TCR engagement and costimulatory signals mediated by CD80/86 ligand interaction with CD28 receptors as well as cytokine secretion, result in lymphocyte differentiation and expansion of effector populations (Figure 6). These populations are primarily made up of T helper 1 (T_H1) cells, which characteristically produce IFNγ and and support $CD8^+$ T cell activation, and T helper 2 (T_H2) cells that produce IL-4 and support B cell proliferation. Other helper T cells (T_H17) aid in extracellular pathogen clearance (Fazilleau et al., 2009). Activated helper T cells facilitate antigen-specific B cell proliferation and maturation into antibody-secreting plasma cells. During this process, a small subset of long term antigen-specific T and B memory cells are also generated. These memory cell populations are long lived and ready to respond upon secondary Ad exposure. It is these memory populations that provide rapid protection during subsequent infection.

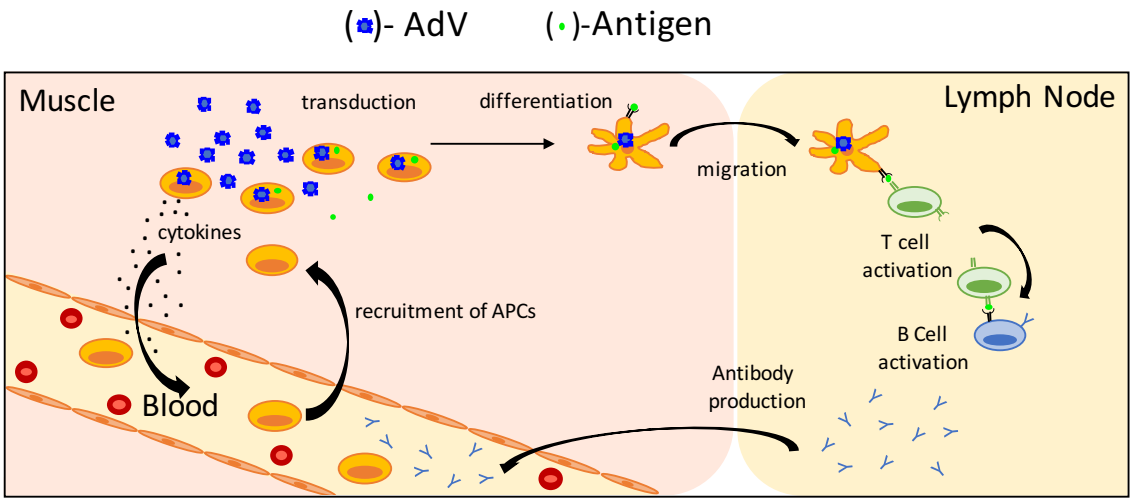


Figure 5. AdV stimulation of Adaptive immunity. AdVs administered through intra-muscular injection are able to enter both the surrounding muscle cells as well as tissue resident antigen presenting cells this leads to a local immune response in which pro-inflammatory cytokines and chemokines are secreted leading to the recruitment of additional Immune cells. Adenovirus containing APCs traffic to the lymph node where their antigen presentation leads to the activation of Both T and B cell responses and in turn the generation of antigen specific T and B cells that can provide protection in the event of exposure to the antigen being vaccinated against. Adapted from (Seubert et al., 2008).

At the site of infection $CD8^+$ T cells recognize MHCI-presented antigen, and upon co-stimulation with CD80/86 and cytokines, produced by $CD4^+$ T cells, mediate effector cytotoxic responses. These cytotoxic responses result in targeted-cell killing of infected cell populations. While Ad stimulates both arms of the adaptive immune response, cellular and humoral, the $CD8^+$ T cell response specifically mediates immunity to intracellular pathogens. The ability of Ad to induce the generation of antigen-specific $CD8^+$ T cells is primarily what makes Advs a desirable vaccine vehicle. Other vaccine approaches often result in stimulation of humoral immunity alone. Generation of antigen-specific $CD8^+$ T cell populations are considered the main correlate of protection in AdV vaccine trials. Therefore, I focus my studies on understanding an aspect of the innate immune response, type I IFN production, that is known to have an effect on the

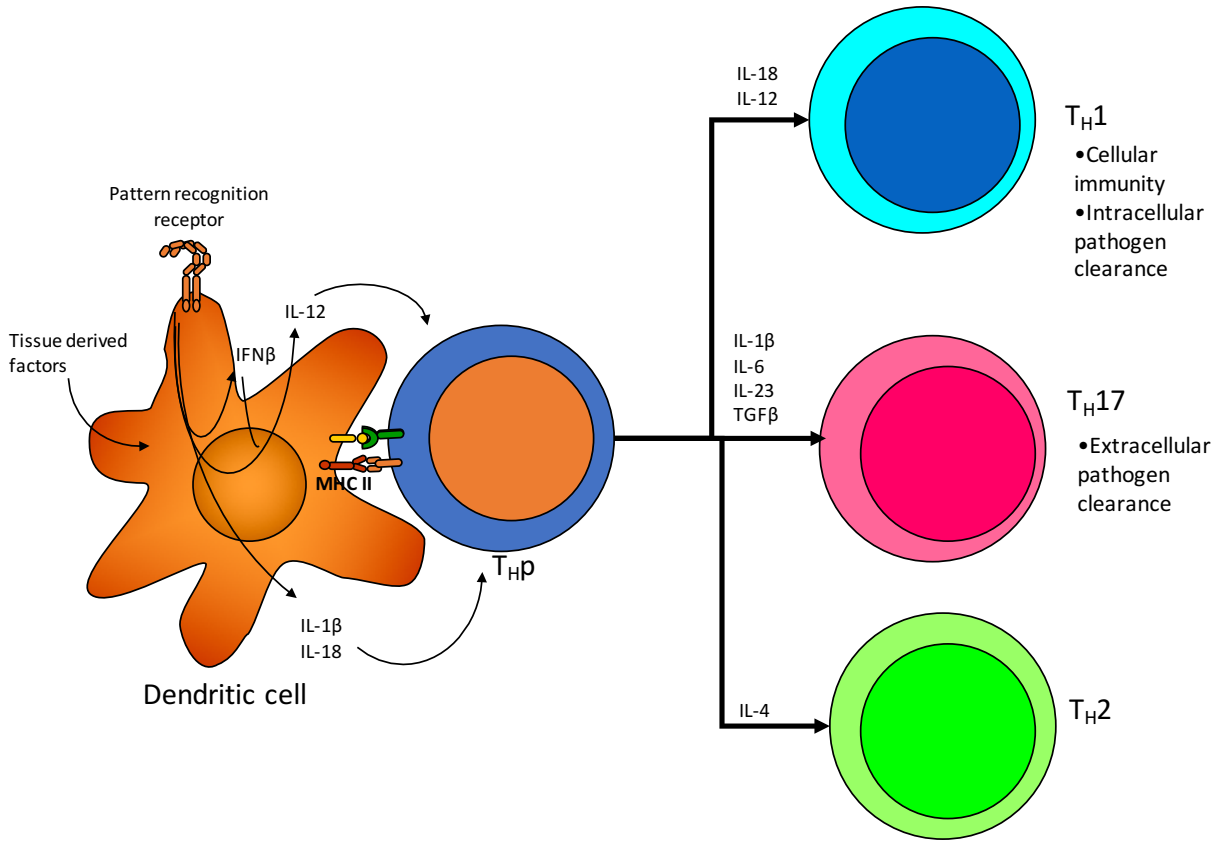


Figure 6. Effect of immune environment on the generation of effector T cell populations Recognition of Ad by PRRs stimulate dendritic cells (APCs) to produce various cytokines. These cytokines, in conjunction with antigen presentation via MHCII molecules can direct the T cell to differentiate into T_H1, T_H2 or T_H17 cells. Il-12 and IL-18 drive differentiation to T_H1 cells. IL-1β derived from inflammasome activation, as well as IL-6, IL-23 and TGF-β drive differentiation to T_H17 cells.

generation of $CD8^+$ T cell populations.

Adenovirus As Vaccine Vectors

Vectors constructed from adenovirus class C, serotype 5 (AdV-5) have been well studied and used to generate recombinant, replication-defective vaccine vectors (AdVs). These vectors are an ideal vaccine platform because they can be easily modified to encode genes or display protein that correspond to different vaccine antigens. Additionally, AdVs can be mass-produced at low cost and they remain relatively stable at room temperature, making it feasible to

use AdVs as a tool for widespread vaccine applications across the world. Ad5 based AdVs (AdV-5) effectively generate strong, transgene-specific immune responses that confer protective immunity in multiple disease models (Johnson et al., 2014; Johnson et al., 2012; Kahl et al., 2010; Teigler et al., 2012; Teigler et al., 2014). This is thought to be due to the inherent viral properties of AdVs, which allow for efficient cellular delivery and stimulation of anti-viral immune responses that help boost the immunogenicity to vaccine antigen transgenes.

However, despite promising results in animal models, the clinical utility of AdV-5 is compromised by pre-existing immunity in the human population. 40-80% of the world's population is seropositive for Ad5 specific antibodies (Kahl et al., 2010). Therefore, newer vaccine strategies focus on using AdVs derived from rarer Ad serotypes. class B and D serotypes are considered rare with Ad class D, serotype 28 (AdV-28) seroprevalence estimated to be less then 10% (Kahl et al., 2010).

Unfortunately, rare AdVs have proven less potent, than AdV-5, at generating transgene-specific immunity (Kahl et al., 2010). While the mechanisms responsible ultimately remain unclear, the superior transgene-specific immunity generated by AdV-5 correlates with higher levels of transgene expression and reduced activation of interferon (IFN) pathways (Johnson et al., 2014; Johnson et al., 2012; Kahl et al., 2010; Teigler et al., 2012; Teigler et al., 2014). Transgene expression following AdV-5 immunization has been linked to the maintenance of $CD8^+$ T-cell populations, with memory populations not sustained when transgene expression is lost prior to 30 days post immunization (Finn et al., 2009). Transgene expression levels could be impacted by AdV factors involved in initial antigen delivery, *de nova* gene expression, or immune mediated vector elimination. While AdV innate immune stimulation and IFN induction

is required to adjuvant transgene-specific adaptive immunity, excessive innate stimulation can lead to anti-viral immune responses that limit vaccine potency (Johnson et al., 2012; Kahl et al., 2010; Teigler et al., 2012; Zhu et al., 2007). Additionally, the high levels of IFN induced by rare serotype AdV may be directly responsible for reduced transgene levels as type I IFN responses have been shown to down-regulate expression from viral promoters (Acsadi et al., 1998; Papadakis et al., 2004). While the different immune profiles induced by various AdV serotypes have been studied, little is known about the mechanisms responsible for these differences and how they ultimately impact vaccine efficacy. My goal is to elucidate how AdV serotype-specific properties contribute to the differences observed in immunogenicity. This will allow for novel AdVs to be generated that stimulate an optimal immune response while avoiding pre-existing antibodies.

The genetic differences between Ad serotypes influence both external components of the viral capsid and internal viral component that are revealed following nuclear delivery. These differences impact tropism, receptor usage, intracellular trafficking, and cellular activation, yet the extent to which precise factors contribute to differences in AdV immunogenicity are not well understood. I chose to focus on how AdV-5 capsid proteins regulate anti-viral host defenses as changes in capsid proteins alone is known to impact the host IFN response to AdVs. I will further discuss the contribution capsid proteins have in host immunity to AdVs in the next section.

Adenovirus-Capsid Immune Regulation

One problem with AdV use is that hosts (i.e., future patients) would become completely immune to any subsequent AdV use, due to the generation of anti-vector neutralizing antibodies

(Roberts et al., 2006). This would make it so there is only a single opportunity to use an AdV vaccine in a patient. There are, however, possible remedies to this problem. AdV neutralizing antibodies primarily target epitopes on the structural capsid proteins, hexon and fiber (Roberts et al., 2006). Pre-existing anti-vector immunity can be overcome by exchanging capsid components between serotypes or using alternative serotypes to create a tool box of different AdVs.

However, capsid modified AdV-5s have failed to stimulate transgene-specific immune responses of the same magnitude as unmodified AdV-5 in the absence of neutralizing antibodies (Roberts et al., 2006; Teigler et al., 2012). Capsid-specific interactions were shown to be partially responsible for the discrepancies in type I IFN level elicited between serotypes (Teigler et al., 2012). However, the *in vivo* mechanism by which different Ad capsid proteins contribute to vector immunity remains unclear, likely due to the large impact structural proteins have on vector delivery and entry in complex tissue environments. Therefore, the ability of different Ad capsid components to influence innate immune activation and the subsequent generation of adaptive immunity warrants further investigation.

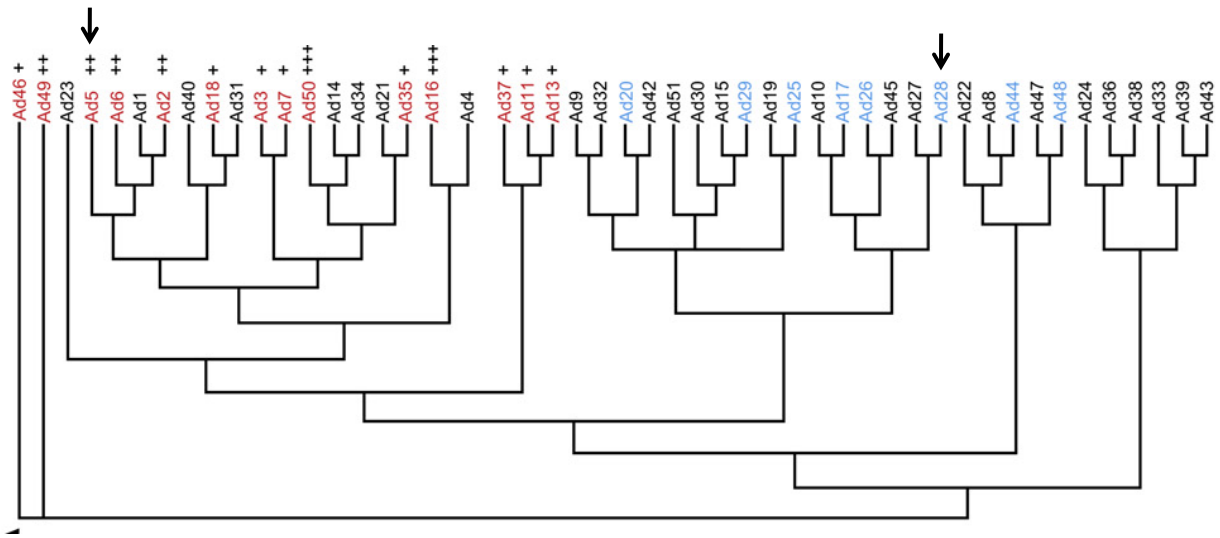
Adenovirus-Serum Factor Interactions

My dissertation research focused on adenovirus interactions with serum factors. Adenovirus serotypes differentially interact with host factors including primary receptors, attachment factors, and serum proteins. Recently, Ad5 virions were demonstrated to bind, multiple members of the vitamin K-dependent protein family including, coagulation (VKD) factors (F) FIV, FX, Protein C, and FVII to bridge interaction with alternative cellular receptors (Alba et al., 2009; Coughlan et al., 2012; Parker et al., 2006; Waddington et al., 2008). Ad-5 interaction with FX has been extensively studied and has been linked to both innate immune

activation and cellular transduction events (Alba et al., 2009; Coughlan et al., 2012; Doronin et al., 2012; Parker et al., 2006; Waddington et al., 2008). FX binding was shown to be serotype-specific, with rare serotypes binding capacity being either dramatically reduced or undetectable by surface plasmon resonance (SPR) (Waddington et al., 2008) (Figure 7A). Cryoelectron microscopy allowing for 23Å resolution enabled identification of the FX γ-carboxyglutamic acid-rich (Gla) domain and Ad-5 hexon hypervariable regions (HVRs) as necessary binding components (Alba et al., 2009). FX is mainly produced in the liver and while AdV-FX interactions must certainly be considered in intravascular (*i.v.*) AdV delivery models, AdV-FX binding capacity has proven inconsequential during intramuscular immunization (*i.m.*), which is the desirable method for vaccine delivery in humans (Waddington et al., 2008). Therefore, differential FX interactions cannot explain the differences seen in the immunogenicity of i.m. delivered AdVs.

However, the role more ubiquitously expressed serum proteins have during AdV immunization remains largely unknown. The Gla domain, bound by AdV-5 during FX interaction, is highly conserved between members of the VDK protein family (Figure 7B). While FX, protein C, FVII, and FIX are known to bind non-enveloped adenovirus virions two others Gas6 and protein S have been shown to bind multiple enveloped viruses (Morizono et al., 2011). Interestingly, Gas6 virion interaction has been demonstrated to bridge interaction with the cellular receptor tyrosine kinases Axl, Tyro3, and Mer (TAMs) leading to enhanced viral uptake or activation of signaling cascades that dampen type I IFN responses during infection (Bhattacharyya et al., 2013; Morizono et al., 2011). Therefore, I chose to investigate the role of

A.



B.

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          **   *.***** ** *. *****:*::  .*  *:  *
 Gas6:  EEAKQGHLERECVEELCSREEAREVFENDPETDYFYPRYLDC
ProtS:  EETKQGNLERECIEELCNKEEAREVFENDPETDYFYPKYLV-
   FX:  EEMKKGHLERECMEETCSYEEAREVFEDSDKTNEFWNKYKDG
 FVII:  EELRPGSLERECKEEQCSFEEAREIFKDAERTKLFWISYSDG
  FIX:  EEFVQGNLERECMEEKCSFEEAREVFENTERTTEFWKQYVDG
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Figure 7. The Gla domain of FX differentially binds adenovirus serotypes and shares sequence homology with the Gla domain of Gas6
(A.) Phylogenetic tree based on the alignment of hexon HVR amino acid sequences performed and described (Waddington et al., 2008) serotypes that bind FX are indicated in red and serotypes that fail to bind FX are indicated in blue. Arrows indicate AdV-5 and AdV-28. **(B.)** Alignment of human Gas6, protein S, FX, FVII and FIX amino acid sequences. The Gla domain is highly conserved across members of the VKD protein family as indicated by star and dot markings. Gas6 and FX Gla domain sequences are highlighted in red for emphasis.

Gas6 and TAM receptors in Ad infection and resulting immunity. I will further discuss how Gas6:TAM signaling occurs and what is known about Gas6-enveloped virion interactions in the following section.

Receptors Tyrosine Kinases Tyro3, Axl, And Mer (TAMs)

The TAM family of receptors including Tyro3, Axl, and Mer, is a unique class of 1 out of 20 subfamilies of receptor tyrosine kinases (Robinson et al., 2000). While for the purpose of this study I will use their most commonly referenced names, all three of these receptors have been referred to through other names as well, Tyro3 is also called (Brt, Dtk, Etk-2, Rek Rse, Sky and Tif,) Axl is also called (Ark, Tyro7, and UF0), and Mer is also called (c-Eyk, Mertk, Nyk, and Tyro12) (van der Meer et al., 2014). TAM receptors are comprised from N' to C'-terminus of 2 immunoglobulin-like (Ig) domains connected to 2 fibronectin type III repeats making up their extracellular domain which is connected to a single-pass transmembrane domain and a cytoplasmic protein tyrosine kinase (van der Meer et al., 2014). TAMs are predominantly expresses in myeloid-derived hematopoietic cells but can also be found in epithelial and endothelial cell types (Tsou et al., 2014b). TAMs are often found overexpressed in cancer cells and are therefore considered an oncogene that is associated with activation of cell survival pathways.

Upon ligand binding, TAM receptor dimerization occurs resulting in trans-autophosphorylation of their kinase domains that in turn results in the activation of complex signaling cascades that mediate diverse cellular functions (Figure 8). TAM activation has been shown to play roles in cell growth, proliferation, survival (Goruppi et al., 1996), efferocytosis (Anderson et al., 2003), migration, differentiation, modulation of immune homeostasis (Angelillo-Scherrer et al., 2005) and regulation of inflammation (Camenisch et al., 1999). These diverse functions are thought to occur via both cell specific and ligand specific interactions. TAM receptors bind to two known host ligands, growth arrest specific protein 6 (Gas6) and

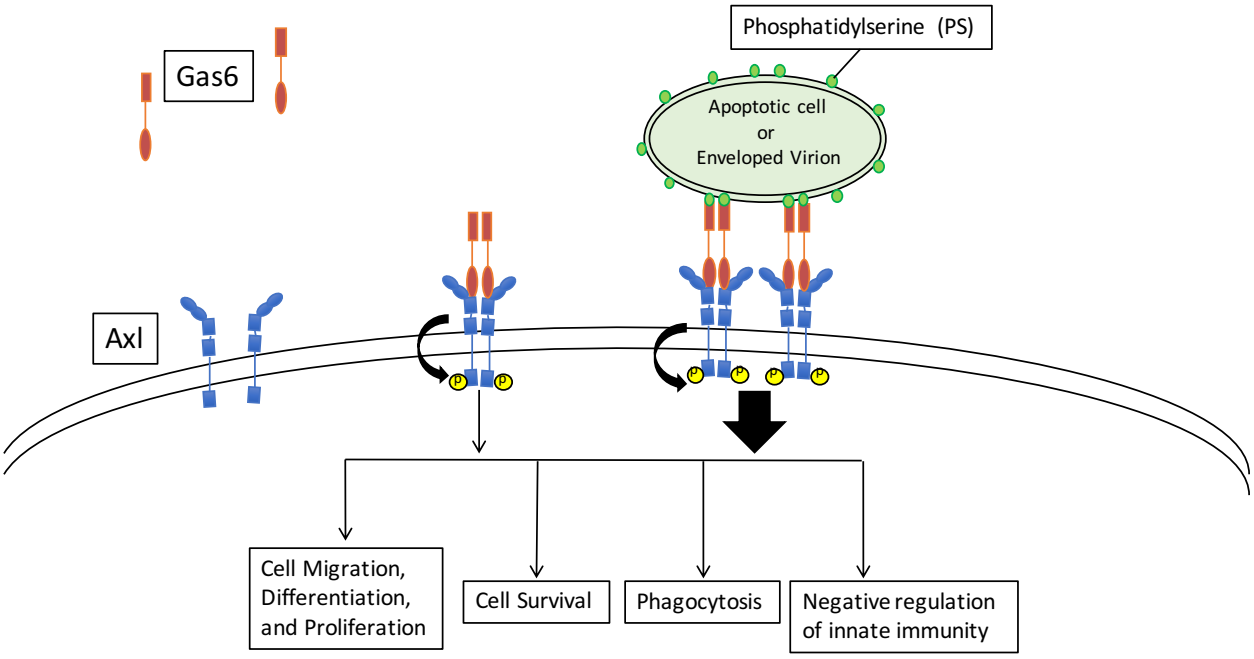

Figure 8. Gas6 activation of cellular TAMs
Free Gas6 ligands can bind cellular TAM receptors either alone or when bound to PS present on the membranes of apoptotic cells or enveloped virions. Gas6 bound TAM receptors oligomerize on the cell surface leading to their auto-phosphorylation and activation of cell signaling cascades that result in a variety of described cell functions. While Gas6 alone can activate TAMs, tripartite binding to PS containing vesicles enhances TAM activation.

protein S. Both Gas6 and protein S are highly conserved between their human and murine homologues but while Gas6 has been demonstrated to bind both human and murine TAM receptors protein S has demonstrated little affinity for human TAMs (Hafizi and Dahlback, 2006). Therefore, I decided to focus my studies on the interactions between TAMs and Gas6.

Gas6 Ligand.

Gas6 is a 75-kDa serum protein that is a member of the vitamin K dependent (VKD) protein family. Gas6 is widely secreted by most tissues including capillary endothelial cells and vascular smooth muscle cells with unregulated expression during injury or inflammation (Lee et al., 1999; Morizono et al., 2011). The structure of Gas6 consists of a glutamic acid rich domain, four

epidermal growth factor-like (EGF) domains, and two lamin G-like (LG) domains (Sasaki et al., 2006) (Figure 9). The Gas6 N-terminal glutamic acid residues are post translationally carboxylated in a vitamin K-dependent reaction to create γ-carboxyglutamic acid. The γ-carboxylation of Gas6 results in an N-terminal Gla domain whereas in the absence vitamin K or exposure to chemicals such as warfarin, that inhibits function of vitamin K epoxide reductase (VKOR), post-translational carboxylation does not occur and Gas6 has an N-terminal glutamic acid (Glu) domain (Rost et al., 2004). The Gla domain has been shown to be required for Gas6 to be considered biologically active, and facilitate activation of cellular TAM receptors (Stenhoff et al., 2004) (Figure 9). However, Gla-less truncated versions of Gas6, that lack Glu or Gla domains, have been shown to still stimulate TAM pathways to some extent (Lew et al., 2014; Zagorska et al., 2014). Gas6 binds TAM receptors with variable affinity Axl > Tyro3 > >Mer, all within the nM range (Nagata et al., 1996). Although a crystal structure of full-length Gas receptor interactions have not been resolved, the use of a truncated Gas6 forms suggests that Gas6:TAM interactions occur via a 2:2 stoichiometry (Sasaki et al., 2006) One Gas6 molecule contains both a high affinity and low affinity binding site in its LG domain for the TAM Ig1 and Ig2 domains respectively. Dimerization of two Gas6 molecules bound to the Ig domains of two separate TAM receptors is thought to drive TAM receptor oligomerization and activation (Meyer et al., 2015). Optimal TAM activation occurs when Gas6 is spatially concentrated at locations on the cell surface. High concentrations of Gas6 alone can prevent receptor oligomerization and actually result in signal inhibition, though to be due to low affinity Ig2 domain binding preventing subsequent high affinity Ig1 domain binding (Meyer et al., 2015). TAM activation is

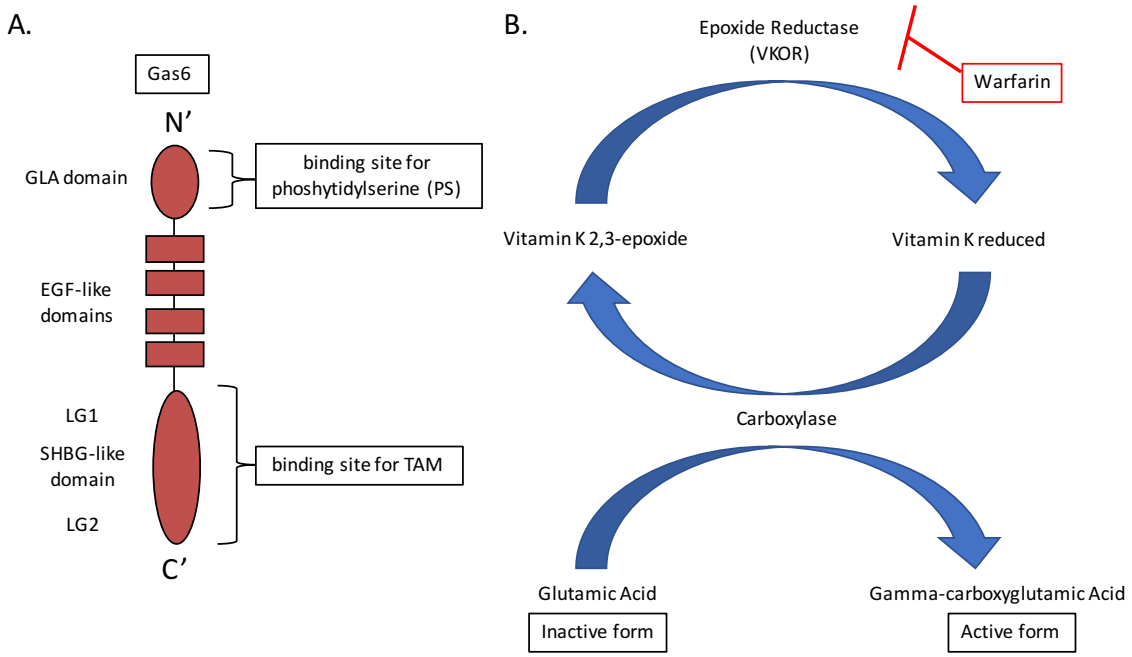

Figure 9. Structure of Gas6 and vitamin K dependent processing
(A.) Domain structure of Gas6 with N' Gla domain serving as the binding site for phosphatidylserine and the LG domains LG1 and LG2 both serving as TAM receptor binding sites. Adapted from (van der Meer et al., 2014) **(B.)** Vitamin-K dependent post translational processing cycle of VDK proteins. This process can be specifically inhibited a VKOR blocking drug Warfarin.

not only influenced by TAM receptor engagement by the Gas6 LG domain but also tripartite Gas6 interactions facilitated by the Gas6 Gla domain, which are thought to aid in concentrating Gas6 at specific cellular locations ideal for increasing receptor avidity-driven interactions.

The Gla domain associates with calcium ions (Ca^{2+}) and mediates Ca^{2+}-dependent binding to negatively charged membrane phospholipids exposed on the surface of apoptotic cells or enveloped virions. This allows for TAM-mediated cellular clearance of apoptotic cells and their debris from the extracellular environment and for a process recently coined "apoptotic mimicry" by which enveloped virions, containing phosphatidylserine (PS) in their lipid bilayer, exploit the TAM signaling process to aid in their uptake and inhibit anti-viral innate immune

responses (Mercer, 2011; Morizono and Chen, 2014; Morizono et al., 2011). Ca^{2+} incorporation and PS binding is believed to cause a conformational change in the GLA domain that is necessary for optimal TAM activation. It is thought that the LG domains of the Gas6 dimer form a V-shaped structure stabilized by the calcium-binding site that help to mediate ligand–receptor interactions (Mark et al., 1996; Sasaki et al., 2002). Gas6 γ-carboxylation has been shown to be essential for full TAM activation and required for the activation of Axl (Tsou et al., 2014b). However, weak activation of Tyro3 and Mer still occurs in the presence of Gas6 ligands that lack a Gla domain entirely, demonstrating that Gla domain interactions are not necessarily required to mediate activation of some TAM receptor pathways that utilize Tyro3 and Mer.

TAM Mediated Immune Regulation.

TAM receptor stimulation can result in diverse cellular functions that depend on ligand-TAM interactions, cell type and cellular environment. While TAM receptors were originally thought to have unique receptor functions, and therefore studied individually, it has become increasingly appreciated that TAM receptors have overlapping functions that are potentially cooperative. All three TAM receptors have previously been implicated in immune homeostasis as loss of function studies result in immune response dysregulation (Rothlin et al., 2007). TAM-receptor knockout mice develop autoimmune diseases. While individual TAM receptor loss is sufficient to render susceptibility to autoimmunity, disease phenotypes are more striking in double knockouts and the most extreme in triple knockouts (Lemke and Lu, 2003; Linger et al., 2008). This autoimmune phenotype is believed to be due to the loss of TAM receptor expression on macrophage and dendritic cells which function to phagocytose apoptotic cells and their debris and control cytokine signaling responses (Lemke and Lu, 2003). In the absence or phagocytosis,

extracellular cell debris accumulates and results in constitutive activation of the immune system. Similarly, in the absence of cytokine response regulation, overactive inflammatory response can be detrimental to host and result in autoimmunity.

TAM mediated clearance of apoptotic cells varies depending on cell type and environment. Macrophage mediate clearance primarily via Mer and to a lesser extent via Axl and Tyro3. Alternatively, dendritic cells mediate clearance primarily via Axl and Tyro3 but independently of Mer (Behrens et al., 2003; Seitz et al., 2007). Cellular environmental cues may also dictate TAM function. Mer mediated phagocytosis seems to function in immunosuppressive environments whereas Axl mediated phagocytosis occurs in response to inflammatory stimulus. As immunosuppressive agents upregulate macrophage Mer expression and inhibit Axl expression while proinflammatory agents conversely upregulate Axl expression and inhibit Mer expression (Zagorska et al., 2014).

TAM receptors are also important for controlling the innate immune response exhibited by APCs, specifically regulating cytokine secretion. TAM knockout dendritic cells demonstrate hyperactivation following stimulation with innate immune TLR agonists (Rothlin et al., 2007). Mer activation has been shown to stimulate the PI3K/Akt pathway to negatively regulate activation of NFκB and decrease production of proinflammatory cytokines, such as TNFα, IL-6, and IL-1, in monocytes, macrophage and dendritic cells stimulated with the TLR4 agonist LPS (Alciato et al., 2010; Sen et al., 2007). Additionally, Axl has been shown to reduced TNFα production following macrophage stimulation with type I IFN by blocking NFκB transcription (Sharif et al., 2006). Additionally, Gas6 treatment of macrophage or dendritic cells following immune stimulation with TLR agonists activates a negative feedback loop that inhibits further

production of proinflammatory and type I IFN cytokines (Bhattacharyya et al., 2013; Morizono et al., 2011).

Gas6 activated TAM receptors hijack the type I IFN receptor (IFNAR) to alternatively induce a STAT1 mediated signaling cascade that induces the expression of suppressor of cytokine signaling proteins (SOCS), SOCS1 and SOCS3 (Rothlin et al., 2007) (Figure 10). SOCS proteins are inhibitors of cytokine signaling pathways and key regulators of macrophage and dendritic cell activation that are essential for T cell development and differentiation (Yoshimura et al., 2007). SOCS proteins have multiple cellular effects that influence immunity. SOC1 can directly interact with the microtubule organizing complex (MTOC) and target proteins including those involved in JAK/STAT cytokine signaling pathway for degradation by the MTOC-associated 20S proteasome (Vuong et al., 2004). Alternatively, both SOCS1 and SOCS3 can associate with JAK kinases and directly inhibit their activity by activating their kinase inhibitory region (Kubo et al., 2003). SOCS1 can also directly bind the type I IFN receptor (IFNAR) which may inhibit IFN signaling (Fenner et al., 2006). Both SOCS1 and SOCS3 proteins have been shown to play a role in DC maturation, differentiation and stimulation and differentiation of T cells (Yoshimura et al., 2007). Therefore, SOCS protein induction by TAMs may not only influence the innate immune profiles that shape adaptive immunity but also directly influence adaptive immune responses themselves.

TAM receptor innate immune regulation is necessary to prevent overactive inflammatory responses during infection. However, enveloped virions have evolved to bind Gas6 to enhance infection by bridging interaction between PS in the viral envelope with the TAM receptors (Morizono et al., 2011). Most studies have focused on enveloped virion binding to Axl receptors

as initial studies found ectopic Axl expression alone to be sufficient to render endothelial cells susceptible to viral entry whereas Mer and Tyro3 expression did not enhance viral entry (Morizono et al., 2011). Axl has been reported to be the cellular receptor for ZIKA virus allowing entry in human glial cells and endothelial cells (Liu et al., 2016; Meertens et al., 2017). Additionally, Tyro3 was show to bind Dengue virus (Meertens et al., 2012) and all three TAMs Axl, Tyro3, and Mer were shown to enhance entry of pseudotyped lentiviruses (Bhattacharyya et al., 2013). While it is agreed that TAMs bind enveloped virions via PS, the role TAMs play in enhancing viral infection remains controversial. TAMs have been attributed to acting solely as attachment factors that aid in cellular attachment and enhance the ability of virions to come in contact with other primary cellular receptors. Others have shown that virion mediated TAM activation inhibits anti-viral type I IFN mediated signaling pathways that limit viral infection, thereby enhancing cell infectivity and replication (Bhattacharyya et al., 2013; Meertens et al., 2017) (Figure 10). TAM binding could influence aspects of cellular attachment, endocytic trafficking and innate immunity. Additionally, the anti-viral state, generated by both pathogen activation and TAM regulation, can prevent cellular uptake by influencing receptor expression, cellular activation, migration, and differentiation. Studies have sought to tease out the role of TAMs during infection using mutant TAM receptors and temporal studies of infection. Use of a catalytically inactive Axl mutant has demonstrated that Axl tyrosine kinase activity is not required for the initial uptake of Dengue particles but is required at to enhance viral proliferation at 48 hours (Meertens et al., 2012). This is further supported by findings that ZIKA cellular binding and initial uptake is independent of Axl kinase activity but kinase signal inhibition via use of kinase dead Axl or treatment with a kinase inhibitor reduced post-entry steps of ZIKA

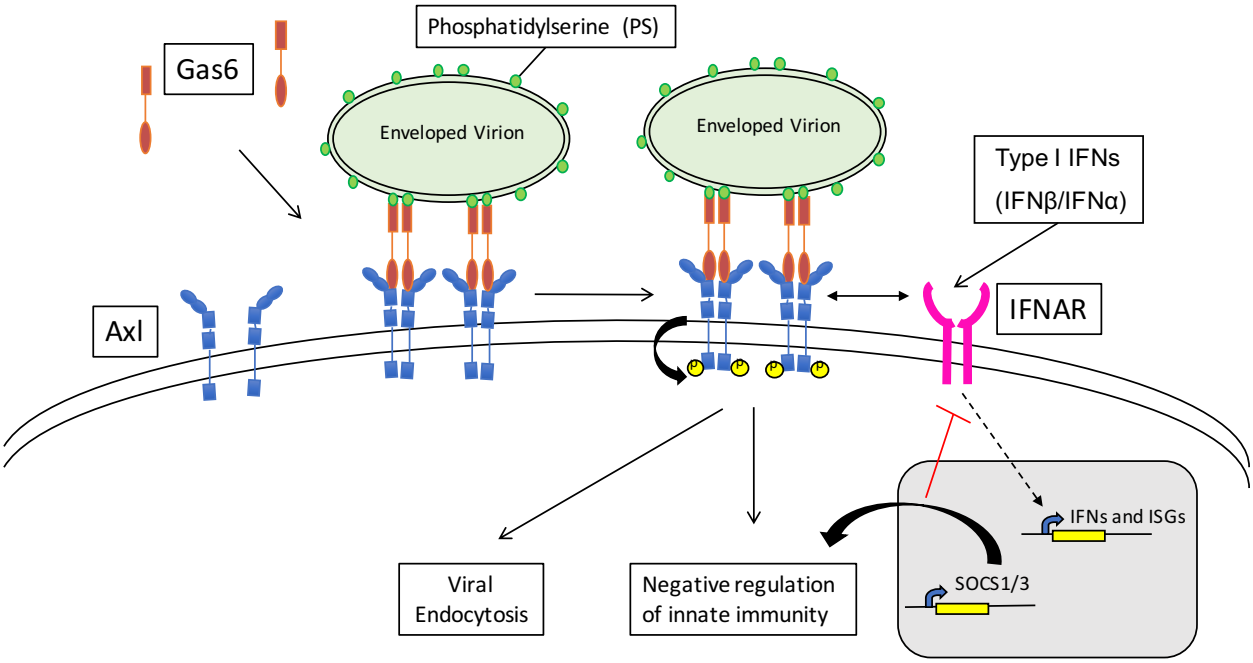

Figure 10. Role of TAM receptors during enveloped viral infection
Enveloped viruses bound to Gas6 mediate exhibit enhanced virus internalization via currently unknown mechanisms. Additionally, Gas6 bound enveloped virions activate TAM receptors and mediate immune suppression. One mechanism by which TAMs regulate immunity is shown. Gas6-enveloped virus complexes activate TAMs and recruit IFNAR. This blocks type I IFNs from inducing a positive feedback loop of cytokine production. TAMs also induce activation of SOCS1/3 expression which inhibits innate antiviral responses. This figure is modified from (Perera-Lecoin et al., 2013).

infection (Meertens et al., 2017). TAM activation can first be detected at the level of phosphorylation and both Mer and Axl tyrosine kinase phosphorylation in response to Gas6 treatment is enhanced in the presence of VSVg-pseudotyped lentivirus (Bhattacharyya et al., 2013). Post-entry roles of TAM all converge, resulting in decreased production of type I IFNs in response to infection despite conflicting reports of SOCS expression.

Currently, our knowledge of Gas6 binding and activation of TAM receptors is restricted to PS-dependent interactions between enveloped virion, liposomes, or apoptotic cells. It is reported that non-enveloped virions do not interact with Gas6 as murine adenovirus (MAV-1)

did not demonstrate detectable binding (Bhattacharyya et al., 2013). MAV-1, like many human adenovirus serotypes, has a distinct viral capsid made up of proteins that are known to differentially interact with other VKD serum proteins. The structural similarities between Gas6 and other VKD serum proteins led us to ask whether Gas6 binds to adenovirus capsids from serotypes known to bind other Gla-domain containing proteins, specifically Ad5. I reasoned that differential Gas6 binding by AdV serotypes may contribute to AdV immunogenicity by regulating host IFN responses.

Purpose of Dissertation

The goal of this proposal is to develop adenovirus vaccine vectors that can bypass prevalent human antibodies and can also elicit appropriate proinflammatory responses, sufficient for durable immunization. This will be accomplished by utilizing an AdV platform that is derived from a "low seroprevalence" serotype, and by identifying the AdV components responsible for differences in vector immunogenicity. Specifically, I focus on identifying the mechanisms by which different Ad serotypes stimulate host immune responses that result in the production of type I IFN. I aim to determine how AdV-5 stimulates IFN responses that are reduced in magnitude when compared to AdV-28. Further I aim to characterize the minimal vector requirements sufficient for the functional discrepancies between AdVs. This will allow genetically modifying rare serotype AdVs to be optimize for use as recombinant vaccines.

CHAPTER II

EXPERIMENTAL METHODS

Cell Lines and Culture

THP-1, A549, Hela, and 293T cells were purchased from ATCC. 293β5, stably expressing human β5 integrin were a gift from Glen Nemerow (The Scripps Research Institute (Smith and Nemerow, 2008)). J774-Dual cells were purchased from InvivoGen. Hela cells stably over-expressing VKORC1 (Hela-VKORC1) were created by co-transfecting 293T cells with pLX304 lentiviral vectors containing the VKORC1 gene which were purchased from DNASU plasmid repository (HsCD00440968) along with the packaging plasmids pHEF-VSVG (cat# 4693, NIH AIDS research and reagent program), pRAV-REV (Addgne #12253) and pMDLg/pRRE (Addgene #12251). Lentivirus produced and isolated from these cells were then used to transduce Hela cells by spinoculation and incubation for 5 hours. Cells were rested for 48 hours, then selected for transgene integration with 5μg/mL blasticidin. Hela cells stably over-expressing VKORC1 and hGas6 were similarly created by generating transducing particles carrying a pBMN-I-GFP vector (addgene #1736) containing the hGas6 gene and transducing Hela-VKORC1 cells. Hela-VKORC1 cells stably expressing hGas6 (Hela-VKORC1-hGas6) were isolated by cell-sorting for GFP positive transductants and maintained by monitoring GFP expression and selecting for blasticidin resistance. THP-1 cells were maintained in RPMI16040 supplemented with 10% fetal bovine serum (FBS), 100IU/ml penicillin, 1mg/ml streptomycin, 0.25 μg/ml amphotericin B, non-essential amino acids, 1mM sodium pyruvate, 10mM HEPES

buffer and 2mM glutamine. A549, Hela, 293T, 293β5, and J774-Dual cells were maintained in Dulbecco modified Eagle medium (DMEM) with the same supplements as RPMI1640.

Virus Preparation

E1, E3-deleted AdV-5 viruses expressing EGFP or firefly luciferase (Luc) were previously described (Wodrich et al., 2010) Ad5f16 chimeric viruses were previously described (Hsu et al., 2005; Rea et al., 2001). El, E3-deleted AdV-28 viruses expressing EGFP were generated by recombineering to insert a shuttle vector pAdAdpt28 containing the EGFP gene into the Ad28 E1/E3-deleted genome. A BAC containing the Ad28 E1/E3-deleted genome and pAdAdpt28 shuttle vector were kind gifts from Dr. Dan Barouch's Lab. EGFP was cloned into the pAdApt28 shuttle vector before being linearized with BstXI and co-transformed into E. coli (SW102) with the Ad28 backbone plasmid. Full genomes were isolated from recombinants and transfected into 293β5 cells. All viruses were propagated in 293β5 cells and purified from cellular lysates by double banding on cesium chloride gradients and dialyzed in 40 nM Tris, 150 mM NaCl, 10% glycerol, and 1mM $MgCl_2$ (pH 8.2) (Wiethoff et al., 2005). Viral concentrations were determined by Bradford assay (Bio-Rad Laboratories, Inc.) and aliquots were flash frozen in liquid nitrogen and stored at -80^{o}C. VSV based pseudovirus particles were produced by methods previously described (Whitt, 2010). Briefly, 293T cells were transfected with plasmids encoding the VSV-glycoprotein (pHEF-VSVG). Two days later, cells were inoculated for 2 hours with VSVΔG-luciferase, rinsed extensively and incubated for one day. Supernatants were collected, centrifuged at 800 x g for 10 min to remove cellular debris, and stored in aliquots at -80^{o}C.

Reagents and Antibodies

hGas6 conditioned media was isolated from Hela-VKORC1-hGas6 cells grown in serum-free media with or without 2uM warfarin for 72 hours. Purified recombinant human Gas6 and recombinant mouse Gas6 were purchased from R&D Systems (885-GSB-050 and 8310-GS-050). Phospho-Axl (D12B2) rabbit mAb was purchased from sell signaling technology (#5724). Axl (M-20/sc-1097) polyclonal goat antibody was purchased from Santa Cruz biotechnology, Inc.

Isolation Of Virion-Gas6 Complexes

100ul AdV-5 ($1.24x10^{11}$ VP) or VSV control was incubated either alone or together with 2μg Gas6 for 30min. at room temperature prior to being layered on top of a step gradient composed of 20%: 40%: 80% Histodenz diluted in HEPES buffer [25mM HEPES, 130mM NaCl, 1mM $mgCl_2$, 1mM $CaCl_2$]. Gradient containing, SW60 tubes were spun at 100,000 x g (30,000 RPM) for 2 hours in Beckman coulter ultracentrifuge. 500μl fractions were collected and assessed for the presence of Gas6 with goat anti-Gas6 antibody (R&D systems, AF885) and donkey anti-goat HRP (R&D systems, HAF109). Pre-spin lanes represent 1:10 of the inoculum ran through the gradient and post-spin lanes represent 1:10 of the inoculum recovered.

Isolation Of Adenovirus Capsid Proteins

Hexon and fiber proteins were purified from AdV-5 infected HEK293 cells as previously described (Smith et al., 2008). Penton base recombinant proteins were generated and purified from E.coli according to methods previously described (Wiethoff et al., 2005).

Immunoblotting

Cells were lysed in a solution containing 25mM Tris, 25mM NaCl, 0.1mM EDTA, 1% triton-100, 0.5% (w/v) NaDeoxycholate, 0.1% (w/v) β-mercaptoethanol and 1mM PMSF, ran on 10 or 15% SDS gels and transferred to PVDF membranes (Millipore) or nitrocellulose membranes (Bio-Rad). Membranes were probed either overnight at 4^{o}C or at room temperature for 1-2 hours in TBST 0.1% or PBST 0.05% with 5% BSA. Mouse and rabbit HRP-conjugated secondary antibodies (Thermo Scientific) or goat HRP-conjugated secondary antibody (R&D systems) were used. Membranes were developed with Dura Super Signal substrates (Thermo Scientific) on FluofChem digital imager.

Dot Blot

Dot Blot assays were performed similarly to previously described methods (Short et al., 2010). In Breif, Polyvinyl pyrrolidon (PVDF) membranes (Bio-Rad) were exposed to virus particles ($4x10^9$ vp/sample) using a dot blot apparatus. The PVDF membrane was washed five times with TBST and then incubated with TBS plus 5% BSA for 30 minutes at room temperature. The membrane was incubated with purified hGas6 (1μg/mL) diluted TBS plus 5% BSA, Gas6 conditioned media, or anti-Ad5 hexon antibody (DSHB, 9c12) for 1 hour at room temperature. The membrane was washed five times with TBST between every incubation. Gas6 incubated samples were incubated with anti-hGas6 antibodies (R&D Systems, AF885) followed by anti-Goat HRP antibodies (R&D Systems). Anti-Ad5 incubated samples were incubate with anti-mouse HRP (Fisher). Membranes were developed with Dura Super Signal substrates (Thermo Scientific) on FluofChem digital imager.

Type I IFN Bioassays

J774-dual reporter cells were stimulated with AdV-5 (3000vp/cell), AdV-28 (3000vp/cell), or Poly I:C (10μg/mL) in the presence or absence (-) of Gas6 at (20,200,2000ng/mL) concentrations for 20 hours. Cell supernatants were collected and luciferase activity was measured with QUANTI-Luc substrate (InvivoGen) on a luminometer and expresses as relative light units (RLU).

Quantification Of IFN-β Secretion By ELISA

Murine Bone Marrow Derived Macrophage (BMDM) cells were a kind gift from Dr. Francis Alonzo. BMDM cells were stimulated with AdV-5 (3000vp/cell), AdV-28 (3000vp/cell), or Poly I:C (10μg/mL) in the presence or absence (-) of Gas6 (200ng/mL) for 20 hours. Cell supernatants were collected and IFNβ levels were measured by ELISA using reagents purchased from R&D Systems (DY8234-05).

Viral Transgene Expression Assays

THP-1 cells were transduced with AdV-5 (3000vp/cell) encoding a luciferase transgene (Ad5V-luc) in the presence or absence of Gas6 (200ng/mL). Cell lysates were collected at indicated time points (2-72 hours) following transduction and luciferase expression was measured with Luciferase assay substrate (Promega) on a luminometer and expresses as relative light units (RLU).

Viral Transduction Assays

THP-1 or J774-dual cells were transduced with AdV-5 (3000vp/cell) encoding a green fluorescent protein (GFP) transgene (Ad5V-GFP) in the presence or absence of Gas6 (200ng/mL). Cells were collected 20 hours following transduction and GFP expression was

measured as a function of GFP florescence by flow cytometery and expresses as mean florescent intensity (MFI).

Statistical Analysis

Statistical significance ($p < 0.05$) was assessed using the Student's t-test whenever two groups were compared. Data are presented as mean ± SEM. Calculations were performed with Microsoft Excel (Microsoft, Inc.)

CHAPTER III

RESULTS

SECTION 1: Gas6 Interacts With the Ad5 Fiber Protein

Gas6 Is Co-Purified Bound To Ad Virions.

To determine if Gas6 interacts with AdV particles, I incubated purified AdV-5 with recombinant, purified Gas6 and separated virus particles from unbound Gas6 using a histodenz step gradient. Fractions were collected at the 40%-80% histodenz interface (1.21-1.43g/mL) where AdV particles band (1.34g/mL) (Tikchonenko et al., 1979). On a parallel gradient, free Gas6 without AdV-5 was centrifuged and the same density fractions were collected. Only the virus-containing fractions contained Gas6 (Fig. 11). As enveloped viruses are known to bind Gas6, I similarly co-incubated VSV with Gas6 and subjected complexes to density gradient centrifugation. VSV: Gas6 complexes were detected at VSV particle densities (1.16g/mL) (McCombs et al., 1966), at the 20%-40% histodenz interface (1.11-1.21g/mL) (Figure 11). These data indicate that Gas6 binds to both enveloped virions (VSV) and to a non-enveloped adenovirus.

It is known that Gas6 binds to enveloped viruses through phosphatidylserine lipids that reside within delimiting virion membranes (Morizono et al., 2011). Since non-enveloped virions by definition lack this lipid layer, I next sought to address how Gas6 interacts with the AdV-5 capsid. Adenovirus capsid exteriors are mainly composed of three major capsid proteins: fiber, penton, and hexon. Therefore, I assessed if Gas6 binding to one of these three proteins occurs.

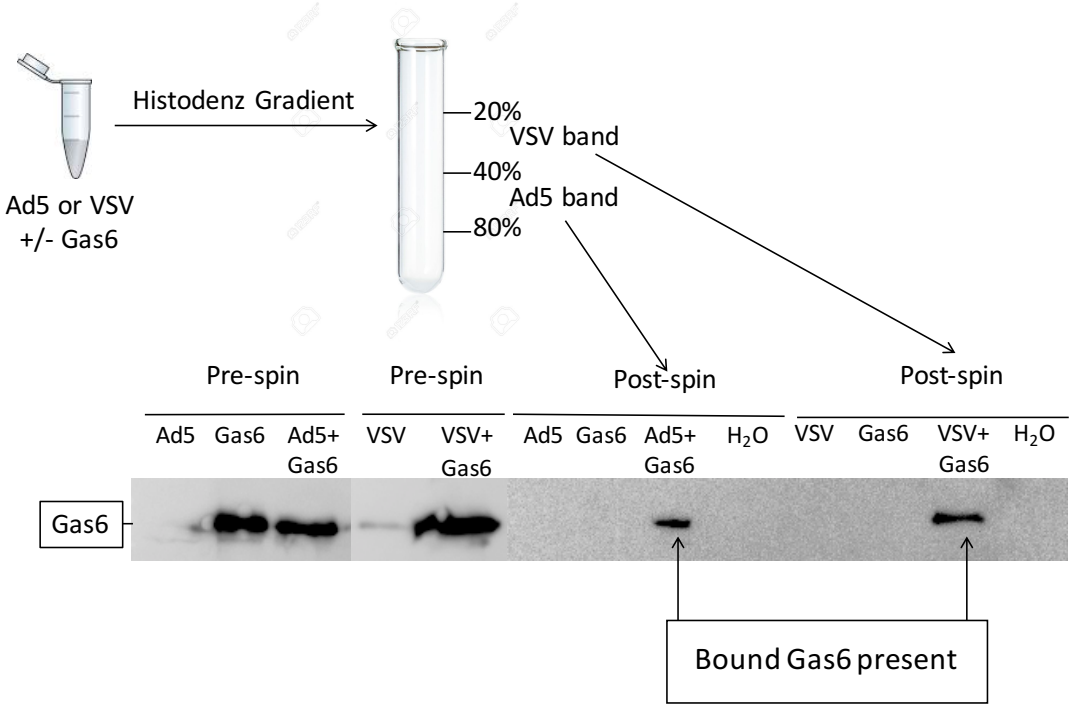

Figure 11. Interaction between AdV and Gas6
Detection of Gas6 in virion density fractions (AdV-5=1.21:1.43g/mL; VSV=1.11-1.21g/mL) isolated from 20/40/80% histodenz step gradient purified samples by Immunoblotting.

Gas6 Interacts With The Ad Fiber Protein.

We individually isolated fiber and hexon proteins through anion exchange chromatography of infected cell lysates, as previously described (Smith et al., 2008). Penton base was expressed in *E. coli* as a 6xHis tagged protein and purified by Ni NTA chromatography (Bai et al., 1993). Each isolated protein had its expected molecular weight in both multimeric and monomeric forms, as indicated by mobilities on a SDS-PAGE gels (Figure 12). To determine whether Gas6 binds to a particular AdV-5 component, I separated either authentic virion or purified capsid proteins on SDS-PAGE gels under either non-reducing or reducing conditions. The proteins were subsequently immobilized onto membranes and incubated with purified Gas6

protein. Using immunoblotting, I detected Gas6 in lanes containing virus or fiber protein but not in lanes containing penton or hexon (Figure 12). Gas6 bound to two major molecular weight proteins ~186kDa and ~62kDa (Figure 12). These sizes correspond to the molecular weights of trimeric and monomeric fiber proteins (van Oostrum and Burnett, 1985). Thus, Gas6 binds both native and denatured forms of the fiber protein.

Alteration Of Ad5 Fiber Protein Eliminates Gas6 Binding To Ad5 Virions.

Adenovirus fiber proteins vary between the 65 human serotypes comprising the seven known adenovirus classes (A-G) (Teigler et al., 2012). Differences in fiber protein dictate receptor specificity and cell tropism (Zhang and Bergelson, 2005). To determine if Gas6 binds to a specific subset of fiber proteins, I made use of a chimeric AdV-5 virus in which the fiber proteins were replaced with those from adenovirus type 16 (Ad5f16). These viruses were captured onto wells of an ELISA plate, Gas6 was overlaid, and bound Gas6 was subsequently detected using anti-Gas6 antibodies. Both AdV-5 and Ad5f16 bound equivalently to the ELISA plate wells, as measured by virion detection using an anti-hexon antibody (Figure 13). However, only the AdV-5 demonstrated measurable Gas6 binding (Figure 13). These data demonstrate that Gas6 binding varies between adenovirus serotypes and requires residues specific to the AdV-5 fiber protein.

SECTION 2: Gas6 Differentially Binds AdV Serotypes

Ad Binding To Gas6 Is Serotype-Specific.

Chimeric Ad5F16 failed to bind Gas6 showing that not all adenovirus serotypes bind Gas6. To determine whether other AdV serotypes bind Gas6, I performed dot-blot binding assays using adenovirus species representative of classes A (Ad-12), B (Ad16), C (Ad5 and Ad2)

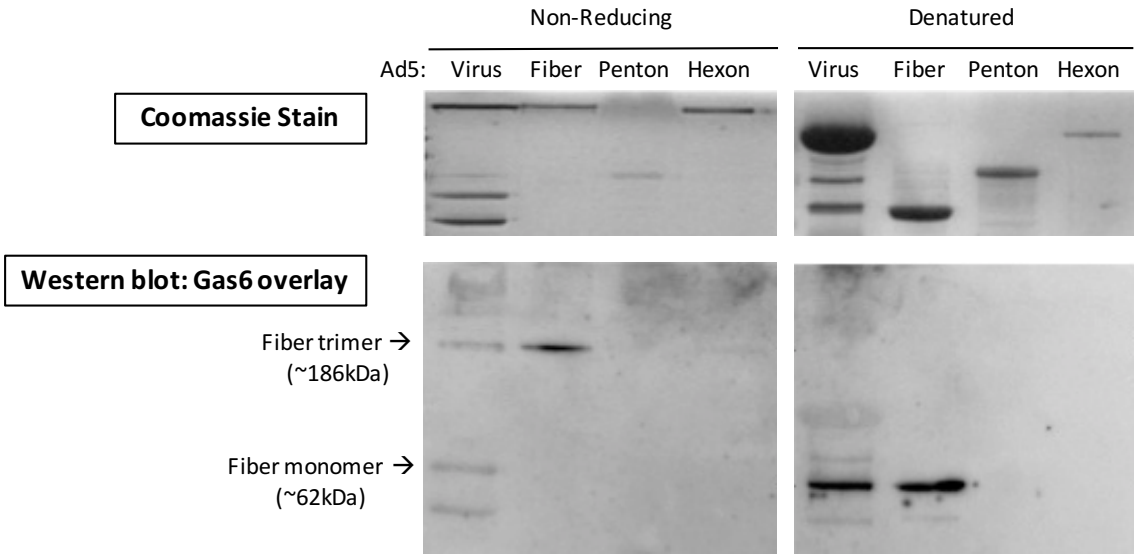

Figure 12. Interaction between Ad capsid proteins and Gas6
Detection of Gas6 bound to AdV-5 capsid proteins, AdV-5 particles or individual capsid proteins were separated by SDS-PAGE under non-reducing or denaturing conditions prior to transfer to nitrocellulose membranes. Membranes were overlaid with Gas6 medium and, bound Gas6 was detected by Immunoblotting. Total protein from each lane corresponds to gels ran in duplicate and stained with Coomassie Blue.

and D (Ad28). While I was able to detect Gas6 bound to class A and C adenoviruses, class B and D adenovirus failed to bind Gas6 to similar levels, suggesting that Gas6 must interact with a common fiber feature found in class A and C adenoviruses (Figure 14).

Ga6 Binding Is Observed Among AdVs That Bind Cellular CAR.

In order to better delineate which Ad fiber features dictate Gas6 binding, I aligned the fiber protein amino acid sequences of the AdVs that I tested for Gas6 binding above (Figure 15). Surprisingly, similarity in amino acid sequence alone did not result in clustering of Gas6 bind Ads (Figure 16). One similarity that did stand out was that Ads that bound Gas6 all bind to the primary cellular receptor CAR and therefore contain a conserved CAR binding domain in their fiber knob.

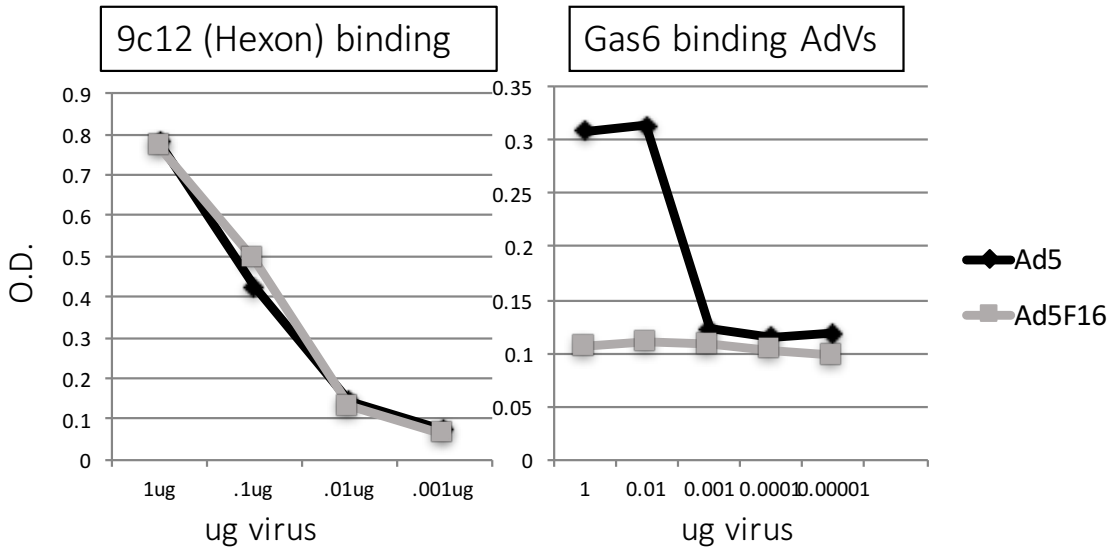

Figure 13. Ad Fiber specific Gas6 binding
Micro titer wells coated with ($1x10^{-5}$-1μg) of AdV-5 or Ad5F16 were overlaid with Gas6 medium. The concentration of AdV particles per well or bound Gas6 was measured by ELISA with antibodies specific to AdV-5 hexon protein (9c12) or Gas6.

Recombinant Ad5 Fiber Knobs Did Not Bind Gas6.

To assess whether residues within the fiber knob of Gas6 binding Ads were sufficient to mediate binding to Gas6, I generating purified recombinant Ad fiber knob proteins and determined their ability to bind Gas6. I was able to successfully purify recombinant fiber knobs corresponding to Ad28 and Ad5 but I was unable to detect Gas6 binding to either (Figure 17). As I previously demonstrated Gas6 binding to Ad5 fibers, my inability to detect binding to Ad5 fiber knobs suggests that residues outside the Ad5 fiber mediate Gas6 binding.

SECTION 3: γ-Carboxylation Of Gas6 Is Required For Adenovirus Binding

Glutamic acid residues within the N-terminal Gla domain of Gas6 are post-translationally γ-carboxylated by a vitamin K-dependent γ-carboxylase. The γ-carboxylated Gla domain enables interaction with phosphatidylserine on apoptotic cells and enveloped virions, which are believed

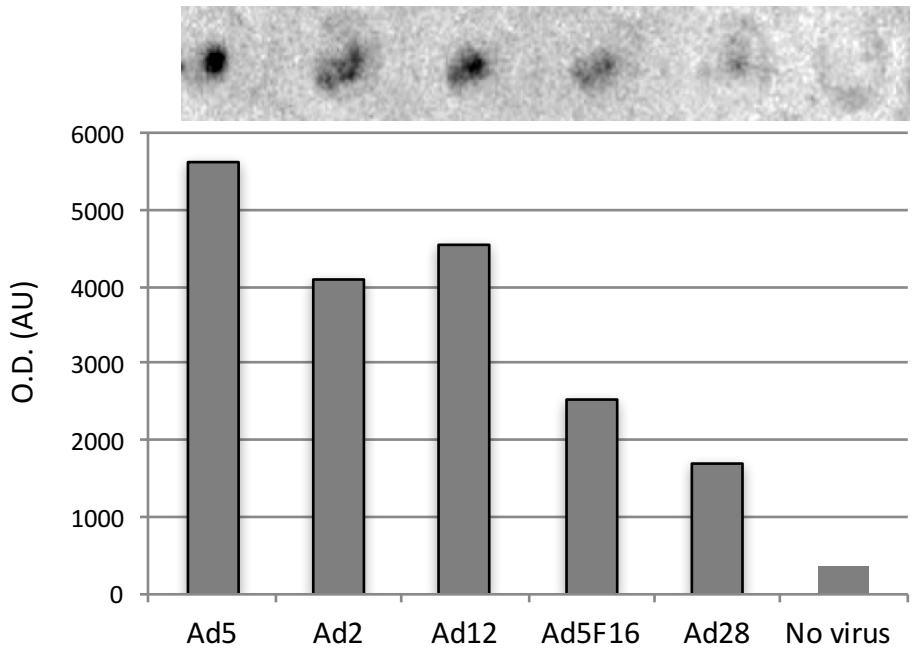

Figure 14. Interactions between diverse AdV serotypes and Gas6
1ug of AdV (AdV-5, AdV-2, AdV-12, Ad5F16 or AdV-28) particles or no treatment control were immobilized on Polyvinyl pyrrolidon (PVDF) membranes and exposed to Gas6 medium using a dot blot apparatus, and followed by Immunoblotting with Abs against hGas6. Blot intensities were measured using ImageJ software with densitometry indicated as optical density (O.D.).

to serve as a nucleation force that facilitates cellular tyrosine receptors Axl, Tyro3, and Mer (TAM) oligomerization and enhanced downstream signaling (Meyer et al., 2015; Tsou et al., 2014a). The Gla domains of other serum proteins have been found to interact with adenovirus capsid proteins (Alba et al., 2009; Coughlan et al., 2012; Parker et al., 2006; Waddington et al., 2008). Therefore, I next sought to determine if γ-carboxylation of Gas6 is necessary for AdV binding. I generated Gas6 in the presence of vitamin K or in the presence of warfarin, the latter blocking vitamin K epoxide reductase dependent γ-carboxylation (Tsou et al., 2014a). The Gas6 I generated in the presence of vitamin K is mostly present in its γ-carboxylated form, as detected with a γ-carboxyglutamic acid specific antibody that only recognizes Gla residues and

Figure 15. Alignment and annotation of Ad fiber protein amino acid sequences
The amino acid sequence corresponding the AdV-5 fiber protein is annotated based of the distinct fiber domains and motifs.

not glutamine (Figure 18). While I generated similar levels of Gas6 in the presence of warfarin, as detected by anti-Gas6 antibody, it was not γ-carboxylated (Figure 18A). I detected virus-bound Gas6 when vitamin K was present during Gas6 production, but not when warfarin was present (Figure 18B). These data demonstrate that Gas6 **γ**-carboxylation is required for adenovirus binding.

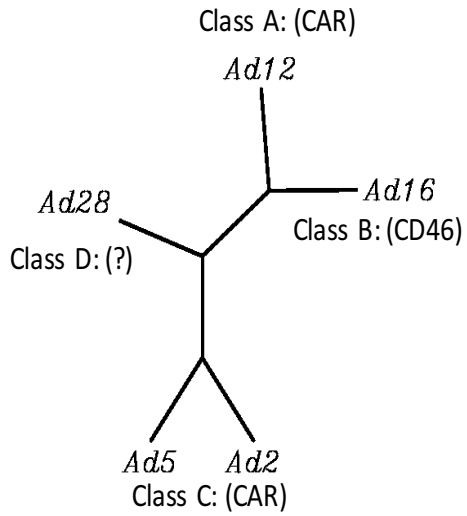

Figure 16. Alignment of fiber protein sequences from representative Ad Serotypes
Phylogenetic tree based on alignment of fiber protein amino acid sequence. Cellular receptor use based on Ad class is noted.

SECTION 4: Gas6 Inhibits the AdV-Induced Innate Immune Response

Gas6 Inhibits the Innate Immune Response To Ad5 And Not Ad28.

Rare serotype-based AdVs such as AdV-28 are desirable vaccine vectors as they are not neutralized by pre-exisiting human antibodies. However, AdV-28 stimulates high levels of type I IFN, which is thought to limit its ability to generate antigen-specific immunity to vector-encoded antigens. As Gas6 inhibits innate immune signaling, including the generation of type I IFNs, I sought to determine whether the high Ad28-mediated IFN response is due to its failure to bind Gas6. Thus, I initially determined whether Gas6 suppresses an AdV-stimulated IFN response. I transduced reporter cells that carry an interferon stimulated response element (ISRE) upstream of a luciferase gene (ISRE-luc) with either AdV-5 or AdV-28, both in the presence or absence of Gas6. I found that Gas6 significantly reduced the virus-induced luciferase reporter gene expression (Figure 19 B-C), implying a Gas6-directed suppression of IFN responses. However,

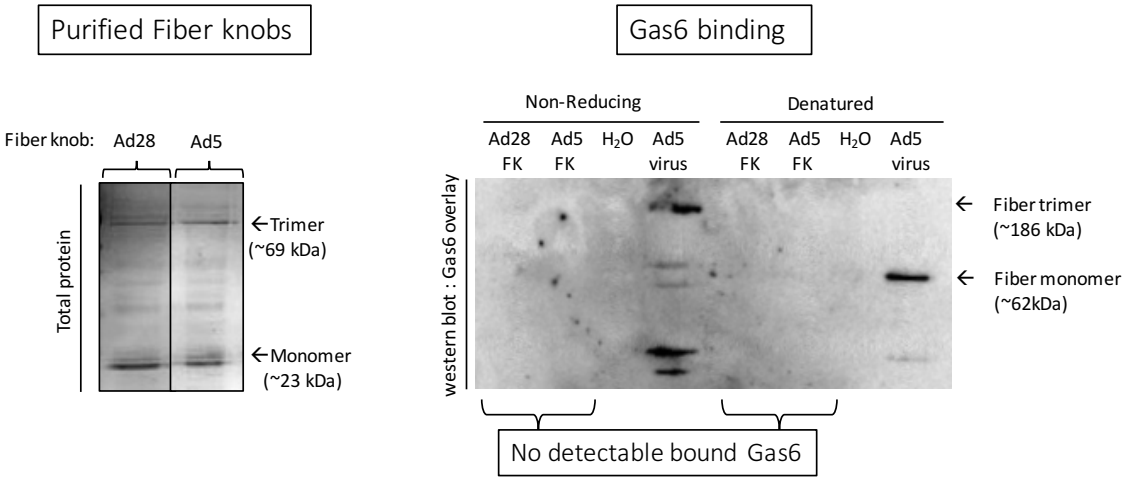

Figure 17. Ad Fiber knob binding of Gas6
1ug of purified recombinant Ad5 or Ad28 fiber knob proteins or authentic AdV-5 virions were separated by SDS-PAGE under non-reducing or denaturing conditions prior to transfer to nitrocellulose membranes. Membranes were overlaid with Gas6 medium and, bound Gas6 was detected by Immunoblotting. Total protein from fiber knob lanes corresponds to gels ran in duplicate and stained with Coomassie Blue.

Gas6 had no effect on the luciferase expression that was stimulated by AdV-28 or a synthetic PolyI:C ligand (Figure 19 B-C). While "free" Gas6 (uncomplexed with viruses or lipid vesicles) has previously been shown to be a pleotropic inhibitor of innate immunity, I did not see that it reduced ISRE-derived luciferase after polyI:C exposures, at least not at the concentrations that were able to suppress ISRE-luciferase expression following AdV-5 transduction (Figure 19 B-C).

Gas6 Directly Inhibits AdV-5 Induced IFNβ Production.

As the ISRE-luciferase reporter assays only indirectly assess type I IFN responses, I sought to directly measure the levels of type I IFN induced by AdV-5 and AdV-5:Gas6 complexes in the context of primary cells. I performed similar experiments using primary bone marrow derived macrophage (BMDM) cells and directly assessed IFNβ levels by ELISA (Figure 19D). Similar to my bioassay results, I saw that Gas6 suppressed AdV-5, but not AdV-28

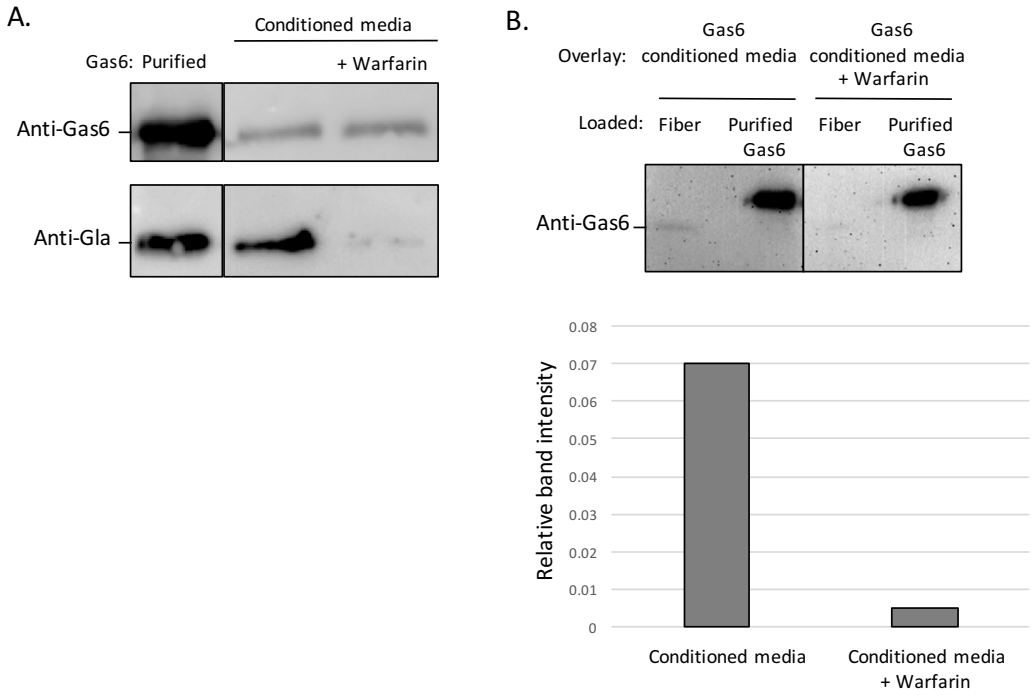

Figure 18. Role of Gas6 Gla domain in Ad5 fiber binding
(A.) Hela cells constructed to stably express vitamin K 2,3-epoxide (VKORC1) and human Gas6 (hGas6) were grown in serum-free media with or without 2μM warfarin for 72 hours. The Conditioned media were collected and resolved by SDS-PAGE along with commercially available purified hGas6 (R&D systems) followed by Immunoblotting with Abs against hGas6 and γ-carboxyglutamic acid (Sekisui Diagnostics). Sample preparations were performed under reducing conditions. **(B.)** 1μg AdV-5 was resolved by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were overlaid with different Gas6 mediums, described above, and followed by Immunoblotting with Abs against hGas6. Band intensities were measured using ImageJ software with densitometry indicated as optical density (O.D.)

induced IFNβ production.

Gas6 Inhibits AdV-5 Induced Immune Responses Without Effecting Entry.

Many enveloped viruses use Gas6 as adaptors to cellular receptors, that enabling viral-cell entry. To determine whether Gas6 increases AdV-cell entry, I transduced J774-dual cells with AdV5-GFP, in the presence or absence of Gas6. At 20 hours post-transduction, both Gas6-

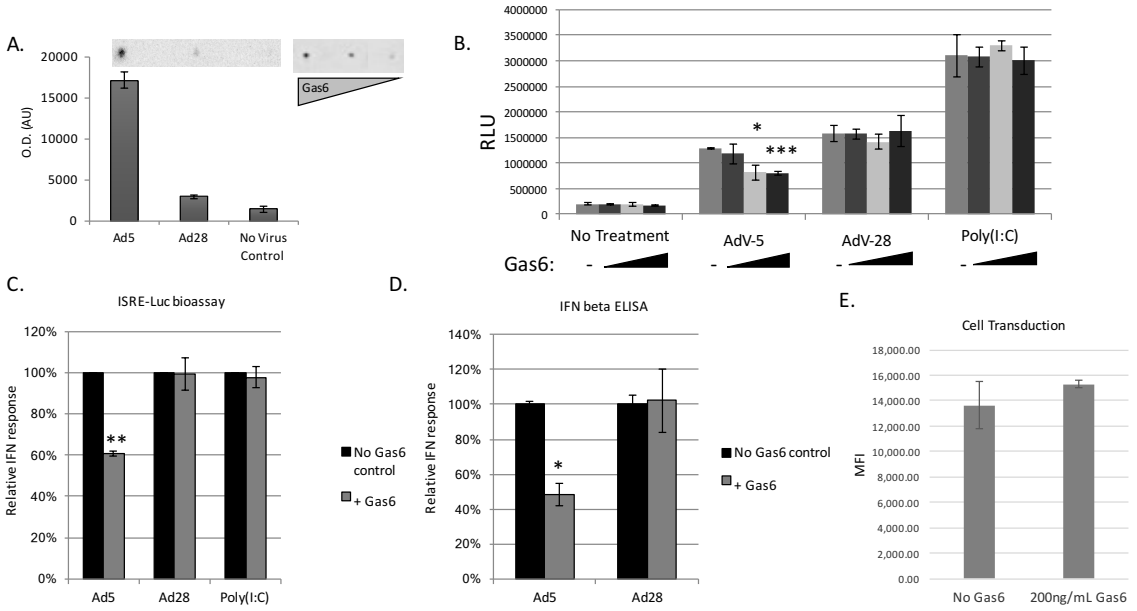

Figure 19. AdV binding of Gas6 correlates with reduced IFN stimulus
(A.) 1μg of AdV (AdV-5 or AdV-28) particles or no treatment control were immobilized on Polyvinyl pyrrolidon (PVDF) membranes and exposed to Gas6 medium using a dot blot apparatus, and followed by Immunoblotting with Abs against. Blot intensities were measured using ImageJ software with densitometry indicated as optical density (O.D.) **(B-C.)** J774-dual reporter cells were stimulated with AdV-5 (3000vp/cell), AdV-28 (3000vp/cell), or Poly I:C (10μg/mL) in the presence or absence (-) of Gas6 (0, 20, 200 or 2000ng/mL) for 20 hours. Cell supernatants were collected and luciferase activity was measured using a luminometer and expresses as relative light units (RLU). **(C.)** Relative IFN response was calculated by normalizing RLU values from 200ng/mL Gas6 treatments to no Gas6 control treatments. Results depict the average and standard error of the mean across three independent experiments performed in triplicate. **(D.)** Murine Bone Marrow Derived Macrophage (BMDM) cells were stimulated with AdV-5 (3000vp/cell), AdV-28 (3000vp/cell), or Poly I:C (10μg/mL) in the presence or absence (-) of Gas6 (200ng/mL) for 20 hours. Cell supernatants were collected and IFNβ levels were measured by ELISA. Relative IFN response was calculated by normalizing pg/mL IFN beta to no Gas6 control treatments. Error bars depict standard error of the mean across three samples. Significant differences between control and Gas6 stimulation are denoted (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). **(E.)** J774-dual cells were transduced with AdV-5 expressing a GFP transgene (AdV5-GFP) at 3000vp/cell in the presence or absence of 200ng/mL Gas6. GFP expression was measured by flow cytometry after 20 hours and the mean fluorescent intensities (MFI) plotted.

treated and control cells exhibited similar GFP transgene expression levels (Figure 19 E). These data suggest that Gas6 affects AdV-5 induced immune responses via a mechanism that does not impede with cellular entry.

SECTION 5: Gas6 Increases Adenovirus Transgene Expression

High levels of type I IFN have been shown to inhibit expression from viral promoters. Therefore, I sought to determine if Gas6 could enhance AdV-5 transgene expression. I transduced cells with either AdV5-luc alone or in the presence of Gas6. Although Gas6 had no effect on the early expression of AdV5-luc encoded luciferase, luciferase expression was markedly increased at late time points (~3days) post transduction (Figure 20). Together these data support a model by which Gas6 binding reduces host IFN responses to AdV and allows for long-term transgene expression.

SECTION 6: Gas6-AdV Complex Activation Of the TAM Receptor Axl

As TAM receptor activation by Gas6 has previously been shown to be potentiated in the presence of possible oligomerizing agents (apoptotic cells and enveloped virions), I sought to determine if AdVs bind Gas6 and dampen IFN responses by enhancing TAM activation. I specifically focused on Axl, as Axl activation is known to be enhanced by enveloped virions and leads to activation of SOCs proteins that disable cellular production of type I interferons via a well understood signaling cascade. To determine if Axl activation is enhanced in the presence of AdV, I treated A549 cells with increasing concentrations of Gas6 in the presence or absence of AdV or VSV control. After 10 minutes, I lysed cells and assessed Axl phosphorylation by immunoblotting using phospho-Axl specific and total Axl specific antibodies. While Gas6 stimulated greater Axl phosphorylation in the presence of VSV, I was unable to detect a

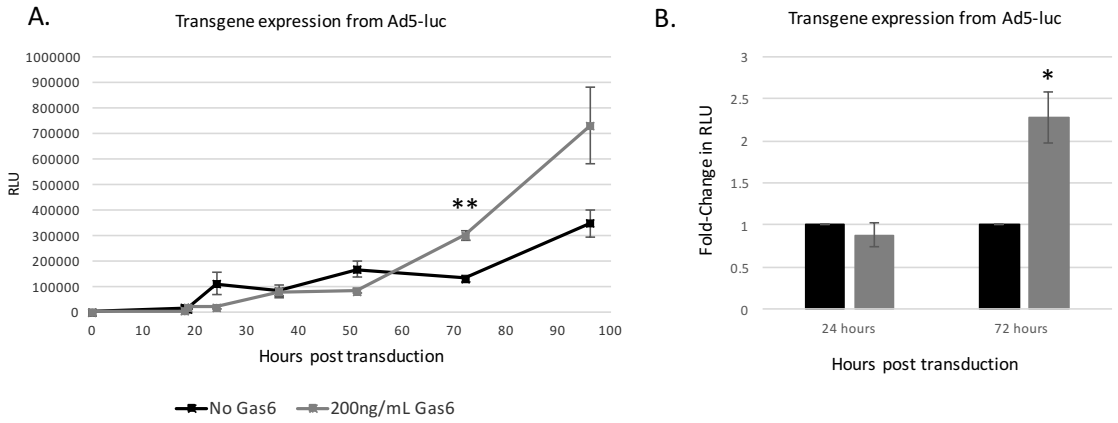

Figure 20. AdV binding of Gas6 correlates with reduced expression of viral transgenes (A-B.) THP-1 cells were transduced with AdV-5 (3000vp/cell) encoding a luciferase transgene (AdV5-luc) in the presence or absence of Gas6 (200ng/mL). Cell lysates were collected at indicated time points (0-96 hours) following transduction and luciferase expression was measured using a luminometer and expressed as relative light units (RLU) **(B.)** Average fold change in RLUs compared to no Gas6 control across three independent experiments each performed in triplicate. Error bars depict standard error of the mean of the averages of three independent experiments. Significant differences between control and Gas6 stimulation are denoted (* = $p < 0.05$, ** = $p < 0.01$).

difference in Axl phosphorylation under these conditions in the presence of AdV (Figure 21). These data suggest that AdV binding may not be enhancing Axl phosphorylation. There are two other distinct TAM receptors Tyro3 and Mer. I suggest that AdV binding could be activating signaling cascades to dampen IFN responses via these alternative receptors. Therefore, Tyro3 and Mer activation status warrants further investigation.

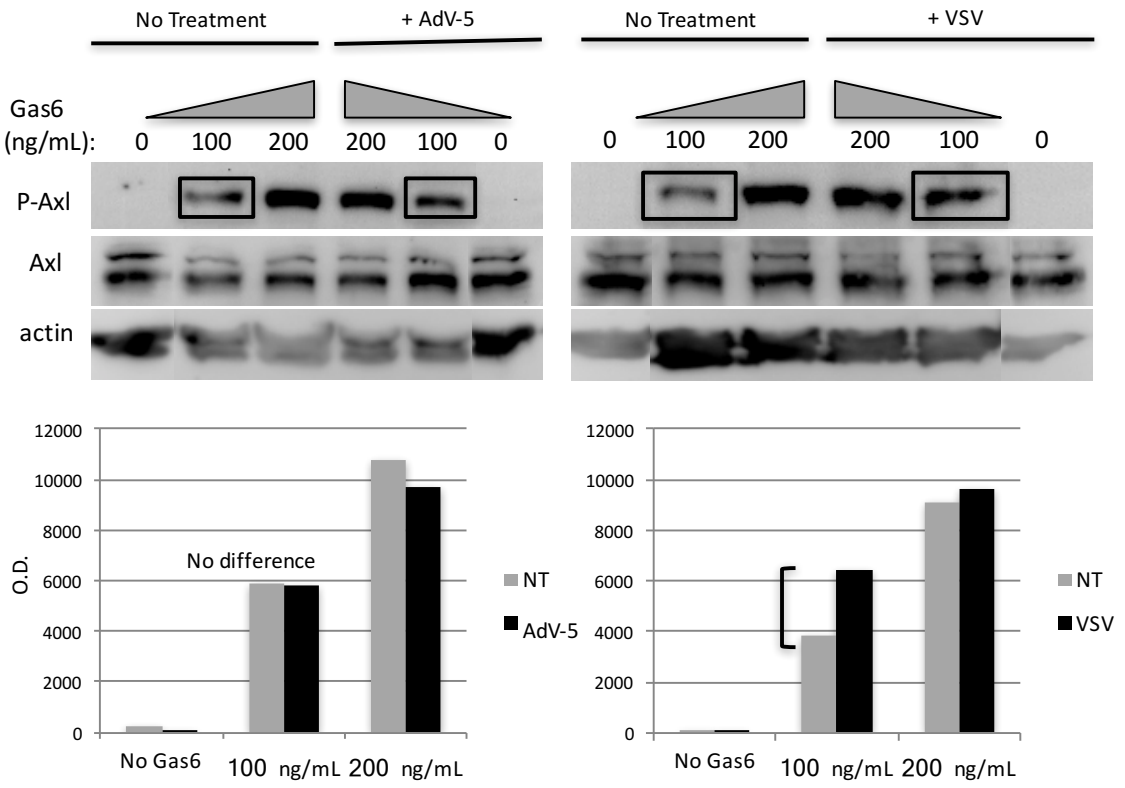

Figure 21. Activation of Axl by Gas6 in the presence or absence of virions
A549 cells were stimulated with Gas6 at the indicated concentration either alone (No Treatment) or concomitantly with AdV-5 or VSV control. After 10 min. cells were lysed and phosphorylated Axl and total Axl was detected by immunoblotting with pAxl and Axl specific antibodies. Band densities were calculated with ImageJ software and indicated as O.D. below blot images.

CHAPTER IV

DISCUSSION

Summary and Significance

I sought to identify and characterize the serotype-specific properties of recombinant adenovirus vectors (AdVs) that contribute to the ability of these vectors to induce potent innate immune responses. These studies will advance our understanding of adenovirus biology and the host immune response. My findings will allow for the optimization of gene transfer vectors. High levels of type I IFN are known to negatively impact the ability of rare serotype-vectors to express antigenic genes and stimulate antigen-specific immune responses. Therefore, I focused my studies on determining how different AdV serotypes stimulate unique innate immune responses that vary in type I IFN production. I found that AdV serotypes differentially interact with a serum protein, Gas6, that is known to negatively regulate innate immunity during viral infection. My studies show that non-enveloped adenoviruses interact with Gas6 in a manner that is mediated by interactions between the Gas6 Gla domain and AdV-5 fiber protein shaft domain. My data highlight a novel mechanism of Gas6 binding that is mediated by viral capsid proteins rather than anionic lipids. This suggests that non-enveloped virions and enveloped virions have co-evolved to utilize Gas6 ligands to subvert anti-vrial immunity. Further, I demonstrate that Gas6 reduces the IFN response stimulated by AdV-5 and enhances AdV-5 encoded transgene expression. My studies suggest that Gas6-fiber interactions contribute to AdV immunogenicity. I reason that rare-serotype based AdVs engineered to mediate Gas6 binding will have enhanced

vaccine efficacy without impacting vector tropism.

AdV Immunogenicity and Gas6

AdVs can be effective, even in the face of population-wide adenovirus seropositivity, by developing chimeras in which common AdV-5 components are replaced with those from other rare adenovirus serotypes. This process overcomes the restrictions by pre-existing antibodies. However, there are yet additional hurdles to overcome. Capsid-modified AdVs fail to stimulate transgene-specific immune responses to the same magnitude as unmodified vectors, and they also stimulate increased levels of anti-viral cytokines (Roberts et al., 2006; Teigler et al., 2012). The mechanism by which capsid proteins impact host cytokine responses and AdV immunogenicity remain largely undefined (Johnson et al., 2012; Teigler et al., 2012), and may be related to ways that capsid modifications influence receptor usage, tropism, intercellular trafficking and cellular activation. Here I provide data arguing that Gas6, an immunomodulatory serum protein, is central to the mechanism by which AdV impacts cytokine responses.

Interactions Between Adenovirus and Gas6

I found that Gas6 interacted with non-enveloped adenoviruses, as demonstrated by Gas6 co-purification with either AdV-5 or VSV particles on density gradients (Figure 11). Further dissection of adenovirus capsids indicated that Gas6 specifically bound the AdV-5 fiber protein within the C-terminal 522 amino acids (Figure 12 and Figure 13). Gas6 binding was found to be serotype-dependent, which may explain why mouse adenovirus-1 did not demonstrate Gas6 binding in previous publications (Bhattacharyya et al., 2013). Of note, one common feature among the adenovirus classes that exhibited binding to Gas6 is the presence of a coxsackie-

adenovirus receptor (CAR) binding domain within the fiber knob. However, I was unable to detect binding of Gas6 to AdV-5 fiber knob proteins (Figure 17). Therefore, I reasoned that Gas6 must be interacting with residues within the fiber shaft domain. This hypothesis is in line with my data that Gas6 binds outside of the adenovirus receptor binding domain, present in the fiber knob, since Gas6 does not influence initial virus transduction of cells (Figure 19E and Figure 20). Although a previous report implicated the fiber protein in binding to the VKD serum protein FIX (Shayakhmetov et al., 2005), more recent reports have concluded that the adenovirus hexon protein mediates binding of Gla-domain containing proteins (Alba et al., 2009; Waddington et al., 2008). While I similarly found that the Gla domain is required for Gas6 binding to AdV-5, my data indicate that Gas6 binds to the fiber protein and not hexon (Figure 12 and Figure 13). Therefore, fiber shaft-Gas6 interactions are occurring via a novel binding mechanism.

I speculate that Gas6 could be interacting with fiber shaft motifs rich in charged amino acids as the Gas6 Gla domain is known to associate with positively charged ions that in turn bind negatively charged phospholipids. I expect these motifs to be conserved between the adenovirus serotypes that I found bound to Gas6 with relatively high affinity: Ad5, Ad2 and Ad12 (Figure 14). One unique fiber motif rich in charged amino acids is the KKTK motif that has been shown to enhance liver tropism of Ads, abide via a controversially unknown mechanism (Rogee et al., 2008). While both Ad5 and Ad2 contain a conserved KKTK motif. Ad12 lacks a KKTK motif and yet still binds Gas6 (Figure 14). Therefore, I suggest that the KKTK motif is not essential for Gas6 binding. Upon alignment of the fiber amino acid sequences no specific regions rich in charged amino acids appears to be uniquely conserved between Ad5, Ad2, and Ad12 (Figure

15). However, I do highlight some area that are richer in charged amino acids compared to non-Gas6 binding serotypes (highlighted in blue; Figure 15). Perhaps these small discrete regions richer in positively charged amino acids facilitate binding to the Gas6 Gla domain. I believe further generation of shaft chimeric AdVs would allow for the shaft residues responsible for Gas6 binding to be further narrowed down and identified. Once the precise mechanisms of this Gas6 interaction are elucidated. Rare serotype based AdVs can be generated that bind Gas6. This will allow for the physiological relevance of Gas6 in AdV immunogenicity to be evaluated through *in vivo* vaccine and gene delivery studies.

Physiological role Gas6 binding has on Adenovirus infection

Future directions should focus on engineering AdV-5 and AdV-28 fiber shaft mutants to assess Gas6 binding and determine the minimal residues required for Gas6 interaction. Once these residues are known it will be possible to assess the role Gas6 binding has *in vivo* for both adenovirus infection and use as a vaccine vehicle. These studies are important to more fully understanding adenovirus biology and host interactions. This knowledge would aid in the design of improved gene delivery and vaccine vehicles. Additionally, these studies would determine if Gas6 is a host factor that influences adenovirus infection and disease. This could potentially highlight Gas6 as a target that could be used for the generation of novel antiviral treatments for adenovirus infection.

While Adenovirus infections are usually self-limiting in healthy individuals they can be life threatening to the elderly and immunocompromised (Gray et al., 2007; Hierholzer, 1992; Leen and Rooney, 2005). Currently, no specific treatments or antiviral therapies exist to combat

adenovirus infection. Therefore, there is a need to identify ways to combat adenovirus infection. I show that Gas6 reduces the host cytokine response to AdVs capable of interacting with Gas6 (Figure 19). Therefore, it is possible that Gas6 binding may represent a mechanism adenoviruses have evolved to combat host immune responses. Gas6 binding may aid in adenovirus infection and subsequent pathogenesis and if so elimination of this interaction could serve as a potential treatment for disease.

In order to determine if Gas6 binding has a physiological role in adenovirus infection. I suggest that *in vivo* infection studies should be done using isogenic strains of adenovirus such as Ad5 that we know binds Gas6 as well as an Ad5 strain minimally genetically modified to eliminate Gas6 binding potential. As classical mouse models are not permissive for infection by human adenoviruses, I suggest these studies to be carried out utilizing either previously described syrian hamster models of infection (Radke et al., 2015; Tollefson et al., 2017) or a humanized mouse model of infection (Rodriguez et al., 2017). Each model has its benefits and drawbacks. Syrian hamster models of infection allow for Ad5 to be administered intranasally or intratracheally. These methods are more representative of the way in which Ad5 would be acquired naturally and results in respiratory symptoms and lung pathology that mimics those observed clinically with Ad5 infection in humans. However, Ad5 is not pathogenic in immunocompetent syrian hamsters (Tollefson et al., 2017). Therefore, this model requires the assessment of alternative Ad strains for Gas6 binding or use of immunocompromised syrian hamsters which eliminate potential effects that Gas6 binding may have on adaptive immunity to be observed. Alternatively, Ad5 is pathogenic in a humanized mouse model but this has only

been observed following intravenous inoculation methods and results in liver pathology. Therefore, future directions may require assessment of Gas6 binding activity during adenovirus infection across multiple model systems to gain a full understanding of its role.

If Gas6 binding has evolved to enhance adenovirus pathology by reducing host cytokine responses during infection, thereby enabling immune evasion, then I expect that adenoviruses unable to bind Gas6 would induce a greater innate cytokine response following infection. This should result in more rapid viral clearance, substantially reduced viral load, and decreased pathology compared to infection with an isogenic stain capable of binding Gas6. However, viruses often utilize multiple approaches to modify the host cell environment to promote viral replication and subvert host immunity. Therefore, other adenovirus proteins, besides the fiber shaft which binds Gas6, could serve redundant functions to inhibit adverse host cytokine responses. If this is the case we may fail to see an effect of Gas6 binding during adenovirus infection *in vivo*.

Wild type adenoviruses contain the E1 and E3 genes that are typically deleted in replication defective adenovirus vectors. These genes are known to encode proteins that function to suppress antiviral host responses. E1 encodes proteins E1A and E1B 55K which both inhibit type I IFN-inducible gene expression (Araujo et al., 2005; Hendrickx et al., 2014). E1 also encodes the protein E1B 19K which inhibits infected cell death and proinflammatory cytokine responses (Radke et al., 2014). Additionally, E1B 55K forms a E3-ubiquitin ligase complex with E4 proteins to target host proteins involved in the DNA damage response and anti-viral signaling for proteasome degradation (Araujo et al., 2005; Hendrickx et al., 2014). E3 proteins are also

involved in immune suppression. For example E3-gp19K block MHC I transport and E3-6.7K blocks NF-κB signaling (Hendrickx et al., 2014). In turn, these proteins act to suppress host cytokine responses that may overlap with the role Gas6 has on immune suppression. This could impede with the ability to detect the effects Gas6 binding may have during wildtype Ad infection *in vivo*.

Replication defective adenovirus vectors that lack E1 and E3 may highlight the immunomodulatory functions of Gas6 binding. I suggest future studies should determine whether AdV-Gas6 interactions contribute to the immunogenicity of AdVs *in vivo* by first generating an AdV-28 based vector that has been modified to promote Gas6 binding (AdV-28G) as well as an AdV-5 based vector that is inversely modified to eliminate its ability to bind Gas6 (AdV-5G). Mice can be immunized with AdV-28, AdV-28G, AdV-5 or AdV-5G, intramuscularly and at 10 days post immunization AdV immunogenicity can be quantified by the generation of antigen specific T-cells, serum IFN levels, and transgene expression in the hind leg and lymph nodes tissues by RT-qPCR. If AdV-Gas6 interactions promote vaccine potency by antagonizing IFN responses then I expect AdV-28G to elicit higher levels of antigen-specific T-cells, reduced IFN levels and increased transgene expression in tissue compared to AdV-28 alone. Additionally, I would expect the immunogenic differences seen in AdV-28G to be inversely mirrored when comparing AdV-5 and AdV-5G.

Alternatively, AdV-Gas6 interactions discovered *in vitro* might not be physiologically relevant in i.m. immunization models. Since AdVs are also under investigation for use in gene therapy, i.v. immunization routes should also be investigated. However, as previously described

other serum proteins such as FX are highly abundance in the bloodstream and may interfere with the observable effects or functions of Gas6. FX binds to the Adenovirus hexon protein and functions to both enhance the transduction of liver cells by bridging interaction with cellular receptors and block antiviral responses by serving as a physical shield which prevents antibody and complement binding (Alba et al., 2009; Coughlan et al., 2012; Doronin et al., 2012; Parker et al., 2006; Waddington et al., 2008). Therefore, the functions of FX could mask the effect of Gas6 antiviral activity or sterically hinder the ability of Gas6 to access its binding residues. The ladder possibility can be assessed by performing similar binding experiments to those shown above in the presence or absence of FX. Alternatively, FX and Gas6 could serve redundant functions in vivo at either the same or in different tissue environments.

Effect Gas6 Has On the Innate Immune Response Induced By AdVs

With pre-existing immunity precluding AdV-5 vaccine vector use, newer rare-serotype based vectors, such as AdV-28, have become desirable candidates for vaccine technologies. However, AdV-28 vectors have proven less potent at providing vaccine immunity, as they stimulate high levels of the anti-viral cytokine type I IFN. Here I found that AdV-28 bound far less Gas6 than did AdV-5. I reasoned that AdV-5 could oligomerize Gas6 ligands, increasing avidity for clustered TAM receptors, to thereby inhibit innate immune signaling pathways and limit type I IFN. This model was previously proposed for Gas6-mediated immunomodulation during enveloped virus infection (Bhattacharyya et al., 2013).

Adenoviruses that fail to bind Gas6, such as Ad28, may be unable to dampen vector-induced anti-viral immune responses and therefore stimulate high levels of type I IFN. In line

with my hypothesis, I found that Gas6 specifically inhibited type I IFN production following AdV-5 transduction but failed to reduce IFN levels following AdV-28 transduction (Figure 19). Thus, when specific Gas6-binding fiber residues are mapped, it should be possible to tailor AdVs for evasion of pre-existing antibodies, and also for specific levels of Gas6 binding and resultant IFN induction. Engineered fiber motifs may promote greater immune responses to vector-expressed vaccine antigens by reducing excessive innate immune stimulation, all without impacting overall vector tropism.

While previous studies have traditionally focused on immune signaling events mediated by the TAM receptor Axl, I was unable to detect increased Axl activation in the presence of AdV-5 (Figure 21). These data suggest that Gas6 mediates immune suppression during AdV-5 transduction via a mechanism independent of Axl activation. However, to fully understand the role TAM receptors have in mediating immune suppression by Gas6:AdV-5 complexes it is necessary to determine if TAMs are required for Gas6 medicated immune suppression during AdV-5 transduction.

I alternatively sought to determine the role of TAM receptors on AdV immunogenicity by generating TAM knockout cell lines. I first attempted to generate a lentiviral vector capable of knocking out all three TAM receptors using the multiplex CRISPR/Cas9-based genome engineering approaches previously described (Kabadi et al., 2014). In short, I generated plasmids containing guide RNA (gRNA) sequences targeted against the individual genes that encode each TAM receptor. Following confirmation of these plasmid constructs I attempted to use restriction enzyme digest and ligation procedures to assemble and insert my plasmids into one lentiviral

construct that contained the Cas9 gene. However, despite multiple attempts I was unable to isolate transformed *E. coli* containing my desired lentiviral plasmid. I originally imaged this genetic approach would streamline cloning procedures and produce a single lentiviral vector capable of efficiently knocking out all three TAMs without the need for transfection of multiple plasmids. However, as my attempts to utilize this engineering platform proved unsuccessful I sought alternative methods to knockout TAM by generating individual lentiviral plasmids that only contain one gRNA and knocking out one TAM receptor at a time. This approach would allow me to both determine if TAMs are required for Gas6: AdV-5 mediated immune suppression as well as determine which TAMs are sufficient for Gas6: AdV-5 immune suppression. As Axl is most commonly associated with immune signaling I first attempted to make Axl knockout (KO) cells. I constructed lentiviral vectors that expressed gRNA targeting the Axl gene, expressing the Cas9 gene, and containing puromycin resistance gene. I transduced THP-1 cells and selected by treated cells with puromycin. However, despite multiple attempts Axl KO always resulted in cell death. This could be due to the fact that Gas6 is also known to signal via Axl to stimulate cellular growth (van der Meer et al., 2014). Therefore, Axl KO cells maybe more likely to undergo cell death. However, as Axl KO mice have previously been generated and described (van der Meer et al., 2014) we believe that further efforts can result in successful generation of Axl KO cells.

I suggest that these efforts should be continued and extended as individual TAM KO cells could reveal the mechanism by which Gas6 immune suppression in the context of adenovirus infection occurs. Alternatively, TAM KO mice could be used to elucidate these mechanisms.

However, in order to confirm if any effects seen in murine models are relevant in human cells it would be important to utilize human TAM KO cells. If CRISPR/Cas9 KO continue to prove problematic I suggest that TAM function could alternatively be impeded by generating knockdown (KD) cells using siRNAs or by blocking TAM function itself by treating cells with blocking antibodies or kinase inhibitors.

I hypothesize that TAM receptor signaling events are responsible for the Gas6 mediated suppression of IFN production to AdVs that I observed. As my preliminary data suggest that Axl activation is not enhanced by AdV oligomerization I hypothesize that either Mer or Tyro3 are primarily responsible for Gas6 mediated immune suppression in the presenc of AdV-5. Mer activation has also been shown to be enhanced by enveloped virions (Bhattacharyya et al., 2013). Therefore, future directions should focus on determining if Mer or Tyro3 phosphorylation is enhanced in the presence of Gas6:AdV complexes compared to free Gas6 alone.

Alternatively, Gas6 binding could be inhibiting IFN production by impeding with host immune recognition of AdV-5 thereby lowering innate immune stimulation. This could occur via similar mechanisms mediated by FX, in which serum proteins coat adenovirus virions and shield them from immune factors. These mechanisms can be elucidated by further studying the temporal events of AdV induced innate immune signaling in the presence or absence of Gas6. If TAM receptor blockade, KD, or KO does not impact Gas6 mediated immune suppression during adenovirus transduction then future directions should focus on utilizing previously described models of Gas6 KO (van der Meer et al., 2014) to determine the role of Gas6 on AdV immunogenicity and adenovirus infection. However, the studies presented here primarily focus

on understanding how IFN modulation by Gas6 binding impacts the ability of AdVs to express viral encoded genes following cellular transduction. Long-term gene expression from AdVs is essential for generating protective immunity to vaccine antigens and for delivery of essential genes in gene therapy applications. I discuss my efforts to determine the effect of Gas6 on AdV gene expression in the following section.

Effect Of Gas6 During AdV Transduction

The superior transgene-specific immunity generated by AdV-5 not only correlates with reduced activation of IFN pathways but also with higher levels of transgene expression (Johnson et al., 2012). I sought to determine if the reduced innate immune responses that were mediated by Gas6 also correlate with increased AdV-5 encoded transgene expressions. I found that while Gas6 had no effect on early gene expression from AdV-5, there were Gas6-associated increases in expression at late time points post transduction (~3days) (Figure 20). Together these data support a mechanism by which Gas6 binding to adenovirus fiber proteins reduces type I IFN induction and enhances long-term transgene expression.

This increased gene expression could be due to Gas6 enhancing aspects of cell survival, differentiation, or directly enhancing expression from viral promoters following AdV transduction. Visual assessment of our cell cultures did not reveal any changes in cellular phenotype, indicative macrophage differentiation, or differences in cell number, indicative of cell survival. Therefore, I suggest that Gas6 is altering viral gene expression by suppressing production of type I IFNs that directly inhibit transcription of viral genes. This is in line with other studies that show that high levels of IFN directly down-regulate gene expression from viral

promoters (Acsadi et al., 1998; Papadakis et al., 2004). However, my *in vitro* results do not take into account aspects of the immune environment *in vivo* where type I IFNs regulate the function and interplay of multiple cell types. Others have shown that the high levels of type I IFN induced by rare AdV serotypes activate NK cell mediated clearance of vector containing cells and stimulate DC maturation processes that prevent generation of antigen-specific $CD8^+$ T cells (Johnson et al., 2014; Johnson et al., 2012). Thereby, the effects that I report Gas6 having on AdV gene expression are likely an underestimate of the cumulative effect Gas6 has on AdV gene expression and immunogenicity *in vivo*.

Implication Gas6 Binding Has On AdV Vaccines Applications

I suggest that Gas6-fiber interactions contribute to AdV immunogenicity by lowering the magnitude of the type I IFN response to AdVs. I reason that rare-serotype based AdVs engineered to mediate Gas6 binding will have enhanced vaccine efficacy. Gas6 binding rare-serotype based AdVs should bypass issues of neutralization from preexisting antibodies and stimulate an optimal IFN response that is sufficient to adjuvant immunity to the antigenic genes encoded by the vector while preventing excessive IFN responses that inhibit antigenic gene expression and persistence. As type I IFNs can influence the expression of hundreds of downstream genes that further augment immunity these new vectors should elicit unique immune profiles. Future directions should focus on investigating how Gas6 impacts other immune functions related to rare serotype based AdVs such as, the production of other proinflammatory cytokines (e.g. IL-6, IL-1, and TNFα) or activation and maturation of DCs. This knowledge will

enhance our understanding of how different AdVs stimulate diverse immune responses and how those responses impact immunogenicity.

Additionally, since Gas6 binding most likely occurs via residues present in the fiber protein shaft domain rare-vectors engineered to bind Gas6 will retain their ability to bind to their primary cellular receptors. This is important as it will allow for aspects of AdV tropism and its relevance to immunogenicity to dissected independently of the effects of Gas6 on immune regulation. While the role CD46 binding has on conferring differences in AdV induced IFN responses has been controversial, my data are in agreement with findings that class B and D adenoviruses stimulate higher cytokine levels compared to AdV-5 via a CD46-independent mechanism (Kahl et al., 2010). Further, my data suggest a potential mechanism in which class B and D adenoviruses stimulate higher levels of type I IFN due to an inability to bind Gas6 and regulate IFN production (Figure 14 and Figure 19).

Implication Gas6 Binding Has On AdV Gene Therapy Applications

The ability to generate rare AdVs with reduced type I IFN responses will also allow for the potential design of improved gene therapy vectors. Currently, AdVs stimulate immune responses that can be detrimental in immunocompromised patients. If we can inhibit immune stimulation during AdV delivery by enhancing interaction with Gas6 during treatment or by pre-treating with TAM activating Gas6-complexes we may be able to prevent induction of harmful immune responses and effectively deliver AdV encoded genes. Additionally, the broad tropism of AdVs is one aspects that makes them highly desirable as gene delivery vehicles. I have defined residues of the fiber protein that should not impact vector tropism. This will allow for

AdVs to be generated that still maintain their unique serotype-specific tropism that could aid in directing gene delivery to specific cell types. Class B and D adenoviruses uniquely have increased tropism for immune cells (macrophage and DCs) which can be maintained even with the modification to Gas6 binding sites that I suggest. Alternatively, continued investigation of how Gas6-AdV binding bridges interaction with TAM receptors could lead to the development of AdVs that have altered tropism for TAM receptor expressing cells.

Conclusions

In conclusion, my studies have shown that non-enveloped adenoviruses interact with Gas6 in a serotype-specific manner that is mediated by interactions between the Gas6 Gla domain and Ad fiber protein shaft domain. I show that Gas6 reduces the IFN response stimulated by AdV-5 vectors and enhances AdV-5 vector encoded transgene expression. I suggest that Gas6-fiber interactions contribute to AdV immunogenicity. I reason that rare-serotype based AdVs engineered to mediate Gas6 binding will have enhanced vaccine efficacy without impacting vector tropism.

APPENDIX A

ROLE OF E4ORF3 IN ADENOVIRUS VECTOR IMMUNOGENICITY

This dissertation focuses primarily on identifying the mechanisms responsible for the differences in immunogenicity between AdV-5 and AdV-28. As previously discussed, many different factors could contribute to serotype-specific differences in immunogenicity. External virion structural proteins influence many aspects of AdV function including: vector tropism, endosomal trafficking, nuclear delivery, and cellular activation. Additionally, viral gene products expressed following transduction can impact downstream viral gene expression and restriction of host immune responses. Therefore, differences in either external or internal virion components between AdV serotype could be responsible for their variances in immunogenicity. Additionally, multiple factors, both external and internal, could ultimately be contributing to the unique immune profiles induced by different AdV serotypes. While I previously discuss how the Gas6 binding potential of AdV-5 fiber proteins contribute to the favorable restriction of adverse host immune responses and prolonged viral gene expression I also investigated other potential mechanisms by which virally expressed gene products could contribute to vector immunogenicity. My preliminary findings suggest that differences in the adenovirus gene product of early region 4 open reading frame 3 (*E4orf3*) may also contribute to the superior immunogenicity elicited by AdV-5 vectors compared to AdV-28. I further discuss the function of E4ORF3 and its potential role in vector immunogenicity below.

The early region 4 (*E4*) of adenovirus is essential for efficient virus production and encodes six gene products, E4 open reading frame (ORF) 1-6 (Stracker et al., 2005). E4ORF3, a protein expressed early after adenovirus entry, is required for prolonged AdV-mediated transgene expression (Lusky et al., 1999; Vink et al., 2015). Interestingly, of the E4 gene products, E4ORF3 is the only one shown to enhance the longevity of transgene expression from

AdVs *in vivo* (Lusky et al., 1999; Thomas et al., 2013). E4ORF3 is a small 116-residue (13 kDa) protein that self assembles to form a complex nuclear polymer capable of binding and relocalizing host proteins to promote viral replication (Ou et al., 2012; Vink et al., 2015). Specifically, E4ORF3 sequestration disrupts host DNA damage and interferon-mediated antiviral response pathways (Lusky et al., 1999; Stracker et al., 2005; Vink et al., 2015). For example, Ad5 E4ORF3 is responsible for the sequestration and disruption of the MRN complex and PML bodies during infection (Ou et al., 2012). The generation of transgene specific immunity following AdV immunization correlates with both the duration of transgene expression following vector transduction (Johnson et al., 2014) and innate cytokine induction (Kahl et al., 2010; Teigler et al., 2012). As both viral gene expression and innate immunity are impacted by the function of E4ORF3, I reason that difference in E4ORF3 function between serotypes could directly impact AdV immunogenicity.

While E4ORF3 is one of the most highly conserved Ad proteins, the reorganization of specific host proteins has been shown to be serotype-specific with AdV-5 E4ORF3 capable of host-protein interactions lacking in other serotypes (Evans and Hearing, 2003; Forrester et al., 2012; Stracker et al., 2005). However, the extent to which E4ORF3 functionally differs across Ad species remains largely undefined as well as the impact serotype-specific interactions have on AdV immunogenicity. I hypothesize that species-specific AdV-5 E4ORF3 host protein interactions promote vaccine potency by enhancing viral gene expression and antagonizing anti-viral immunity.

To determine whether serotype-specific E4ORF3 functions impact the immunogenic differences elicited by AdV-5 or AdV-28 administration, I first generated a chimeric AdV-28

that has its *E4orf3* gene replaced with the *E4orf3* sequence of AdV-5 (AdV28-543). By encoding a GFP transgene into AdV-5, AdV-28, and AdV28-543 I am able to monitor infectivity *in vitro* as a function of fluorescence as well as assess immunogenicity *in vivo* as a function of GFP specific cellular responses. Preliminary observations suggest that Ad-5 E4ORF3 enhances AdV replication in E1 complementing cell lines with AdV28-543 infection demonstrating more rapid plaque growth compared to AdV-28 (Figure 22A-B). These data show that differences in E4ORF3 between serotypes can contribute to differences during AdV infection.

I next sought to determine whether E4ORF3 contributes to AdV immunogenicity *in vivo*. I immunized Balb/C mice intramuscularly (i.m.) with $2x10^7$ infectious units (IFU) of AdV-5, AdV-28, or AdV28-543 and assessed antigen specific T-cell activity, a classic indicator or AdV potency. Splenocytes were obtained from mice 10 days post immunization and cultured in the presence of recombinant purified GFP. Supernatants collected 24 hours later were pooled and antigen-specific T-cell activity, measured as a function of IFN-γ production, quantified by ELISA. Splenocytes from AdV28-543 immunized mice elicited increased production of the T-cell cytokine IFN-γ in response to GFP compared to splenocytes collected from AdV-28 immunizations (Figure 22C). These data support our hypothesis that E4ORF3 differences contribute to AdV immunogenicity. Unexpectedly, splenocytes from AdV28-543 immunized mice produced more IFN-γ then those from AdV-5 immunized mice (Figure 22C). This could suggest that other virion factors contribute to AdV induced antigen-specific T-cell activity.

Future generation of a reverse chimera AdV-5 encoding Ad28 E4ORF3 (AdV5-2843) would be useful to confirm our observed phenotypes. I would expect splenocytes from AdV28-543 immunized mice to elicit higher IFN- γ production in response to antigen compared to

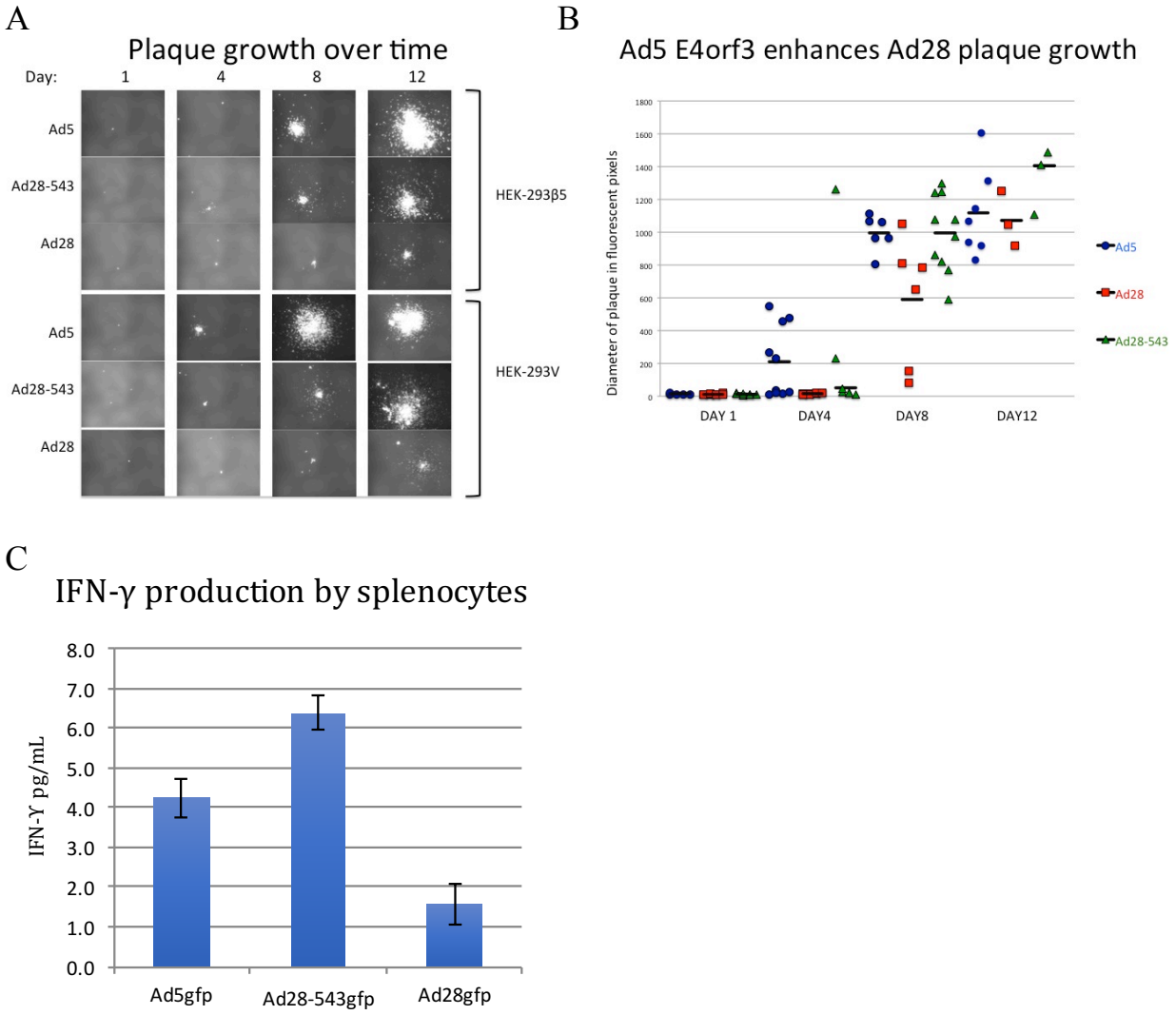

Figure 22. Ad5 E4orf3 expression enhances AdV-28 based vectors.
(A) Plaque growth on both HEK-293β5 and HEK-293V cells infected with AdV-5, AdV-28, or AdV28-543 **(B)** Quantification of average plaque diameters over time from experiments in A. **(C)** IFN-γ production by splenocytes obtained from mice 10 days post immunization of $2x10^7$ IFU of either AdV-5, AdV-28, or AdV28-543 after 24-hour stimulation of recombinant GFP.

splenocytes from AdV-28 immunized mice. Additionally, I expect the effect that Ad-5 E4ORF3 has on the immunogenicity of AdV-28 to be inversely mirrored in AdV5-2843. Results from

these experiments will determine if E4ORF3 differences impact the *in vivo* immunogenicity of AdVs.

While my preliminary results suggesting a serotype-specific E4ORF3 function capable of generating AdV immunogenic variance warrant further investigation. I further sought to determine how different E4ORF3 proteins could be capable of facilitating serotype specific host protein interactions and immune evasion. Alignment of the amino acid sequences of E4ORF3 proteins highlights a two amino acid difference in AdV-28 E4ORF3 compared to AdV-5, where residue 101 is changed from a valine to a cysteine and residue 104 is changed from an isoleucine to an arginine (Figure 23A). This small change appears to slightly alter protein structure (Figure 23A). Interestingly, these residues were previously determined to be important in Ad5 E4ORF3 interactions with host proteins involved in the DNA Damage Response (Ou et al., 2012). Specifically, these residues are part of an emergent MRN binding interface, V^{101}-D^{105}, created by higher order assembly of E4ORF3 dimers (Ou et al., 2012). Conversely, resides shown to be important for self-assembly remain conserved between Ad5 E4ORF3 and the E4ORF3 proteins from rare serotypes Ad28 and ChadOX-1 (a chimpanzee adenovirus also associated with reduced immunogenicity as a AdV). Therefore, I predict Ad28 E4ORF3 is capable of polymer formation but that polymer assembly fails to generate the same host-protein binding sites as Ad5 E4ORF3. In order to determine whether E4ORF3 proteins from Ad28 and ChadOX-1 sufficiently assemble into nuclear polymers. I transfected cells with plasmids encoding HA-tagged E4ORF3 from Ad-5, Ad-28, or ChadOX-1 and visualized nuclear polymers by IFA. All E4ORF3 proteins successfully formed a nuclear polymer, which supports further investigation of differences in emergent binding site generation (Figure 23B).

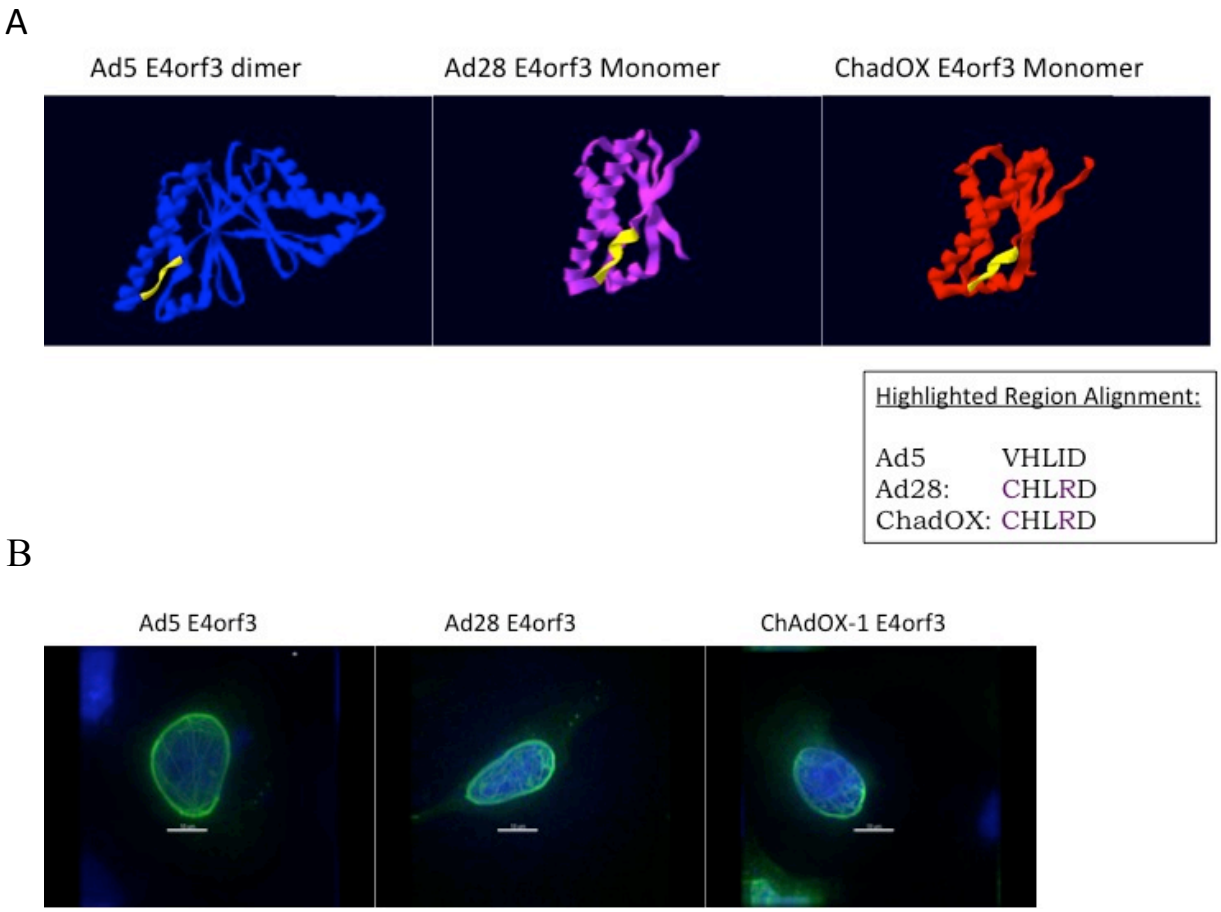

Figure 23. Comparison of E4ORF3 proteins from Ad5 vs. rare serotypes Ad28 and ChAdOX-1. (A) Crystal structure of the Ad5 E4ORF3 dimer along with predicted models of Ad28 E4ORF3 and ChadOX-1 E4ORF3 monomer structure generated by threading amino acid sequences on the solved Ad5 E4ORF3 crystal structure. Below, sequence alignment of the highlighted E4orf3 residues 101-105. **(B)** Visualization of E4ORF3 polymer formation, hela cells were transfected with HA-tagged-E4ORF3 constructs and detected by IFA 24 hours later.

Future directions should investigate serotype-specific E4ORF3 protein interactions in order to identify the critical residues required for protein interaction. E4ORF3 is highly conserved between Adenovirus species and previously defined variations in host protein binding mapped to small changes in the amino acid sequence. Therefore, I expect it is possible to identify potential residues involved in species-specific host protein interactions through E4ORF3

sequence alignment of binding and non-binding Ad serotypes. Use of protein-modeling software could narrow down candidate residues structurally before examining their requirement by mutagenesis. Ultimately, these studies could lead to the production of genetically engineered rare AdV-28 based vectors that have been minimally modified to be sufficient in serotype-specific E4ORF3 functions that promote immunogenicity.

Together our findings suggest that both interaction with host Gas6 and functional activities of Ad5 E4orf3 contribute to the superior immunogenicity elicited by AdV-5 vaccines compared to rare serotypes in the absence of pre-existing neutralizing antibodies. These findings advance our understanding of adenovirus biology and will contribute to the future engineering and used of rationally designed AdVs.

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VITA

Natalie Frances Nidetz was born in Edina, MN on April 12, 1988. In May, 2010 Natalie received a B.S. in Biology from Indiana University in Bloomington, IN. During her undergraduate studies, Natalie first gained laboratory experience washing and drying plant roots for graduate students in the Ecology department.

Following her undergraduate education, Natalie began work as an animal care technician at Wuxi Apptec in St. Paul, MN. There she gained experience providing animal care and learned to preform research tests in compliance with GLP, USDA, and AAALAC guidelines. In 2011, Natalie moved to Boulder CO, where she worked as a chemist with Microbac laboratories Inc. There she performed analytical chemistry assessing the properties of potential biofuels and consumer food products.

In August 2012, Natalie joined the Interdisciplinary Program in Biomedical Sciences at Loyola University Chicago where she began her work in the Microbiology Immunology department in the laboratory of Christopher M. Wiethoff Ph.D. Under the continued supervision of Tom Gallagher Ph.D., Natalie completed her doctoral work focused on understanding the molecular mechanisms by which adenoviral vectors stimulate immune responses when used in vaccine applications. Natalie will continue working in the field of research as she pursues her career.