This is to certify that the
dissertation entitled

EVALUATING AND MODIFYING ADENOVIRUS VECTOR INTERACTIONS WITH MULTIPLE ARMS OF THE INNATE IMMUNE SYSTEM

presented by

Sergey Seregin

has been accepted towards fulfillment
of the requirements for the

PhD degree in Microbiology and Molecular Genetics

Major Professor's Signature

04-02-2010

Date

EVALUATING AND MODIFYING ADENOVIRUS VECTOR INTERACTIONS WITH MULTIPLE ARMS OF THE INNATE IMMUNE SYSTEM

By

Sergey Seregin

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Microbiology and Molecular Genetics

2010

ABSTRACT

EVALUATING AND MODIFYING ADENOVIRUS VECTOR INTERACTIONS WITH MULTIPLE ARMS OF THE INNATE IMMUNE SYSTEM

By

Sergey Seregin

A single, universally adaptable gene transfer vector cannot be envisioned for use in all human clinical gene transfer applications. However, Adenovirus (Ad) based vectors offer several important benefits compelling their potential for use in a wide range of gene transfer applications. Ad vectors can be readily produced to cGMP guidelines, which allowed their safe usage in thousands of clinical trial subjects. Unfortunately, upon contact with the circulatory system and/or cellular membranes, Ads induce several, innate, complement dependent toxicities that limit more widespread utilization of this promising platform. In addition, Ad derived transgene expression is greatly diminished when pre-existing Ad immunity is present in the host. These limitations have driven initiation of several distinct approaches to improve the safety/efficacy profiles of Ad-based vectors, including: the generation of chemically modified Ad capsids and/or chimeric Ads; the complete replacement of common Ad-based serotypes with alternative (human and non-human origin) Ad serotypes; genome modification of common (Ad5) serotype in attempts to improve the efficacy of the platform as well minimize acute innate responses to the vector itself.

In this dissertation, I describe the pivotal role that the complement system plays in regulating Ad safety and efficacy. I unveil the mechanism underlying the complement dependent induction of neutralizing antibodies to Ad capsids as a

C3 and CR1/2-dependent phenomenon that correlates with B-cell activation. Moreover, I have constructed several novel Ad5-based vectors, "capsid-displaying" complement regulatory peptide (COMPinh), as fiber or pIX fusion proteins. These novel Ads dramatically minimize Ad-dependent activation of the human and non-human primate complement systems. We have also demonstrated that a simple, pre-emptive and transient glucocorticoid pre-treatment is a viable approach to reduce Ad associated acute toxicities, without reducing efficacy of Ad mediated gene transfer, suggesting that glucocorticoid therapy can be combined with the use of novel capsid-displaying Ads to further improve the outcome.

Previous studies have demonstrated that several signaling pathways are triggered by Ads, inclusive of TLR dependent pathways. Here, I have unveiled an important role for the G-protein coupled receptor adaptors β-arrestin1 (β-Arr1) and β-arrestin2 (β-Arr2) in Ad5 vector-induced inflammatory responses, thereby identifying them as new potential targets in attempts to improve Ad vector safety/efficacy profile.

Future studies will expand upon these findings. I leave readers with the view that utilizing a combination of the approaches, summarized herein will likely improve the capabilities of this important vector platform for expanded use in a number of additional human and agricultural applications.

DEDICATION

I would like to dedicate this dissertation to my parents. My parents, who are both scientists, are happy that one of their four children followed their path. I'm extremely grateful for their paramount and unbelievable support, unconditional love and care, throughout all my educational steps. My parents live in Russia and I miss them very much – I have not seen my Father for over four years. I'm happy that upon Graduation I will finally have an opportunity to visit them in summer 2010. I would like to express my love to my beautiful sisters, Dina and Maria and my brother Alexander. I love my family so much, and I would not have made it this far without their support.

ACKNOWLEDGEMENTS

I would like to take the opportunity to thank everyone who contributed to my development as an independent investigator – the path I continue to follow to become one. First and foremost, I express my profound gratitude to my mentor Dr. Andrea Amalfitano, a leading researcher in a field of Adenovirus-based gene transfer. He kindly allowed me to join his newly formed lab, here at Michigan State University in early 2006, and since then constantly supported and encouraged me to develop scientific thinking, writing skills, critical data analysis and planning of experiments. His sincere guideless, patience and continuous support created, in my opinion, productive and positive atmosphere in our lab. Dr. Amalfitano has a unique ability to sense a "golden middle" in supervising graduate students, allowing them to reach an answer / accomplish a task by themselves and only when person is puzzled, he provides precious scientific advice. This "smart guideless" takes a lot of his time, patience and effort, and I'm extremely happy, Dr. Amalfitano utilized this approach and feel that I have greatly improved all my skills, associated with future scientific career. Dr. Amalfitano is my best role model for a scientist, scientific advisor, teacher and person. It is my privilege to have an opportunity to work with him. In my future career I will be trying to preserve and elaborate upon these important personal characteristics.

I greatly appreciate the help provided by my guidance committee: Dr. Michele M. Fluck, Dr. Robert A. Roth and Dr. Ian York. Their insightful comments

and constructive discussions have significantly improved my research progress as well as development of critical scientific thinking.

My sincere thanks to all current and former members of Dr. Amalfitano lab: Dan Appledorn, Yasser Aldhamen, Sarah Godbehere Roosa, Jeannine Scott, Nathaniel Schuldt, Aaron McBride, Tyler Voss, Joyce Liu, Dionisia Quiroga, William Nance, Mathew Bugold, William De Pas, Megan Hoban, Jenny Zehnder, Brandy Burke, Anthony Sisk, Ryan Stringer. All of them are genuinely nice and eager to help each other, and I'm glad, I have worked and interacted with them. Together we have created wonderful positive friendly atmosphere in a lab, an environment with a space for fruitful scientific and science unrelated discussions, productive team work and, of course, fun and jokes!

I will forever be thankful to Jeannine Scott, who contributed greatly in my initial training here at MSU, she was the one who taught me how to design, construct, propagate and purify high titer Adenovirus vectors as well as other numerous techniques. She also provided advices many times during my graduate school career.

I would like to give my particularly special thanks to the most extraordinary person (in all aspects), I have ever met in my life – Dan Appledorn. He possesses amazing skills in countless science related issues, he is enthusiastic, and energetic and definitely one of the smartest people I know. He is my primary resource for getting my science questions answered and was instrumental in helping me to proofread this thesis. We had countless fruitful discussions, which helped me greatly to improve my thinking skills. I know that I could always ask him

for advice and opinion on any issue. He is a wonderful person, and I admire his positive outlook and his ability to smile despite the situation. What makes Dan even more unique is together with all this great scientific thinking, he is the main source of fun and jokes, creating positive environment in our lab. It is very critical to get some good laughs in a tough, 12-hour day. I'm sure that Dan will soon become great and productive PI.

I also thank my friend and co-worker, Yasser Aldhamen, who is a wonderful person. It is a pleasure to work with him, in particular as a team on some projects. He is honest, smart and responsible; he has unique ability to dig out foundation for future experiments or projects, the skill, I tried to improve, as it is critical piece during my future independent career.

My special thanks to Sarah Godbehere Roosa, who is a wonderful technician and exceptionally pleasant person to work with. If not for all Sarah's help, my work would have taken me twice as long. I apologize that I can't individually thank all members of Dr. Amalfitano lab, as this dissertation should contain at least a little bit of science.

My sincere thanks go to our collaborators from Duke University Medical Center: Dr. Michael Frank, Zachary Hartman, Anne Kiang, Junping Wei, Delila Serra, Fang Xu, Xiao Yi Zhao, Hai Xiang Jiang. We had several papers published together and their contribution was significant, especially in complement assays. Anne and Zack are former Dr. Amalfitano Graduate students, who had started several projects we continued to develop. In particular, I have finished several projects, Zack has initiated back in 2003. Despite the fact that I never met him

personally, we have exchanged numerous e-mails and he helped me greatly during my research and I know that he a great person.

I would like to emphasize my paramount gratitude to Adenovirus – the best and the most pragmatic vector platform for numerous future gene transfer applications and all people who contributed in discovery of Ads and who started to design Ad-based vectors. Thank you!

Special thanks to all wonderful MSU core facilities, including: ULAR and, in particular, all employees of BPS/biochemistry animal housing facility; MSU Histopathology labs (Amy S. Porter, Kathy A. Joseph, Rick A. Roseburry); MSU electron microscopy facility (Ralph Common); MSU Genomics Technology Support Facility: Flow Cytometry (Louis King), quantitative RT-PCR (Jeff Landgraf), sequencing (Christi Hemming), Proteomics (Douglas Whitten). Without their great performance and skilled assistance our research would not have been possible.

I'm thankful to all faculty members and employees of the Department of Microbiology and Molecular Genetics, especially the Chair of the department (Dr. Walter J. Esselman), the Director of MMG Graduate program (Dr. Robert Hausinger) and Graduate Secretary (Suzanne Peacock) for their kindness and all their help and advises, provided during these years and their patience, when they had to deal with my numerous questions. I would like to give my special thanks to Administrative Assistant Coreena K. Spitzley for her countless assistance in issues associated with International student status, health insurance, and Grants

submission. She is the best and the smartest administrative employee, I have ever worked with.

American Heart Association is one of the few agencies, which accept applications from international applicants. I'm extremely thankful to AHA for their policy and support, which they provided to me as 2-year pre-doctoral fellowship.

My special warm thanks for my Aunt Julia Busik, who helped us a lot during all these years, especially during first year and upon arriving to MSU: new culture, new country, with 11-month old child. Our first couple of months were extremely tough and I don't know how we would of got by if not her constant and unconditional help. I'm thankful to her husband Denis, who used to be semi-professional downhill skier, for teaching us how to ski and enjoy the slopes.

I thank with all my heart to my parents for their encouragements, love and support which motivated me to accomplish all my educational aims. My special thanks go to my dear wife, Maria Tikhonenko and my wonderful daughter Anastasia, who have been a source of motivation and inspiration during all those tough moments. Maria has been a true and great supporter; she has unconditionally loved me during my good and bad times. I feel her support and the faith she has in me and I will continue to give her my absolute support and love to provide her with patience and power to complete her Graduate Studies. I feel that we became much wiser and calmer after these years; life has strengthened our commitment and determination to our family as a whole.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Images in this dissertation are presented in color

LIST OF ABBREVATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
ADAR	Adenosine deaminase-RNA-specific (IFN-inducible)
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AP	Alternative complement pathway
ATCC	American type culture collection
β-Arr-1, β-Arr-2	beta-Arrestins 1 and 2
CAR	Coxsackie and adenovirus receptor
C3	Complement component 3
C3a-desArg	stable protein, produced upon complement activation
CMV	Cytomegalovirus
COMPinh	peptide with ability to inhibit human complement
CR	Complement receptor
CRAD	Conditionally replicative adenoviruses
CTL	Cytotoxic T lymphocyte
CXCL-9	Chemokine, induced by IFNγ
DAF	Decay accelerating factor
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
dpi	Days post injection
EC	Endothelial cells

EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethylene-glycol-tetra-acetic acid
EM	Electron microscopy
fD, fH, fI	Factor D, Factor H, Factor I (complement)
FDAd	Fully deleted adenovirus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
FDA	Food and drug administration
HDAd	Helper-dependent adenovirus
HVR	Hypervariable region of Ad hexon protein
hpi	Hours post injection
ICAM-1	Inter-Cellular adhesion molecule 1
IFNα, IFNβ	Interferons α and β (type I IFNs)
Ig	Immunoglobulin
IL-6, IL-12p40	Interleukins 6 and 12 (pro-inflammatory cytokines)
IRF3, IRF7, IRF8	Interferon Regulatory Factors 3, 7 and 8
JAK-1, JAK-3	Janus kinases 1 and 3
KC (CXCL-1)	Keratinocyte derived chemokine, murine analog of human IL-8
KO	Knockout
LPS	Lipopolysaccharides

LacZ	Bacterial β-galactosidase
ml	Milliliter
mM	Milli-molar
mg	Milligram
µl	Micro-liter
µM	Micro-molar
µg	Microgram
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MBL	Mannose lectin binding pathway (complement)
MCP-1 (CCL-2)	Monocyte chemotactic protein 1
MIP-1β (CCL-4)	Macrophage inflammatory protein 1 beta
MyD88	Myeloid differentiation factor 88 (TLR adaptor)
NAb	Neutralizing antibody
NFκB	Nuclear factor kappa B
NHS	Normal human serum
NHPS	Non-human primate serum
NOD-1, NOD-2	Nucleotide-binding oligomerization domains 1 and 2
OAS-1a	2'-5' Oligoadenylate synthetase (IFN-inducible)
OD	Optical density
qRT-PCR	Reverse transcription and quantitative real-time PCR
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PTA	Phosphotungstic acid (for EM staining)
PRR	Pattern recognition receptor
RANTES	Normal T-cell Expressed, and Secreted
RNA	Ribonucleic acid
SD	Standard deviation
SOCS-1, SOCS-3	Suppressors of cytokine signaling 1 and 3
TCID	Tissue culture infectious dose
TBK-1	TANK-binding kinase (TBK)
TLR	Toll-Like Receptor
TNFα	Tumor necrosis factor alpha
TRAF2bp	TNF receptor associated factor 2 binding protein
TRAF6	TNF receptor associated factor 6
TRIF	Toll/Interleukin-1 receptor (TIR)-domain-containing adaptor-inducing interferon-β
VCAM-1	Vascular cell adhesion molecule 1
VP	Viral particle
WT	Wild-type

Chapter I

Introduction

1.1. Adenovirus (Ad) based vectors: the most pragmatic vectors for gene transfer applications

Ads are non-enveloped icosahedral viruses of 60-100 nm in diameter, that contain an ~36 kb double-stranded linear DNA genome. The human *Adenoviridae* family is comprised of more than 50 Ad serotypes (based primarily upon the lack of cross reacting antibody neutralization between serotypes), which are categorized into six subgroups (A-F), primarily based upon different red blood cell agglutinating capabilities of the various subgroups. Classification of Ad serotypes have been historically based upon biological, biochemical as well as structural properties, including the lack of cross-neutralization between serotypes from different species. Subsequently, such parameters as oncogenicity, length of fiber protein and genomic sequence similarity were taken into consideration to further sub-classify Ads (1, 2). Ads have a worldwide distribution and account for ~5% of all respiratory infections in childhood (3). The viral capsid contains total of eleven structural proteins, of which hexon (protein II), penton base (protein III) and fiber (protein IV) are termed major capsid proteins, and proteins IIIa, VI, VIII and IX minor capsid proteins, four additional proteins are packaged with the DNA inside the core (4-6). The capsid proteins hexon, and fiber are the major targets of Ad specific antibodies, many of which become neutralizing (7-12). Transcription and processing of Ad RNA occurs on both strands utilizing early (E) and late (L) regions, resulting, mainly by mechanism of alternative splicing, in production of over 30 non-structural proteins (13) (Figure 1). Early region (E1-E4), is transcribed and translated first, and resultant proteins assist is subsequent stages of Ad replication and packaging: E1a (immediate early gene),

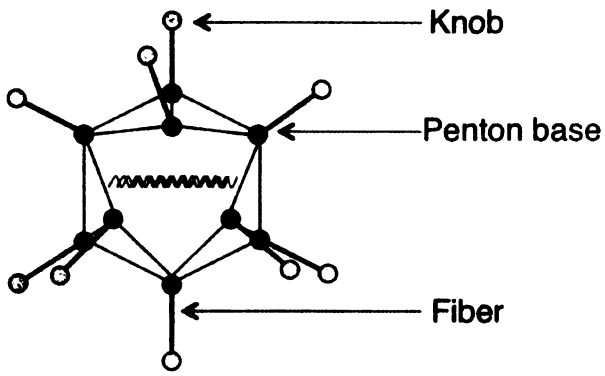
encodes transcription factor necessary for activation of early genes; E1b – encodes protein, blocking apoptosis, as well together with E1a, E3 and E4 products modulate cellular transcriptional machinery to transcribe predominantly viral genes and evade host immune responses; E2a – encodes DNA binding protein, E2b – encodes preterminal protein and viral polymerase. Late region (L1-L5) predominantly encode structural proteins, necessary for virion assembly, for example, L2 region encodes core proteins V, VII and X as well as penton base (pIII), L5 encodes fiber protein (2, 14).

Several generations of recombinant Ad vector has been generated. First generation Ad vectors lack E1 region ([E1-] Ad) or E1 and portion of E3 ([E1-, E3-] Ad). These Ad vectors are specifically propagated on transcomplementing cell lines such as HEK293 or PER.C6, which supply the essential E1a and E1b proteins in trans (2, 14). Advanced generation vectors encompassing additional deletions in the E2b or E4 regions (as well as fully deleted "gutless" Ads) have also been developed. These vectors retain the capabilities of large-scale production noted with E1 deleted vectors. However, transcomplementing cell lines unique to each vector (or an additional helper virus) must also be provided to support their growth (15, 16).

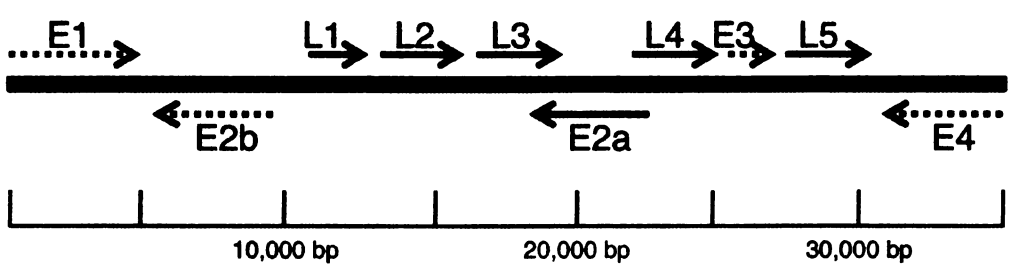
Recombinant, Ad-based gene transfer vector platforms are heavily utilized in both gene therapy and vaccine applications. This utility is based on a number of positive attributes inherent to the use of recombinant Ads. Recombinant Ads are capable of transducing foreign genes into a wide range of cell types inclusive

Figure 1: Diagram of Adenovirus and its genome. (A) Adenoviruses are icosahedral, non-enveloped viruses that contain multiple features including a double stranded DNA genome, the fiber protein that terminates in the knob region (required for binding to the coxsackie adenovirus receptor (CAR)), and the penton base (required for facilitating viral entry). **(B)** Adenoviruses contain a ~36 kb genome generally organized into regions encoding the Early (E) expressed genes and Late (L) genes. Various regions of the Ad genome can be removed and replaced with an expression cassette containing a "gene of choice" and its associated promoter and enhancer elements. Important regions of the Ad genome that have been removed to improve the persistence and/or safety of the vector are indicated with dotted arrows. These viruses can be grown to high viral titers using cell lines expressing the proteins encoded by these regions in *trans*.

A


Knob
Penton base
Fiber

B


E1
L1
L2
L3
L4
E3
L5
E2b
E2a
E4
10,000 bp
20,000 bp
30,000 bp

of antigen presenting cells; allow for efficient transgene expression, have large cloning capacities (8-36 Kb); and can be easily produced to extremely high titers in a current good manufacturing practices (cGMP) compliant fashion (up to $1x10^{13}$ vp/ml). The ability to easily "scale-up" traditional Ad vectors in a manner that is cGMP compliant, has resulted in thousands of patients safely receiving recombinant, Ad5-based gene transfer vectors. In fact, it is the author's opinion that the proven ability to mass-produce Ad-based vectors makes them one of the most pragmatic of all gene transfer vectors currently proposed for direct *in vivo* human use. For example, the lack of simple, efficient, and large-scale cGMP production capabilities has limited the clinical utilization of a number of other proposed gene transfer platforms (i.e.: Adeno-associated virus (AAV) or lentivirus based systems) to a handful of human clinical trials (see: http://www.wiley.co.uk/wileychi/genmed/clinical/).

There are, however, several limitations to the use of recombinant Ad vectors as a gene transfer platform, primarily: the decreased efficacy of these platforms in Ad-immune individuals, and vector-associated innate immunogenic toxicities. Many in the lay press and, in fact, the scientific theater wrongly hold the view that Ad-based clinical trials are no longer being conducted or pursued due to these important issues. Thus, it is very important to note the fact that Ad5-based vectors are currently the most widely used gene transfer vector in human clinical trials (http://www.wiley.co.uk/wileychi/genmed/clinical/). More recently, a major advantage to the use of Ad-based vectors has also become apparent, with the confirmation that integrating viral vectors (i.e.: retrovirus, lentivirus, possibly

AAVs) can cause insertional mutagenesis and cancer in animal models and in human clinical trial subjects, therefore such vectors may subject human recipients to similar and/or additional risks (17-21). The natural biology of the recombinant Ad genome to not integrate into the host chromosome as a nuclear episome largely mitigates insertional mutagenesis risks (22).

1.2. Primary problem: Ad-triggered innate immune responses

The main problem with using Ad vectors in current applications is the frequent need for use of high doses of the vector to achieve successful evidence of gene transfer in several specific tissues, a problem that increases unwanted side effects, such as vector-associated innate immune responses (23-28). Importantly, these Ad-triggered responses are clinically relevant, unfortunately as primarily noted in the Ornithine transcarbamylase gene transfer tragedy (29). In that trial, the tragic death of a patient, who received $6x10^{11}$ vp/kg of an Ad vector, coincided with induction of a "cytokine storm" – a massive release of pro-inflammatory cytokines (including IL-6), causing a systemic inflammatory response syndrome. Although not replicated in other patients receiving similar doses of the vector (both in this trial and in other subsequent studies (29-31), the trial highlighted the need for greater study of Ad vector induced innate immune responses. The lack of efficient vector platforms for systemic targeting of the liver has primarily driven the need more detailed investigation of Ad vector interactions with multiple arms of the innate immune system in attempts to find a way of improving the safety profile of this promising vector in these sorts of applications. It is now known that Ad vectors elicit multiple innate immune

responses after systemic administration due to several processes: complement system activation, anaphylotoxins release, macrophage activation, cytokine/chemokine release, induction of granulocyte and mast cells infiltration, endothelial cell activation, generalized transcriptome dysregulation in multiple tissues, and thrombocytopenia (23-28). Several of the innate immune systems, known to interact with Ads are described in greater detail below.

1.2.1. Toll-like receptors and Adenovirus

In the late eighteen hundreds, the famous Russian microbiologist Ilya Ilyich Mechnikov, Nobel prize laureate, described phagocytosis and suggested the term "innate immunity" to define an initial, nonspecific defensive mechanism that was present for defense against newly encountered pathogens. Almost a whole century passed before the modern scientific community discovered that pathogen recognition receptors (PRRs) function to recognize microbes and viruses at both the intra- and extracellular levels, triggering the induction of multiple innate immune responses. PRRs are able to recognize pathogen-associated molecular patterns (PAMPs), which are expressed on foreign agents. Bacterial lipopolysaccharides (LPS), viral/bacterial nucleic acids, lipoproteins are all examples of PAMPs. Since these molecules are absent in host cells, they are recognized as non-self motifs by PRRs. The most studied PRRs include β2 integrins (CD11/CD18), Nucleotide Oligomerization Domains (NODs), complement receptors (CR1/CD35, CR2/CD21), RIG-I-like receptors and Toll-Like Receptors (TLRs). TLRs were discovered in late 1990s, giving rise to tremendous amount of studies, which shed light on TLRs signaling mechanisms

associated with the innate (and downstream adaptive) immune responses (32, 33). TLRs are expressed in many cell types: non-hematopoietic epithelial and endothelial cells, macrophages, neutrophils and dendritic cells; 13 mammalian TLRs (10 in humans) have been identified to date. TLRs predominantly utilize conserved leucine rich repeat (LRR) at the N-termini for pathogen recognition, although amino acid insertions and non-consensus LRRs remarkably diversify TLR's ability to recognize a wide range of PAMPs (34).

Ad vectors have been confirmed to activate multiple PRRs both *in vitro* and *in vivo*, including RIG-I-like receptors, TLR2, TLR4 and TLR9 (for more detailed review please see (25)). These activations ultimately results in Ad-triggered innate toxicities. Specifically, we have shown that acute innate (and downstream adaptive) immune responses developed following systemic injection of Ad vectors, are significantly mediated by TLR adaptor proteins MyD88 and TRIF, as shRNA-mediated stable knockdown of these molecules resulted in diminished pro-inflammatory cytokine (IL-6) releases *in vitro* in response to Ad injection (35); moreover, cytokine/chemokine activation (G-CSF, IL-5, IL-6, IL-12p40, MCP-1, RANTES) and massive, pro-inflammatory genes induction was blunted in MyD88 knockout mice, as compared to identically Ad-treated WT mice (23). Ad-triggered activation of MAPK and NFkB signaling pathways, systemic cytokine release and generation of Ad-capsid specific neutralizing antibodies (NAb) are at least partially TLR2/TLR9/MyD88 dependent (36). Therefore, it is confirmed that TLRs play a pivotal role in mediating Ad-triggered innate immune responses both *in*

vitro (35, 37, 38) and *in vivo* (38, 39), however, continued investigations of Ad-induced innate immune responses and specific signaling pathways are required.

β-Arrestins 1 and 2 (β-Arr-1 and -2) are ubiquitously expressed G-protein coupled receptor adaptors, known to have pivotal roles in regulating TLR signaling pathways (40). β-Arr-1 and -2 were originally discovered to play a major role in G protein coupled receptor desensitization, due to their ability to bind to phosphorylated G protein coupled receptors and sterically block their ability to signal downstream (40). Subsequent studies revealed more versatile roles of β-Arr1 (arrestin-2) and β-Arr2 (arrestin-3), including broader regulation of cell signaling in general, serving as scaffolds, as well as having roles in transcriptional regulation (40). Recent studies demonstrate β-arrestins as negative regulators of TLR-stimulated NFκB and ERK signaling pathways in macrophages and fibroblasts (41, 42). Consistent with this role of β-arrestins, Wang et. al. showed that deficiency of β-Arr2 significantly enhances endotoxic mortality in mice (41). In contrast to these studies, Fan et. al. (43) demonstrated differential effects of β-Arr1 and -2 in lipopolysaccharide signaling in mouse embryo fibroblasts, suggesting that β-arrestin's effect on TLR signaling might depend on the cellular context. Although a role for β-arrestins has been established using selective TLR ligands, their role in microbial infections (which activate multiple TLRs) is not known.

Innate immune responses associated with systemic injection of Adenovirus (Ad) based vectors remain to be one of the most important obstacles limiting the usage of these vectors in numerous clinically important applications,

requiring high level vector administrations, for example for liver targeted therapy. Systemic (intravenous) administration of Ads results in acute thrombocytopenia (26, 44), activation of the complement, TLR and IFN systems, a robust cytokine release into the circulation sometimes referred to as a "cytokine storm" (24, 26, 27, 36), activation of endothelial cells (45) as well as massive inductions of pro-inflammatory gene expression in multiple tissues, including the liver, spleen and lung (23, 26, 46-50). Therefore, understanding the mechanisms mediating Ad induction of these processes may allow for focused efforts to interfere with these mechanisms to foster improved safety and/or efficacy of Ad mediated gene transfer applications. Because TLRs significantly modulate Ad vector responses, and since β-arrestins play a crucial role in TLR signaling, we analyzed the role of β-arr-1 and -2 in Ad5-induced innate immune responses both *in vitro and in vivo*. Our results confirm that multiple Ad5 induced innate immune responses are mediated by β-arrestin functionality. Moreover β-Arr1 and β-Arr2 differentially mediate Ad5 induced innate responses, having somewhat opposite functions: β-Arr2 clearly down-regulates Ad5 induced gene induction and cytokine responses, whereas β-Arr1 enhances portions of these responses, as detailed in chapter II of the dissertation.

1.2.2. The complement system: first line of defense against pathogens

The complement system, which is composed of over 30 membrane-bound as well as fluid phase proteins, represents the main non-cellular part of the innate immune system (Figure 2). Most complement components are synthesized by hepatic parenchymal cells in the liver, however, some may also be synthesized

by macrophages (51). Complement proteins can bind and facilitate opsonization of different types of pathogens including those not previously encountered by the host. There are three major complement pathways:

1) **Classical Complement Pathway** is activated as a result of specific antibody interactions with a previously encountered pathogen. Pre-existing antibodies (IgG or IgM) bind to the microbe, which allows C1q complement protein to bind to the antibody/pathogen complex. This in turn triggers C1r and C1s binding, creating an active C1qrs serine protease (Ca^{2+} dependent), which cleaves proteins C4 (releasing C4a and C4b) and C2 (releasing C2a and C2b). Reactive C2a and C4b associate with each other into a complex on the pathogen, giving rise to the classical pathway C3 convertase: C4b2a (Mg^{2+} dependent). C3 convertase cleaves large amounts of C3, generating C3a anaphylotoxin and C3b. C3b protein can activate endothelial cells (ECs) by binding to specific receptors on EC surfaces (49). C3b can also directly bind to pathogens, facilitating targeting of pathogens to complement receptors on phagocytic cells (macrophages, granulocytes) (49, 52). The C4b2a3b complex is a C5 convertase cleaving C5 into C5a (a potent anaphylotoxin) and C5b. C5b protein binds to the surface of a pathogen (or infected cell), which triggers membrane attack complex (MAC) formation by recruitment of complement proteins C6-C9. The MAC can form a pore in bacterial or enveloped viral pathogens, thereby initiating their lysis. The potent anaphylotoxins C3a and C5a efficiently bind to their receptors (C5aR, C3aR) on mast cells, granulocytes and

macrophages facilitating their activation (49), (Figure 2), thereby activating the cellular part of the innate immune system.

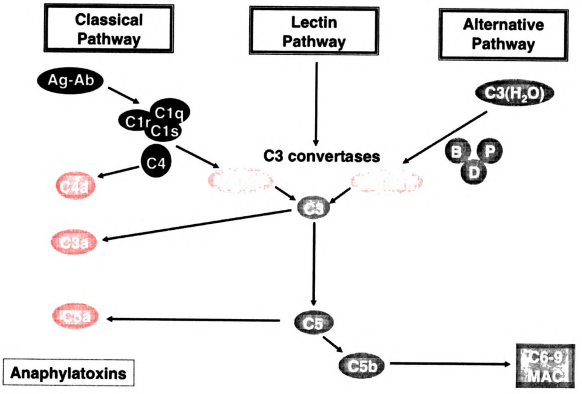
2) **The lectin pathway** for complement activation is homologous to the classical pathway but is initiated by the binding of the mannan-binding lectin to mannose residues on the pathogen surface. This causes activation of the MBL-associated serine proteases, MASP-1, MASP-2, MASP-3, which cleave proteins C4 and C2, generating the C3 convertase C4b2a.

3) **The alternative complement pathway** has a role in combating pathogens not previously encountered by the host immune system (i.e. there are no pre-existing antibodies to the pathogen). Protein C3 possesses an internal thioester bond between a cysteine and the γ-carboxyl group of a glutamine residue allowing low levels of reactive fluid phase C3b-like molecules to be spontaneously produced and rapidly inactivated in the blood via a "tick-over" mechanism. However, upon pathogen encounter, pre-existing C3-OH can bind stably to the surface of the pathogen, facilitating binding of factor B (Fb) to the C3b/pathogen complex. The complex is recognized by factor D, and Fb is cleaved to generate the alternative pathway-derived C3 convertase C3bBb (Mg^{2+} dependent). Numerous C3 molecules are cleaved by this alternative pathway-derived C3 convertase. The alternative pathway can also serve as an amplification loop for classical pathway-initiated complement system activation (52-54).

Excessive complement activation can cause a number of disorders including adult respiratory distress syndrome (ARDS), hypotensive shock,

anaphylactoid reactions, systemic inflammatory response syndrome, and thrombocytopenia. Many of these toxicities have been observed after high dose Ad administration into rodents, non-human primates, and humans (48-50). Our published data confirms that the intact Ad capsid interacts with human and murine complement immediately after delivery *in vitro* and *in vivo* thereby consuming complement components and inducing several toxicities, toxicities that can be avoided by complement inhibition (24, 26, 46, 55), as fully detailed in several chapters of this dissertation.

Figure 2: The complement system. Three major complement pathways are shown. Activation of either of the pathway leads to formation of C3-convertase, which cleaves main complement protein C3 and allows to generate C5-convertase, capable of cleaving C5 into C5a and C5b. Potent anaphylotoxins, released during complement activation, activate cellular arm of the innate immune system. C5b facilitates formation of membrane attack complex (MAC), capable of lysing infected cells and pathogens.


Classical Pathway
Lectin Pathway
Alternative Pathway
Ag-Ab
C1q
C1r
C1s
C4
C3(H2O)
B
P
D
C3 convertases
C4a
C3
C3a
C5
C5a
C5b
C6-9
MAC
Anaphylatoxins

1.2.3. Complement and Adenovirus

Ad-based vectors are capable of activating human complement via both classical and alternative complement pathways (24, 26, 46, 47, 55). While it was well established that Ad5 can activate the classical complement pathway in the presence of Ad-specific antibodies, our group first confirmed that immobilized Ad5 capsids can mediate the activation of the alternative complement pathway in Ad-naïve serum (47, 55). Ad5 is also capable of activating murine complement, justifying the use of a mouse model to investigate the role of complement in Ad-triggered responses (26). In this regard, intravenous administration of high doses of Ad5-based vectors in mice deficient in various complement factors including C1q and C4 (classical pathway), FB (alternative pathway), or C3 (shared pathway) has revealed that Ad-triggered induction of thrombocytopenia is dependent upon a functional complement system and is alternative pathway dependent (24, 26, 46). It was also confirmed that the induction of several important pro-inflammatory cytokines/chemokines by Ads is also complement dependent. The induction by Ads of some cytokines is dependent upon alternative pathway proteins, while the induction of other cytokines by Ads is depended upon classical pathway activation. However, the induction of most cytokines and chemokines by Ads was found to be primarily dependent upon the presence C3 – the complement pathway component that is a key converging point of both the classical and alternative complement pathways (24, 26, 46, 56).

It is also known that complement system activation acts to optimize induction of pathogen specific antibody responses (57-59). Subsequent to

opsonization by activated C3, pathogens are bound to B cells and dendritic cells via binding of the pathogen bound C3 to the complement receptors (CRs). The human CR1 and CR2 receptors have well known roles in modulating both innate and adaptive immune responses. Human CR1 (hCR1) is a potent inhibitor of complement activation, having both decay-accelerating and cofactor activities. Furthermore, hCR1 has a critical role in the clearance of immune complexes, and B cell maturation, as thoroughly reviewed elsewhere (60, 61). Human CR2 (hCR2) expression is restricted to the surface of B cells, follicular dendritic cells and thymocytes. When hCR2 binds C3d opsonised pathogens and becomes associated with CD19, it lowers the threshold for B cell activation by up to 1000 fold (62). HCR1 and hCR2 also play a role in T cell biology, for example, crosslinking of hCR1 inhibits T cell proliferation and IL-12 production (63).

In contrast to their human counterparts, the murine complement receptors (mCRs) 1 and 2 (CD35/CD21) are products of alternative splicing from the same gene. mCR1 contains 21 complement control protein repeats (CCPRs), whereas the smaller mCR2 contains only 15 C-terminal CCPRs of mCR1 (64). mCR1/2 is known to be expressed on B cells and dendritic cells (64). Therefore, the expression pattern of mCR1/2 resembles that of hCR2, but not hCR1. Similar to their human homologues, the functions of mCR1/2 related to generation of maximal humoral responses have also been well described (65-69). Interestingly, mCR1/2 functionality prevents excessive myocardial tissue damage subsequent to coxsackievirus B3 infection (70), as well prevents lethal *Streptococcus*

pneumoniae infection, a role potentially indirectly reflective of the complement inhibitory activities of the CRs (71).

While the role of murine CR1/2 protein has been extensively studied in regards to adaptive immune responses, its function in inhibiting/regulating murine complement dependent responses has not been intensely investigated, possibly since in most mouse models, the Crry protein was suggested to play the predominant role in controlling complement activation. Our studies, however, now confirm that the role of murine CR1/2 protein in innate immune responses (including the ones which are known to be complement dependent) may be more important than previously considered, as suggested by our present studies of Adenovirus mediated gene transfer into mCR1/2-KO mice. Our results in murine models revealed dual roles for mCR1/2; roles that include down-regulation of multiple aspects of the Ad induced innate immune responses, while also playing the major role in the complement dependent induction of neutralizing antibody responses to Ads.

1.3. Approaches directed to minimize Ad-triggered immune responses

Innate toxicities, rapidly developed in response to systemic Ad injections, significantly limit more widespread utilization of this gene transfer platform. Additionally, Ad-based gene transfer approaches can be limited due to adaptive immune responses to the virus or the transgene it encodes. These adaptive immune responses can limit the duration of transgene expression and/or limit the ability to re-administer the vector, although this highly depends on the immunogenicity of the transgene delivered (14). Though it is often noted that Ad

mediated gene delivery is transient due to these responses, there are multiple examples that even first generation Ads, and certainly advanced generation Ads, can allow for long-term gene expression *in vivo* (14). For example, first generation Ad mediated delivery of non-immunogenic transgenes can persist for long periods of time in both murine and non-human primate models. Similar levels of improved efficacy are more likely to occur with the use of the advanced generation Ad-based vectors (16, 72, 73).

An important problem in regards to potential use of Ads in human clinical applications is that the majority of the human population has been infected by wild type Ads in childhood, and often these individuals develop pre-existing immunity to a number of common Ad serotypes (i.e.: serotypes 2, 4, 5, and 7). The percentage of the human population with medium to high Ad specific antibody titers largely depends upon geographic location, with 30-50% of the United States population, and up to 90% of the sub-Saharan African populations having significant Ad5 antibodies with an overall trend to having higher titers in developing countries (74-76). Pre-existing immunity to Ad can include both neutralizing antibodies (NAb), as well as Ad protein specific T cell responses. Additionally, recent studies highlight a critical role for $CD8^+$ T cells in pre-existing Ad immunity (9, 77). This latter point is very important, as these pre-existing responses can in fact still be harnessed when a host is exposed to a novel (previously unencountered) Ad serotype (78-81).

Therefore, several strategies have been proposed to improve the efficacy or utility of Ad-based gene transfer vectors (78, 82, 83). Below we discuss three

approaches, developed to minimize Ad vector induced innate toxicities. Importantly, these approaches have shown improved efficacy in Ad-immune hosts and/or allowed for efficient re-administration of Ad vector, generating significantly blunted Ad-specific adaptive immune responses, including NAb.

1.3.1. Ad capsid modifications

1.3.1.1. Chemical modifications of the Ad capsid

Several groups have chemically modified the Ad5 capsid (i.e.: in a non-covalent fashion) in an attempt to shield or hide highly antigenic epitopes on the viral capsid (mainly hexon protein) from the immune system of the Ad-immune host. This strategy primarily involves the addition of synthetic polymers onto the Ad capsid. These polymers can include polyethylene glycol (PEG) (84), polyactic glycolic acid (PLGA) (85) or other lipids (86).

PEGylation appears to be the most promising approach for non-covalent modification of Ad capsids. Ad5 PEGylation may not only reduce Ad-triggered toxicities, but also allow for re-administration of such modified Ads in mice. For example, animals were intravenously injected with an Ad5 vector expressing an irrelevant antigen, and then re-injected with conventional or PEGylated Ad5 vectors each expressing the bacterial β-galactosidase gene (β-Gal) (87). This simple study revealed high levels of β-Gal expression only in Ad5-immune mice re-injected with the PEGylated Ad5 vector (87). Moreover, it was shown that PEGylated Ad5 vectors also reduce the induction of Ad5-triggered innate toxicities, such as thrombocytopenia, plasma ALT elevations, and release of pro-

inflammatory cytokines such as IL-6 (87, 88). PEGylation with a small PEG (5 kDa) moiety increases transduction efficiency of the Ad5 capsid in Ad-immune mice (89). Use of a 5 kDa PEGylation of Ad5 can also reduce Ad5 specific neutralizing antibody (NAb) titers generated after intranasal injection into Ad-naive BALB/c mice (90). How PEGylation may affect the efficiency or tropism of Ad5 mediated gene transduction *in vivo* has not been fully delineated. For example, a dramatic reduction of liver transduction was reported after Ad PEGylation (91), while other studies reported equal and/or increased liver transduction by PEGylated Ads (87, 88).

In summary, characterization of how PEGylation affects the ability of so modified Ad vectors relative to tropism changes, ability to reduce Ad-triggered innate and adaptive immune responses, is still required to determine if these strategies afford improved utility of PEGylated Ad-based platforms.

1.3.1.2. Insertion of novel peptides into Ad capsid proteins

Several Ad capsid proteins, including the fiber, penton, protein IX and hexon proteins have been exploited for genetic insertion of foreign peptides into these specific capsid proteins, either as "in-frame" insertions within the proteins, or as "in-frame" C-terminal fusions. If the insertion does not cause a deleterious change in the biology of the so-modified protein, (i.e.: causes misfolding, lack of stability, lack of trimerization or lack of incorporation into the tertiary structure of the Ad capsid) novel, recombinant Ad vectors "capsid-displaying" the respective foreign peptide can be isolated. The penton and hexon proteins tolerate relatively small peptide insertions: up to 18 amino acids (aa) for penton base (92, 93), and

up to 36 aa within the hypervariable region 5 (HVR5) of hexon (94, 95), whereas the HI loop of the fiber knob and C-terminus of protein IX allow for incorporation of peptides of substantial size: up to 83 aa for fiber (96) and up to 1018 aa for pIX (97). Issues regarding capability to generate these recombinant viruses consistently, to very high titers, or in a cGMP compliant fashion have many times not been addressed in studies published to date, but will need to be if clinical deployment of these vectors is to be achieved.

The insertion of foreign peptides into capsid proteins in attempts to change the natural tropism of the Ad vector have been well-described (98, 99). Ad5-based vectors depend on coxsackievirus–adenovirus receptor (CAR) receptor for cell transduction: fiber protein, consisting of three domains (tail, shaft, and trimeric knob), is responsible for initial cell attachment. Fiber knob / CAR interaction is followed by the binding of the penton proteins to αvβ3 or αvβ5 integrins through a conserved Arg–Gly–Asp (RGD) consensus motif. In addition, factor X Gla domain interactions with hypervariable region of Ad hexon were recently identified as critical factor mediating liver Ad transduction via Heparan-sulfate proteo-glycans (100).

Based on this simple Ad biology, fiber domains (and knob in particular) as well as RGD motif from penton and hypervariable region of hexon protein are the best targets in attempts to modify Ad5 vector tropism. Therefore, several studies described the incorporation of polylysine or RGD motifs into different locations of Ad5 capsid, resulting in modified tropism. Specifically, incorporation of a polylysine motif into the pIX was shown to augment Ad fiber knob independent

infection of CAR-deficient cell types *in vitro* (101). RGD and polylysine motifs have been incorporated into the fiber knob, providing an ability to infect CAR-deficient cells *in vitro* (102-105). Additional studies, utilizing *in vitro* comparative analysis of Ads, capsid-displaying RGD peptide at HI loop of fiber knob, C-terminus of fiber knob, C-terminus of pIX or HVR5 of hexon, revealed that the most efficient transduction was achieved when HI loop of fiber was used for RGD incorporation (99). It has been subsequently confirmed that RGD motifs incorporated into C-terminus of protein IX allowed for transduction of CAR-deficient cell types *in vitro* (106). The use of 30-75 Angstrom α-helical spacers for incorporation of foreign peptides in pIX was suggested (106), but it is still unclear, however, if these spacers provide any detectable benefit, since numerous studies showed promising results incorporating peptides in pIX without spacers (99, 101). More detailed direct comparison between several alternative pIX-displaying Ads (with and without linkers) is required to answer this question.

A major limitation of these targeting approaches is that numerous promising *in vitro* results fail to provide any significant benefit *in vivo,* primarily due to inability to overcome natural liver tropism of Ad-based vectors. However, several studies provide optimism by showing reduced metastasis formation and increase survival in mice with usage of E1a CRAD (107) or 2-fold increased tumor uptake of targeting Ad-displaying $\alpha v\beta 6$ integrin-selective peptide as compared to intact capsid-Ad, which was in parallel with reduced liver transduction by targeting Ad (108). Future development of advanced targeting Ads, possibly by combining approaches undertaken up to date, should be

continued in order to more fully determine the applicability of targeting Ad for clinical applications (109).

Note that in this dissertation we describe a novel class of Ad5-based vectors, capsid-displaying specific complement inhibitory peptide (COMPinh). These novel Ads minimize complement activation (with a potential to minimize Ad-induced complement dependent toxicities) and represent a promising tool for numerous future gene transfer applications.

1.3.2. Immunosuppressive glucocorticoid dexamethasone

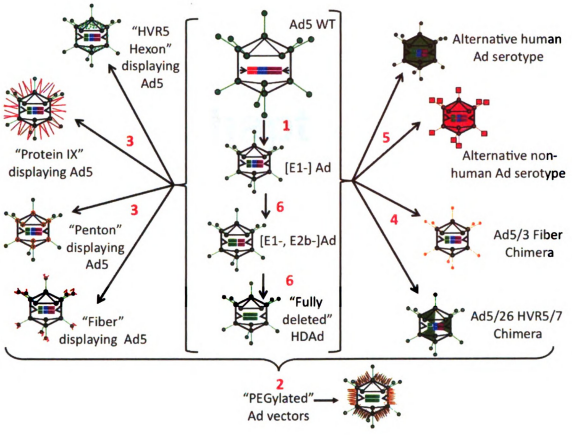
The synthetic anti-inflammatory glucocorticoid dexamethasone (DEX) is an FDA approved drug widely used to treat a number of transient and/or chronic inflammatory conditions (110-115). Importantly, mechanisms of action of DEX include preventing the activation of NFkB and AP1 transcription factors, as well as MAP kinases, all of which have been shown by us and others to also be important mediators of the Ad induced inflammatory response complex (116-118). IL-8 and IFNβ production have also been shown to be inhibited by DEX (117, 119). As a result, the maturation of mast cell progenitors, as well GM-CSF and TNFα secretion by mast cells are also altered by DEX treatment (120).

Very limited data is available regarding the effect of DEX treatment on animals treated with gene transfer agents. Use of glucocorticoids has been shown to increase the efficacy of gene transfer *in vitro,* while i*n vivo* studies showed that dexamethasone could improve non-viral gene and virus derived gene expression in experimental animal models (121-125). Relative to Ad

mediated gene transfer, DEX can improve Ad vector derived gene expression when these vectors are directly administered into the spinal cord, nasal mucosa, inner ear or lungs (124-128). Pulmonary delivery of Ad vectors into mice pre-treated with Dexamethasone/spermine resulted in a reduced activation of limited portions of the lung specific innate and adaptive immune response to the Ad vector utilized (128). These results prompted us to evaluate the efficacy of DEX to prevent the numerous innate toxicities induced by systemic administration of Ad vectors, as detailed in chapter V.

In summary, all of the approaches, summarized on figure 3 have shown promising results in terms of improving some aspects of Ad vector safety and/or efficacy. Further development of genome modified Ad vectors, "capsid-displaying" Ads, chemically modified Ads, chimeric Ads, as well as Ad vectors, derived from alternative Ad serotypes is now justified. Possibly, a combination of some (or all) of these approaches may result in construction of safer and a more efficacious Ad vector platform. In this dissertation we describe a novel class of Ad vectors, "capsid-displaying" specific peptides, inhibiting complement activation, and outline critical role of complement in mediating Ad-triggered innate and adaptive immune responses. We also propose to use an FDA approved potent immunosuppressive glucocorticoid Dexamethasone to block Ad-induced immune responses, potentially in combination with other approaches to further improve the outcome of gene transfer applications.

Figure 3: Current approaches to design improved Adenovirus based gene transfer vectors. Group 1. Starting with a wild-type Ad5 virus the E1 region is deleted to generate [E1-] Ad5 vectors; Group 2. Adenovirus vectors of any group can be PEGylated; Group 3. Capsid "display" of antigens at various locations on the Ad capsid where indicated; Group 4. Chimeric Adenovirus vectors, that contain capsid proteins derived from 2 different Ad serotypes; Group 5. Adenovirus vectors derived completely from alternative serotypes; Group 6. Genome Modified Adenovirus vectors, that retain the parental serotype capsid, but are deleted for portions of the parental genome. All of Group 1-6 vectors can be utilized in combination with transient pre-treatment with synthetic immunosuppressive glucocorticoid Dexamethasone to further improve the outcome of an Ad-based gene transfer. Note, that we describe Group 3 approach in this dissertation.


"HVR5 Hexon" displaying Ad5
Ad5 WT
Alternative human Ad serotype
"Protein IX" displaying Ad5
3
1
5
[E1-] Ad
Alternative non-human Ad serotype
3
6
4
"Penton" displaying Ad5
[E1-, E2b-]Ad
Ad5/3 Fiber Chimera
6
"Fiber" displaying Ad5
"Fully deleted" HDAd
Ad5/26 HVR5/7 Chimera
2
"PEGylated" Ad vectors

Chapter II

Ad5-triggered signaling pathways: critical roles of βArr-1 and βArr-2 adaptor proteins

This chapter is the edited version of a research article that was published in the Journal of Virus Research, Volume 147, Issue 1 (123-134), November 10, 2009.

Authors: Seregin, S. S., Appledorn, D. M., Patial, S., Bujold, M., Nance, W., Godbehere, S., Parameswaran, N., and A. Amalfitano.

2.1. Introduction

β-Arrestin-1 and -2 (β-Arr1 and 2) were originally discovered to play a major role in G protein coupled receptor desensitization, due to their ability to bind to phosphorylated G protein coupled receptors and sterically block their ability to signal downstream (40). Subsequent studies revealed more versatile roles of β-Arr1 (arrestin-2) and β-Arr2 (arrestin-3), including broader regulation of cell signaling in general, serving as scaffolds, as well as having roles in transcriptional regulation (40). Recent studies demonstrate β-arrestins as negative regulators of TLR-stimulated NFκB and ERK signaling pathways in macrophages and fibroblasts (41, 42). Consistent with this role of β-arrestins, Wang et. al. showed that deficiency of β-Arr2 significantly enhanced endotoxic mortality in mice (41). In contrast to these studies, Fan et. al. (43) demonstrated differential effects of β-Arr1 and -2 in lipopolysaccharide signaling in mouse embryo fibroblasts, suggesting that β-arrestin's effect on TLR signaling might depend on the cellular context. Although a role for β-arrestins has been established using selective TLR ligands, their role in microbial infections (which activate multiple TLRs) is not known.

Innate immune responses associated with systemic injection of Adenovirus (Ad) based vectors remain to be one of the most important obstacles limiting the usage of these vectors in numerous clinically important applications. For example, systemic administration of Ads results in acute thrombocytopenia (26, 44), activation of the complement, TLR and IFN systems, a robust cytokine

release into the circulation sometimes referred to as a "cytokine storm" (24, 26, 27, 36), activation of endothelial cells (45) as well as massive inductions of pro-inflammatory gene expression in multiple tissues, including the liver, spleen and lung (23, 26, 46-50). Therefore, understanding the mechanisms mediating Ad induction of these processes may allow for focused efforts to interfere with these mechanisms to foster improved safety and/or efficacy of Ad mediated gene transfer applications. Because TLRs significantly modulate Ad vector responses, and since β-arrestins play a crucial role in TLR signaling, in this study we analyzed the role of β-arr-1 and -2 in Ad5-induced innate immune responses both *in vitro and in vivo*. Our results confirm that multiple Ad5 induced innate immune responses are mediated by β-arrestin functionality. Moreover β-Arr1 and β-Arr2 differentially mediate Ad5 induced innate responses, having somewhat opposite functions: β-Arr2 clearly down-regulates Ad5 induced gene induction and cytokine responses, whereas β-Arr1 enhances portions of these responses.

2.2. Results

β-Arrestins differentially mediate Ad5 induced cytokine and chemokine release from peritoneal macrophages *in vitro.* Macrophages participate in the first line of defense against invading pathogens by functioning as phagocytes, antigen presenting cells, and activators of both innate and adaptive immune responses via secretion of cytokines and chemokines that serve as co-activating factors. Numerous studies have indicated that the induction of cytokines and chemokines by Ad5 injection is dependent upon the presence of macrophages, specifically liver resident macrophages known as Kupffer cells. We have shown that the inductions of KC, MCP-1, IL-12(p40) and RANTES are Kupffer cell dependent *in vivo* (36). To determine if β-Arrestins also play a role in the secretion of cytokines and chemokines from macrophages, we isolated peritoneal macrophages from wild type, β-Arr1, and β-Arr2 knockout mice, exposed them to Ad5-LacZ, and measured the concentration of pro-inflammatory cytokines in the growth media at various time points post Ad exposure (Figures 4-5). Interestingly we observed that levels of MIP-1β, MCP-1 and RANTES were significantly lower ($p<0.05$) in the β-Arr-1-KO macrophages compared to the wild types. We also observed that the decrease was time-dependent, i.e. MIP1β was lower at 6 h.p.i., whereas MCP-1 and RANTES were decreased at 24 h.p.i., suggesting that these are potentially regulated by distinct mechanisms (Figure 4). In contrast to these results, levels of IL-12(p40) and MCP-1 (at 24 and 48 h.p.i.) were significantly enhanced ($p<0.05$) in

Figure 4: Functional β-arrestin-1 acts as positive regulator for several chemokines released in response to Ad infection *in vitro*. Peritoneal macrophages derived from WT or β-Arr1-KO C57BL/6 mice were isolated, plated and infected with Ad5-LacZ as described in Materials and Methods. Media samples were collected at 6, 24 and 48 hours post virus infection (hpi) and assayed using a multiplexed bead array based system. The bars represent Mean ± SD. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=4 for all groups of peritoneal macrophages, including mock-infected groups. *, ** - indicate media cytokine values that are statistically different from those in Mock-infected groups of the same genotype at the same time point, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in the same treatment group at the same time point, $p<0.05$, $p<0.001$ respectively.

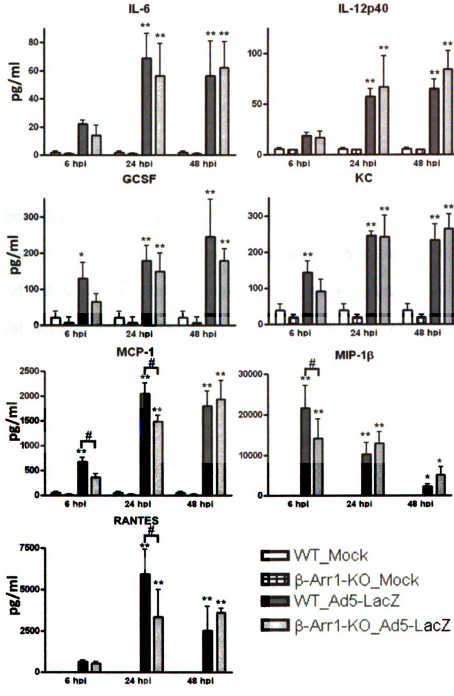
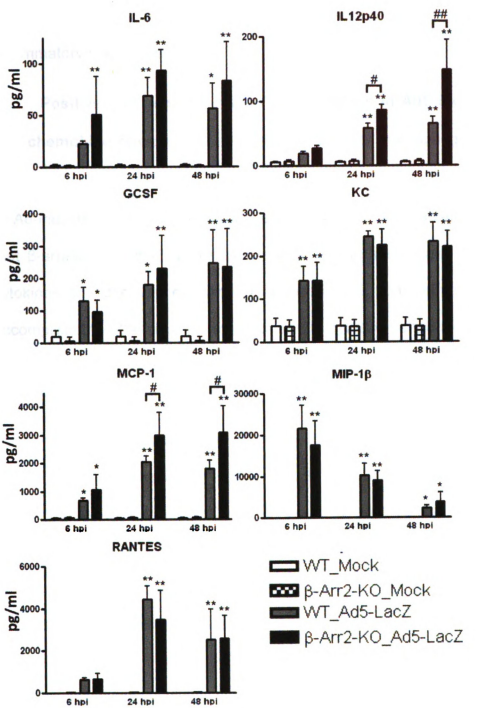

IL-6
IL-12p40
GCSF
KC
MCP-1
MIP-1β
RANTES
pg/ml
6 hpi
24 hpi
48 hpi
WT_Mock
β-Arr1-KO_Mock
WT_Ad5-LacZ
β-Arr1-KO_Ad5-LacZ

Figure 5: Functional β-arrestin-2 acts as negative regulator for several chemokines released in response to Ad infection *in vitro*. Peritoneal macrophages derived from WT or β-Arr2-KO C57BL/6 mice were isolated, plated and infected with Ad5-LacZ as described in Materials and Methods. Media samples were collected at 6, 24 and 48 hours post virus infection (hpi) and assayed using a multiplexed bead array based system. The bars represent Mean ± SD. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=4 for WT_Mock and WT_Ad5-LacZ groups, N=6 for β-Arr2-KO_Mock and β-Arr2-KO_Ad5-LacZ groups. *, ** - indicate media cytokine values that are statistically different from those in Mock-infected groups of the same genotype at the same time point, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in the same treatment group at the same time point, $p<0.05$, $p<0.001$ respectively.


IL-6
IL12p40
GCSF
KC
MCP-1
MIP-1β
RANTES
pg/ml
6 hpi
24 hpi
48 hpi
WT_Mock
β-Arr2-KO_Mock
WT_Ad5-LacZ
β-Arr2-KO_Ad5-LacZ

macrophages, derived from β-Arr-2-KO mice compared to WT macrophages (Figure 5). These results, taken together, suggest that β-Arr1 may act as a positive regulator, and β-Arr2 as a negative regulator of Ad5-induced inflammatory responses.

Positive and negative roles for β-Arrestins in Ad5 induced cytokine and chemokine release. Our results using primary macrophages from β-Arr-1 and -2 KO mice clearly demonstrate important but differential roles for β-arrestins in Ad-induced inflammatory responses. Therefore, we evaluated the potential that β-arrestin-1 and -2 also play a pivotal role in the release of inflammatory cytokines and chemokines following systemic Ad5 administrations *in vivo*. To accomplish this, the plasma levels of seven cytokines/chemokines, commonly induced following Ad5 injection were evaluated, following systemic delivery of 7.5×10^{10} vps Ad5-LacZ. As expected, all seven cytokines/chemokines tested were significantly induced within 1 h.p.i. (IL-6, MCP-1, MIP-1β, and KC, $p<0.001$, $p<0.05$), and/or 6 h.p.i (IL-12(p40), G-CSF, MCP-1, MIP-1β, and RANTES; $p<0.001$, $p<0.05$) in wild-type C57BL/6 mice (Figures 6-7). Identically injected β-Arr-1-KO mice also exhibited significant inductions of MCP-1 and MIP-1β at 1 h.p.i. ($p<0.05$ and $p<0.001$, respectively). However, at this time point, levels of IL-6 and KC were significantly lower than levels found in the wild types ($p<0.05$) and were not statistically induced over mock levels. Furthermore, the induction of IL-12(p40) was significantly lower at 6 h.p.i. in Ad5-injected β-Arr-1-KO as compared to wild-type animals. Although levels of G-CSF and MCP-1 in β-Arr1-

Figure 6: Functional β-arrestin-1 acts as positive regulator of Ad mediated systemic cytokine and chemokine release in C57BL/6 mice. WT or β-Arr1-KO C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples were collected at 1 and 6 hours post virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array based system. The bars represent Mean ± SD. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=3 for Mock (PBS) injected animals, N=4 for virus injected mice. *, ** - indicate plasma cytokine values that are statistically different from those in Mock-injected animals of the same genotype at the same time point, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in the same treatment group at the same time point, $p<0.05$, $p<0.001$ respectively.

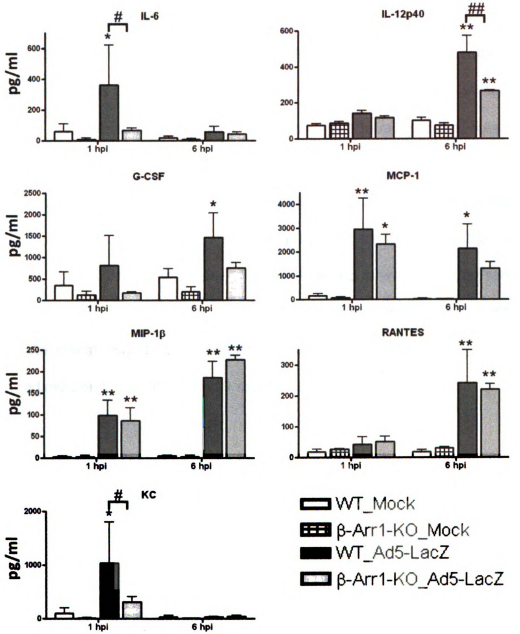
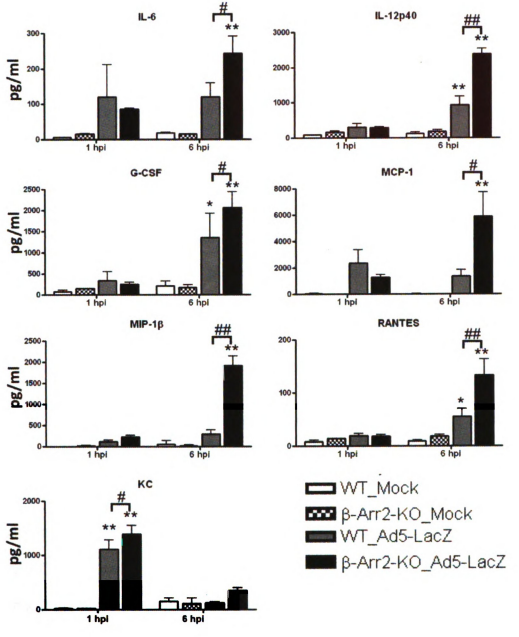

IL-6
IL-12p40
G-CSF
MCP-1
MIP-1β
RANTES
KC
pg/ml
1 hpi
6 hpi
WT_Mock
β-Arr1-KO_Mock
WT_Ad5-LacZ
β-Arr1-KO_Ad5-LacZ

Figure 7: Functional β-arrestin-2 acts as potent suppressor of Ad mediated systemic cytokine and chemokine release in C57BL/6 mice. WT or β-Arr2-KO C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples were collected at 1 and 6 hours post virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array based system. The bars represent Mean ± SD. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=3 for Mock (PBS) injected animals, N=4 for virus injected mice. *, ** - indicate plasma cytokine values that are statistically different from those in Mock-injected animals of the same genotype at the same time point, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in the same treatment group at the same time point, $p<0.05$, $p<0.001$ respectively.


IL-6
IL-12p40
G-CSF
MCP-1
MIP-1β
RANTES
KC
pg/ml
1 hpi
6 hpi
WT_Mock
β-Arr2-KO_Mock
WT_Ad5-LacZ
β-Arr2-KO_Ad5-LacZ

KO did not reach statistically significant differences as compared to identically injected C57BL/6 mice, the levels of these factors were not statistically induced over background levels in β-Arr1-KO mice in contrast to WT mice. No significant differences in either MIP-1β or RANTES were observed (Figure 6). Overall, the results demonstrate that β-Arr1 regulates induction of an important subset of Ad5-induced cytokines and chemokines following systemic injection of Ad5 vectors *in vivo*.

In contrast to observations in β-Arr1-KO mice, levels of all cytokines and chemokines tested in the plasma of Ad5-injected β-Arr2-KO mice were significantly enhanced when compared to identically injected wild type mice. Specifically, at the peak of the KC response, significantly higher levels of this chemokine were detected in Ad-injected β-Arr2-KO animals as compared to wild-type mice. Additionally, significantly higher levels of IL-6 (2-fold), IL-12(p40) (2-fold), G-CSF (1.4-fold), and MCP-1 (6-fold) were detected in the plasma of β-Arr2-KO compared to wild type mice at 6 h.p.i. (Figure 7). Importantly, the absolute numbers of Ad5 genomes present in the liver in either β-Arr1-KO or β-Arr2-KO mice were not different from WT mice as measured by Ad5-genome levels (Figure 8A-B). While there were differences in Ad genome content of the spleens, these differences did not result in differential transduction of these tissues as measured by LacZ protein expression, as determined using a qualitative analysis of X-gal staining and quantitative β-Gal activity

Figure 8: Functional β-arrestin-1 and β-arrestin-2 have modest impact upon liver and spleen transduction efficiency by Ad5 in C57BL/6 mice. qPCR based quantification of Ad5 genomes in liver and spleen tissues harvested from **(A)** WT and β-Arr1-KO C57BL/6 mice or **(B)** WT and β-Arr2-KO C57BL/6 mice at 6 hpi was performed. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare each β-Arr-KO with WT group of virus injected animals. # - indicate statistically different values in β-Arr1-KO_Ad5-LacZ group or β-Arr2-KO_Ad5-LacZ group as compared to WT_Ad5-LacZ group, $p<0.05$. Experiments were performed in duplicate; representative experiment is shown. N=4 for all groups of mice, including Mock-injected animals.

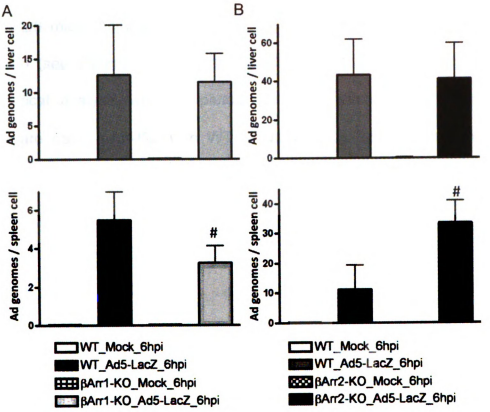
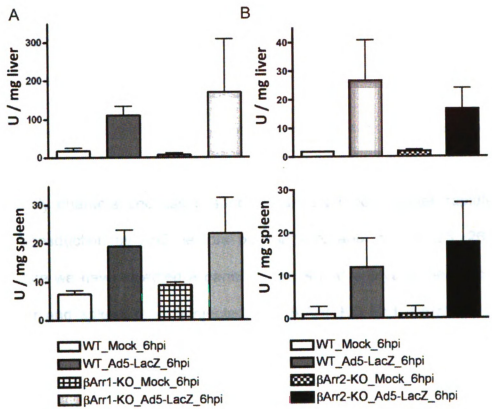

A
B
Ad genomes / liver cell
Ad genomes / spleen cell
#
WT_Mock_6hpi
WT_Ad5-LacZ_6hpi
βArr1-KO_Mock_6hpi
βArr1-KO_Ad5-LacZ_6hpi
WT_Mock_6hpi
WT_Ad5-LacZ_6hpi
βArr2-KO_Mock_6hpi
βArr2-KO_Ad5-LacZ_6hpi

Figure 9: Functional β-arrestin-1 and β-arrestin-2 have modest impact upon Ad derived transgene expression in both liver and spleen tissues in C57BL/6 mice. Bacterial β-galactosidase activity levels were analyzed in liver and spleen protein homogenates prepared at 6 hpi from **(A)** WT and β-Arr1-KO C57BL/6 mice or **(B)** WT and β-Arr2-KO C57BL/6 mice. Activity levels were presented as Units per mg of total protein (see Materials and Methods). The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare each β-Arr-KO with WT group of virus injected animals. No significant differences were detected.


A
B
U / mg liver
U / mg spleen
300
200
100
0
40
30
20
10
0
30
20
10
0
WT_Mock_6hpi
WT_Ad5-LacZ_6hpi
βArr1-KO_Mock_6hpi
βArr1-KO_Ad5-LacZ_6hpi
WT_Mock_6hpi
WT_Ad5-LacZ_6hpi
βArr2-KO_Mock_6hpi
βArr2-KO_Ad5-LacZ_6hpi

measurements in both liver and spleen tissues (Figure 9A-B). Therefore the results presented here were most likely not attributable to differential transduction of murine tissues *per se*. Together these data indicate that β-Arr1 positively regulates the induction of a subset of Ad5-induced cytokines and chemokines, while β-Arr2 functions more globally as a negative regulator of the induction of these innate immune factors.

β-Arrestins differentially mediate the induction of pro-inflammatory genes in livers and spleens following systemic Ad5 injection. We have previously characterized tissue specific transcriptome changes rapidly induced after transduction by Ad5 vectors both *in vitro* and *in vivo* (23, 26, 28, 35). Therefore we have selected a panel of genes and analyzed their expression in the liver and spleen of Ad5-injected WT, β-Arr1-KO and β-Arr2-KO mice at 6 h.p.i. (Tables 1-4). Selected genes include those involved in innate immune responses, such as pattern recognition receptors (TLRs, NODs), TLR signaling pathways (MyD88, TRIF, TRAF6, TRAF2bp, TBK1), markers of endothelial cells activation (e-Selectin, ICAM-1, VCAM-1), interferon responsive genes (OAS1a, IRF7, IRF8), negative regulators of cytokine signaling (SOCS-1, SOCS-3), and dsRNA editing enzymes (ADAR) (23, 28, 35). Our results demonstrate that Ad5-induced activation of a number of genes in the liver of Ad5-injected β-Arr1-KO mice were significantly reduced compared to corresponding Ad treated WT mice (Table 1). Specifically, β-Arr1-KO mice completely lacked Ad5-induced ADAR, CD14, TLR6 and VCAM-1 transcripts. However, baseline levels of TLR6 were significantly lower in the tissues of β-Arr1-KO mock-injected animals, which may

have partially obscured these results. In contrast to Ad5-injected WT mice, NOD-2 and TRIM-30 were not induced in Ad5 treated β-Arr1-KO mice. We also observed a significant reduction in TLR3 transcripts in the liver of Ad5-injected β-Arr1-KO mice as compared to identically injected WT mice. A transcriptome profile observed in spleens isolated from these same animals, revealed significantly reduced levels of TLR3 transcript in β-Arr1-KO mice (Table 2).

Opposite results were obtained in Ad5-injected β-Arr2-KO mice, results that positively correlated with the increased cytokines and chemokines levels observed in these same mice. Over half of the genes tested were induced to significantly higher levels in livers of Ad-injected β-Arr2-KO as compared to identically injected WT mice (Table 3). Specifically, we detected significantly higher amounts of CXCL-9, ICAM-1, IRF-7, MyD88, SOCS-1, SOCS-3, TBK-1, TLR-2, and TRAF-2bp transcripts in livers of Ad5-injected β-Arr2-KO mice, as compared to wild type mice. Moreover, several genes, including ADAR, IRF8, Oas1a, which were not induced in Ad5-injected WT mice, were significantly induced in Ad5-injected β-Arr2-KO mice. The levels of these same transcripts in the spleen followed a similar trend, as IRF-7, IRF-8 and MyD88 were present in significantly higher levels in spleens of Ad5-injected β-Arr2-KO compared to wild type mice. In addition, CXCL9, IFNα, SOCS-1 and TLR9, which were not induced in spleens of wild type C57BL/6 mice, were significantly induced in β-Arr2-KO mice following systemic Ad5 injection (Table 4). These results further support the notion that β-Arr2 functions as a negative regulator of Ad5-induced innate

Table 1: β-Arr1-KO mice had significantly reduced Ad5-triggered activation of a number of genes in livers, as compared to WT mice. Values represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed statistically significant. N=3 for Mock-injected groups, N=4 for Ad5-LacZ injected groups. Significant differences compared to WT_Mock are highlighted in light grey color. Significant differences in transcriptional activation in β-Arr1-KO (Ad5-LacZ) group compared to WT (Ad5-LacZ) group are indicated in table by black frame and boldface font. * Indicate significant differences between mock-injected animals. For full gene names please refer to abbreviation list.

Ad5-LacZ induced gene expression in the liver (fold over C57BL/6 Mock, 6 h.p.i.)				
	C57BL/6 (Mock)	β-Arr1-KO (Mock)	C57BL/6 (Ad5-LacZ)	β-Arr1-KO (Ad5-LacZ)
ADAR	1.0 ± 0.2	0.7 ± 0.1	2.2 ± 0.5	**1.6 ± 0.2**
CD14	1.0 ± 0.2	0.7 ± 0.1	2.3 ± 0.5	**1.3 ± 0.7**
CXCL-9	1.0 ± 0.1	1.0 ± 0.1	10.3 ± 2.4	9.9 ± 4.7
DAF	1.0 ± 0.2	0.9 ± 0.1	1.2 ± 0.1	1.4 ± 0.2
e-Selectin	1.0 ± 0.1	0.6 ± 0.2	2.1 ± 0.3	2.0 ± 0.7
ICAM	1.0 ± 0.2	0.7 ± 0.1	2.2 ± 0.2	2.2 ± 0.4
IFNα	1.0 ± 0.2	0.6 ± 0.1	1.2 ± 0.3	0.7 ± 0.2
IFNβ	1.0 ± 0.4	0.8 ± 0.2	1.6 ± 0.4	1.0 ± 0.1
IRF-3	1.0 ± 0.2	0.9 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
IRF-7	1.0 ± 0.1	0.9 ± 0.2	15.3 ± 1.5	**18.0 ± 1.5**
IRF-8	1.0 ± 0.1	0.8 ± 0.1*	2.1 ± 0.3	2.3 ± 0.4
Jak-1	1.0 ± 0.2	0.8 ± 0.1	1.4 ± 0.2	1.1 ± 0.1
Jak-3	1.0 ± 0.1	0.7 ± 0.2	1.3 ± 0.1	1.3 ± 0.1
MyD88	1.0 ± 0.1	0.8 ± 0.2	4.1 ± 0.5	4.6 ± 0.9
NFkB-RelA	1.0 ± 0.1	1.0 ± 0.1	1.7 ± 0.2	1.6 ± 0.2
NOD-1	1.0 ± 0.1	0.7 ± 0.1*	1.7 ± 0.2	1.4 ± 0.4
NOD-2	1.0 ± 0.1	0.6 ± 0.2*	1.3 ± 0.3	1.0 ± 0.2
OAS-1a	1.0 ± 0.3	0.7 ± 0.1	3.1 ± 0.3	2.6 ± 0.5
SOCS-1	1.0 ± 0.2	0.7 ± 0.1	4.6 ± 0.6	4.6 ± 1.4
SOCS-3	1.0 ± 0.2	0.3 ± 0.1	2.3 ± 0.2	2.5 ± 1.4
TBK-1	1.0 ± 0.1	0.9 ± 0.1	3.9 ± 0.2	3.8 ± 1.0
TLR-2	1.0 ± 0.1	0.7 ± 0.1	21.7 ± 7.0	25.9 ± 10.9
TLR-3	1.0 ± 0.2	0.7 ± 0.1	11.6 ± 1.4	**7.9 ± 3.9**
TLR-6	1.0 ± 0.1	0.6 ± 0.1*	2.0 ± 0.5	**1.5 ± 0.2**
TLR-9	1.0 ± 0.2	0.6 ± 0.1	2.0 ± 0.3	1.8 ± 0.2
TRAF2bp	1.0 ± 0.2	0.6 ± 0.2	6.1 ± 1.1	5.9 ± 3.3
TRAF6	1.0 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
TRIF	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.3	1.7 ± 0.1
TRIM30	1.0 ± 0.2	0.6 ± 0.1	11.4 ± 1.5	7.0 ± 5.1
VCAM	1.0 ± 0.2	0.7 ± 0.1	1.5 ± 0.2	**1.0 ± 0.1**

Table 2: β-Arr1-KO mice had similar levels of Ad5-triggered activation of genes in spleens, as compared to WT mice. Values represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed statistically significant. N=3 for Mock-injected groups, N=4 for Ad5-LacZ injected groups. Significant differences compared to WT_Mock are highlighted in light grey color. Significant differences in transcriptional activation in β-Arr1-KO (Ad5-LacZ) group compared to WT (Ad5-LacZ) group are indicated in table by black frame and boldface font. * Indicate significant differences between mock-injected animals. For full gene names please refer to abbreviation list.

Ad5-LacZ induced gene expression in the spleen (fold over C57BL/6 Mock, 6 h.p.i.)				
	C57BL/6 (Mock)	β-Arr1-KO (Mock)	C57BL/6 (Ad5-LacZ)	β-Arr1-KO (Ad5-LacZ)
ADAR	1.0 ± 0.6	1.5 ± 0.4	2.7 ± 0.2	3.0 ± 0.2
CD14	1.0 ± 0.5	1.9 ± 0.7	2.0 ± 0.1	1.5 ± 0.5
CXCL-9	1.1 ± 1.0	3.3 ± 1.0	12.4 ± 2.9	8.9 ± 3.5
e-Selectin	1.0 ± 0.2	1.5 ± 0.4	1.2 ± 0.1	1.5 ± 0.3
ICAM	1.0 ± 0.4	1.3 ± 0.1	4.1 ± 0.2	4.3 ± 0.6
IFNα	1.0 ± 0.6	2.0 ± 0.8	1.8 ± 0.6	1.7 ± 0.3
IRF-7	1.0 ± 0.3	1.4 ± 0.3	34.3 ± 4.7	38.4 ± 1.4
IRF-8	1.0 ± 0.3	1.2 ± 0.2	2.6 ± 0.4	2.4 ± 0.3
Jak-1	1.0 ± 0.5	1.9 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
MyD88	1.0 ± 0.1	1.5 ± 0.4	3.3 ± 0.3	3.4 ± 0.4
NFkB-RelA	1.0 ± 0.4	0.8 ± 0.1	0.9 ± 0.1	1.0 ± 0.4
OAS-1a	1.0 ± 0.5	2.6 ± 0.4*	13.4 ± 2.6	11.4 ± 0.3
SOCS-1	1.1 ± 0.8	1.3 ± 0.2	3.9 ± 0.2	3.4 ± 1.0
SOCS-3	1.1 ± 0.9	2.2 ± 0.1	30.4 ± 3.6	33.3 ± 10.3
TBK-1	1.0 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	2.1 ± 0.3
TLR-2	1.0 ± 0.4	1.6 ± 0.1	3.8 ± 0.1	4.3 ± 1.0
TLR-3	1.0 ± 0.3	1.9 ± 0.2*	10.0 ± 0.6	**7.6 ± 0.9**
TLR-6	1.0 ± 0.5	1.6 ± 0.5	0.9 ± 0.1	1.0 ± 0.1
TLR-9	1.1 ± 0.8	1.1 ± 0.1	4.3 ± 0.7	5.2 ± 1.1
TRAF2bp	1.0 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.5

Table 3: β-Arr2-KO mice had significantly higher levels of Ad5-triggered activation of a number of genes in livers, as compared to WT mice. Values represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed statistically significant. N=3 for Mock-injected groups, N=4 for Ad5-LacZ injected groups. Significant differences compared to WT_Mock are highlighted in light grey color. Significant differences in transcriptional activation in β-Arr2-KO (Ad5-LacZ) group compared to WT (Ad5-LacZ) group are indicated in table by black frame and boldface font. * Indicate significant differences between mock-injected animals. For full gene names please refer to abbreviation list.

Ad5-LacZ induced gene expression in the liver (fold over C57BL/6 Mock, 6 h.p.i.)				
	C57BL/6 (Mock)	β-Arr2-KO (Mock)	C57BL/6 (Ad5-LacZ)	β-Arr2-KO (Ad5-LacZ)
ADAR	1.0 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	**1.6 ± 0.1**
CD14	1.0 ± 0.2	0.9 ± 0.1	2.1 ± 1.2	1.9 ± 1.2
CXCL-9	1.0 ± 0.3	0.6 ± 0.1	**5.2 ± 0.7**	**9.9 ± 2.7**
DAF	1.0 ± 0.1	0.7 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
e-Selectin	1.0 ± 0.2	0.7 ± 0.2	**2.0 ± 0.8**	**3.0 ± 1.1**
ICAM	1.0 ± 0.2	0.7 ± 0.2	**1.9 ± 0.2**	**3.2 ± 0.6**
IFNα	1.0 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
IFNβ	1.0 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
IRF-3	1.0 ± 0.1	0.6 ± 0.1*	0.7 ± 0.1	0.6 ± 0.1
IRF-7	1.0 ± 0.1	0.7 ± 0.1	**9.1 ± 2.4**	**13.2 ± 1.3**
IRF-8	1.0 ± 0.1	0.7 ± 0.1*	1.6 ± 0.4	**2.8 ± 0.6**
Jak-1	1.0 ± 0.1	0.7 ± 0.1*	0.7 ± 0.2	0.7 ± 0.1
Jak-3	1.0 ± 0.1	0.7 ± 0.1	1.0 ± 0.3	1.1 ± 0.1
MyD88	1.0 ± 0.1	0.6 ± 0.1*	**2.7 ± 1.0**	**4.1 ± 1.0**
NFkB-RelA	1.0 ± 0.1	0.7 ± 0.1	1.0 ± 0.3	1.4 ± 0.2
NOD-1	1.0 ± 0.3	0.8 ± 0.1	1.1 ± 0.1	1.4 ± 0.1
NOD-2	1.0 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
OAS-1a	1.0 ± 0.2	0.6 ± 0.1	1.5 ± 0.4	**2.2 ± 0.3**
SOCS-1	1.0 ± 0.1	0.8 ± 0.1	**3.9 ± 1.4**	**7.5 ± 1.4**
SOCS-3	1.0 ± 0.4	0.9 ± 0.6	**2.2 ± 0.7**	**4.5 ± 0.5**
TBK-1	1.0 ± 0.1	0.8 ± 0.1	**2.7 ± 0.4**	**3.4 ± 0.5**
TLR-2	1.0 ± 0.1	0.9 ± 0.3	**18.6 ± 8.5**	**75.8 ± 28.1**
TLR-3	1.0 ± 0.1	0.6 ± 0.1*	**5.9 ± 1.3**	**9.0 ± 3.0**
TLR-6	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.2	1.6 ± 0.4
TLR-9	1.0 ± 0.1	0.6 ± 0.1*	1.2 ± 0.3	1.2 ± 0.1
TRAF2bp	1.0 ± 0.3	0.6 ± 0.1	**3.7 ± 1.5**	**11.5 ± 2.7**
TRAF6	1.0 ± 0.1	0.8 ± 0.1*	0.9 ± 0.1	0.8 ± 0.1
TRIF	1.0 ± 0.1	0.9 ± 0.1*	0.9 ± 0.2	1.1 ± 0.2
TRIM30	1.0 ± 0.1	0.6 ± 0.1*	**6.7 ± 2.3**	**8.7 ± 1.0**
VCAM	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.9 ± 0.1

Table 4: β-Arr2-KO mice had significantly higher levels of Ad5-triggered activation of a number of genes in spleens, as compared to WT mice. Values represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed statistically significant. N=3 for Mock-injected groups, N=4 for Ad5-LacZ injected groups. Significant differences compared to WT_Mock are highlighted in light grey color. Significant differences in transcriptional activation in β-Arr2-KO (Ad5-LacZ) group compared to WT (Ad5-LacZ) group are indicated in table by black frame and boldface font. * Indicate significant differences between mock-injected animals. For full gene names please refer to abbreviation list.

Ad5-LacZ induced gene expression in the spleen (fold over C57BL/6 Mock, 6 h.p.i.)				
	C57BL/6 (Mock)	β-Arr2-KO (Mock)	C57BL/6 (Ad5-LacZ)	β-Arr2-KO (Ad5-LacZ)
ADAR	1.1 ± 0.8	0.9 ± 0.3	1.3 ± 0.5	1.6 ± 0.1
CXCL-9	1.1 ± 0.9	1.2 ± 0.9	3.6 ± 3.3	4.6 ± 1.9
e-Selectin	1.0 ± 0.2	1.0 ± 0.3	0.7 ± 0.1	1.0 ± 0.2
ICAM	1.0 ± 0.5	0.5 ± 0.2	1.3 ± 0.4	2.3 ± 0.7
IFNα	1.0 ± 0.1	1.6 ± 1.1	1.5 ± 1.0	2.3 ± 0.1
IRF-7	1.0 ± 0.1	0.7 ± 0.2	21.4 ± 5.3	**30.9 ± 3.0**
IRF-8	1.0 ± 0.1	0.7 ± 0.4	0.9 ± 0.2	**1.7 ± 0.1**
MyD88	1.0 ± 0.4	1.0 ± 0.1	2.6 ± 0.1	**3.2 ± 0.1**
NFkB-RelA	1.0 ± 0.3	0.7 ± 0.6	0.7 ± 0.4	1.0 ± 0.2
OAS-1a	1.0 ± 0.1	3.4 ± 3.1	12.9 ± 3.3	15.9 ± 2.1
SOCS-1	1.0 ± 0.4	0.11 ± 0.04*	1.9 ± 1.0	3.3 ± 0.7
SOCS-3	1.0 ± 0.4	2.9 ± 0.4*	12.5 ± 4.4	12.6 ± 4.0
TBK-1	1.0 ± 0.2	0.7 ± 0.4	0.9 ± 0.5	1.3 ± 0.2
TLR-2	1.0 ± 0.3	0.9 ± 0.3	2.0 ± 0.2	4.1 ± 1.6
TLR-3	1.0 ± 0.3	2.2 ± 1.4	6.1 ± 0.9	5.4 ± 0.3
TLR-6	1.0 ± 0.3	2.0 ± 1.4	0.9 ± 0.1	1.0 ± 0.2
TLR-9	1.0 ± 0.2	0.6 ± 0.3	1.5 ± 1.3	1.8 ± 0.2
TRAF2bp	1.0 ± 0.7	1.1 ± 0.5	1.0 ± 0.2	1.1 ± 0.1

immune responses, whereas similar responses are positively mediated by β-Arr1.

2.3. Discussion

Ad-based vectors possess an enormous potential for numerous gene transfer applications based upon their broad tropism, highly efficient transductional capability, and their ease for scalable production. Based upon these facts, Ad vectors have been the most utilized gene transfer vector in humans to date (http://www.wiley.co.uk/wileychi/genmed/clinical/). However, the robust innate immune response elicited shortly after intravascular Ad delivery poses a significant limitation to the use of this vector for numerous applications that could benefit from such administrations, such as gene transduction into the liver. Intravascular delivery of Ads into animal models facilitates detection of Ad induced innate immune responses, which may not be detectable when other routes of administration (such as intramuscular) are utilized. These innate responses include acute thrombocytopenia (26, 44), robust cytokine and chemokine releases (24, 26, 28, 36), activation of endothelial cells (45, 129) and inductions of inflammatory gene networks in multiple tissues, including both the liver and the spleen (23, 26, 46).

It is well established that Ad5-induced innate immune responses are mediated by the TLR adaptor proteins MyD88 and TRIF. As a receptor that utilizes both MyD88 and TRIF, TLR4 is activated by LPS (41-43). Because both β-Arr1 and β-Arr2 proteins have been shown to modulate LPS induced TLR4

mediated signaling, we sought to determine if these proteins also played a role in Ad5-induced innate immune responses. Utilizing β-Arr1-KO and β-Arr2-KO mouse models, we show that whereas β-Arr1 served as positive regulator of a portion of Ad5 induced innate immune responses, β-Arr2 appears to function as a negative regulator of Ad5 induced innate immune responses.

For example, following intravenous Ad5 injection into β-Arr1-KO mice, we observed significantly reduced levels of pro-inflammatory mediators including IL-6, IL-12(p40) and KC, relative to levels measured in Ad5-injected WT mice. Furthermore, we observed significantly reduced transcription of innate immune response genes in transdcued tissues of Ad5-injected β-Arr1-KO mice. We additionally detected reduced expression levels of MCP-1, MIP-1β, and RANTES in peritoneal macrophages from β-Arr1-KO mice following exposure to Ad5 vectors *in vitro*. The results suggest that β-Arr1 may serve as a mediator of Ad-induced immune responses downstream of TLR signaling, specifically those that require MyD88 as an adaptor. Consistent with this notion, we have found significantly reduced activation of IRF7, TLR3 and TLR6 genes in livers of β-Arr1-KO mice, which further suggest the role of β-Arr1 in mediating TLR/MyD88/IRF7 pathways. The findings also highlight a relatively underappreciated role for β-Arr1 as a positive regulator of pro-inflammatory responses, a role that contrasts the previously reported role for β-Arr1 as playing a negative role in the induction of cytokine responses following LPS challenge (41). Our present results however, are consistent with our recent study where we found that β-Arr1 is necessary for

the sustained production of some cytokines/chemokines after LPS challenge *in vivo* (Porter et al, 2009. Manuscript submitted for publication).

The role of β-Arr2 in the induction of innate immune responses by Ad vectors was diametrically opposite to that of β-Arr1. This was evidenced by significantly higher inductions by Ads of multiple cytokines and chemokines, and inflammatory gene expression responses after injection into β-Arr2-KO mice. These responses were corroborated *in vitro* as detected in Ad5 infected peritoneal macrophages, suggesting that this cell type may play an important role in mediating this response *in vivo*. Interestingly, the profile of innate immune responses observed in β-Arr2-KO mice paralleled the role that TLR4 plays in innate immune responses noted after systemic Ad5 injection(130). Our previous work indicated that TLR4 plays a negative role in the induction of IL-12(p40) and G-CSF following intravenous Ad5 injection. It is possible that the negative role that TLR4 plays in Ad5 induced innate immune responses is mediated by β-Arr2, such that loss of either protein results in a more pronounced innate immune response triggered by Ad5. Our results also suggest that β-Arr2 may act as a negative regulator of TLR2/MyD88/IRF7 pathways. However, it is also clear that other factors must also play a role in these responses, since the phenotype in Ad5-injected β-Arr2-KO animals did not completely mimic that found in Ad5-injected TLR4-KO mice (130). Interestingly, in a recent study, we found that β-Arr-2 also mediates LPS/TLR4-induced cytokine responses *in vivo* (Porter et al, 2009, Manuscript submitted for publication).

In summary, our data demonstrate important roles for both β-Arr1 and β-Arr2 as positive and negative regulators in modulating Ad5 induced innate immune responses. β-Arr-1 and -2 interaction partners that mediate β-arrestin's actions in response to Ad5 administration may include proteins of the TLR pathways or other unique proteins of the Ad5 responsive signaling pathways.

Acknowledgements

We wish to thank Michigan State University Laboratory Animal support facility for their assistance in the humane care and maintenance of the animals utilized in this work. S.S.S. was supported by American Heart Association Midwest Affiliate Fellowship 0815660G. A.A. was supported by the National Institutes of Health grants RO1DK-069884, P01 CA078673, the MSU Foundation and the Osteopathic Heritage Foundation. N.P. is supported by the National Institutes of Health grants R01AR056680, R01HL095637, and R21AR055726.

Chapter III

Ad interactions with the complement system are pivotal in understanding how to maximize the safety and potency of Ad mediated gene transfer for both gene therapy and vaccine applications

This chapter is the edited version of a research article that was published in the Gene Therapy Journal, Volume 16, Issue 10 (1245-1259), June 25, 2009.

Authors: Seregin, S. S., Y. A. Aldhamen, D. M. Appledorn, N. J. Schuldt, A. J. McBride, M. Bujold, S. Godbehere, and A. Amalfitano.

3.1. Introduction

Adenovirus (Ad) based vectors offer tremendous capabilities as gene transfer platforms. However, Ad-triggered innate immune responses and Ad-specific neutralizing antibodies hinder the ability to repeatedly administer the vector and preserve transgene expression for long periods of time, prompting multiple efforts to develop alternative Ad vectors to overcome this problem. It is with these realities in mind that studies investigating the mechanisms underlying the induction of neutralizing antibodies to the well-characterized Ad5 vector platform are required. Importantly, the Ad5 serotype is the only Ad serotype utilized in all human gene transfer clinical trials (>367 as per September 2008, comprising 24.9% of all gene therapy trials to date), please see http://www.wiley.co.uk/wileychi/genmed/clinical/).

Our previous studies have confirmed that the induction of neutralizing antibodies to Ads is dependent upon the presence of a functional complement system in the host (24). Although we have discovered that the protein C3 is essential in this response, the mechanisms underlying C3-dependent induction of neutralizing antibody by Ad vectors is currently unknown. Our previous results confirm that lack of Ad interactions with the C3 protein results in a diminished induction of pro-inflammatory cytokines and acute phase responses early after Ad administration, suggesting that a lack of this initial response may contribute to diminished induction of neutralizing antibody responses. This hypothesis is supported by recent studies in IFN receptor KO mice that also showed

diminished induction of neutralizing antibody titers after Ad treatments (131). We have utilized Ad treated CR1/2-KO mice in this study to test this hypothesis.

It is known that complement system activation acts to optimize induction of pathogen specific antibody responses (57-59). Subsequent to opsonization by activated C3, pathogens are bound to B cells and dendritic cells via binding of pathogen bound C3 to the complement receptors (CRs). The human CR1 and CR2 receptors have well known roles in modulating both innate and adaptive immune responses. Human CR1 (hCR1) is a potent inhibitor of complement activation, having both decay-accelerating and cofactor activities. Furthermore, hCR1 has a critical role in the clearance of immune complexes and B cell maturation, as thoroughly reviewed elsewhere (60, 61). Human CR2 (hCR2) expression is restricted to the surface of B cells, follicular dendritic cells and thymocytes. When hCR2 binds C3d opsonised pathogens and becomes associated with CD19, it lowers the threshold for B cell activation by up to 1000 fold (62). HCR1 and hCR2 also play a role in T cell biology, for example, crosslinking of hCR1 inhibits T cell proliferation and IL-12 production (63).

Murine complement receptors (mCRs) 1 and 2 (CD35/CD21) are products of alternative splicing from the same gene. mCR1 contains 21 complement control protein repeats (CCPRs), whereas the smaller mCR2 contains only 15 C-terminal CCPRs of mCR1 (64). mCR1/2 is known to be expressed on B cells and dendritic cells (64). Therefore, the expression pattern of mCR1/2 resembles that of hCR2, but not hCR1. Similar to their human homologues, the functions of mCR1/2 related to generation of maximal humoral responses have been well

described (65-69). Interestingly, mCR1/2 functionality prevents excessive myocardial tissue damage subsequent to coxsackievirus B3 infection (70), as well prevents lethal *Streptococcus pneumoniae* infection, a role potentially indirectly reflective of the complement inhibitory activities of the CRs (71).

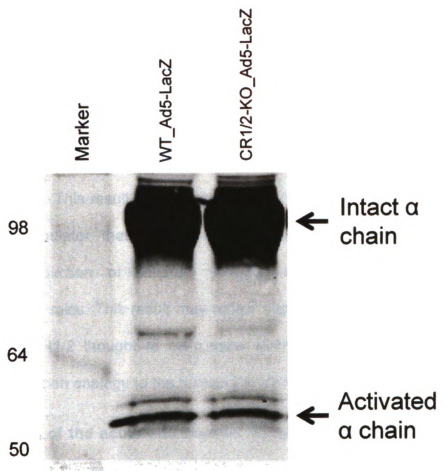
While the role of murine CR1/2 protein has been extensively studied in regards to adaptive immune responses, its function in inhibiting/regulating murine complement has not been demonstrated, possibly since in most mouse models, the Crry protein was suggested to play the predominant role in controlling complement activation. We think that the role of murine CR1/2 protein in innate immune responses (including the ones which are known to be complement dependent) may be more important than previously considered, as suggested by our present studies of Adenovirus mediated gene transfer into mCR1/2-KO mice. Our results in murine models revealed dual roles for mCR1/2; roles that include down-regulation of multiple aspects of the Ad induced innate immune responses, while also playing the major role in the complement dependent induction of neutralizing antibody responses to Ads.

3.2. Results

Murine Complement Receptor 1/2 regulates Ad mediated cytokine and chemokine release in C57BL/6 mice. To study the role of mCR1/2 protein in Ad induced innate and adaptive immune responses, we utilized mCR1/2-KO mice. These mice have been previously demonstrated to completely lack expression of CR1/2 on B cells (71). It is also known that CR1/2 activities also impact upon levels of activated C3, by virtue of CR1/2's decay accelerating properties. Utilizing western blotting with C3-specific antibodies, we confirmed that mock-injected CR1/2-KO mice have normal overall levels of C3 no different than wild-type mice, and equivalent amounts of C3 cleavage products were present in the plasma of virus injected WT and CR1/2-KO mice, as investigated both at 10 minutes post injection and 6 hours post injection (Figure 10). These results suggest that CR1/2-KO mice do not have significant alterations in the ability of C3 to initially interact with Ads, an interaction that we have previously confirmed mediates many Ad induced innate and adaptive immune responses (23-26, 46).

Inflammatory cytokines and chemokines are rapidly released after systemic Ad injection. We have identified 7 cytokines and chemokines (KC or CXCL1, MCP-1, MIP-1β, G-CSF, RANTES, IL-6, IL-12p40) that become significantly elevated within hours of systemic administration of Ad vectors, some of which are elevated in a C3-dependent fashion (24, 26). We investigated the role that mCR1/2 has in the induction of these cytokines by administering Ads into wild type and mCR1/2 knockout mice. Plasma samples, collected at 1 and 6

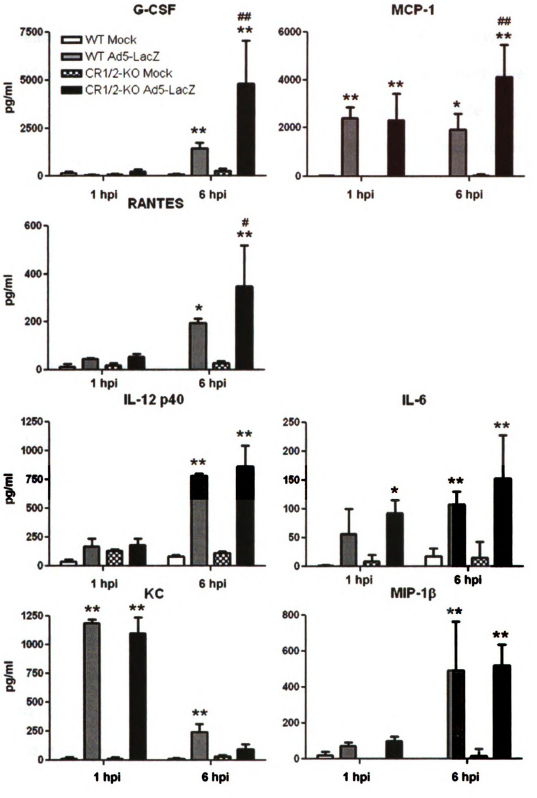
Figure 10: Plasma basal levels and Ad dependent activation of complement protein C3 were identical between C57Bl/6 WT and CR1/2-KO mice. Plasma samples from WT C57BL/6 and CR1/2-KO mice were collected and Western blotting was performed as described in Materials and Methods utilizing quantitative Licor's Odyssey system. Intact and activated α-chains of C3 protein are shown. There were no significant differences in levels of basal C3 protein or C3 protein cleavage detected.


Marker
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
98
64
50
Intact α chain
Activated α chain

hours post intravenous Ad administration confirmed that KC and MCP-1 chemokines are rapidly released in response to Ad injections and reach maximum levels by 1 hpi. Ad-injected mCR1/2-KO mice had identical levels of activation of these 2 chemokines at 1 hpi (Figure 11), suggesting that Ad induction of these very early mediators of the inflammatory responses is not dependent upon mCR1/2 functionality. Interestingly, Ad-injected mCR1/2-KO mice exhibited significantly higher plasma levels of G-CSF, MCP-1 and RANTES at 6 hours after systemic Ad injection, as compared to identically treated WT mice (Figure 11). This result identifies the role of functional mCR1/2 protein as a complement regulator that suppresses complement activation, resulting in diminished production of several cytokines released subsequent to Ad administration in mice. This result may reflect a complement decay accelerating property of mCR1/2 thought to be present in the extracellular portion of the protein, based upon analogy to the human CR1/2 homologs.

Portions of the acute and chronic cellular responses to Ad vectors are Complement Receptor 1/2 dependent. Activation of vascular endothelium is a critical step during initiation of inflammatory immune responses, since many inflammatory cells (i.e. platelets, neutrophils, macrophages, mast cells) utilize activated endothelial cells (EC) as a means to localize to damaged sites. This response is mediated by activated EC over-expression of E-selectin, ICAM-1 and/or VCAM-1 adhesion molecules on their surface, molecules that facilitate the migration of inflammatory cell types into damaged organs (45, 132, 133). Soluble

Figure 11: Murine Complement Receptor 1/2 mitigates Ad mediated cytokine and chemokine release in C57BL/6 mice. C57BL/6 WT and CR1/2-KO mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples were collected at 1 and 6 hours post virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array based system. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=4 for Mock (PBS) injected animals, N=6 for virus injected mice) and 6 hpi (N=4 for Mock, N=12 for virus treated mice. The bars represent Mean ± SD. *, ** - indicate plasma cytokine values that are statistically different from those in Mock-injected animals of the same treatment at the same time point (i.e. CR1/2-KO_Ad-LacZ group from CR1/2-KO_Mock group), $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group at the same time point, $p<0.05$, $p<0.001$ respectively.


G-CSF
WT Mock
WT Ad5-LacZ
CR1/2-KO Mock
CR1/2-KO Ad5-LacZ
pg/ml
7500
5000
2500
0
1 hpi
6 hpi
MCP-1
6000
4000
2000
RANTES
600
400
200
IL-12 p40
1250
1000
750
500
250
IL-6
250
200
150
100
50
KC
MIP-1β
800
600
400

forms of these cell adhesion molecules are also produced by activated ECs (134). Interestingly, while the levels of sE-selectin in Ad treated WT mice were increased 3-fold, sE-selectin levels in Ad treated mCR1/2-KO mice were at least 7-fold higher, as compared to mock-injected mCR1/2-KO mice, these levels were also significantly higher than levels noted in Ad treated WT mice (Figure 12). Induction of both sVCAM-1 and sICAM-1 was also slightly more robust in Ad treated mCR1/2-KO mice as compared to Ad-injected WT mice, although these levels did not reach a significant difference (data not shown). We have also tested the level of ICAM-1, VCAM-1 and E-selectin mRNA transcripts in murine liver at 6 hpi after systemic Ad injection and found significantly increased levels of all 3 transcripts in mCR1/2-KO mice (Table 5). These results confirm that lack of mCR1/2 functionality results in a more robust induction of cellular adhesion molecules in Ad treated mice, suggesting that another role of mCR1/2 is to modulate (suppress) the extent of complement dependent induction of EC activation.

We next wished to correlate EC activation with cellular elements known to be mobilized subsequent to Ad infections or Ad vector administrations. Acute thrombocytopenia and/or lymphopenia can occur within hours of natural Ad infections, and Ad induction of thrombocytopenia is a complement (C3) dependent event (24, 26, 29). However, at 24 hours after systemic Ad injection, mCR1/2-KO mice exhibited levels of thrombocytopenia no different than that noted in Ad-injected WT mice (data not shown). Ad-injected mCR1/2-KO mice

Figure 12: Murine Complement Receptor 1/2 reduces Ad dependent activation of endothelial cells in C57BL/6 mice. C57BL/6 WT and CR1/2-KO mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples, collected at 6 hpi (N=6 for virus treated groups, N=4 for Mock-injected groups) were analyzed using a multiplexed bead array based quantitative system. The bars represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test. Fold difference over WT_Mock group is shown. *, ** - indicate values, statistically different from those in Mock-injected animals of the same genotype, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.

e-Selectin
FOLD
0
2
4
6
8
*
#
*
C57BL/6_WT_Mock
C57BL/6_WT_Ad5-LacZ
CR1/2-KO_Mock
CR1/2-KO_Ad5-LacZ

Table 5: mCR1/2 is an important suppressor of Ad5-LacZ induced gene expression in livers of C57BL/6 mice. The numbers represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. Note, when significant $p<0.001$ was observed in majority of cases. N=4 for Mock-injected groups, N=6 for virus injected groups was used. Significant differences compared to C57BL/6_WT_Mock are highlighted in grey color. Significant inductions of transcriptional activation in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group are indicated in table with black frame and boldface font. Note that there were no significant differences detected between Mock-injected WT and CR1/2-KO mice. For full gene names please refer to abbreviation list.

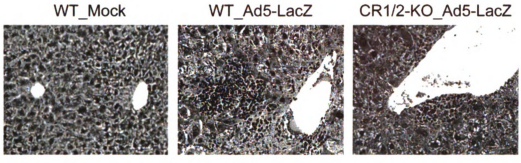
Ad5-LacZ induced gene expression in a liver (fold over C57BL/6_WT_Mock)				
	C57BL/6 WT 6 hpi Mock	CR1/2 -/- 6 hpi Mock	C57BL/6 WT 6 hpi Ad5-LacZ	CR1/2 -/- 6 hpi Ad5-LacZ
ADAR	1.0 ± 0.2	1.0 ± 0.1	7.1 ± 1.2	8.7 ± 1.8
CXCL-9	1.0 ± 0.1	1.8 ± 0.6	27.9 ± 6.0	34.2 ± 19.9
DAF	1.0 ± 0.4	1.9 ± 0.1	1.8 ± 0.3	2.1 ± 0.6
GATA-3	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.4	2.0 ± 0.3
ICAM	1.0 ± 0.5	1.6 ± 0.4	5.3 ± 1.0	7.7 ± 1.6
IFNα	1.1 ± 0.5	0.9 ± 0.2	1.9 ± 0.5	1.7 ± 0.6
IFNβ	1.0 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.2
IRF-7	1.1 ± 0.7	1.2 ± 0.4	22.4 ± 4.9	22.7 ± 7.7
IRF-8	1.1 ± 0.7	2.0 ± 0.5	5.9 ± 0.7	6.4 ± 2.4
Jak-1	1.0 ± 0.3	1.0 ± 0.2	1.3 ± 0.2	1.6 ± 0.4
Jak-3	1.0 ± 0.3	1.2 ± 0.3	1.7 ± 0.3	2.4 ± 0.3
MyD88	1.0 ± 0.4	1.05 ± 0.03	6.0 ± 1.2	9.9 ± 2.0
NFkB-RelA	1.0 ± 0.3	1.1 ± 0.1	2.2 ± 0.3	2.4 ± 0.4
NOD-1	1.0 ± 0.3	1.2 ± 0.3	2.5 ± 0.4	3.5 ± 1.1
NOD-2	1.1 ± 0.5	0.2 ± 0.1	2.6 ± 0.8	0.6 ± 0.2
OAS-1a	1.0 ± 0.4	1.2 ± 0.5	20.7 ± 6.4	27.7 ± 6.1
SOSC-1	1.0 ± 0.4	1.1 ± 0.5	70.5 ± 19.1	115.4 ± 22.6
SOSC-3	1.1 ± 0.5	0.6 ± 0.2	2.8 ± 0.6	3.2 ± 1.0
TBK-1	1.0 ± 0.2	1.2 ± 0.1	4.6 ± 0.6	7.3 ± 2.2
TLR-2	1.0 ± 0.1	2.7 ± 0.3	169.2 ± 54.2	251.8 ± 71.8
TLR-3	1.0 ± 0.2	0.8 ± 0.2	14.8 ± 2.6	28.5 ± 6.1
TLR-6	1.0 ± 0.4	1.2 ± 0.4	6.0 ± 1.8	7.0 ± 1.9
TRAF2bp	1.1 ± 0.6	1.3 ± 0.5	18.8 ± 4.8	62.6 ± 9.2
TRAF6	1.1 ± 0.6	1.9 ± 0.3	1.8 ± 0.3	2.1 ± 0.4
TRIF	1.0 ± 0.4	1.4 ± 0.2	1.8 ± 0.3	2.5 ± 0.7
VCAM	1.0 ± 0.1	1.0 ± 0.1	2.0 ± 0.2	3.3 ± 0.7

trended towards elevated levels of neutrophils and reduced levels of lymphocytes at 24 hpi, though the changes did not reach statistical significance when compared to identically treated WT mice. There was also no difference in levels of Kupffer cell necrosis in virus treated WT and mCR1/2-KO mice, suggesting that mCR1/2 is not involved in Kupffer cell necrosis, an event that occurs within hours of Ad injection (data not shown) (135).

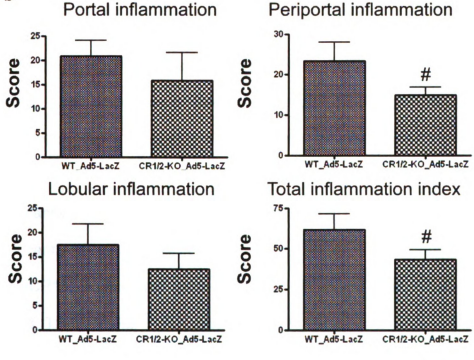
Systemically administered Ad vectors induce significant leukocyte infiltration into the liver (136, 137). The infiltration becomes apparent at 3 dpi, continuously increases with the generation of an adaptive immune response to the virus and/or antigen it expresses, and reaches maximal levels by 21-56 dpi (137, 138). We have quantified monocellular (leukocyte) infiltration into the livers of Ad treated WT and mCR1/2-KO mice at 28 dpi. Both WT and mCR1/2-KO Ad-injected mice exhibited significant infiltration of inflammatory cells into their livers, as compared to mock-injected animals. Despite the previously noted enhanced pro-inflammatory cytokine responses, as well as enhanced activation of ECs in Ad-injected mCR1/2-KO mice, Ad-injected mCR1/2-KO mice had significantly reduced periportal inflammation as well as total levels of hepatic inflammation relative to Ad-injected WT mice (Figure 13A-B). Portal and lobular inflammation were somewhat reduced in virus treated mCR1/2-KO mice as compared to identically treated WT mice, although these differences did not reach statistical significance. These results suggest that maximal induction of cellular inflammation after Ad vector administration is directly dependent upon mCR1/2,

Figure 13: mCR1/2-KO mice exhibit significantly reduced leukocyte infiltration into the liver of Ad treated mice at 28 dpi. Mice injections and morphometric evaluation of liver sections was performed as described in Materials and Methods. **(A)** Representative H&E stained liver sections obtained at 28 dpi from three groups of mice: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5) are shown. Note the lack of any inflammation in WT_Mock, the large number of inflammatory cells in WT_Ad5-LacZ and moderate infiltration in CR1/2-KO_Ad5-LacZ. **(B)** Representative sections from each treated animal were analyzed, scored and averaged for the levels of portal, periportal and lobular inflammation, as described in Materials and Methods. The sum of averages for each category was computed to obtain a total inflammation index score. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.

a


WT_Mock
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ

b


Portal inflammation
Score
25
20
15
10
5
0
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
Periportal inflammation
Score
30
20
10
0
#
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
Lobular inflammation
Score
25
20
15
10
5
0
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
Total inflammation index
Score
75
50
25
0
#
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ

despite enhanced activation of the innate immune system in the same animals earlier.

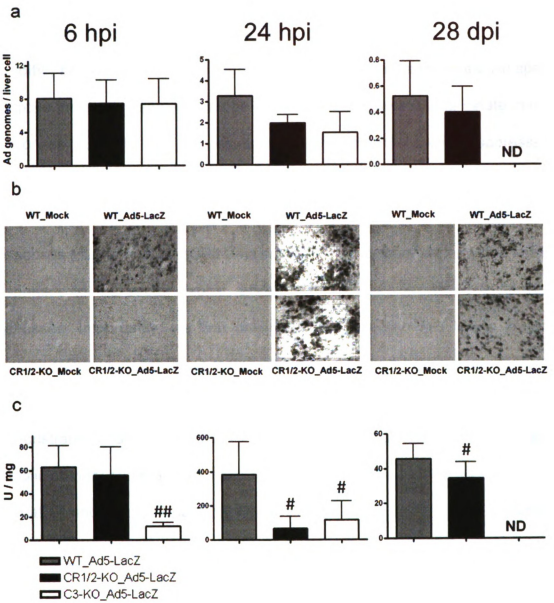
Murine Complement Receptor 1/2 downregulates Ad induction of pro-inflammatory genes in livers of Ad treated C57BL/6 mice. We have previously confirmed that systemic administrations of Ad vectors results in a rapid and profound transcriptome response in the murine liver, responses that are mediated by Ad interactions with the murine complement system (23, 26, 35). Based in part upon those studies, we analyzed expression levels of several liver gene transcripts, including those participating in innate immune responses, i.e.: pattern recognition receptors (TLRs, NODs), TLR signaling pathways (MyD88, TRIF, TRAF6, TRAF2bp, TBK1), markers of EC activation (ICAM1, VCAM1, E-selectin), interferon responsive genes (ADAR, OAS1a, IRF7, IRF8), all listed in Table 5. Note, that none of the genes tested showed any differences in basal transcription levels between mock-injected WT and mCR1/2-KO mice liver derived transcripts. However, the levels of 13 liver transcripts, although significantly induced in Ad-injected WT mice at 6 hpi, were induced to significantly greater levels in Ad treated mCR1/2-KO mice. These Ad induced, but mCR1/2 suppressed genes included ADAR, ICAM1, VCAM1, E-selectin, MyD88, TBK1, TLR2, TLR3, JAK3, SOCS1. Importantly, 5 gene transcripts, that were not induced in Ad-injected WT mice (as compared to Mock-injected animals) also revealed significant inductions in mCR1/2-KO Ad treated mice (Table 5). These observations allowed us to conclude that mCR1/2 plays a significant role in down-regulating Ad induction of pro-inflammatory gene

expression. Importantly, the Ad induction of most of these genes has also been previously shown by us to be a complement protein C3-dependent event, suggesting that mCR1/2 downregulates Ad activation of pro-inflammatory genes by suppressing and/or regulating the overall levels of Ad induced, C3-dependent activation of the complement system (24, 26).

Ad mediated transduction *in vivo* is not dependent upon murine Complement Receptor 1/2, but the levels of Ad derived transgene expression are complement and mCR1/2-dependent. We next confirmed that Ad mediated liver transduction of Ad genomes was not diminished at any time point tested during our studies (Figure 14A), in part confirming our previously published results that complement does not significantly mediate Ad transduction efficacy *in vivo* (24).

Ad derived transgene expression in livers of treated mice was then tested by two independent techniques: qualitatively by staining liver sections with X-Gal (Figure 14B) and by quantitative measurement of β-galactosidase activity at 6 hpi, 24 hpi and 28 dpi (Figure 14C). We found a significant reduction of β-Gal activity in mCR1/2-KO mice as compared to WT mice both at 24 hpi and 28 dpi, but not at 6 hpi (Figure 14B-C). Ad-injected C3-KO mice also exhibited significantly reduced β-Gal activity both at 6 hpi and 24 hpi, a trend that we and others have noted previously (24, 26, 139). Our observations confirm that lack of mCR1/2 functionality does not dramatically alter the ability of Ads to transduce their genomes into the murine liver *per se*, but rather results in a more rapid

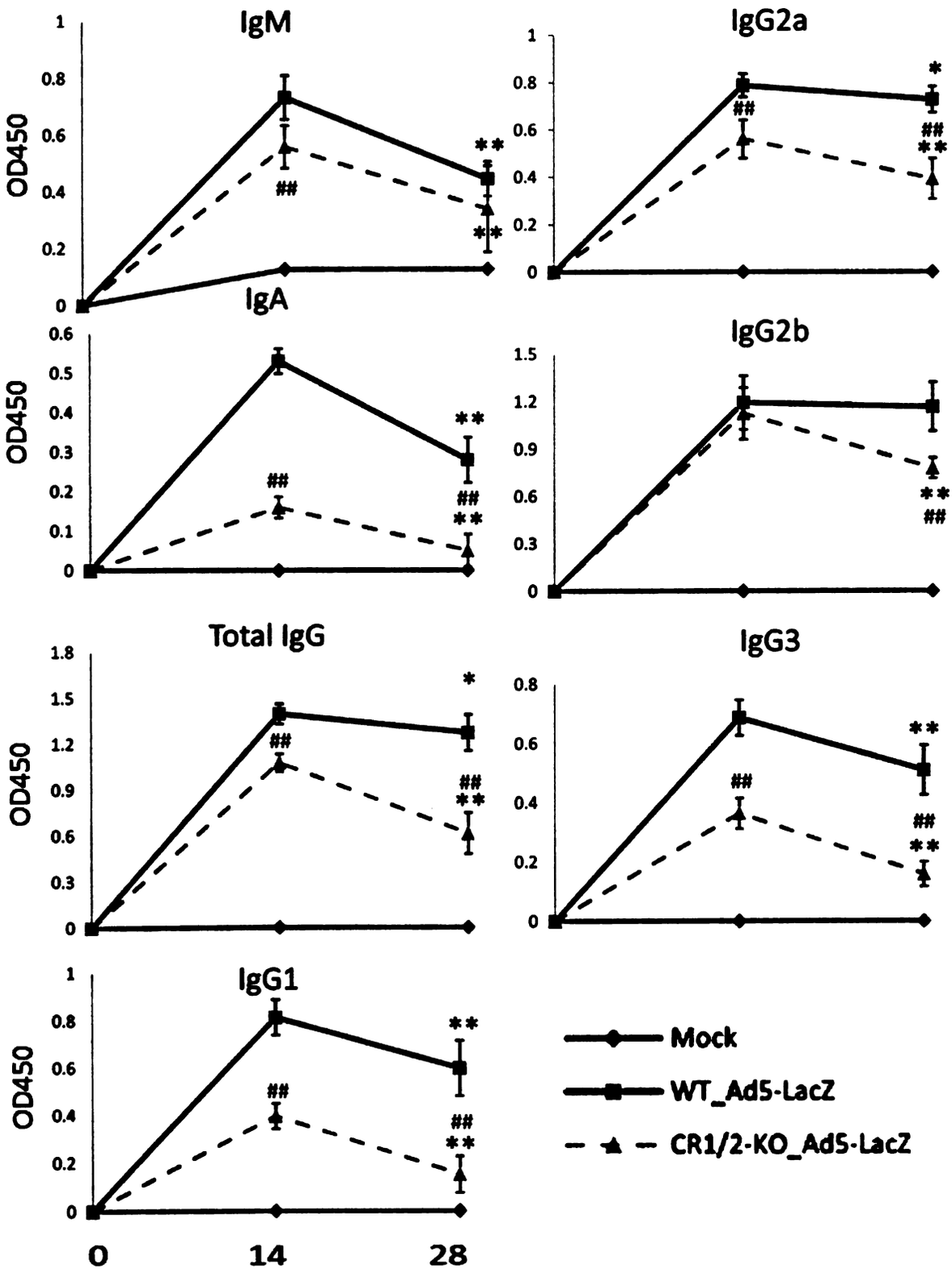
Figure 14: The efficacy of Ad transduction of the liver of C57BL/6 mice is not dependent upon murine Complement Receptor 1/2, but the levels of Ad derived transgene expression are complement and mCR1/2-dependent. **(A)** qPCR based quantification of Ad5-LacZ genomes in livers harvested from C57BL/6 WT and CR1/2-KO mice at 6 hpi, 24 hpi, 28 dpi. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. Note the difference in scale for different time points. **(B)** *In situ* visualization of bacterial β-galactosidase in liver of Ad5-LacZ treated C57BL/6 WT and mCR1/2-KO mice. Representative sections for each of the groups are shown. Total magnification of 200X was used to obtain images. N=6 for all virus injected groups at 6 hpi, N=4 for all virus injected groups at 24 hpi, N=5 for all virus injected groups at 28 dpi, N=4 for all Mock-injected groups at all time points. **(C)** Bacterial β-galactosidase activity levels were analyzed at 6 hpi, 24 hpi and 28 dpi from four groups of mice. Activity levels were presented as Units per mg of total protein. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.


a
6 hpi
24 hpi
28 dpi
Ad genomes / liver cell
ND
b
WT_Mock
WT_Ad5-LacZ
CR1/2-KO_Mock
CR1/2-KO_Ad5-LacZ
c
U / mg
##
#
ND
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
C3-KO_Ad5-LacZ

shutdown of CMV enhancer dependent transgene expression from Ad vectors, a complex finding elaborated upon further in the discussion section of this chapter.

Murine Complement Receptor 1/2 is required for generation of Ad vector specific, but not transgene specific adaptive immune responses. Ad specific adaptive immune responses can be primed by the robustness of innate immune responses (63, 131, 140). Our results confirm that mCR1/2 protein suppresses or down-regulates many Ad induced innate immune responses, therefore we wished to determine whether enhanced induction of Ad induced innate immune responses (due to lack of mCR1/2 function) altered subsequent adaptive immune responses to the vector capsid, and/or the transgene product the Ad vector expresses. Importantly, we first confirmed that baseline IgG levels in mock-injected WT and mCR1/2-KO mice were identical (data not shown), confirming previously published observations (63, 65, 66, 140). However, our results demonstrated that Ad-injected mCR1/2-KO mice had diminished Ad-capsid specific primary humoral responses (as compared to identically treated WT mice) both at 14 and 28 dpi (Figure 15). IgA, total IgG, IgG1, IgG2a, IgG3 subtypes of Ad capsid specific antibodies tested in Ad treated mCR1/2-KO mice were significantly reduced at least at one of the two time points tested as compared to Ad-injected WT mice, moreover all were diminished both at 14 and 28 dpi in mCR1/2-KO mice (Figure 15). Additionally, Ad-injected mCR1/2-KO mice did not generate significant titers of Ad-capsid neutralizing antibodies, as determined both at 14 and 28 dpi (Figure 16). These observations strongly parallel our

Figure 15: mCR1/2-KO mice exhibit significantly reduced Ad vector capsid specific humoral immune responses. Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples, collected at 14 dpi and 28 dpi, were analyzed for anti Ad capsid specific total IgM, IgA and IgG antibodies and various IgG subclasses. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. *, ** - indicate values, statistically different from animals of the same group at different time point, $p<0.05$, $p<0.001$ respectively.

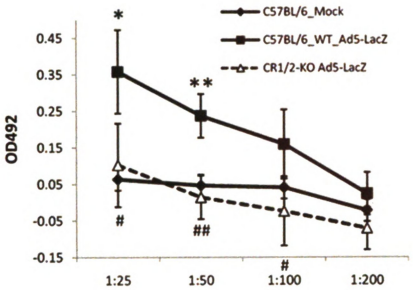

IgM
IgG2a
IgA
IgG2b
Total IgG
IgG3
IgG1
OD450
0
14
28
Mock
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ

previously published data noting that Ad-injected C3-KO mice also exhibit a diminished capacity to generate anti-Ad capsid specific humoral responses, as well as neutralizing antibody responses (24).

Since B cells are known to express high levels of mCR1/2, and depend upon mCR1/2 for induction of antigen specific B cell activation, we quantified B cell activation 48 hours after systemic Ad injection into WT, C3-KO and mCR1/2-KO mice. We confirmed that the number of activated B cells (i.e.: $CD19^{+}/CD69^{+}$ splenocytes) was significantly increased in WT mice injected with Ad vectors as compared to uninfected WT mice (Figure 17). Interestingly, the levels of B cell activation were significantly and identically reduced in both Ad treated C3 and mCR1/2-KO mice, again as compared to identically injected WT mice, suggesting that the C3-dependent portion of induction of neutralizing antibody responses to Ad capsids may be entirely mediated by interactions mediated by the CR1/2 protein (Figure 17). This result positively correlates with our findings that functional mCR1/2 protein is required to generate Ad capsid specific humoral immune responses (Figures 15-16), a response that is at least in part a complement dependent event (24). The overall results suggest that induction of neutralizing antibodies to Ads is dependent upon C3 opsonized Ads interacting with mCR1/2 present on B cells, resulting in their activation.

Finally, we have previously published that the lack of complement protein C3 did not reduce the induction of transgene specific humoral responses in Ad-injected mice (24). Interestingly, mCR1/2-KO mice injected with Ad5-LacZ

Figure 16: mCR1/2-KO mice exhibit significantly reduced Ad capsid specific neutralizing antibodies titer. Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples were collected at 28 dpi and assayed for neutralizing antibodies using successive dilutions (see Materials and Methods). The error bars represent ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. *, ** - indicate values, statistically different from those in WT_Mock-injected animals, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. The bottom bar graph shows endpoint neutralizing antibody titer. Note significant difference in endpoint NAb titer detected between WT and CR1/2-KO virus injected groups by two-tailed Student t-test.


C57BL/6_Mock
C57BL/6_WT_Ad5-LacZ
CR1/2-KO Ad5-LacZ
OD492
0.45
0.35
0.25
0.15
0.05
-0.05
-0.15
*
**
#
##
#
1:25
1:50
1:100
1:200

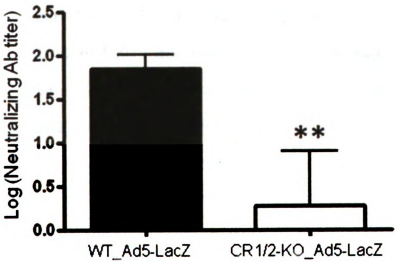
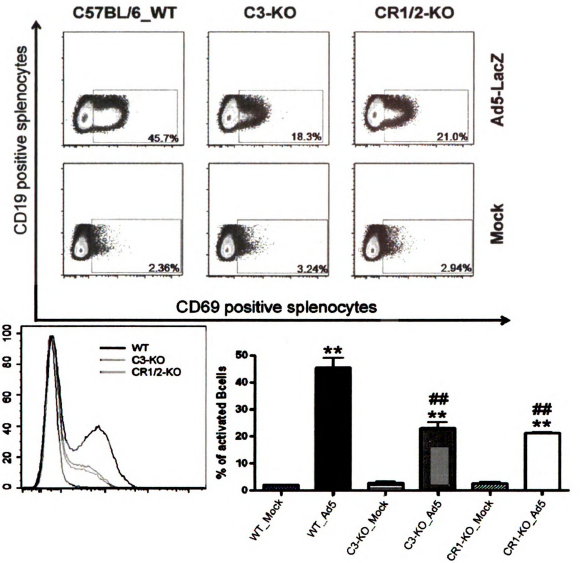
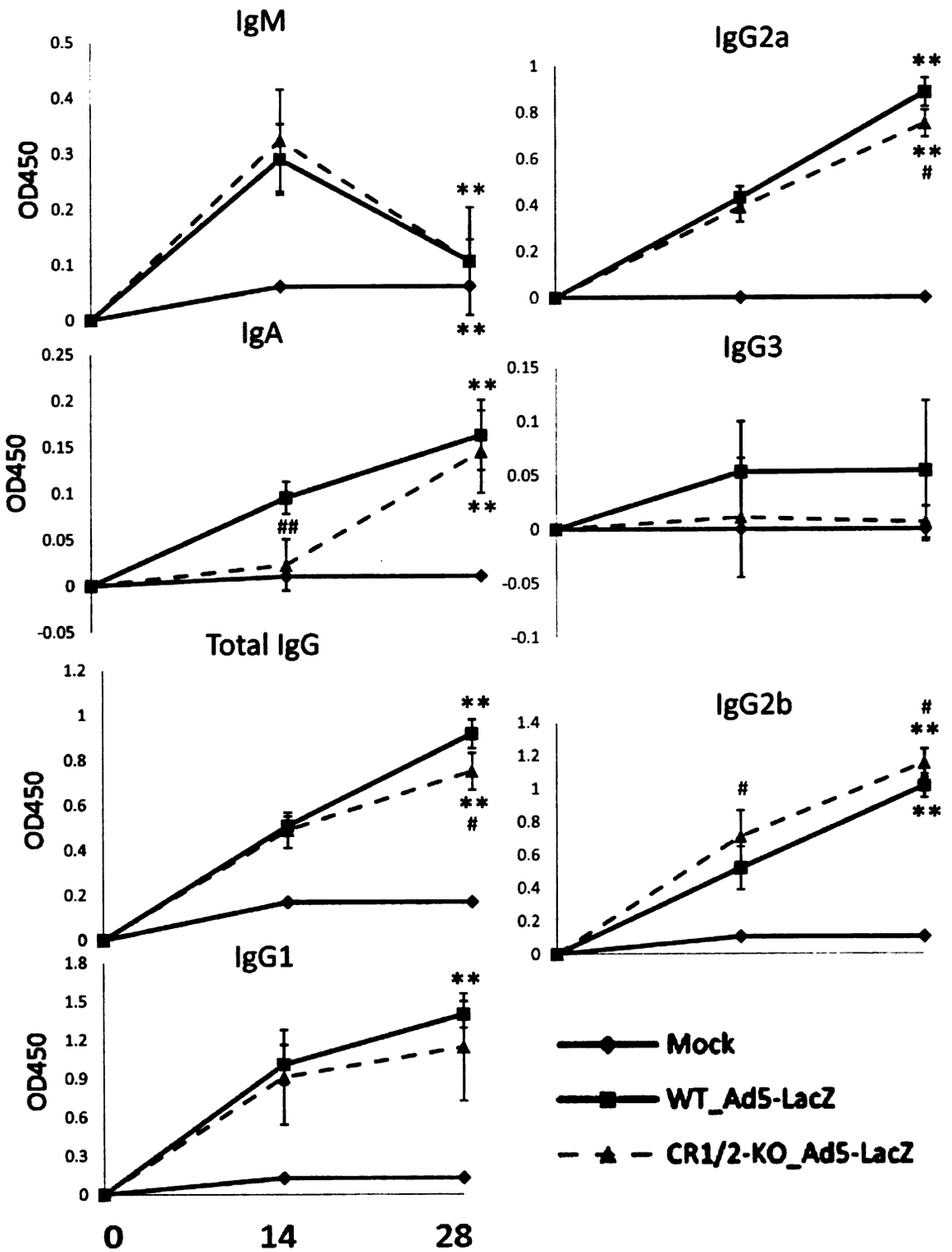

Log (Neutralizing Ab titer)
2.5
2.0
1.5
1.0
0.5
0.0
**
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ

Figure 17: mCR1/2 and C3-KO mice have impaired B cell activation in response to systemic Ad injection. C57BL/6_WT, C3-KO and CR1/2-KO mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Splenocytes were isolated at 48 hpi and processed as described in Materials and Methods. Percentage of activated B cells ($CD19^+/CD69^+$ splenocytes) was determined by flow Cytometry based methods. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. The bars represent Mean ± SD. *, ** - indicate values statistically different from those in Mock-injected animals of the same treatment (i.e. CR1/2-KO_Ad-LacZ group from CR1/2-KO_Mock group), $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in virus injected complement deficient mice groups compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.


C57BL/6_WT
C3-KO
CR1/2-KO
CD19 positive splenocytes
Ad5-LacZ
Mock
45.7%
18.3%
21.0%
2.36%
3.24%
2.94%
CD69 positive splenocytes
WT
C3-KO
CR1/2-KO
% of activated B cells
**
##
**
##
**
WT_Mock
WT_Ad5
C3-KO_Mock
C3-KO_Ad5
CR1-KO_Mock
CR1-KO_Ad5

generated levels of transgene specific (β-Gal) antibodies that paralleled those noted in identically treated WT mice (Figure 18). Only β -Gal specific IgA, total IgG and IgG2a reached significant differences, and this occurred at only one of the two time points tested. These results suggest that Ad expressed, transgene specific adaptive immune responses are not dependent upon mCR1/2.

Figure 18: mCR1/2-KO mice have minimally reduced Ad vector derived transgene specific humoral immune responses as compared to WT mice. A) Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples, collected at 14 dpi and 28 dpi, were analyzed for anti β-Gal (Ad derived transgene) specific total IgM, IgA and IgG antibodies and various IgG subclasses. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. *, ** - indicate values, statistically different from animals of the same group at different time point, $p<0.05$, $p<0.001$ respectively.


IgM
IgG2a
IgA
IgG3
Total IgG
IgG2b
IgG1
OD450
0
14
28
Mock
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ

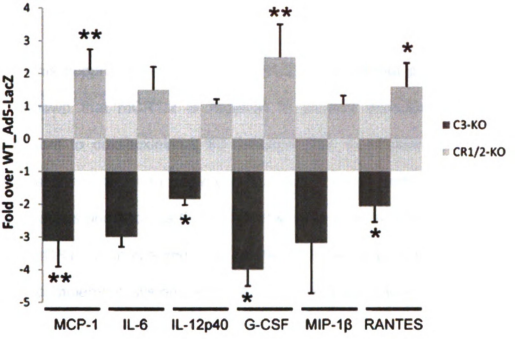
3.3. Discussion

Despite the fact that the role of murine Complement Receptor 1/2 (mCR1/2) in modulating adaptive immune responses has been extensively studied (65, 66, 141, 142), very little information is available regarding the role of mCR1/2 in regulating aspects of the innate immune system, including complement mediated responses. In this study we have investigated the role of mCR1/2 protein in regulating Ad vector induced innate immune responses and also assess what impact these roles have upon downstream adaptive immune responses to the Ad vector, and/or the transgene the vector expresses.

We have previously described the pivotal role the complement system has in generating robust innate immune responses subsequent to Ad treatment, including systemic pro-inflammatory cytokines/chemokine release, EC activation, acute thrombocytopenia, and liver transcriptome dysregulation (24, 26). In this study we have shown that many of these complement dependent responses are modulated (suppressed) by mCR1/2.

To highlight how mCR1/2 specifically down-regulates Ad dependent induction of pro-inflammatory cytokines and chemokine responses we have combined data obtained in this study with our previously published data in Ad treated C3-KO mice (24), (Figure 19). Note, that MCP-1, IL-12p40, G-CSF and RANTES are all induced in a complement dependent manner (evident by the lack of induction of these cytokines/chemokines in C3-KO mice). Moreover, mCR1/2 protein down-regulates the induction of these very chemokines (MCP-1,

Figure 19: mCR1/2 protein plays a significant role in down-regulation of Ad mediated complement dependent pro-inflammatory cytokines production. This graph summarizes data obtained in this study (Figure 11) and our previously published data on Ad mediated cytokine release in C3-KO mice (24). Plasma levels (at 6 hpi) of pro-inflammatory cytokines and chemokines in Ad-injected CR1/2-KO and C3-KO mice are normalized to the levels of WT_Ad5-LacZ. Levels higher than the ones observed in WT mice have positive fold induction (CR1/2-KO mice), whereas reduced levels compared to the levels of WT mice (C3-KO) have negative fold induction. Shaded area represents the levels of cytokines release in Ad-injected WT mice. *, ** - represent a statistically significant differences between Ad-injected WT and complement (CR1/2 or C3) knockout mice, $p<0.05$, $p<0.001$ respectively. Note, that MCP-1, IL-12p40, G-CSF and RANTES are all induced in a complement dependent manner (evident by the lack of induction of these cytokines/chemokines in C3-KO mice). Moreover, mCR1/2 protein down-regulates the induction of these very molecules (MCP-1, G-CSF and RANTES).


Fold over WT_Ad5-Lacz
4
3
2
1
0
-1
-2
-3
-4
-5
**
**
*
**
*
*
*
MCP-1
IL-6
IL-12p40
G-CSF
MIP-1β
RANTES
C3-KO
CR1/2-KO

G-CSF and RANTES) as compared to Ad treated wild-type mice. These overall results reveal that mCR1/2 plays a very important role in suppressing overall innate immune responses induced by complement system activation by Ad vectors.

This suppressive activity of mCR1/2 is not without precedent, as it has been shown that mCR1/2 is required to prevent excessive tissue damage subsequent to coxsackievirus B3 infection (70). In addition, lack of mCR1/2 functionality results in an enhanced susceptibility (i.e.: lethality) subsequent to *Streptococcus pneumoniae* (71). Note, that none of these studies focused on the role of mCR1/2 in innate immunity, as most studies regarding modulation of the murine complement system and the innate immune responses have focused upon the complement receptor-1 related protein (Crry) (143, 144). Those studies indirectly suggest that murine Crry has taken over some hCR1 functions, i.e.: those roles relative to mCR1/2 acting as a murine complement inhibitor (64, 145). We think that our studies confirm a significant role for mCR1/2 in suppressing the activation of the murine complement system, and innate immune responses derived from that activation. Why the mouse has what appears to be a redundant complement inhibiting activities (present in Crry and mCR1/2) will require future experimentation, but this redundancy may have contributed as to why humans lack Crry.

Pre-existing immunity to Adenoviruses remains a main hindrance for numerous applications utilizing Ad-based vectors as a gene transfer platform. In light of this fact, it is of critical importance to study the mechanisms underlying

generation of Ad capsid specific antibody responses. Increased innate immune responses are generally thought to positively correlate with enhanced inductions of humoral, and/or cellular adaptive immune responses (63, 131, 140). The complement system is well known to impact significantly upon the generation of humoral responses to pathogens by facilitating interactions between the innate and adaptive response systems (60-62).

Our previous results demonstrated that Ad vector administrations into C3-KO mice resulted in diminished cytokine and chemokine responses, and a diminished induction of neutralizing antibodies to Ad (24, 26). However, despite enhanced induction of pro-inflammatory cytokines and chemokines rapidly after Ad vector administrations into CR1/2-KO mice, we still found a dramatically reduced induction of neutralizing antibodies to the Ad vector capsid. These results positively correlated with a lack of activation of B cells in Ad-injected C3-KO and mCR1/2-KO mice. The combined observations suggest that lack of induction of pro-inflammatory cytokine responses early after Ad administrations into C3 -KO mice is not the reason for lack of neutralizing antibody induction in the Ad treated C3-KO mice (24). Rather, C3 opsonized Ad interactions with mCR1/2 on murine B cells is primarily responsible for induction of neutralizing antibodies to the Ad vector capsid. This insight suggests that further investigation of this interaction may promote strategies to proactively modulate the induction of neutralizing antibodies to Ad vectors.

Zaiss et al. detected evidence of Ad induction of neutralizing antibodies in an alternative CR1/2-KO mouse model (56). That strain of CR1/2-KO mouse has

a different portion of the murine Cr2 gene disrupted, relative to the CR1/2-KO mouse strain utilized in our studies, which may be relevant to the different results. Additional technical caveats including the use of a significantly different assay system may also explain the differing results. Our multiple findings demonstrating a lack of significant induction of Ad capsid neutralizing antibodies in both Ad treated C3-KO *and* CR1/2-KO mice, as well as our finding that Ad induction of B cell activation is also significantly decreased in these same strains of mice strongly support our conclusion that CR1/2 does play an important role in the induction of neutralizing antibodies to Ad vectors. While Zaiss *etal.* did not report whether or not induction of neutralizing antibodies to Ads occurred in Ad treated C3-KO mice, they did find that mCR1/2 functions were also necessary for induction of neutralizing antibodies to AAV based vectors. The latter results, combined with ours, suggest that mCR1/2 may be playing a role in the induction of neutralizing antibodies to several commonly utilized gene transfer vectors. Interestingly, activation of type I interferons (IFNα/β), have also been shown to play a role in the induction of Ad specific humoral adaptive immune responses. Whether these responses are due to or a result of Ad interactions with the complement system, and CR1/2 specifically, will require future investigations (131).

In a similar vein, we found that an increased induction of Ad induced innate immune responses in mCR1/2-KO mice did not correlate with increased induction of adaptive immune responses to a foreign antigen expressed by an Ad vector. The reasons for this observation are not due to a diminished ability of the

Ad vector to transduce cells in mCR1/2-KO mice, but rather appear to correlate with reduced transgene expression levels in Ad vector treated mCR1/2-KO mice both at 24 hpi and 28 dpi. Possibly, the globally altered cytokine and chemokine responses noted after Ad administration into CR1/2-KO mice indirectly decreases activity of the CMV derived enhancer/promoter element utilized to drive expression of the expression foreign transgene encoded by the vector, a possibility that will require experiments beyond the scope of this study.

We previously reported that transgene specific adaptive immune responses were higher in Ad treated C3-KO mice as compared to identically Ad treated WT mice (24). At the least, those results suggested that lack of complement activation does not significantly interfere with generation of transgene specific antibody responses by Ad vectors. Thus, targeted blockade of complement system activation that prevents C3 opsonized Ads from interacting with the CR1/2 receptor may minimize induction of antibody responses to the Ad capsid while simultaneously preserving or enhancing the ability of the Ad to induce beneficial immune responses to Ad expressed foreign antigens.

Our results may also suggest a possible mechanism as to how non-covalent modifications of Ad vectors, (such as Ad PEGylation) may allow for avoidance of induction of neutralizing antibodies by indirectly minimizing their induction of the complement system (146-148). We also suggest that these findings may allow for improved use of Ad vectors as a gene transfer platform, though such studies are beyond the scope of this dissertation. However, several previous studies indirectly confirmed roles for the complement system in

generation of robust Ad induced adaptive immune responses, in particular in Ad vaccine settings utilizing C4-binding protein or C3d as novel adjuvants (149-153).

In conclusion, we have confirmed the role of mCR1/2 in regulating and/or suppressing several Ad induced innate immune responses, as well confirmed that mCR1/2 modulates the induction of adaptive immune responses to the Ad vector. While the former observation now confirms that in mice, mCR1/2 can play an important role in down-regulating complement dependent innate immune responses in a manner that is independent of Crry, the latter observations suggests that Ad vector interactions with mCR1/2 also figure prominently in induction of neutralizing antibodies to Ads.

Acknowledgements:

We wish to thank Michigan State University Laboratory Animal support facility for their assistance in the humane care and maintenance of the animals utilized in this work and Michigan State University Investigative Histopathology Laboratory for performing H&E stains of liver tissues. S.S.S. was supported by American Heart Association Midwest Affiliate Fellowship 0815660G. A.A. was supported by the National Institutes of Health grants RO1DK-069884, P01 CA078673, the MSU Foundation as well the Osteopathic Heritage Foundation.

Chapter IV

Ad5-based vectors "capsid-displaying" specific complement inhibitor: a novel approach to improve Ad vector safety profile

This chapter is the edited version of a research article that was published in the Journal of Innate Immunity, DOI: 10.1159/000284368, February 11, 2010.

Authors: Seregin, S. S., Hartman, Z. C., Appledorn, D. M., Godbehere, S., Jiang, H., Frank, M. M., and A. Amalfitano.

4.1. Introduction

The complement system is one of the first lines of innate defense against invading pathogens. Pathogen interactions with pre-existing antibodies and/or circulating complement proteins results in activation of one or more of the three major complement pathways: the Classical (antibody dependent), Alternative (antibody independent), and the Lectin (antibody independent) complement pathways. Activation of each of the pathways results in the rapid production of C3-convertases. This activation eventuates in the direct production of potent anaphylotoxins (i.e.: C3a, C5a), indirectly promotes the high level production of several cytokines and chemokines, and thereby initiates the recruitment of cellular elements of the innate and adaptive immune systems to the site of infection (49, 52-54).

Inappropriate or excessive complement activation can, however, also cause thrombocytopenia, anaphylactoid reactions, systemic inflammatory responses, adult respiratory distress syndrome (ARDS), and/or death. Many of these same toxicities have been observed after high dose administrations of Ads into rodents and non-human primates, as well in human trial subjects (24, 26, 28, 46-50, 55). Ad capsids are now known to interact with and activate complement proteins *in vitro* and *in vivo*, interactions that induce several complement dependent toxicities in several species. Importantly, Ad capsids are non-enveloped and therefore are not thought to be directly lysed by terminal complement C6-$C9_n$ Membrane Attack Complexes, however, it has been confirmed that Ads become opsonized by complement components, mainly C3b

and C4b, both *in vitro* (55, 146) and *in vivo* (154). Complement opsonization facilitates phagocytosis of Ad virions by cells of macrophage origin, if the latter contain proper complement receptors (CR1 (60)) on their membranes. These important interactions with the complement system lead to Ad vector triggered, complement dependent release of pro-inflammatory cytokines and chemokines. These responses also augment activation of the cellular part of the innate immune system, resulting in the recruitment of macrophages and granulocytes, and activation of endothelial cells. Importantly, many of these toxicities are avoided when Ad vectors are administered into complement deficient (C3-KO) mice (24, 26, 27, 46, 55). Based upon these observations, we have attempted to provide the Ad capsid with an inherent complement inhibitory activity, in an effort to mitigate Ad capsid activation of human complement, and/or complement dependent toxicities.

A 13 amino acid synthetic peptide with complement inhibitory activity (COMPinh), was initially identified by others in a high-throughput, phage-based screening effort (155-157). Importantly, COMPinh, amino acid sequence: ICVWQDWGAHRCT, is an optimized version of the originally described peptide (156). COMPinh is able to bind and inhibit complement component C3 of human and non-human primate origin, but not C3 from other species (155-157). In this report, we confirm that we can successfully construct and isolate high titers of these novel Ad vectors "displaying" COMPinh directly on the Ad capsid surface as a fusion protein with several Ad capsid proteins. Based upon this observation,

we have performed relevant testing of the novel Ads utilizing several human model assays of Ad dependent complement activation.

4.2. Results and Discussion

We constructed two Ad5-based vectors capsid-displaying a 13 amino acid sequence with known complement inhibitory properties (COMPinh). The COMPinh DNA sequence was inserted in-frame into two sites of the Ad genome, forcing expression of the COMPinh peptides as (1) a carboxy-terminal fusion displayed from the cement capsid protein IX (Ad5-LacZ-IX-dCOMPinh), and (2) embedded within the HI loop of the Ad fiber protein (Ad5-LacZ-Fiber-dCOMPinh) as diagrammed in Figure 20. The viability and infectivity of all Ad vectors utilized in our studies was confirmed by electron microscopy of purified Ads (Figure 21), infectious unit titering assay (TCID50) and transducing unit titering assays. Viral particle titers were determined by spectrophotometry and validated by SDS-PAGE electrophoresis of purified Ads followed by silver staining and/or western blotting. COMPinh displaying Ads preserve VP/TCID ratios typically observed for conventional Ad vectors (Table 6). Direct sequencing of DNA derived from all CsCl purified Ad vectors and capsid thermostability assays further confirmed the integrity of the constructs (data not shown).

To investigate the potential of COMPinh displaying Ads to diminish complement activation, we utilized two different Normal Human Serum (NHS) based assays: the AP50 and C3a-desArg ELISA. For example, identical amounts of control or COMPinh displaying Ads were incubated with NHS (+EGTA) and the residual complement activity remaining in the human serum was then measured in the AP50 assay. NHS pre-incubated with WT_Ad5, Ad5-LacZ and

Figure 20: Schematic diagram of all Ad vectors constructed and utilized in our study. Genome maps of all Ads constructed are shown. Ad vectors were designed as described in Materials and Methods. Capsid protein IX and fiber are outlined as Ad capsid proteins utilized for fusion with COMPinh. Genome sizes are shown relative to WT Ad5 genome (top). Letter "d" prior to COMPinh or GFP defines that this peptide is "capsid-displayed". Note: genomes are not drawn to scale.

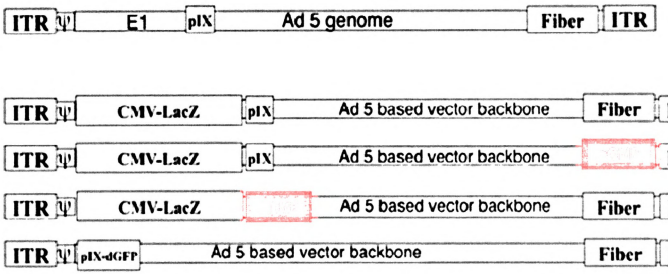
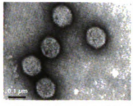
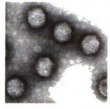

ITR
Ψ
E1
pIX
Ad 5 genome
Fiber
ITR
ITR
Ψ
CMV-LacZ
pIX
Ad 5 based vector backbone
Fiber
ITR
ITR
Ψ
CMV-LacZ
pIX
Ad 5 based vector backbone
ITR
ITR
Ψ
CMV-LacZ
Ad 5 based vector backbone
Fiber
ITR
ITR
Ψ
pIX-dGFP
Ad 5 based vector backbone
Fiber
ITR

Figure 21: Electron microscopy of purified Ad5 vectors. Cesium chloride purified Ad vectors were stained with 1% PTA and electron micrographs were taken, exactly as described in Materials and Methods. Icosahedral virion structures approximately 100 nm in diameter are clearly visible for all Ads. There is no significant amount of damaged capsids and/or free capsid proteins detected. Photographs were taken from representative areas from each sample.

Ad5-LacZ



Ad5-LacZ-IX-dCOMPinh



Ad5-LacZ-Fiber-dCOMPinh

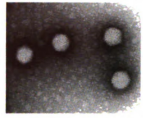


Table 6: Novel "capsid-displaying" Ads can be propagated to high titers, similar to conventional Ad vectors. Capsid modifications do not impair infectivity and transduction efficiency of novel Ads.

VP – viral particles

TU – transducing units

BFU – blue-forming units (for Ads expressing β-Gal)

TCID – tissue culture infections dose (measure of Ad infectivity)

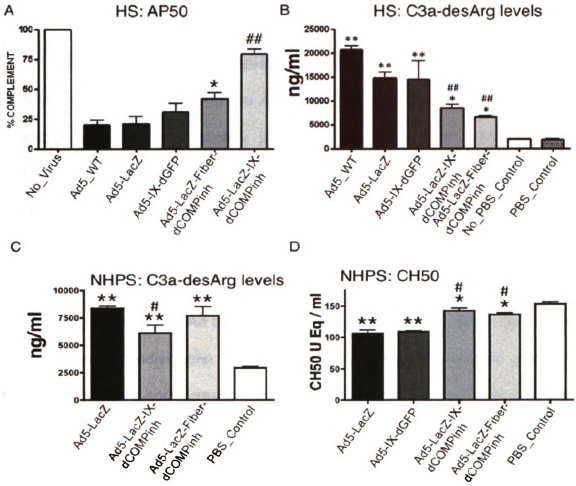
TEM – transmission electron microscopy

Virus	VP titer (vp/ml)	TU titer (BFU/ml)	TCID titer (TCID/ml)	VP/TU/ TCID ratio	Silver Stain	TEM
Ad5-CMV-LacZ	$2.86x10^{12}$	$1.8x10^{11}$	$1.6x10^{10}$	179/11/ 1	Intact	Intact
Ad5-IX-dGFP	$3.54x10^{12}$	NA	$6.3x10^{10}$	56/NA/1	Intact	Intact
Ad-LacZ-IX-dCOMPinh	$4.75x10^{12}$	ND	$6.3x10^{10}$	75/ND/1	Intact	Intact
Ad-LacZ-Fiber-dCOMPinh	$2.06x10^{12}$	$7.0x10^{10}$	$1.0x10^{10}$	206/7/1	Intact	Intact

Ad5-IX-dGFP control viruses revealed significant complement consumption (~80%) as compared to NHS samples pre-incubated with PBS, confirming that conventional Ad vectors significantly activate the alternative complement pathway. Note that the Ad5-IX-dGFP virus displays a non-specific peptide sequence (GFP) from pIX, confirming that random display of foreign peptides on the Ad capsid does not result in complement inhibitory activity in this assay. Complement consumption was dramatically reduced in NHS incubated with similar particle numbers of the COMPinh displaying Ads (Figure 22A) relative to conventional Ad vectors. Specifically, when NHS was incubated with Ad5-LacZ-IX-dCOMPinh particles, only 20% of the available complement activity in the NHS was consumed. Notably, the display of COMPinh from the pIX protein facilitated a significantly improved ability to prevent complement activation, relative to the fiber protein display of COMPinh. This may be due to several reasons, inclusive of the simple fact that the pIX protein is present in 240 copies on each Ad capsid, vs. 36 copies for the fiber protein.

To further investigate the properties of COMPinh displaying Ads, we incubated NHS with the novel Ad vectors and measured C3a-desArg protein levels generated after the incubations. Control Ad vectors including WT_Ad5, Ad5-LacZ and Ad5-IX-dGFP significantly activated complement, as the levels of C3a-desArg detected after these incubations were 8-10 times higher than those observed in NHS pre-incubated with PBS (Figure 22B). Novel Ad vectors

Figure 22: Novel COMPinh displaying Ads minimize Ad mediated complement activation in several serum-based assays. (A) AP50 was performed and residual complement activity was normalized to human serum (HS), incubated with media (no_virus control). The error bars represent ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test. * - indicate values, statistically different from WT_Ad5 and Ad5-LacZ, $p<0.05$; ## - indicate statistically different values from WT_Ad5 and Ad5-IX-dGFP, and Ad5-LacZ, $p<0.001$. **(B)** Overall complement activation mediated by Ads in HS was determined by C3a-desArg specific ELISA. Negative control was HS incubated with PBS prior to ELISA. *, ** - indicate values, statistically different from no_virus controls, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values from Ad5_WT, Ad5-IX-dGFP and Ad5-LacZ, $p<0.05$, $p<0.001$ respectively. **(C)** Overall complement activation mediated by Ads in NHPS was determined by C3a-desArg specific ELISA. ** - indicate values, statistically different from PBS control, $p<0.01$; # - indicate statistically different values from Ad5-LacZ, $p<0.05$. **(D)** Activation of classical complement pathway mediated by Ads was performed. After NHPS/Ad incubations, residual CH50 activity was measured and graphed in CH50 units equivalent per ml. *, ** - indicate values, statistically different from PBS control, $p<0.05$, $p<0.01$ respectively. # - indicate statistically different values from Ad5-LacZ and Ad5-IX-dGFP, $p<0.05$.

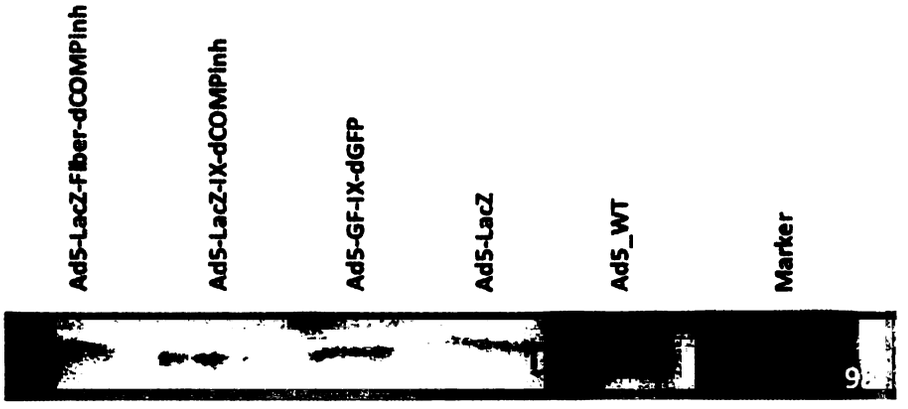

A
HS: AP50
%.COMPLEMENT
100
75
50
25
0
No_Virus
Ad5_WT
Ad5-LacZ
Ad5-IX-dGFP
Ad5-LacZ-Fiber-dCOMPinh
Ad5-LacZ-IX-dCOMPinh
B
HS: C3a-desArg levels
ng/ml
25000
20000
15000
10000
5000
0
Ad5_WT
Ad5-LacZ
Ad5-IX-dGFP
Ad5-LacZ-IX-dCOMPinh
Ad5-LacZ-Fiber-dCOMPinh
No_PBS_Control
PBS_Control
C
NHPS: C3a-desArg levels
ng/ml
10000
7500
5000
2500
0
Ad5-LacZ
Ad5-LacZ-IX-dCOMPinh
Ad5-LacZ-Fiber-dCOMPinh
PBS_Control
D
NHPS: CH50
CH50 U Eq / ml
150
100
50
0
Ad5-LacZ
Ad5-IX-dGFP
Ad5-LacZ-IX-dCOMPinh
Ad5-LacZ-Fiber-dCOMPinh
PBS_Control

displaying COMPinh in either protein IX or fiber were able to significantly reduce the production of C3a-desArg in this assay (Figure 22B).

Subtle differences in the levels of C3a-desArg produced after incubation with WT_Ad5 as compared to the Ad5-LacZ first generation Ad vector may be possibly explained by a slightly increased number of viral particles in the WT_Ad5 virus preparations as compared to Ad5-LacZ, as was validated by silver staining (Figure 23). We did not detect any significant differences in CH50 activity, when control or COMPinh-displaying Ads were incubated with NHS (data not shown). This result may have been due to the presence of pre-existing neutralizing antibodies to Ad being present in the NHS, (as the latter was derived from numerous individuals) complicating the efficacy of capsid displayed COMPinh to inhibit complement activation in this assay, in contrast to COMPinh mediated inhibition of complement activation in the C3a-desArg assay or NHP based assays.

Since COMPinh has been proven to be effective in inhibiting complement activation not only in human serum, but also in non-human primate serum (NHPS) (155-157), we evaluated the properties of COMPinh displaying Ads upon incubation with Rhesus monkey serum. High homology between human and Rhesus monkey complement proteins allowed using human specific antibodies to detect the level of complement activation in NHPS (158). We found that overall complement activation was significantly reduced when Ad5-LacZ-IX-dCOMPinh was incubated with NHPS as compared to Ad5-LacZ

Figure 23: Silver staining of purified Ad vectors revealed marginal differences in spectrophotometry determined vp titer. Total of 10^{10} vp of purified virions of each Ad vector were separated by 10% SDS-PAGE and subsequently stained with silver nitrate as described in Materials and Methods. Amount of hexon protein (above 98 kDa) was quantified for each vector by scanning densitometry, as shown in table. Note, that VP titers determined by spectrophotometry fall within ~1.13 fold window confirming that capsid-displaying Ad5 vectors did not contain less virions as compared to conventional first generation Ad5 vectors.

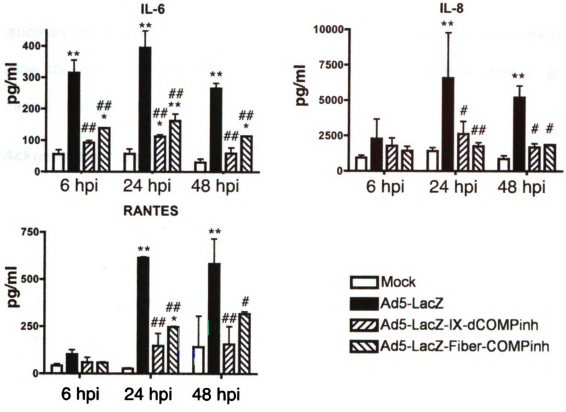


Adenovirus vector	Hexon integrated density	Fold over Ad5_WT
Ad5_WT	14847	1.00
Ad5-LacZ	13968	0.93
Ad5-LacZ-IX-dCOMPinh	14908	1.00
Ad5-LacZ-Fiber-dCOMPinh	16739	1.13
Ad5-IX-dGFP	11108	0.75

first generation vector, as measured by C3a-desArg levels in NHPS exposed to the respective vectors (Figure 22C). Moreover, both pIX-dCOMPinh and Fiber-dCOMPinh capsid-displaying Ads caused a reduced activation of classical complement pathway as compared to conventional Ad vectors, as evident by significantly higher CH50 unit equivalents being measured in NHPS exposed to COMPinh capsid-displaying Ads as compared to NHPS exposed to control Ads (Figure 22D). Finally, to determine if COMPinh displaying Ads can minimize Ad-triggered cytokine releases from human cells, we have infected human peripheral blood mononuclear cells (PBMCs) with control or experimental Ad5 vectors, and quantified the cytokine/chemokine released from those cells into the media at 6, 24 and 48 hours post infection (hpi). Our results demonstrated that human PBMCs exposed to pIX-dCOMPinh and Fiber-dCOMPinh displaying Ads triggered significantly reduced IL-6 (at all time points tested), IL-8 and RANTES (at 24 and 48 hpi) levels as compared to similar exposures of the PBMCs to the control Ad vector (Figure 24). These studies highlight previously published studies demonstrating that membrane-localized complement components can mediate Ad-triggered innate inflammatory responses. This previously described mechanism may be responsible for our results utilizing COMPinh displaying Ads *in vitro* (146).

Overall, our results confirm that the functional activity of a peptide to specifically inhibit portions of an important innate immune response can be retained when displayed from the Ad5 capsid. Specifically, the COMPinh peptide

Figure 24: Novel COMPinh displaying Ads reduce Ad-triggered activation of pro-inflammatory cytokines and chemokines in PBMCs. Peripheral blood mononuclear cells were cultured and stimulated by adding PBS (Mock), Ad5-LacZ control first generation Ad, Ad5-LacZ-IX-dCOMPinh or Ad5-LacZ-Fiber-dCOMPinh at MOI=5000 vp/cell, N=4 for all groups. At indicated time points media was collected and cytokine concentrations were analyzed using a multiplexed bead array based quantitative system. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The bars represent Mean ± SD. *, ** - indicate cytokine values that are statistically different from those in Mock samples at the same time point, $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values from Ad5-LacZ group at the same time point, $p<0.05$, $p<0.001$ respectively.


IL-6
pg/ml
400
300
200
100
0
6 hpi
24 hpi
48 hpi
IL-8
10000
7500
5000
2500
RANTES
750
500
250
Mock
Ad5-LacZ
Ad5-LacZ-IX-dCOMPinh
Ad5-LacZ-Fiber-COMPinh

can retain its anti-complement activity when displayed as a genetic fusion peptide in several locations on the Ad capsid. As these vectors can be propagated to high titer without need for post-purification chemical modifications (such as PEGylation, or other potentially non-scalable manipulations) they may allow for more widespread, and safer use of so-modified Ad vectors in gene transfer applications.

Acknowledgements:

We wish to thank Michigan State University Electron Microscopy support facility for their assistance in performing electron microscopy of purified Ad vectors. S.S.S. was supported by American Heart Association Midwest Affiliate Fellowship 0815660G. A.A. was supported by the National Institutes of Health grants RO1DK-069884, P01 CA078673, the MSU Foundation and the Osteopathic Heritage Foundation.

Chapter V

Simple, pre-emptive and transient glucocorticoid pre-treatment reduces Ad5-associated acute toxicities

This chapter is the edited version of a research article that was published in the Molecular Therapy Journal, Volume 17, Issue 4 (685-696), January 29, 2009.

Authors: Seregin, S. S., D. M. Appledorn, A. J. McBride, N. J. Schuldt, Y. A. Aldhamen, T. Voss, J. Wei, M. Bujold, W. Nance, S. Godbehere, and A. Amalfitano.

5.1. Introduction

Adenovirus (Ad) based vectors continue to be the most commonly utilized gene transfer vector for a variety of potential applications. Ad vectors can be easily produced to high titers (scalability is a critical point when considering potential human applications), possess the ability to transduce dividing and non-dividing cells without the need for chromosomal integration, and have an extremely broad tropism. These advantages have resulted in the initiation of 342 human clinical trials utilizing Ad vectors since the first Ad clinical trial in 1993 (http://www.wiley.co.uk/wileychi/genmed/clinical/). Furthermore, first generation Ad vectors have been repeatedly demonstrated to persist for long periods of time when transducing non-immunogenic transgenes (159, 160). Limitations to long term persistence of first generation Ads transducing immunogenic transgenes has been largely overcome with the development of multiply deleted, helper independent, or fully deleted helper virus dependent advanced generation Ad-based vectoring systems (14). Despite these encouraging facts, safety concerns regarding Ad vector associated innate toxicities, responses that often prime subsequent adaptive immune responses, has severely limited progress as to the use of this important vector class for systemic applications, such as gene transfer to the liver.

Several approaches have been studied to minimize the inflammatory responses acutely induced by systemic exposure to Ad vectors. These approaches include genetic modification of the Ad capsid to alter the tropism of the vector for liver cells; pre-emptive depletion (or blockade) of Ad sequestration

by liver macrophages to minimize induction of macrophage dependent inflammatory responses, use of immunosuppressive drugs (such as anti-TNF blockers, TLR-9 inhibitors, ERK inhibitors and others) to transiently block acute inflammatory responses, as well surgical isolation procedures to minimize systemic distribution of recombinant Ads (36, 38, 46, 161-170). All of these approaches have an ability to reduce portions of the multi-faceted Ad induced innate immune response, but their ability to impact upon the full inflammatory response induced by recombinant Ads is either limited, or has not been fully determined. Furthermore, many of these approaches have inherent problems of their own, such as known toxicities prohibiting their use in human applications (i.e.: clodronated liposomes for liver macrophage depletions, Ad capsid changes that may result in enhanced innate toxicity, and/or technical difficulties associated with moderate to significantly invasive surgical procedures (46, 163, 171, 172). What is needed is a safe, simple, transient, inexpensive, and widely accepted method for the reduction and/or elimination of the myriad Ad vector induced inflammatory responses induced after systemic administration.

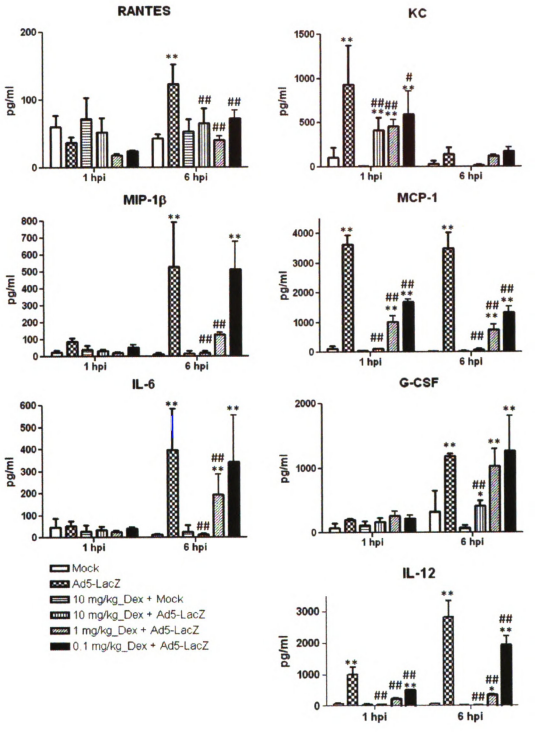
We have, therefore, tested if pre-treatment with anti-inflammatory glucocorticoid Dexamethasone can be utilized to address these issues. In our study we intravenously injected high doses of a first generation (E1-deleted) Ad5 vector encoding a highly immunogenic transgene (Bacterial B-galactosidase (LacZ)) into either control or DEX pre-treated C57BL/6 mice, and investigated the subsequent innate and adaptive immune responses to the Ad and the transgene product expressed by the vector. Our results demonstrate that multiple aspects

of the systemic inflammatory response induced rapidly after systemic delivery of Ads can be obliterated by simple, transient pre-treatment with a potent glucocorticoid.

5.2. Results

Dexamethasone blocks Ad mediated cytokine and chemokine release in C57BL/6 mice in a dose dependent manner. We quantified the release of inflammatory cytokines and chemokines in murine plasma at 1 and 6 hours after systemic Ad delivery utilizing a multi-plexed, bead array based detection system. Based on our previous publications, as well those of others, we focused upon seven cytokines and chemokines that are known to be rapidly (within 1-6 hours) induced following systemic injection of Ad vectors (26, 46). KC (CXCL1) and MCP-1 are the first inflammatory chemokines to be induced (within 1 hour of Ad injection). These murine chemokines are known to recruit neutrophils and monocytes to the sites of tissue damage, comprising one of the first defensive barriers to Ad vectors (26, 46). At 6 hpi, plasma levels of IL-6, IL-12p40, G-CSF, MIP-1β and RANTES also reach a peak, further activating the cellular arm of the innate immune system (26, 46). As expected, these 7 cytokines and chemokines were induced after Ad injection into WT mice (Figure 25). To investigate the impact of DEX pre-treatment (15 hours and 2 hours prior to intravenous Ad5-LacZ injection) in the induction of these pro-inflammatory factors, we completed a dose response study where mice were pre-treated with 10 mg/kg, 1 mg/kg, or 0.1 mg/kg of DEX. At 1 hour post injection of Ad5-LacZ, the level of KC in the serum of DEX pretreated mice (all dosages) was significantly lower compared to mice identically injected with Ad. RANTES levels at 6 hpi were also equivalently reduced by DEX. Interestingly, we did not observe a dose dependent reduction of

Figure 25: Dexamethasone (DEX) blocks Ad-mediated systemic cytokine and chemokine release in C57BL/6 mice in a dose dependent manner. C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Dexamethasone (DEX) pre-treatment was performed via intraperitoneal injection at 15 and 2 hours before virus injection. Plasma samples were collected at 1 and 6 hours post virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array based system. The bars represent Mean ± SD. *, ** - indicate plasma cytokine values that are statistically different from those in Mock-injected animals of the same treatment at the same time point (i.e. WT_DEX_Ad5-LacZ group from WT-DEX-Mock group), $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group at the same time point, $p<0.05$, $p<0.001$ respectively. Statistical analysis was completed using Two Way ANOVA with a Bonferroni post-hoc test. The N=4 for Mock (PBS) injected animals, N=6 for virus injected mice.


RANTES
KC
MIP-1β
MCP-1
IL-6
G-CSF
IL-12
pg/ml
1 hpi
6 hpi
Mock
Ad5-LacZ
10 mg/kg_Dex + Mock
10 mg/kg_Dex + Ad5-LacZ
1 mg/kg_Dex + Ad5-LacZ
0.1 mg/kg_Dex + Ad5-LacZ

these chemokines, as the 0.1 mg/kg dose was as efficacious as the 10 mg/kg dose in modulating the induction of both KC and RANTES.

At a dose of 10 mg/kg, DEX completely blocked the release of IL-6 and MIP-1β ($p<0.001$) at 6 hpi, and MCP-1 and IL-12(p40) at both 1 and 6 hpi ($p<0.001$). The levels of IL-12(p40) and MCP-1, at both time points, were also significantly reduced when mice were pre-treated with either 1.0 or 0.1 mg/kg DEX. Serum levels of MIP-1β and IL-6 were also significantly lower in mice pre-treated with 10.0 or 1 mg/kg DEX, but were not significantly reduced in Ad-injected mice pre-treated with 0.1 mg/kg DEX. Finally, plasma levels of G-CSF were only significantly reduced when Ad-injected mice were pre-treated with 10 mg/kg DEX (Figure 25). These results demonstrate that DEX pre-treatment of animals can abrogate Ad induction of the "cytokine storm" noted after systemic delivery of Ad5-based vectors (24). Because pre-treatment of mice with 10 mg/kg DEX maximally suppressed these responses, we evaluated the ability of this dose to further prevent the induction of other detrimental responses typically observed following systemic Ad-vector administrations.

Dexamethasone prevents Ad induction of thrombocytopenia in C57BL/6 mice. Next, we determined if DEX treatment could prevent the acute, consumptive thrombocytopenia typically observed within 24 hours after systemic Ad injection, an event that we have shown to be due to Ad activation of the alternative complement pathway (24, 26, 44). As expected, thrombocytopenia developed in WT mice treated with Ad5-LacZ (Figure 26). DEX pre-treated mice

did not develop thrombocytopenia after Ad injection, as their platelet levels were not different from mock-injected animals (Figure 26).

Dexamethasone minimizes Ad dependent activation of endothelial cells in C57BL/6 mice. Endothelial cell activation facilitates the infiltration of inflammatory cells, including macrophages, granulocytes and mast cells, into the parenchyma of inflamed tissues and organs. When endothelial cells become activated, they overexpress surface as well as soluble forms of adhesion molecules such as e-Selectin, ICAM-1, VCAM-1 (45, 132, 133). Intravenous injection of Ad vectors is known to induce endothelial cell activation, an event that is mediated by Ad interactions with Kupffer cells, and can result in significant hypotension (129). We have measured plasma levels of soluble ICAM-1 and e-Selectin molecules in Ad-injected mice at 6 hpi. Ad treated WT mice had at least 3-fold higher levels of soluble ICAM-1 and e-Selectin molecules, than levels measured in mock-injected animals (Figure 27). DEX pre-treatment completely blocked Ad dependent activation of synthesis of these 2 molecules, indirectly demonstrating that DEX pre-treatment can reduce the Ad dependent activation of endothelial cells (Figure 27).

Dexamethasone pre-treatment minimizes the induction of pro-inflammatory genes in livers of Ad treated C57BL/6 mice. We have previously and extensively characterized the cell or tissue specific transcriptome changes rapidly induced after transduction by Ad vectors both *in vitro* and *in vivo* (23, 26, 35). These studies revealed that Ads induce the expression of genes

Figure 26: Dexamethasone prevents Ad mediated thrombocytopenia in C57BL/6 mice. C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. DEX pre-treatment and platelets enumerations were performed as described in Materials and Methods. Four groups of wild type mice were analyzed: WT_Mock (N=4), WT_DEX_Mock (N=4), WT_Ad5-LacZ (N=5), WT_DEX_Ad5-LacZ (N=5). The bars represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. *, ** - indicate values, statistically different from those in WT_Mock-injected animals, $p<0.05$, $p<0.001$ respectively. Note: Normal range levels were adapted from Jackson laboratories studies on C57BL/6 mice (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=projects/details&sym=Peters3).

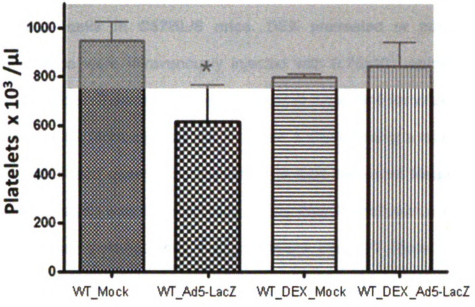
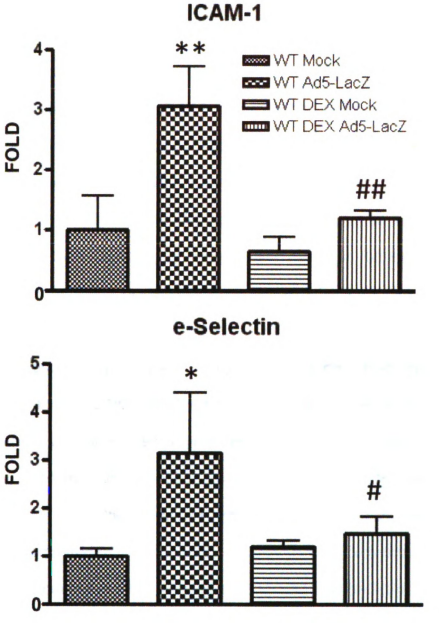

Platelets x 10³ /µl
1000
800
600
400
200
0
*
WT_Mock
WT_Ad5-LacZ
WT_DEX_Mock
WT_DEX_Ad5-LacZ

Figure 27: Dexamethasone minimizes Ad dependent activation of endothelial cells in C57BL/6 mice. DEX pretreated or non-treated C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. Plasma samples, collected at 6 hpi (N=6 for virus treated groups, N=4 for Mock-injected groups) were analyzed using a multiplexed bead array based quantitative system. The bars represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test. Fold difference over WT_Mock group is shown. *, ** - indicate values, statistically different from those in Mock-injected animals of the same treatment,- $p<0.05$, $p<0.001$ respectively. #, ## - indicate statistically different values in any group compared to WT_Mock group, $p<0.05$, $p<0.001$ respectively.


ICAM-1
FOLD
4
3
2
1
0
**
##
WT Mock
WT Ad5-LacZ
WT DEX Mock
WT DEX Ad5-LacZ
e-Selectin
FOLD
5
4
3
2
1
0
*
#

involved in innate immune responses, such as pattern recognition receptors (TLRs, NODs), TLR signaling pathways (MyD88, TRIF, TRAF6, TRAF2bp, TBK1), markers of endothelial cells activation (e-Selectin, ICAM-1, VCAM-1), interferon responsive genes (OAS1a, IRF7, IRF8), negative regulators of cytokine signaling (SOCS-1, SOCS-3) and dsRNA editing enzymes (ADAR) (23, 35). We have also previously shown that in C57BL/6 mice, systemically administered Ads mediate induction of maximal liver transcription dysregulation at 6 hpi (23). Therefore this time point was selected to determine whether pretreatment with DEX might block and/or minimize Ad vector induction of transcription of these pro-inflammatory genes (Table 7). Besides the genes mentioned above, we interrogated the expression of several additional genes: DAF – potent membrane-bound complement inhibitor; NFkB-RelA – subunit of transcription factor; JAK1, JAK3 – molecules involved in cytokine signaling in this assay. Thus a total 23 genes were tested by qRT-PCR based methods. Our results demonstrate that all of the genes tested (except IFNα) were significantly induced at 6 hpi in the livers of C57BL/6 mice systemically treated with Ad5-LacZ, as compared to mock-injected animals ($p<0.05$), whereas only ADAR and TLR3 were induced in DEX pre-treated Ad5-LacZ injected mice, as compared to mock-injected animals. Importantly, the level of transcription induction of all the genes except one was significantly reduced in virus injected DEX pre-treated mice, compared to the virus treated group ($p<0.05$, in vast majority of cases $p<0.001$). Some of the genes in the DEX treated, mock-infected groups of mice

Table 7: Dexamethasone pre-treatment abrogates Ad5-LacZ induced gene expression in livers of C57BL/6 mice. The numbers represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. Note, when significant $p<0.001$ was observed in majority of cases. N=4 for Mock-injected groups, N=6 for virus injected groups was used. Significant differences compared to WT_Mock are highlighted in grey color. Significant reductions of transcriptional activation in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group are indicated in table with black frame and boldface font. For full gene names please refer to abbreviation list.

Ad5-LacZ induced gene expression in a liver of C57BL/6 mice (fold over Mock, 6 hpi)				
	C57BL/6 WT Mock	C57BL/6 WT Dex_Mock	C57BL/6 WT Dexamethasone Ad5-LacZ	C57BL/6 WT Ad5-LacZ
ADAR	1.0 ± 0.2	0.47 ± 0.03	2.6 ± 0.3	7.1 ± 1.2
CXCL-9	1.0 ± 0.1	0.10 ± 0.01	0.6 ± 0.3	27.9 ± 6.0
DAF	1.0 ± 0.4	0.6 ± 0.1	0.8 ± 0.1	1.8 ± 0.3
ICAM	1.0 ± 0.5	0.6 ± 0.1	1.1 ± 0.3	5.3 ± 1.0
IFNα	1.1 ± 0.5	0.7 ± 0.2	1.3 ± 1.2	1.9 ± 0.5
IRF-7	1.1 ± 0.7	0.16 ± 0.02	5.0 ± 1.2	22.4 ± 4.9
IRF-8	1.1 ± 0.7	0.3 ± 0.1	0.5 ± 0.1	5.9 ± 0.7
Jak-1	1.0 ± 0.3	0.6 ± 0.1	0.6 ± 0.1	1.3 ± 0.2
Jak-3	1.0 ± 0.3	0.3 ± 0.1	0.6 ± 0.1	1.7 ± 0.3
MyD88	1.0 ± 0.4	0.5 ± 0.1	1.4 ± 0.3	6.0 ± 1.2
NFkB-RelA	1.0 ± 0.3	0.6 ± 0.1	0.6 ± 0.2	2.2 ± 0.3
NOD-1	1.0 ± 0.3	0.40 ± 0.04	0.8 ± 0.1	2.5 ± 0.4
NOD-2	1.1 ± 0.5	0.10 ± 0.01	0.2 ± 0.1	2.6 ± 0.8
OAS-1a	1.0 ± 0.4	0.2 ± 0.1	4.5 ± 1.2	20.7 ± 6.4
SOCS-1	1.0 ± 0.4	0.4 ± 0.1	4.3 ± 2.4	70.5 ± 19.1
TBK-1	1.0 ± 0.2	0.5 ± 0.1	1.4 ± 0.2	4.6 ± 0.6
TLR-2	1.0 ± 0.1	0.9 ± 0.3	7.4 ± 4.2	169.2 ± 54.2
TLR-3	1.0 ± 0.2	0.37 ± 0.03	4.4 ± 1.6	14.8 ± 2.6
TLR-6	1.0 ± 0.4	0.7 ± 0.1	3.0 ± 1.3	6.0 ± 1.8
TRAF2bp	1.1 ± 0.6	0.13 ± 0.05	0.6 ± 0.2	18.8 ± 4.8
TRAF6	1.1 ± 0.6	1.0 ± 0.2	1.1 ± 0.1	1.8 ± 0.3
TRIF	1.0 ± 0.4	1.0 ± 0.2	1.20 ± 0.1	1.8 ± 0.3
VCAM	1.0 ± 0.1	0.5 ± 0.1	0.36 ± 0.04	2.0 ± 0.2

also had lower levels of transcription relative to mock-infected mice not treated with DEX, but in only five genes did this reach statistically significant levels: JAK1, JAK3, VCAM-1, NOD-1 and NOD-2, which indicates that several genes non-specifically respond to DEX treatment. These data strongly indicate that despite the huge redundancy inherent to the Ad induced host transcriptome response mechanism, DEX can efficiently block all of these responses. Possibly, DEX can block transcription activation pathways and/or directly downregulate the transcription of pro-inflammatory genes typically responsive to glucocorticoid receptor activation, although posttranscriptional modifications altering RNA stability is a legitimate alternative explanation of DEX mediated blockage of gene induction (173, 174).

Dexamethasone treatment causes an increase in lymphopenia and neutrophilia in C57BL/6 mice, an effect NOT related to Ad treatment. Besides acute thrombocytopenia, high dose, systemic Ad injections can induce lymphopenia and neutrophilia (175). At the dose of Ad we used ($0.75x10^{11}$ vp/mouse) we did not detect any increase in neutrophils and/or decrease in lymphocytes in C57BL/6 mice at 24 hpi as compared to mock-injected mice (Figure 28A-B). Interestingly, both groups of mice treated with DEX experienced neutrophilia and lymphopenia when compared with non-treated WT mice (Figure 28). These phenomena were not related to Ad injection, since there were no significant differences between the DEX pre-treated, mock or Ad5-LacZ injected groups. DEX has been shown to induce lymphopenia and neutrophilia in several animal models, including C57BL/6 mice (176), rabbits (177) and cattle (178).

Dexamethasone pre-treatment preserves the efficacy of Ad mediated gene transduction of the liver of C57BL/6 mice. It is known that systemic injection of high dose Ad vectors results in over 90% of an Ad vector bolus becoming sequestered in liver Kupffer cells, with the remaining virus primarily infecting hepatocytes, as the 100 nm Ad capsid can pass through the fenestrated liver endothelium (46, 179, 180). Having determined that DEX pre-treatment efficiently blocked the vast majority of the acute toxicities typically elicited rapidly after systemic Ad administrations, it was critical to confirm whether DEX pre-treatment impacted upon the efficacy of Ad transduction of the murine liver. With this information, we analyzed the level of Ad derived transgene expression (β-Gal) in the liver hepatocytes of Ad vector treated C57BL/6 mice at 6 hpi, 24 hpi and 28 dpi (Figure 29A-B). The liver activity of Ad vector derived β-Gal was not different between Ad5-LacZ groups that did or did not receive DEX at 24 hpi and 28 dpi, suggesting similar transductional efficacy. Although transgene derived enzyme activity was highest at 24 hpi, enzyme activity was still present at significant levels at 28 dpi.

We noted significant differences ($p<0.001$) in initial transgene expression (at 6 hpi) between virus injected DEX pre-treated mice and mice treated with virus alone (Figure 29B), which were not due to different levels of liver transduction by the Ad in these two experimental groups (Figure 29C). DEX did not diminish the efficacy of liver transduction by Ads, as Ad genome content in the liver was not different in all Ad5-LacZ treatment groups at all time points tested (Figure 29C). Moreover, we noted a mild, though significant, increase in the number of Ad

vector genomes per liver cell at 24 hpi in DEX pre-treated group ($p<0.05$), which possibly suggests a somewhat reduced level of clearance of Ad vectors by the host innate immune system at initial time points. Despite this finding, an increased number of Ad genomes at 24 hpi in DEX pretreated, Ad5-LacZ treated mice did not result in significant increases in enzyme activity, suggesting that LacZ expression was already at maximal levels.

Dexamethasone treatment does not change Kupffer cell loss in livers of Ad-injected mice. Resident liver macrophages, Kupffer cells, comprise a primary innate defensive system, a system known to non-specifically engulf, sequester and destroy Ads prior to hepatocyte encounter (179). It is known that systemic injection of high dose Ad vectors results in Kupffer cell dependent increases in plasma cytokine and chemokine responses (as well endothelial cell activation), which is quickly followed by a rapid Kupffer cells necrosis and loss from the liver (46, 129, 135). Therefore it was important to determine if DEX pre-treatment altered Kupffer cell responses to systemic Ad. We tested if DEX prevented Ad induced Kupffer cell destruction by staining frozen liver sections with the macrophage specific F4/80 antibody at 24 hpi (Figure 30). As expected, a significant decrease of Kupffer cell numbers occurred in the livers of Ad5-LacZ treated mice as compared to mock-infected mice. Similarly, DEX pre-treated, Ad injected mice also had Kupffer cells destroyed at 24 hpi at rates no different from Ad5-LacZ injected mice. Finally, DEX treatment by itself did not have any significant effect on Kupffer cell populations in the murine liver based on this

Figure 28: Dexamethasone treatment causes an increase in lymphopenia and neutrophilia in a blood of C57BL/6 mice, which is NOT related to Ad treatment. C57BL/6 mice were intravenously injected with $0.75x10^{11}$ vp/mouse of Ad5-LacZ vector. DEX pre-treatment and CBC differential count was performed as described in Materials and Methods. Total four groups of mice (N=4) analyzed: WT_Mock, WT_DEX_Mock, WT_Ad5-LacZ, WT_DEX_Ad5-LacZ. The bars represent Mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. Normal ranges for Neutrophils **(A)** and Lymphocytes **(B)** count in male C57BL/6 mice are indicated by light grey shaded boxes. Note: Normal range levels were adapted from Jackson laboratories studies on C57BL/6 mice (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=projects/details&sym=Peters3).

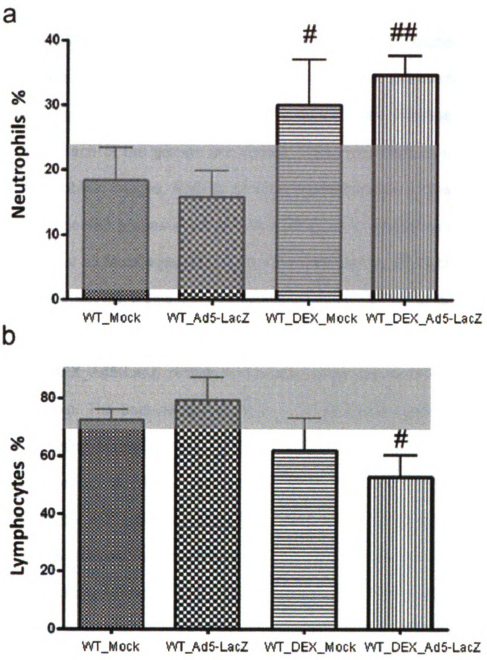

a
Neutrophils %
40
30
20
10
0
#
##
WT_Mock
WT_Ad5-LacZ
WT_DEX_Mock
WT_DEX_Ad5-LacZ
b
Lymphocytes %
80
60
40
20
0
#
WT_Mock
WT_Ad5-LacZ
WT_DEX_Mock
WT_DEX_Ad5-LacZ

Figure 29: Dexamethasone treatment preserves the efficacy of Ad derived transgene expression and Ads genomes persistency in the livers of C57BL/6 mice. (A) *In situ* visualization of bacterial β-galactosidase in liver of Ad5-LacZ treated C57BL/6 mice. Cryosections of liver from all groups of mice were stained for β-Gal *in situ*. Representative sections for each of the groups are shown. Total magnification of 200X was used to obtain images. N=6 for all virus injected groups at 6 hpi, N=4 for all virus injected groups at 24 hpi, N=5 for all virus injected groups at 28 dpi, N=4 for all Mock-injected groups at all time points. **(B)** Bacterial β-galactosidase activity levels were analyzed at 6 hpi, 24 hpi and 28 dpi from four groups of C57BL/6 mice: WT_Mock, WT_DEX_Mock, WT_Ad5-LacZ, WT_DEX_Ad5-LacZ. Activity levels were presented as Units per mg of total protein. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. **(C)** qPCR based quantification of Ad5-LacZ genomes in livers harvested from C57BL/6 mice at 6 hpi, 24 hpi, 28 dpi. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.

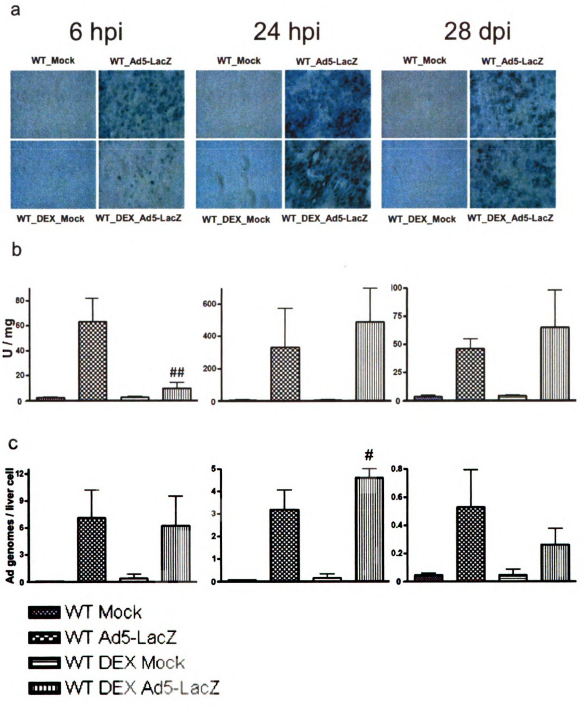
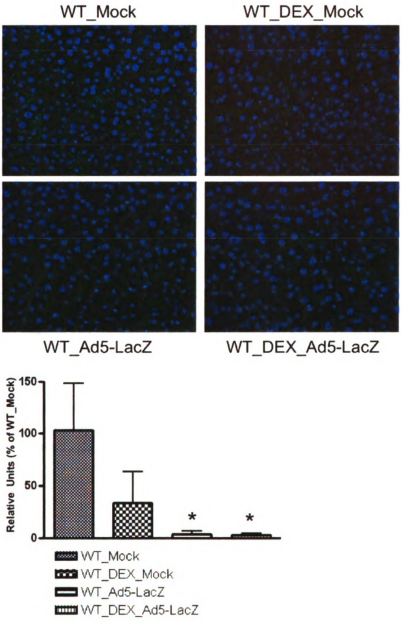

a
6 hpi
24 hpi
28 dpi
WT_Mock
WT_Ad5-LacZ
WT_DEX_Mock
WT_DEX_Ad5-LacZ
b
U / mg
##
c
Ad genomes / liver cell
#
WT Mock
WT Ad5-LacZ
WT DEX Mock
WT DEX Ad5-LacZ

Figure 30: Dexamethasone treatment does not change Ad dependent Kupffer cells degradation in a liver of C57BL/6 mice. 7 μm liver sections obtained at 24 hpi were stained with macrophage specific F4/80 antibody. Pixel density of both Kupffer cell staining and DNA staining (DAPI) was quantified. Kupffer cells values were normalized to DAPI values to control for cell density variation. Values were subsequently divided by WT_Mock average values to give percent difference. Error bars indicate ±SD. N=4 for all groups tested, pictures represent one of at least 12 sections derived from 4 mice in each group: WT_Mock, WT_DEX_Mock, WT_Ad5-LacZ, WT_DEX_Ad5-LacZ. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, $p<0.05$ was deemed a statistically significant difference. *, ** - indicate values, statistically different from those in WT_Mock-injected animals, $p<0.05$, $p<0.001$ respectively.

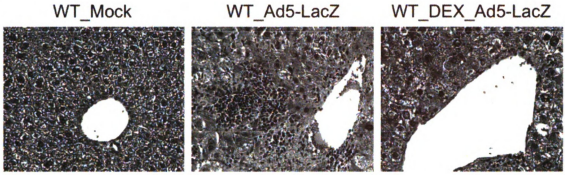

WT_Mock
WT_DEX_Mock
WT_Ad5-LacZ
WT_DEX_Ad5-LacZ
Relative Units (% of WT_Mock)
150
100
50
0
*
*
WT_Mock
WT_DEX_Mock
WT_Ad5-LacZ
WT_DEX_Ad5-LacZ

assay. Therefore, although it is known that the induction by Ads of a number of pro-inflammatory cytokines, chemokines, and markers of endothelial cell activation after systemic Ad injection is Kupffer cell dependent, Kupffer cell destruction by Ads is not directly responsible for many of these responses *per se* (135).

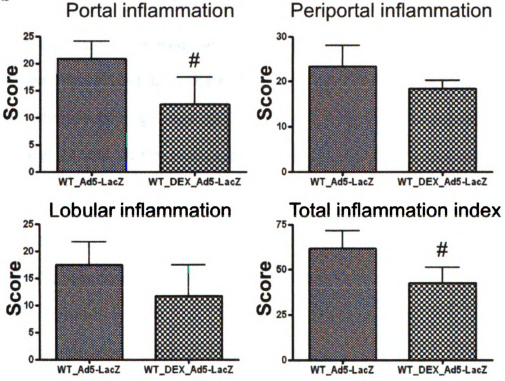
Dexamethasone treatment significantly reduces leukocyte infiltration into the liver of Ad treated mice. To test if DEX pretreatment can impact upon the known propensity of systemically administered Ads to induce leukocyte infiltration into the murine liver, we quantified leukocyte infiltration in liver sections collected at 28 dpi from control and experimental animal groups (Figure 31A-B), (137, 138). Treatment of mice with only Ad5-LacZ induced significant infiltration of macrophages and lymphocytes into the liver (Figure 31A-B). DEX pre-treatment significantly ($p<0.05$) reduced portal inflammation in virus-injected animals, and trended to lower periportal and lobular inflammation at this time point as well. Furthermore, the total inflammation index score was significantly ($p<0.01$) lower in DEX treated, virus injected mice, as compared to mice receiving only the virus. We have also evaluated leukocyte infiltration in livers of Ad treated mice at 6 and 24 hours after Ad injection. We did not detect any significant infiltrations of inflammatory cells by this assay in any of the groups tested (data not shown).

Figure 31: Dexamethasone treatment results in significantly reduced Ad mediated leukocytes infiltration to the liver of C57BL/6 mice at 28 dpi. Mice injections and morphometric evaluation of liver sections was performed as described in Materials and Methods. **(A)** Representative H&E stained liver sections obtained at 28 dpi from three groups of mice: WT_Mock (N=4), WT_Ad5-LacZ (N=5), WT_DEX_Ad5-LacZ (N=5) are shown. Note the lack of any inflammation in WT_Mock, the large number of inflammatory cells in WT_Ad5-LacZ and moderate infiltration in WT_DEX_Ad5-LacZ. **(B)** Representative sections from each treated animal were analyzed, scored and averaged for the levels of portal, periportal and lobular inflammation, as described in Materials and Methods. The sum of averages for each category was computed to obtain a total inflammation index score. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively.

a


WT_Mock
WT_Ad5-LacZ
WT_DEX_Ad5-LacZ

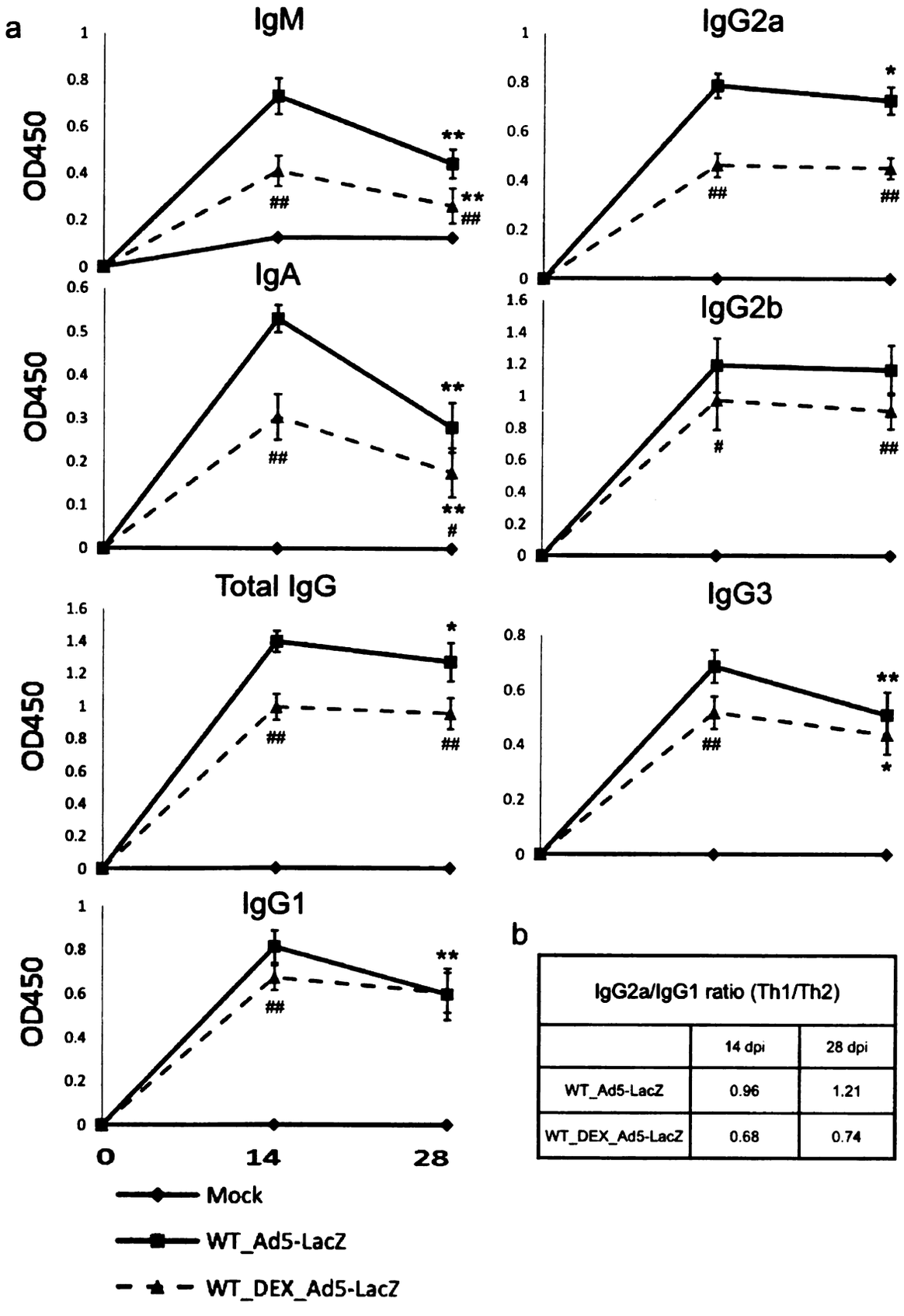
b


Portal inflammation
Periportal inflammation
Lobular inflammation
Total inflammation index
Score
#
WT_Ad5-LacZ
WT_DEX_Ad5-LacZ

Dexamethasone pre-treatment significantly alters Ad vector and transgene specific adaptive immune responses. With the knowledge that pretreatment with DEX minimizes and/or abrogates multiple Ad-triggered innate immune responses, we next determined how these blunted innate responses impacted upon subsequent adaptive immune responses. We and others have previously shown that anti-Ad specific primary humoral responses reach their peak at 7-14 days post-virus treatment, whereas anti-transgene responses peak at 23-28 days (24, 36). We have performed a series of ELISAs to determine the levels of total Ad virus or LacZ specific IgM, IgA, IgG and IgG isotype (IgG1, 2a, 2b and 3) antibody levels at 14 and 28 dpi. Pre-treatment of Ad5-LacZ injected mice with DEX significantly reduced the levels of all antibodies generated against the Ad capsid (Figure 32A). Specifically, IgG1 and IgG3 were significantly reduced at 14 dpi, whereas IgM, IgA, total IgG, IgG2a and IgG2b were all significantly reduced both at 14 and 28 dpi relative to Ad5-LacZ treated mice not treated with DEX (Figure 32A).

The IgG2a/IgG1 ratio is considered to reflect the relative contribution of the Th1/Th2 response. DEX treatment noticeably altered the Th1/Th2 ratio from 1.21 in Ad-injected mice to 0.74 in DEX pre-treated, Ad-injected mice at 28 dpi. Similar reductions were observed at 14 dpi (Figure 32B). Previous reports suggest that glucocorticoids such as DEX can suppress Th1 responses while exaggerating Th2 responses (181, 182). In an attempt to clarify the altered Th1/Th2 balance, we also investigated the systemic induction of Th1 (IFNγ) and

Figure 32: Dexamethasone treatment significantly reduced Ad vector capsid specific humoral immune responses, including capsid-neutralizing antibodies. (A) Plasma samples, collected at 14 dpi and 28 dpi, were analyzed for anti Ad capsid specific total IgM, IgA and IgG antibodies and various IgG subclasses. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. *, ** - indicate values, statistically different from animals of the same group at different time point, $p<0.05$, $p<0.001$ respectively. **(B)** IgG2a/IgG1 ratio, indicative of Th1/Th2 response were calculated based on subclass titering.



b

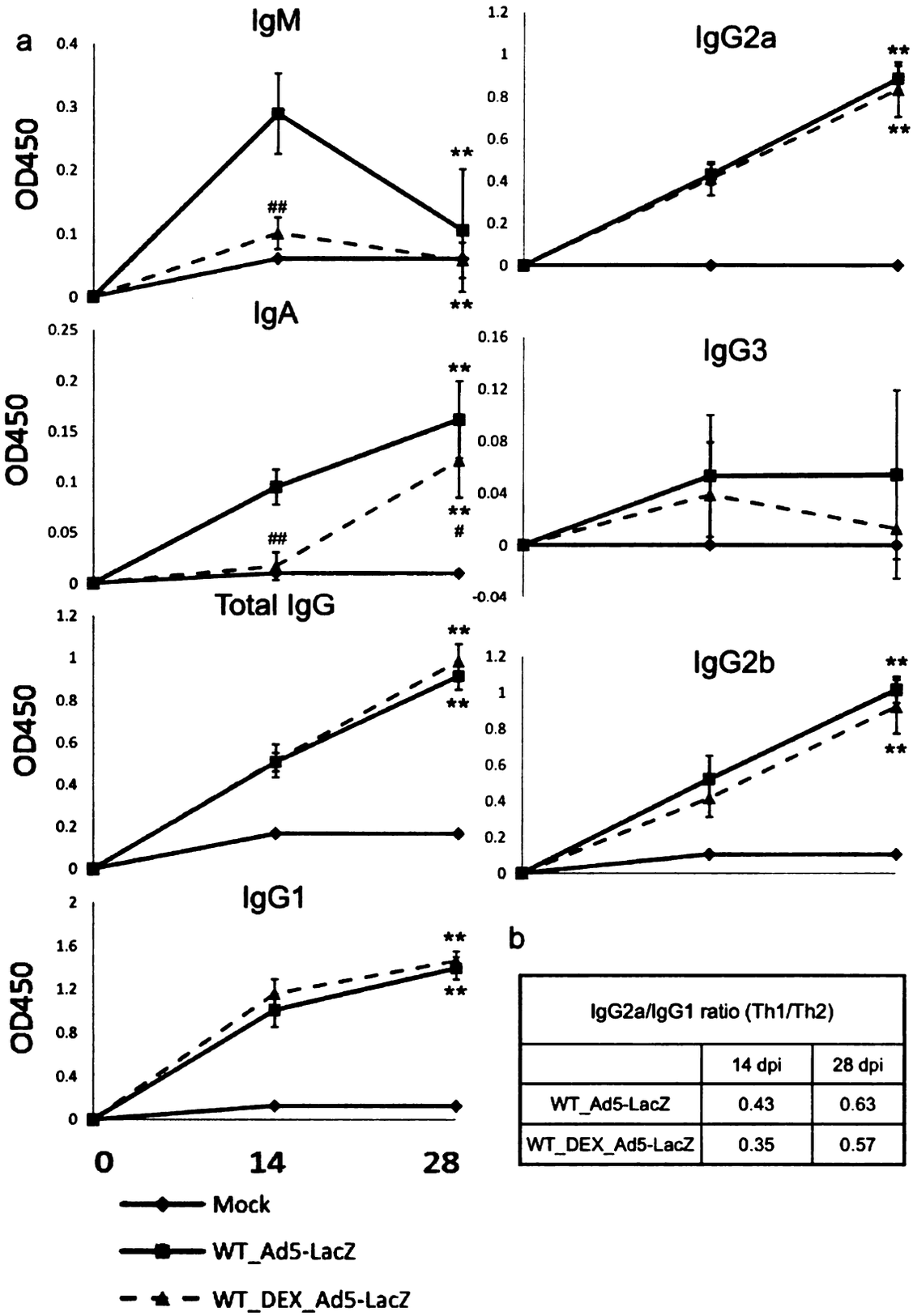
IgG2a/IgG1 ratio (Th1/Th2)		
	14 dpi	28 dpi
WT_Ad5-LacZ	0.96	1.21
WT_DEX_Ad5-LacZ	0.68	0.74

Th2 (IL-4 and IL-5) specific cytokines at both 6 and 24 hpi, however, we were unable to detect significant inductions of any of these factors in the plasma of the treated mice (data not shown). We next determined if these differences in primary humoral immune responses resulted in any significant changes in Ad neutralizing antibody (NAb) titers at 28 dpi. We found that at several plasma dilutions, Ad5-LacZ injected mice had significant amounts of anti-Ad NAb titers, whereas DEX pre-treated, virus injected mice had significantly lower NAb titers (data not shown).

DEX pre-treatment also reduced anti transgene (β-Gal) humoral immune responses since LacZ specific IgM and IgA antibodies titers were significantly lower in DEX pre-treated, Ad5-LacZ treated animals relative to Ad-LacZ treated mice. However, similar levels of LacZ specific IgG antibody isotypes were induced in Ad-injected mice, regardless of DEX pre-treatment status. The LacZ specific Th1/Th2 ratio was slightly reduced in DEX treated animals as compared to WT mice not treated with DEX (Figure 33A-B). Overall, several anti-transgene specific humoral immune responses were reduced with DEX treatment, although less significantly than the DEX induced reductions in anti-capsid responses. Importantly, we have shown that DEX treatment did not interfere with antibody production in C57BL/6 mice, since levels of total non-specific mouse IgG measured in Mock-injected animals were identical in DEX pre-treated mice (Figure 34).

Finally, we determined whether transient pretreatment of mice with DEX had

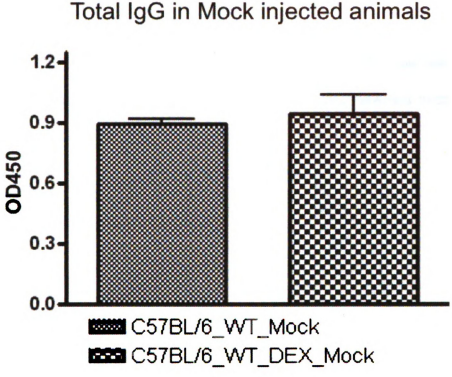
Figure 33: Dexamethasone treatment significantly reduced Ad vector derived transgene specific humoral immune responses. (A) Three groups of mice were treated as described in Materials and Methods: WT_Mock (N=4), WT_Ad5-LacZ (N=5), WT_DEX_Ad5-LacZ (N=5). Plasma samples, collected at 14 dpi and 28 dpi, were analyzed for anti β-Gal (Ad derived transgene) specific total IgM, IgA and IgG antibodies and various IgG subclasses. The error bars represent ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus injected animals. #, ## - indicate statistically different values in WT_DEX_Ad5-LacZ group compared to WT_Ad5-LacZ group, $p<0.05$, $p<0.001$ respectively. *, ** - indicate values, statistically different from animals of the same group at different time point, $p<0.05$, $p<0.001$ respectively. **(B)** IgG2a/IgG1 ratio, indicative of Th1/Th2 response were calculated based on subclass titering.



b

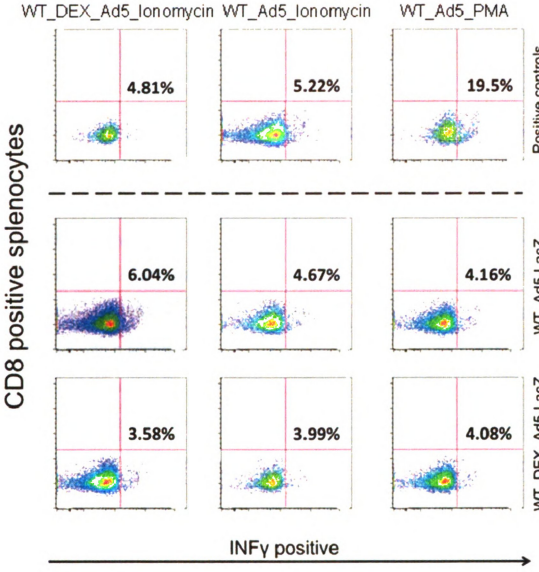
IgG2a/IgG1 ratio (Th1/Th2)		
	14 dpi	28 dpi
WT_Ad5-LacZ	0.43	0.63
WT_DEX_Ad5-LacZ	0.35	0.57

Figure 34: Dexamethasone treatment does not change natural levels of total non-specific IgG antibodies in a blood of C57BL/6 mice. Plasma samples from Mock-injected mice (WT_Mock and WT_DEX_Mock, N=4 for each group) were analyzed for total non-specific IgG antibodies. The bars represent Mean ± SD. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of Mock-injected animals, $p<0.05$ was deemed a statistically significant difference. No significant difference was found.


Total IgG in Mock injected animals
1.2
0.9
0.6
0.3
0.0
OD450
C57BL/6_WT_Mock
C57BL/6_WT_DEX_Mock

any effect on systemically administered Ad vector induction of cellular adaptive immune responses. We derived total splenocytes from virus-injected mice and performed flow cytometry experiments after *ex vivo* stimulation of the splenocytes with a LacZ specific peptide (Figure 35). DEX pre-treated, Ad5-LacZ injected C57BL/6 mice induced similar numbers of IFNγ positive, LacZ specific $CD8^+$ T cells as noted in non DEX treated Ad5-LacZ injected mice (3.6% versus 4.6%).

Figure 35: Dexamethasone treatment does not change Ad dependent CD8 positive T cells activation. Two groups of mice were treated as described in Materials and Methods: WT_Ad5-LacZ (N=3), WT_DEX_Ad5-LacZ (N=3). Total splenocytes were collected, stimulated with LacZ peptide (3 μg/ml) or positive controls PMA or ionomycin for 6 hours. IFNγ secretion assay was performed as described in Materials and Methods. Statistical analysis was completed using two-tailed Student t-test to compare 2 groups of virus-injected animals. No significant differences found.


WT_DEX_Ad5_Ionomycin
WT_Ad5_Ionomycin
WT_Ad5_PMA
4.81%
5.22%
19.5%
Positive controls
6.04%
4.67%
4.16%
WT_Ad5-LacZ
3.58%
3.99%
4.08%
WT_DEX_Ad5-LacZ
CD8 positive splenocytes
INFγ positive

5.3. Discussion

Toxicities that rapidly develop after intravenous injection of Ad vectors is a major problem, limiting the usage of Ads in gene therapy applications requiring systemic administration; i.e.: for high-level liver transduction (46, 78, 183, 184). To improve the risk/benefit ratio of systemic administration of Ad vectors requires simultaneous blockade of several Ad induced innate immune responses, such as acute thrombocytopenia (44), cytokines/chemokine release (26, 185, 186), induction of pro-inflammatory gene expression (23, 25, 26, 46) and activation of endothelial cells (45, 129, 132). In this study, we attempted to block all of these responses with a simple pretreatment with a clinically convenient and the widely utilized glucocorticoid, in this instance, Dexamethasone. Our results confirmed that transient pretreatments of mice with several doses of Dexamethasone can abrogate most innate toxicities attributable to systemic delivery of Ad-based vectors, improving the risk/benefit ratio of this vector class for numerous applications such as liver gene therapy. The drug dosages used in our studies parallel doses routinely utilized in humans, doses that range from those attempting to capitalize upon the anti-inflammatory effects of DEX to treat mild conditions, to doses used to treat complications of sepsis and clinical shock (187-189).

The mechanism of action of glucocorticoids is relatively well known. Glucocorticoid hormones bind to their respective glucocorticoid receptors (GR), which dimerize and translocate to the nucleus, where they bind to specific DNA sequences, the Glucocorticoid response elements (GRE) and either activate

transcription of anti-inflammatory genes (IL-10, IL-1_RA) or indirectly downregulate transcription of a number of pro-inflammatory genes (cytokines, adhesion molecules) through a variety of mechanisms (113-115). Glucocorticoids are known to change chromatin structure allowing for increased (or decreased) accessibility for transcriptional machinery (173). A number of reports confirm the role of high dose glucocorticoids in reducing transcription of pro-inflammatory genes mediated by GRs (113-115), in particular in LPS-induced models (190-192). Systemic activation of the innate immune system can also be minimized by high dose DEX pre-treatment (or other glucocorticoids) at least in part by GR-mediated blocking of pro-inflammatory genes transcription (127, 187, 190, 192, 193).

Based upon this analysis, we thought that many of the same innate immune response pathways that are induced by Ad vectors may also be impacted upon by glucocorticoids. Clearly, our results confirm this notion, as DEX pretreatment can minimize or prevent numerous Ad vector induced innate immune responses. In Figure 36, we have attempted to summarize some of the known innate immune response pathways induced by Ad that appear to be responsive to DEX pretreatment (Figure 36).

We found that DEX pre-treatment significantly diminished the Ad dependent activation of most innate immune responses noted after systemic delivery of Ads (44-46, 129, 132, 185, 186). Specifically, the systemic release of pro-inflammatory cytokines/chemokines within 6 hours of Ad injection was partially or completely blocked by pretreatment of mice with escalating doses of DEX. At

maximal DEX dosages, Ad induction of thrombocytopenia was prevented, the transcriptional activation of multiple, pro-inflammatory liver genes at 6 hpi was completely blocked, and endothelial cell activation were all mitigated by transient DEX pre-treatment of Ad-injected mice. DEX pre-treatment also blocked leukocyte infiltration into the Ad transduced liver at 28 dpi. Our previous studies and those of others suggests that this effect was likely mediated by significantly altering Ad interactions with the TLR and complement systems, since these systems are known to mediate Ad interactions with macrophages, dendritic cells, mast cells, endothelial cells, and/or granulocytes (23-26, 35, 36, 46, 163, 194, 195).

Cellular infiltration of the liver by elements of the mammalian innate immune response is dependent upon activation of endothelial cells. Elevated expression of adhesion molecules on their surface, such as the P and E selectins, mediate tethering and rolling of leukocytes, whereas increased expression of ICAM-1, ICAM-2 and VCAM-1 mediate firm adhesion of leukocytes. It is known that high dose Ad vectors cause rapid activation of endothelial cells both *in vitro* (45) and *in vivo* (45, 129, 132). We have shown that plasma levels of endothelial cell derived activation markers were induced at least 3 fold in Ad-injected mice as compared to mock-injected animals (e-Selectin, ICAM-1). This induction was completely blocked with DEX pre-treatment of Ad-injected mice, a result verified by qRT-PCR analysis of ICAM-1 and VCAM-1 gene transcriptions. Since we confirmed that systemic Ad injection into DEX pretreated mice still resulted in

Figure 36: Dexamethasone pre-treatment mediates blockage of Ad induced innate immune responses: model of action. Ad interactions with the complement system, as and/or Ad capsid triggered TLR signaling results in nuclear translocation of inflammatory Transcription Factors, such as NFkB, IRF7 and others. The expression of a number of genes involved in innate immune responses becomes upregulated in response to systemic injection of Ads. This leads to cytokine and chemokine release and signal amplification by autocrine and paracrine cytokine signaling. DEX, when injected prior to Ad treatment, causes Glucocorticoid Receptor dimerization and nuclear translocation, where GR interacts with genes containing the target DNA sequence (GRE). Thus, GR activation by DEX pretreatment interferes with induction of transcription of pro-inflammatory genes, to block Ad induced transcription of adhesion molecules (ICAM-1, VCAM-1) minimize systemic cytokine release and therefore in lack of induction of IFN responsive genes, such as Oas1a or ADAR. See (23-26, 35, 36, 44-46, 129, 132, 163, 185, 186, 194, 195) for further details.

complement
TLR2
TLR9
MyD88
TRAF6
TBK1
IRF7
NFKB
NFKB
GR
DEX
GR
SOCS1
STAT1
JAK2
JAK2
Cytokine receptor
IRF7
IRF7
Type I IFNs
Pro-inflammatory genes
autocrine
paracrine
IL-6
RANTES
MIP1β
KC
MCP1
IL-12
IFNα
IFNβ
G-CSF
ICAM-1

Kupffer cell necrosis, we have now confirmed that Kupffer cell dependent innate immune responses induced by Ads are likely consequent to immediate interactions with Kupffer cells, rather than due to Ad induced Kupffer cell necrosis *per se* (36, 129, 196). Interestingly, DEX pre-treatment did not have any effect on Ad-dependent Kupffer cell necrosis, which suggests that DEX does not mediate damage associated molecular pattern molecules inflammatory responses.

We further confirmed that pre-treatment with DEX did not prevent Ad vectors from transducing the murine liver with transgenes. Interestingly, DEX pre-treatment increased the detectable number of Ad genomes in the murine liver early after injection, but this did not elevate Ad derived β-Gal expression levels in murine liver at 24 hpi and 28 dpi. This paradox may be due to the fact that in the vectors utilized in this study the LacZ gene was expressed under control of the CMV promoter/enhancer element. This enhancer is known to contain Nuclear factor κB (NF-κB) response elements (197). NF-κB is a key transcription factor activating pro-inflammatory gene expression, and is induced over 2 fold in the livers of Ad5-LacZ treated C57BL/6 mice at 6 hpi compared to Mock-injected animals. In contrast, DEX pre-treated virus injected animals had NF-κB transcript levels no different from mock-injected animals; in fact they had 1.6 fold less NF-κB transcripts compared to WT_Mock group. This over 3.5 fold difference between DEX treated and non-treated Ad5-LacZ injected animals may explain why CMV promoter/enhancer derived LacZ gene transcription did not correlate with Ad vector genome copy numbers (198).

DEX pre-treatment of mice minimized Ad induced innate immune responses, an effect that also resulted in blunted adaptive immune responses. Neutralizing antibody titers, anti-Ad and anti-LacZ antibody titers were significantly reduced in DEX pre-treated Ad-injected mice relative to non-DEX treated, Ad vector treated mice. The fact that DEX pre-treatment diminished the generation of anti-Ad NAb may have significant applicability in Ad re-administration scenarios (199).

In summary, results obtained in this study confirm that the simple pre-emptive and transient use of a potent glucocorticoid prior to systemic delivery of an Ad vector can allow for safer systemic Ad mediated gene transfer. This approach avoids the complications associated with long-term glucocorticoid usage, while simultaneously significantly improving the safety profile of systemic Ad administration for use in gene therapy strategies requiring widespread liver transduction. Furthermore, this method can be simply applied in conjunction with use of either advanced generation Ad vectors, and/or other pre-emptive strategies previously shown to impact on Ad induced innate immune responses to further improve the safety profile of this important gene transfer platform. These benefits, coupled with DEX dependent diminishment of generation of Ad specific neutralizing antibodies further highlights the importance of this study.

Acknowledgements:

We wish to thank Michigan State University Laboratory Animal support facility for their assistance in the humane care and maintenance of the animals utilized in this work. S.S.S. was supported by American Heart Association Midwest Affiliate Fellowship 0815660G. A.A. was supported by the National Institutes of Health grants RO1DK-069884, P01 CA078673, the MSU Foundation as well the Osteopathic Heritage Foundation.

Chapter VI

Materials and Methods

6.1. Adenovirus vector construction

6.1.1. Incorporation of COMPinh in HI loop of the fiber protein

All novel Ad vectors were constructed utilizing pAdEasy based system (200) with modifications. pAdEasy plasmid was digested with *SpeI* and *PacI* restriction enzymes and the 6.20 kb fragment containing the fiber gene was gel purified and subcloned into the pBSX plasmid, giving rise to pBSX-FiberHI. The Fiber HI loop was flanked with in frame, *NotI* and *XbaI* restriction sites, using an approach similar to that described in Fontana et.al. (201) generating pBSX-FiberHI+*NotXba*. Next, 45-mer complementary oliogonucleotides, encoding the 13-mer COMPinh nucleotide sequence were synthesized. When hybridized together, these oligomers yielded *NotI* and *XbaI* compatible overhangs, allowing in-frame subcloning into the HI fiber loop of pBSX-FiberHI+*NotXba*. The plasmid obtained, pBSX-FiberHI+*NotXba*-COMPinh, was digested with *SpeI* and *PacI* and the 6.25 kb fragment was cloned back into the pAdEasy backbone, giving rise to pAd-Fiber-COMPinh. Bacterial homologous recombination of pAd-Fiber-COMPinh with *PmeI* linearised pShuttle-CMV-LacZ yielded the pAd-CMV-LacZ-Fiber-COMPinh plasmid. Further manipulations with this plasmid are identical to the ones described below.

6.1.2. Incorporation of COMPinh in the C-terminus of protein IX

Oligonucleotides encoding COMPinh with *Nhe*I compatible ends were subcloned in-frame into the C-terminus of viral protein IX into pShuttle-IX/*Nhe*I, the latter contains an *Nhe*I site just upstream of the pIX stop codon (101). A LacZ

expression cassette was inserted into the MCS of the pShuttle-IX-COMPinh as previously described (15, 26). pShuttle-LacZ-IX-COMPinh, was linearised with *Pme*I restriction enzyme and homologously recombined with the plasmid pAdEasyI (200), yielding pAd-LacZ-IX-COMPinh plasmid. HEK293 cells were transfected with *Pac*I linearised Ad5-LacZ-IX-COMPinh or Ad5-LacZ-Fiber-COMPinh plasmids. Recombinant viable viruses were isolated, amplified, and purified in $CsCl_2$ gradients as previously described (185, 202). Note that a complete list of primer sequences utilized for construction and validation studies is in table 8. All viruses were designed to be [E1-,E3-] and found to be RCA free (23).

6.2. Adenovirus vector production and characterization

A first-generation, human Adenovirus type 5 derived replication deficient vector (deleted for the E1 and E3 genes) encoding β-galactosidase (LacZ) as a transgene (Ad5-LacZ) was used in these studies. Virus construction, propagation and purification was performed as previously described (27, 28, 185, 202). Briefly, a number of serial passages on HEK293 cells allowed high titer purification of Ad5-LacZ by sequential, cesium chloride density gradient centrifugations. Purified virus was dialyzed against 10 mM Tris (pH 8.0) and stored in 1% sucrose, 1 X PBS at -80° C until use. Viral particle (vp) and transducing unit titers (bfu/ml) were determined as previously described, and were 2.6×10^{12} vp/ml and 1.8×10^{11} bfu/ml respectively (15, 26). The vp to bfu ratio was ~14:1. Infectious titers of all Ads were determined by standard Tissue

Table 8: Complete list of primers and oligos, utilized to construct and validate Ad5-based vectors. Oligos, utilized to subclone COMPinh into pIX and fiber are shown. Important sequencing primers used to confirm integrity of plasmids constructed are also presented. Note, validation primers were used to sequence plasmids at all stages and purified virus derived DNA.

Primer name	Primer sequence (5'–3')	Used for
Comp-Nhe-F	CTAGCatctgcgtgtggcaggattggggcgcccacaggtgcaccG	Cloning compinh in pIX
Comp-Nhe-R	CTAGCggtgcacctgtgggcgccccaatcctgccacacgcagatg	Cloning compinh in pIX
Comp-Xba-Fc	CTAGAatctgcgtgtggcaggattggggcgcccacaggtgcaccCG	Cloning compinh in Fiber
Comp-Not-Rc	GGCCGCggtgcacctgtgggcgccccaatcctgccacacgcagatT	Cloning compinh in Fiber
Knob-F1	AGGCAGTTTGGCTCCAATATCTG	*NotI/XbaI* insertion in Fiber
knob-Xba/Not-R1	**GCGGCCGCA**CCT**CTAGA**TGTGTCTCCTGTTTCCTGTGTA	*NotI/XbaI* insertion in Fiber
knob-Xba/Not-F2	**TCTAGAGGTGCGGCCGC**TCCAAGTGCATACTCTATGTCATT T	*NotI/XbaI* insertion in Fiber
knob-R2	GCTATGTGGTGGTGGGGCTATACTA	*NotI/XbaI* insertion in Fiber
CMV-F	TGGGAGTTTGTTTTGGCACC	Sequencing transgene
SV40polyA-R	TTCATTTTATGTTTCAGGTTCAGGG	Sequencing transgene
pIX-SEQ-F1	GCAAGCAGTGCAGCTTCCCG	Sequencing pIX display
pIX-SEQ-F2	GATCTGCGCCAGCAGGTTTC	Sequencing pIX display
pIX-SEQ-R	CAGGACCCTCAACGACCGAG	Sequencing pIX display
Ad5-Comp-R	CCGCCCTATCCTGATGCACG	Sequencing Fiber display
Cominh-SEQ-F	ATCTGCGTGTGGCAGGATTG	Sequencing compinh
pIX-upstream-R	CCACGCCCACACATTTCAGTACC	Sequencing of Ads to test for RCA

Culture Infectious Dose 50 (TCID50) method (AdEasy Adenoviral vector system manual, Qbiogene, Carlsbad, CA). Infections titer was calculated by using KARBER statistical method: TCID50/ml titer = $10 \times 10^{1 + d(S-0.5)}$, where d is the log(10) of the dilution and S is the sum of ratios from the first dilution. VP/TU/TCID results are summarized in table 6. Note, that VP/TU/TCID ratios of capsid-displaying Ads were not dramatically different from the same ratios for control Ads (table 6). These experiments validated the viability of novel capsid-displaying Ads.

All viruses were found to be RCA free both by RCA PCR (E1 region amplification) and direct sequencing, methods as previously described (23). Ad vectors have also been tested for the presence of bacterial endotoxin as previously described (136) and were found to contain <0.01 EU per injection dose.

6.3. Electron Microscopy of purified Ad vectors

Negative staining of $CsCl_2$ purified Ad vectors was performed as follows. Ads diluted to 10^{12} vp/ml in 10 mM Tris were adsorbed to Formvar/Carbon film 300 mesh Copper grids (Electron Microscopy Sciences, Hatfield, PA) and stained with a freshly prepared, 1% solution of phosphotungstic acid (1 g PTA, 50 µl of FBS, 50 ml miliQ water, pH 6.0, adjusted by KOH) for 30 seconds and examined by using transmission electron microscope (Philips EM410). Photographs were taken from representative areas from each sample (Figure 21).

6.4. Validation of VP titers of Ads

6.4.1. Silver Staining

To verify that particle number quantification was accurate across all Ads constructed, 10^{10} vps of lysed purified virions of each Ad were separated by 10% SDS-PAGE and subsequently stained with silver nitrate utilizing a Silver stain kit for proteins (Sigma, St. Louis, MO). The amount of hexon protein was quantified for each Ad vector by scanning densitometry using ImageJ software, ver. 1.29 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Results from this analysis indicated that the VP titers of all viruses determined by spectrophotometry fall within ~1.13 fold of each other, thus capsid-displaying Ad5 vector preparations did not contain less virions as compared to first generation Ad5 vectors, based on this assay (Figure 23 and data not shown).

6.4.2. Western Blotting

To further verify that particle number quantification was accurate across all Ads constructed, 10^{10} of lysed purified virions of each Ad were separated by 10% SDS-PAGE and Western blotting was performed utilizing hexon specific antibodies (Abcam, Cambridge, MA). Electrophoretically separated capsid protein samples were transferred onto nitrocellulose membranes and probed with rabbit polyclonal Ad5 hexon specific antibody, followed by probing with a fluorescent secondary antibody as previously described (36). Membranes were scanned and hexon concentrations quantified using Licor's Odyssey scanner (36). Results from this analysis indicated that the VP titers determined by

spectrophotometry fall within ~1.3 fold of each other based on this assay, thus capsid-displaying Ad5 vector preparations did not contain less virions as compared to conventional first generation Ad5 vectors based on this assay (data not shown).

6.5. Capsid thermostability assay

It has been shown that Ad capsids containing functional pIXs are more resistant to temperature inactivation as compared to Ad capsids lacking pIX or pIX functionality (203, 204). Therefore, we pre-incubated control Ads, and pIX-COMPinh displaying Ads at 45° C or 56° C, infected HEK293 cells and determined the percentage of LacZ positive HEK293 cells as previously described (15, 26). We did not detect significant differences between the ability of Ad-LacZ-IX-dCOMPinh to transduce 293 cells compared to control vectors Ad-LacZ. This confirms that COMPinh displaying vectors contain a functional protein IX.

Capsid thermostability assay was performed according to previously described protocol (101, 203, 204) with modifications. 400,000 HEK293 cells were plated in each well of a 24 well tissue culture plate. Ads were diluted from $CsCl_2$ purified stocks in complete media ($1.55x10^7$ vp in 500 µl of media) and were either not heat treated, or heated to 45° C or 56° C for 1 hour. Following incubation viruses were added to 293 cells. Following a 12-hour incubation, the cells were stained with X-gal (15, 26) and the percentage of LacZ positive cells was determined for every sample. The results indicated that all Ads retained

about 10-20% of LacZ positive cells after incubation at 45° C as compared to the non-heat treated Ads.

6.6. Animal procedures

Adult C57BL/6 WT and B6.129S4-C3tmlCrr (C3-KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME). mCR1/2-KO mice in C57BL/6 background were a kind gift from Dr. Tedder, Duke University Medical Center (71, 205). β-arrestin-1 and -2 KO mice have been backcrossed to C57BL6 background for more than 10 generations and have been described previously (206). These mice were kindly provided by Dr. Robert Lefkowitz (Duke University).

The Ad vector was injected intravenously (via the retro-orbital sinus) into 8-10 week old male C57BL/6 mice after performing proper anesthesia with isofluorane. A total of 0.75×10^{11} vp in 200 µl of PBS was injected per mouse. Dexamethasone (America Pharmaceutical Partners, INC, Schauburg IL, USA) was administered by intraperitoneal injection (10 mg/kg, 1 mg/kg, 0.1 mg/kg), at 15 hours and 2 hours prior to Ad vector administration. Note, that 0.1 mg/kg and 1 mg/kg DEX was used only in measuring Ad induced cytokines/chemokines release. The highest dose of DEX was selected based upon current dose regimens utilized to treat bacterial sepsis and shock human clinical applications (187-189), Ad vector induced inflammatory responses closely resemble these conditions. These dosages have been widely used in a number of studies on animal models (127, 193).

Four groups of mice were analyzed in DEX study: Wild-type (WT) mice mock-injected with PBS, WT mice pre-treated with DEX and then mock-injected with PBS (to identify DEX mediated responses not directly attributed to Ad), WT mice injected only with Ad5-LacZ, and WT mice pre-treated with DEX and subsequently injected with Ad5-LacZ. Control and experimental mice were sacrificed at different times after mock or virus treatment: 6 hours post injection (hpi), (N=6 for virus injected groups, N=4 for Mock-injected groups), 24 hpi (N=4 for all groups), 28 dpi (N=5 for all groups).

Four groups of mice were analyzed in CR1/2 study: C57BL/6 Wild-type (WT) and CR1/2-KO mice, Mock-injected with PBS and C57BL/6_WT or CR1/2-KO mice injected with Ad5-LacZ. For some of the control experiments C3-KO mice were utilized (N=4 for all C3-KO groups). Mice were sacrificed at different times after mock or virus treatment: 6 hours post injection (hpi), (N=6 for virus injected groups, N=4 for Mock-injected groups), 24 hpi (N=4 for all groups), 28 dpi (N=5 for all groups).

For both β-Arr and β-Arr2 four groups of mice were analyzed: Wild-type (WT) mice mock-injected with PBS, β-Arr or β-Arr2-KO mice mock-injected with PBS, WT mice injected with Ad5-LacZ, and β-Arr or β-Arr2-KO mice injected with Ad5-LacZ. Control and experimental mice were sacrificed at 6 hours after mock or virus treatment: N=4 for virus injected groups, N=3 for Mock-injected groups).

Plasma and tissue samples were collected and processed at the indicated time points in accordance with Michigan State University Institutional Animal

Care and Use Committee. All procedures with recombinant Ads were performed under BSL-2, and all vector treated animals were maintained in ABSL-2 conditions. All animal procedures were reviewed and approved by the Michigan State University ORCBS and IACUC. Care for mice was provided in accordance with PHS and AAALAC standards.

6.7. Cytokine/Chemokine/Endothelial cells activation markers release measurement

Ad induced systemic release of pro-inflammatory cytokines/chemokines in murine plasma was measured in all groups of mice utilizing a multiplex bead array system. Plasma samples were collected using heparinized capillary tubes and EDTA coated microvettes (Sarstedt, Nümbrecht, Germany) and centrifuged at 3400 rpm for 10 min to retrieve plasma samples. Samples were assayed for 7 independent cytokines/chemokines, which we have previously shown to be rapidly induced by systemically injected Ad vectors (MCP-1, KC, MIP-1β, IL-6, IL-12p40, G-SCF, RANTES) (23, 25, 26). All procedures were performed exactly as previously described according to manufacturer's instructions (Bio-Rad, Hercules, CA) via Luminex 100 technology (Luminex, Austin, TX) (23). For *in vitro* experiments utilizing peritoneal macrophages, media collected at specified time points was assayed for the same 7 analytes as per manufacturer's instructions. The measurement of soluble ICAM-1 and e-Selectin molecules (endothelial cells activation markers) in murine plasma (collected at 6 hpi) was performed utilizing mouse cardiovascular disease panel LINCOplex kit (Millipore, Billerica, MA) as per manufacturer's instructions.

6.8. Cytokine quantification in human PBMCs

Human PBMCs were plated as previously described (207). Briefly, PBMCs were resuspended in RPMI-1640 with 10% FBS, 1% PSF and plated into 24-well tissue culture plate at a concentration of 10^6 PBMC/ml. Upon 24 hour incubation, cells were washed two times with HBSS and exposed to the following Ad5 vectors at a multiplicity of infection 5000 ($5x10^9$ vp/well): Ad5-LacZ, Ad5-LacZ-IX-dCOMPinh, Ad5-LacZ-Fiber-dCOMPinh. Media was collected at 6, 24 and 48 hpi, stored at -20° C until use, and levels of human IL-6, IL-8, RANTES, GCSF, IL-10, MCP-1 and MIP-1β measured utilizing a multiplex bead array system exactly as previously described (27, 28). Only levels of IL-6, Il-8 and RANTES are reported, since levels of other analytes were not induced over Mock (IL-10, GCSF) or were not significantly different between Ad-injected groups (MCP-1, MIP-1β).

6.9. Complete blood count analysis and cell type differentiation

Total blood (0.3-0.4 ml) was collected into EDTA coated 1.0 ml Lavender tubes (BD Microtainer, Franklin Lakes, NJ) at 24 hpi. For complete blood counts (CBCs), blood was analyzed on an Advia 120 Hematology System (Bayer, New York) by the Clinical Pathology Laboratory of the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). In addition, all blood samples were examined microscopically and underwent manual differential count.

6.10. Platelet enumeration

To access Ad vector induced thrombocytopenia in mice, platelets were measured 24 hpi after systemic Ad injection by using Unopette (Fisher Scientific) system as previously described (23, 26) as per manufacturer's recommendations. Platelets were subsequently manually counted using Neubauer hemocytometer.

6.11. Ad genome copy number per liver or spleen cell

To determine the number of Ad genome copies per spleen and/or liver cell at different time points post-transduction, tissues (<0.1 g) were snap frozen in liquid nitrogen, crushed to a fine powder using a mortar and pestle and total DNA was extracted from as previously described (208). Ad genome copy numbers were assessed using Real-Time PCR based quantification. PCR reactions were performed on an ABI 7900HT Fast Real-Time PCR System using the SYBR Green PCR Mastermix as described for qRT-PCR technique. Primers generated against the Ad5 Hexon gene have been previously described (46). As an internal control for ensuring adequate DNA amplification, DNA was quantified using primers spanning the GAPDH gene. Standard curves were run in duplicate and consisted of 6 half-log dilutions using total genomic DNA, or DNA extracted from the purified Ad5-LacZ virus. These standard curves were used to determine the number of viral genomes present per liver/spleen cell. Melting curve analysis confirmed the quality and specificity of the PCR (data not shown).

6.12. β-Galactosidase enzyme activity and in situ X-gal staining

Ad mediated expression of the transgene LacZ was measured both qualitatively and quantitatively. Liver and spleen sections from animals sacrificed at 6 hpi, 24 hpi and (in case of DEX and CR1/2 experiments) 28 dpi were embedded in Optimal Cutting Temperature (OCT) compound, frozen and stored at -80° C until use. Frozen samples were sectioned (7 µm sections) on a Leica cryostat and were fixed and *in situ* stained for LacZ expression using 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, 1 mg/ml) as previously described (138). For quantitative assay, enzyme β-Galactosidase (β-gal) activity was measured in snap frozen liver samples. Liver samples (<0.1 g) were homogenized and total protein concentration was determined by bicinchoninic acid (BCA) assay, Pierce (Rockford, IL). β-gal activity was quantified by use of a β-gal activity detection kit (Stratagene, La Jolla, CA) according to manufacturers instructions and as previously described (137). Data were reported as Units of β-gal activity per µg of total protein.

6.13. qRT-PCR Analysis

To determine relative levels of a specific, liver or spleen derived RNA transcript, corresponding tissues were snap frozen in liquid nitrogen and RNA was harvested from ≈100mg of frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) per the manufacturer's protocol. Following RNA isolation, reverse transcription was performed on 180ng of total RNA using SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA) per manufacturer's protocol. RT reactions were diluted to a total volume of 60µl and 2µl was used as the template in the

subsequent PCR reactions. Primers were designed using Primer Bank web based software (http://pga.mgh.harvard.edu/primerbank/). Some primers used for amplification have been previously described (26, 28, 46). Complete list of primers utilized in this study is available in table 9. Q-PCR was carried out on an ABI 7900HT Fast Real-Time PCR System using SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA) in a 15µl reaction. All PCRs were subjected to the following procedure: 95.0° C for 10 minutes followed by 40 cycles of 95.0° C for 15 seconds followed by 60.0° C for 1 minute. The comparative Ct method was used to determine relative gene expression using GAPDH to standardize expression levels across all samples. Relative expression changes were calculated based on comparing experimental levels of a respective liver/spleen transcript to those quantified in liver/spleen samples, derived from Mock-injected animals.

6.14. Kupffer cell staining

Liver blocks, preserved in OCT compound at -80°C, were sliced into 7µM sections using a cryostat, placed on glass slides and frozen at -80°C for future use. Slides were thawed, fixed in 100% ethanol for 15min, and washed in KPBS containing 0.2% gelatin and 0.05% tween-20. Sections were permeabilized in 0.1% triton-X100 and blocked with KPBS containing 0.1% gelatin, 1% tween-20, and 5% BSA for 60 min at RT. To prevent non-specific binding of the secondary antibody, 5% rabbit serum in wash buffer was added and incubated at RT for 30 min. Rat anti-mouse F4/80 antibody (Invitrogen, Carlsbad, CA) diluted to 5.2 µg/mL in wash buffer containing 2% rabbit serum was added to the sections and

incubated overnight at 4° C. Sections were washed and incubated at RT for 45 min with rabbit anti-rat Alexafluor-488 (Invitrogen, Carlsbad, CA) secondary antibody diluted 1:25,000 in wash buffer containing 2% rabbit serum. Sections were then washed, stained with DAPI (Invitrogen, Carlsbad, CA) for 5 min at RT and mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA). Images were obtained using a Leica DMLB microscope, and captured using SPOT software v.3.5.9. Both DAPI and F4/80 stained images were transferred to Adobe Photoshop and converted to grayscale. The background threshold was set based on mock-injected livers, and pixels were quantified. F4/80 fluorescence values were normalized to DAPI fluorescence values to control for cell number differences. Values are reported as percent relative to number of Kupffer cells identically enumerated in liver tissues derived from mock-injected control mice.

6.15. Hematoxilin and Eosin staining

To study Ad vector mediated hepatic inflammation (at 28 dpi), Hematoxilin and Eosin (H&E) staining of mouse liver samples was performed as previously described (137). Briefly, tissues were fixed in 10% neutral formalin for 12 hours, washed in 70% ethanol, embedded in paraffin and 6-μm sectioned were stained with H&E. We have adapted a previously developed semi-quantitative scoring system, which allows the level of hepatic pathology between different liver sections to be quantified and statistically compared (137). For every mouse, 10 liver sections obtained at different portions of the liver (0-1000 μm from liver surface) were analyzed and given a numerical score (0-3) for three different

Table 9: List of primers, utilized in qRT-PCR experiments. A pair of Forward (For) and Reverse (Rev) primers is provided for every transcript tested by qRT-PCR based methods. The primers were designed as described in Materials and Methods section; the length of resulted PCR products was 100-160 nucleotides.

ADAR	ADAR-Mm-For 5' - AGGATTGGTGAGCTCGTCAG ADAR-Mm-Rev 5' - GCCCTCTTTCTTGCTGTGTG
CXCL-9	CXCL9-Mm-For 5' - GCCCCAATTGCAACAAAACTG CXCL9-Mm-Rev 5' - CCTCTTTTGCTTTTTCTTTTGGCTG
DAF	DAF-Mm For 5' GGCCAATGGTCGAGCCACG DAF-Mm-Rev 5' - CGAAATCCTGGCCGACACTC
GAPDH	GAPDH-Mm-For 5' - AGAACATCATCCCTGCATCC GAPDH-Mm-Rev 5' - CACATTGGGGGTAGGAACAC
GATA3	GATA3-Mm-For 5' - CCTACTACGGAAACTCCGTCAGG GATA3-Mm-Rev 5' - GCCGCCATCCAGCCACG
ICAM-1	ICAM1-Mm-For 5' - GGCATTGTTCTCTAATGTCTCCG ICAM1-Mm-Rev 5' - GCTCCAGGTATATCCGAGCTTC
IFNα	IFNα1-Mm-For 5' - GCCTTGACACTCCTGGTACAAATGAG IFNα1-Mm-Rev 5' - CAGCACATTGGCAGACGGAAGACAG
IFNβ	IFNβ-Mm-For 5' - TGGGTGGAATGAGACTATTGTTG IFNβ-Mm-Rev 5' CTCCCACGTCAATCTTTCCTC
IRF-7	IRF7-Mm-For 5' - TGAACGAGGCTCGCACAGTC IRF7-Mm-Rev 5' - GGCAGGTTAACTCCACTAGGTG
IRF-8	IRF8-Mm-For 5' - GTTTACCGAATTGTCCCCGAG IRF8-Mm-Rev 5' - CTCCTCTGGGTCATACCCATGTA
Jak-1	Jak1-Mm-For 5' GCTGTGCATCAGGGCCGCC Jak1-Mm-Rev 5' - GCGGTAGTGGAGCCGGAGAG
Jak-3	Jak3-Mm-For 5' - GTGTGCGAGCTGCCAACG Jak3-Mm-Rev 5' - GCCTGTAGACCAAGACTTGAGTGTCC
MyD88	MyD88-Mm-For 5' - AGAGCTGCTGGCCTTGTTAG MyD88-Mm-Rev 5' - TTCTCGGACTCCTGGTTCTG
NFkB-RelA	NFkB-RelA-Mm-For 5' - GGCGCTCAGCGGGCAGTATTC NFkB-RelA-Mm-Rev 5' - CCACAAGTTCATGTGGATGAGGC
NOD-1	NOD1-Mm-For 5' - CCCCTTCCCAGCTCATTCG NOD1-Mm-Rev 5' - TGTGTCCATATAGGTCTCCTCCA
NOD-2	NOD2-Mm-For 5' - CAGGTCTCCGAGAGGGTACTG NOD2-Mm-Rev 5' GCTACCGATGAGCCAAATGAAG
OAS-1a	OAS1a-Mm-For 5' - CAGGAGGTGGAGTTTGATGTG OAS1a-Mm-Rev 5' - CCGTGAAGCAGGTAGAGAACTC
SOCS-1	SOCS1-Mm-For 5' - CTGCGGCTTCTATTGGGGAC SOCS1-Mm-Rev 5' - AAAAGGCAGTCGAAGGTCTCG
SOCS-3	SOCS3-Mm-For 5' CAAGAACCTACGCATCCAGTG SOCS3-Mm-Rev 5' - CCAGCTTGAGTACACAGTCGAA
TBK-1	TBK1-Mm-For 5' - AGGACCATCAGAAGAAGTACGG TBK1-Mm-Rev 5' - CCCCTCGAAGGGTCTAAACG
TLR-2	TLR2-Mm-For 5' - CCAGACACTGGGGGTAACATC TLR2-Mm-Rev 5' - CGGATCGACTTTAGACTTTGGG
TLR-3	TLR3-Mm-For 5' - GGGGTCCAACTGGAGAACCT TLR3-Mm-Rev 5' - CCGGCGAGAACTCTTTAAGTGG
TLR-6	TLR6-Mm-For 5' - AGGAACCTTACTCATGTCCCC TLR6-Mm-Rev 5' - TGTTGTGGGAGAGTCTCAGAA
TRAF2bp	TRAF2bp-Mm-For 5' - CAGGTTCGGAGAGTATCAGTTCC TRAF2bp Mm-Rev 5' - CTCTGGTATGGGATTCTGTGTTG
TRAF6	TRAF6-Mm-For 5' - GCGAGAGATTCTTTCCCTGACG TRAF6-Mm-Rev 5' - CGTTGGCACTGGGGACAATTC
TRIF	TRIF-Mm-For 5' - AACCTCCACATCCCCTGTTTT TRIF-Mm-Rev 5' - CGGGCACCTGAAATTCCTCA
VCAM-1	VCAM1-Mm-For 5' - AGTTGGGGATTCGGTTGTTCT VCAM1-Mm-Rev 5' - CCCCTCATTCCTTACCACCC

categories of liver pathology:

1. *Portal inflammation:*

   0 – no portal inflammation

   1 – low-moderate number of inflammatory cells (macrophages, lymphocytes) evident in <1/3 of portal tracts

   2 – moderate number of inflammatory cells in 1/3-2/3 of portal tracts

   3 – high number of inflammatory cells in over 2/3 of portal tracts

2. *Periportal inflammation*

   0 – no inflammation

   1 – low-moderate number of inflammatory cells infiltration evident around <1/3 of portal tracts

   2 – moderate number of inflammatory cells infiltrated through 1/3-2/3 of portal tracts, in majority of which they take less that 50% of circumference. Minimum hepato-cellular necrosis observed.

   3 – moderate-high number of inflammatory cells infiltrated through over 2/3 of portal tracts or infiltrated through over 1/3 of tracts but occupy over 50% of circumference in at least 50% of them. Significant hepato-cellular necrosis observed.

3. *Lobular inflammation / damage*

   0 – no inflammation

   1 – minimum-moderate necrosis observed in <1/3 of lobules

   2 – moderate hepatocellular necrosis observed in 1/3-2/3 of lobules

3 – moderate-severe hepato-cellular necrosis observed in over 2/3 of lobules

Two independent researchers have scored all the slides in a blind manner and the averages of their scores were taken. The sum of scores (10 slides) for each mouse was taken and individual category scores were averaged for each group. Total inflammation index was computed by averaging the sum of all three individual category scores for each mouse.

6.16. IFNγ secretion assay (Flow Cytometry)

IFNγ-secreting cells were detected using the APC-IFNγ secretion assay (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, after harvesting splenocytes from individual mice at 28 days post injection, RBCs were lysed by using ACK lysis buffer (Invitrogen, Carlsbad, CA). Splenocytes were subsequently washed two times with RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 1% PSF (penicillin, streptomycin, fungizone), counted, and stimulated for 6 hours with PMA (5 ng/ml), ionomycin (25 ng/ml) or H2b restricted LacZ peptide (DAPIYTNV, 3 µg/ml) constructed by Genscript (Piscataway, NJ) at 37° C under 5% CO2 (or left unstimulated). After stimulation splenocytes ($1x10^6$) were transferred to 5 ml tubes and washed with 3 ml cold FACS-buffer (1600 rpm, 5 min, 4° C). The cell pellet was resuspended in 90 µl of cold complete RPMI and 15 µl IFNγ catch reagent. After 5 min of incubation (labeling) at 4° C, 2 ml of warm (37° C) medium was added to each tube. The cells were then incubated at 37° C and briefly shaken every 10 min to allow cytokine secretion for 55 min.

Following incubation, cells were placed on ice and washed with 4 ml cold FACS-buffer (1600 rpm, 5 min, 4° C), resuspended in 90 µl cold FACS-buffer. The secreted IFNγ was stained with 15 µl APC-conjugated IFNγ-specific antibody (IFNγ detection reagent). After 10 min incubation at 4° C, the cells were washed with cold FACS- buffer, pelleted (1600 rpm, 5 min, 4° C), and resuspended in FACS-buffer. For surface molecules staining, the antibodies were prepared in the supernatant of the 2.4G2 hybridoma cell line, cells were stained with PE-CD8 (6 µg/ml), PerCpCy5.5-CD19 (8 µg/ml) (all BD Biosciences, San Diego, CA), and incubated on ice for 30 minutes. Samples were analyzed on BD LSR II instrument and analyzed using FlowJo software.

6.17. B cell activation assay (Flow Cytometry)

Early activation of B cells was studied by Flow Cytometry based methods. $0.75x10^{11}$ vp/mouse of Ad5-lacZ was injected intravenously into WT, CR1/2-KO or C3-KO mice. Forty-eight hours after Ad injection splenocytes were harvested and $CD19^{+}$ cells examined for expression of CD69 activation marker. Briefly, after harvesting splenocytes at 48 hpi from individual mice, RBCs were lysed by using ACK lysis buffer (Invitrogen, Carlsbad, CA). Splenocytes were subsequently washed two times with RPMI medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 1% PSF (penicillin, streptomycin, fungizone), resuspended and counted. Two million cells were first washed two times with cold FACS buffer and then incubated for 15 minutes with purified rat anti-mouse CD16/CD32 Fc block (BD Biosciences, San Diego, CA). For surface staining, cells were washed one time with FACS buffer and

incubated on ice for 30 minutes with the following antibodies, APC-CD3 (8 µg/ml), PerCpCy5.5-CD19 (8 µg/ml) and FITC-CD69 (10 µg/ml), (all BD Biosciences, San Diego, CA). Samples were analyzed on BD LSR II instrument and analyzed using FlowJo software (Tree Star, San Carlos, CA, USA).

6.18. Antibody Titering Assay

ELISA based titering experiments were essentially completed as previously described (209). Briefly, 5×10^8 vp/well or 0.2 µg recombinant LacZ protein/well (each diluted in PBS) was used to coat wells of a 96 well plate overnight at 4°C. Plates were washed with PBS-Tween (0.05%) solution, and blocking buffer (3% BSA in PBS) was added to each well and incubated for 1-3 hours at room temperature. For titering of total IgG antibodies, plasma was diluted in 1:800 in blocking buffer, added to the wells, and incubated at RT for 1 h. Wells were washed using PBS-Tween (0.05%) and HRP conjugated rabbit anti-mouse antibody (BioRad, Hercules, CA) was added at a 1:4000 dilution in PBS-Tween. TMB (Sigma-Aldrich, St. Louis, MO) substrate was added to each well, and the reaction was stopped with 1 N phosphoric acid. Plates were read at 450nm in a microplate spectrophotometer. Subisotype titering was completed using a hybridoma subisotyping kit (Calbiochem, La Jolla, CA) using plasma at a dilution of 1:200 per manufacturer's recommendations.

6.19. Neutralizing Antibody Assay

2×10^3 HEK293 cells were seeded in microwells in 125µl of complete media (DMEM, 10% FBS, 1% PSF). Cells were cultured overnight in a 37° C, 5%

CO_2 incubator. To inactivate complement, plasma was heat inactivated for 60 min at 56° C and brought to room temperature. Dilutions were made as indicated in complete media in a total volume of 100µl for each well. 1.3×10^6 viral particles of Ad5-LacZ (~650 vp/cell) was next added to each dilution, mixed well and incubated at room temperature (RT) for 1 hour. 100 µl of the medium/plasma/virus mixture was applied to cells and incubated for 2-3 days. Control samples were incubated with either virus alone or complete media alone. CELLTITER 96 AQ_{ueous} One solution (Promega, Madison, WI) was added to each well and incubated for 2 hours in a 37° C, 5% CO_2 incubator. 150 µl of media from each well was removed into a new microtiter plate and read at 492 nm in a microplate spectrophotometer. Blank subtracted OD492 values are reported.

6.20. Western blotting

To determine if mCR1/2 protein has any effect on levels of major complement protein C3 (or its cleavage products) in murine plasma, we have performed Western blotting utilizing murine C3 specific polyclonal antibody, which also recognizes C3 cleavage products (Abcam, Cambridge, MA). Assay was performed as previously described (144). Briefly, 5 µl of murine plasma, collected at 10 minutes post injection or 6 hpi from all 4 groups of mice, utilized in this study was loaded on 7.5% SDS-PAGE, transferred onto PVDF membranes and probed with C3 antibody. Subsequently, the blot was probed with secondary fluorescent antibodies, scanned and quantified utilizing Licor's Odyssey scanner (210).

6.21. Peritoneal macrophages isolation and infection

Murine peritoneal macrophages were elicited by injection of 1 ml of sterile Brewer's thioglycollate medium (4.05 g/100 ml; Sigma) into the peritoneal cavity of C57BL/6 WT, β-Arr1-KO or β-Arr2-KO mice. After 4 days, mice were euthanized. PBS (10 ml) was injected into the peritoneum, and lavage fluid was removed as previously described (211, 212). Peritoneal cells were washed twice by centrifugation, resuspended in 10% fetal bovine serum (US origin, endotoxin tested (less than 0.3 EU/ml), heat inactivated, mycoplasma, virus and bacteriophage tested from Gibco/Invitrogen) supplemented RPMI-1640 medium (Gibco/Invitrogen) and then plated in 12-well plates and allowed to adhere to the wells for 8 h in 5% CO2 at 37 °C. Nonadherent cells were removed by two washes with fresh culture medium (RPMI supplemented with 0.5% FBS), and the remaining adherent peritoneal macrophages were used for the subsequent infection with 20,000 vp/cell of Ad5-LacZ. Perotineal macrophages were extracted from four WT mice, four β-Arr1-KO mice and six β-Arr2-KO mice and plated onto multiple wells at the concentration of $1.5x10^6$ cells/well. For every time point independent wells were utilized: N=4 for WT and β-Arr1-KO, N=6 for β-Arr2-KO. Total media was collected at corresponding time point (6, 24 or 48 hpi) and stored at -80° C until use.

6.22. Complement activation AP50 serum-based assay

Normal adult human serum (pooled from 30 individuals) was purchased from Complement Technology Inc. (Tyler, TX), Rhesus monkey serum was

purchased from Innovative Research (Novi, MI), human PBMCs were obtained from Astarte Biologics (Redmond, WA). Alternative pathway 50 complement activation assay was performed as previously described (24) with modifications. NHS was diluted 1/5 in EGTA-GVBS^{++} (8 mM EGTA) and mixed with the respective Ads ($2x10^{11}$ vp). NHS/Ad mixtures were incubated at 37° C for 30 min., and rabbit erythrocytes were added to each tube ($0.75x10^{8}$ in EGTA-GVBS^{++})

After incubation at 37° C for 1 hour, 2 ml of EDTA-GVBS (10 mM EDTA) was added to each tube and tubes were centrifuged at 3500 rpm for 5 min. Following centrifugation absorbance of all tubes was read at 414 nm. All reagents including NHS (pooled from 30 healthy individuals) were purchased from Complement Technology Inc. (Tyler, TX). AP50 assay experiment was repeated 4 times yielding similar results. Data from one representative experiment are reported. In every experiment we have utilized N=3 technical replicates.

6.23. C3a-desArg ELISA and CH50 assay

200 µl of NHS or NHPS was mixed with $1x10^{10}$ vp of each of the respective Ad vectors (final concentration equal to $5x10^{10}$ vp/ml of serum) and incubated at 37° C for 90 min. The reaction was then stopped by adding EDTA to a final concentration of 10 mM. C3a-desArg was then quantified using ELISA as per the manufacturer's instructions (Fitzgerald Industries Intl, former Research Diagnostic Inc., Concord, MA) or (Quidel Corporation, San Diego, CA). Activation

of Classical complement pathway was measured by CH50 assay using the MicroVue CH50 Eq E1A Kit (Quidel Corporation, San Diego, CA). For this assay, after exposure of the serum to the control or respective Ad5 vectors aliquots were taken, without exposure to EDTA, mixed with complement activator and an ELISA that specifically detects the complement terminal complexes was performed according to the manufacturer's instructions (Quidel Corporation, San Diego, CA). Note, that the residual amounts of terminal complement complexes present in the control or Ad vector exposed serum samples were quantified as CH50 unit equivalents per ml.

6.24. Statistical analysis

For every experiment, pilot trials were performed with 3 mice per group (or N=3 for *in vitro* experiments). This allowed us to determine effect size and sample variance so that Power Analysis could be performed to correctly determine the number of subjects per group required to achieve a statistical Power > 0.8 at the 95% confidence level. Statistically significant differences in toxicities associated with innate immune response (i.e. platelet counts, gene induction, etc.) were determined using One Way ANOVA with a Student-Newman-Keuls post-hoc test (p value < 0.05). Furthermore, a Two Way ANOVA with a Bonferroni post-hoc test was used to analyze the levels of cytokines at 1 and 6 hpi (or other specified time points) to determine significant differences (p value < 0.05) between groups. For antibody titering assays, liver H&E stains, β-Gal activity and Ad genomes in mouse liver, a two-tailed Student t-test was used to compare 2 groups of virus-injected animals (p < 0.05). All graphs are

presented as Mean of the average ± SD. GraphPad Prism software was utilized for statistical analysis.

Chapter VII

Summary and Future Perspectives

The use of Ad5 vectors as a platform for gene transfer applications has been gaining steady momentum. The large number of patients safely treated with the Ad5 platform confirms its high likelihood for acceptance by regulatory bodies, relative to less well tested platforms, an important point when considering the considerable risks and costs involved in developing new therapeutics. Unfortunately, despite this tremendous track record for safe and efficacious delivery of transgenes in numerous applications, conventional, E1 deleted Ad5-based vectors have several confirmed limitations. These include Ad vector triggered innate toxicities and a dramatically reduced efficacy for gene transfer in Ad-immune hosts. These facts indicate a need for development of more efficacious Ad-based vectors. We have summarized how many groups have attempted to address the need to improve the efficacy of Ad-based vectors in general, and/or avoid the problem of pre-existing Ad5 immunity specifically, as summarized in Figure 3. Some groups have proposed the utilization of alternative human Ad serotype based vectors (some derived from non-human primates) as a relatively simple method to avoid pre-existing Ad5 immunity (209, 213-215). However, use of alternative serotype based Ad vectors also poses several new serious problems including: (1) lack of previous usage in humans, (2) significantly altered biodistribution profiles and (3) induction of more deleterious inflammatory immune responses relative to Ad5, in some cases causing excessive morbidity and mortality in animal models (46, 75, 163, 209). All of these caveats may prevent/hinder regulatory approval of alternative serotype based Ad vectors for widespread deployment.

As a result of these considerations, it is clear that each of the modified Ad vectors may be better utilized for some clinical applications, but not for others. Despite this context specific utility, one should also be impressed that the Ad platform appears to be highly "plastic" and capable of tolerating a number of elegant molecular manipulations. In many instances, these modifications not only preserve important benefits inherent to the use of the traditional Ad5-based platform, but also provide for improved efficacy, even in the context of pre-existing Ad5 immunity.

Future studies will expand upon these findings. Incorporation of some of the modifications summarized herein will likely improve the capabilities of this important platform for expanded use in a number of additional human and agricultural applications, not targeted to date. One can also envision combining some of the approaches described in this dissertation. However, theoretical contemplation of these multiply modified vectors is not a guarantee for reduction to practice, as nuances regarding compatibility of multiple manipulations to allow for viability and large scale production of the resultant vector must be respected.

In regards to Ad-triggered innate toxicities, various strategies were designed to minimize these acute immune responses, including non-covalent modification of Ad capsids (PEGylation) (87), usage of immunosuppressive drugs, such as Dexamethasone (28), cytokines (TNFα) blockers (167) or PRR (TLR9) inhibitors (38). They have all shown initial promising results, but may have limited applicability due to either non-specific off target effects, a significant alteration of Ad transductional efficiency, lack of scalability, and/or alteration of

Ad vector biodistribution profiles. As a result, continued investigations of Ad-induced innate immune responses are required in order to more fully understand the mechanisms by which immune responses against Ad vectors are generated within the host.

In attempts to investigate the role of PRRs in mediating Ad-triggered responses, we have studied the roles of β-Arr-1 and β-Arr-2 in modulating these inflammatory responses. We were the first to identify β-Arr-2 as potent inhibitor of Ad-induced immune responses in mice. Although the biochemical mechanisms by which β-arrestins mediate or inhibit the innate immune responses of Ad5 are not known, previously reported interactions of β-arrestins with TRAF6, IκBα and p105 might play a role in this process (41, 42, 216). It is also possible that scaffolding functions of β-arrestins unique to Ad5 signaling pathways might be important. It has recently been shown that Ad5 vectors trigger TLR-independent pathways, such as NALP3/ASC inflammasomes (cytoplasmic NOD-like receptors) (58), resulting in activation of IL-1β and IL-18, IL-1α/IL-1RI mediated, nucleic acid independent signaling, and Ad interactions with mucosal defensins have also been identified as mediating Ad5-induced innate immune responses (217, 218). The potential roles of β-arrestins in mediating these responses remain to be determined and represent a target for future studies.

In this dissertation, we have described the pivotal role the complement system plays in generating robust innate and adaptive immune responses subsequent to Ad treatment, (including systemic pro-inflammatory

cytokines/chemokine release, EC activation, acute thrombocytopenia, and liver transcriptome dysregulation (24, 26)), while identifying that Ad-triggered toxicities are modulated by C3 and CR1/2. Importantly, in our studies we have demonstrated the critical role of these complement components (C3 and CR1/2) in mediating generation of Ad-capsid specific humoral responses, including Ad capsid-specific neutralizing antibodies. Future studies will shed light if the use of C3-blockers or CR1/2 receptor antagonists may be a viable approach to improve the efficacy of Ad-mediated gene transfer, possibly by reducing the generation of Ad capsid specific neutralizing antibodies.

The role of cytokines/chemokines in mediating Ad-triggered cellular immune responses has not been fully investigated. Our studies provide a body of evidence confirming that the systemic release of pro-inflammatory cytokines/chemokines is C3-dependent, and regulated by CR1/2 protein (Figure 19). Specifically, since both MCP-1 and G-CSF are known to activate macrophages (219), and both RANTES and G-CSF activate neutrophils (219), it might be hypothesized that lack of mCR1/2 might result in an enhanced infiltration of these inflammatory cells to the sites normally transduced by Ad vectors (i.e. liver). However, our results were not able to detect substantial increases in acute, Ad induced cellular responses in the livers of Ad-injected mCR1/2-KO mice, suggesting that recruitment of these cells requires additional factors.

In one of the most exciting chapters of this dissertation we have described the construction and analysis of a novel class of Ad5-based vectors, "capsid-

displaying" specific complement inhibitors. We have began to test these novel COMPinh-displaying Ads in several models and reported improved properties of such-modified vectors. Furthermore, the use of COMPinh displaying Ads could be combined with other methods (i.e.: prophylactic glucocorticoid therapy, or surgical bypass techniques) (28, 170) to further reduce aspects of the Ad vector triggered innate toxicities and improve the outcomes of gene transfer applications. Based upon these data, future, more advanced studies are now justified. We leave our readers with the view that despite all pragmatic limitations, fully described in this dissertation, the continued improvement of Ad-based vectors is likely to yield several important vector platforms that will have high utility in a number of clinical and agricultural applications.

References

1. Bailey, A., and V. Mautner. 1994. Phylogenetic relationships among adenovirus serotypes. *Virology* 205:438-452.

2. Russell, W.C. 2000. Update on adenovirus and its vectors. *J Gen Virol* 81:2573-2604.

3. Volpers, C., and S. Kochanek. 2004. Adenoviral vectors for gene transfer and therapy. *J Gene Med* 6 Suppl 1:S164-171.

4. Parks, R.J. 2005. Adenovirus protein IX: a new look at an old protein. *Mol Ther* 11:19-25.

5. Rux, J.J., and R.M. Burnett. 2004. Adenovirus structure. *Hum Gene Ther* 15:1167-1176.

6. Vellinga, J., S. Van der Heijdt, and R.C. Hoeben. 2005. The adenovirus capsid: major progress in minor proteins. *J Gen Virol* 86:1581-1588.

7. Gall, J., A. Kass-Eisler, L. Leinwand, and E. Falck-Pedersen. 1996. Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J Virol* 70:2116-2123.

8. Gall, J.G., R.G. Crystal, and E. Falck-Pedersen. 1998. Construction and characterization of hexon-chimeric adenoviruses: specification of adenovirus serotype. *J Virol* 72:10260-10264.

9. Molinier-Frenkel, V., R. Lengagne, F. Gaden, S.S. Hong, J. Choppin, H. Gahery-Segard, P. Boulanger, and J.G. Guillet. 2002. Adenovirus hexon protein is a potent adjuvant for activation of a cellular immune response. *J Virol* 76:127-135.

10. Roy, S., P.S. Shirley, A. McClelland, and M. Kaleko. 1998. Circumvention of immunity to the adenovirus major coat protein hexon. *J Virol* 72:6875-6879.

11. Wu, H., I. Dmitriev, E. Kashentseva, T. Seki, M. Wang, and D.T. Curiel. 2002. Construction and characterization of adenovirus serotype 5 packaged by serotype 3 hexon. *J Virol* 76:12775-12782.

12. Myhre, S., P. Henning, O. Granio, A.S. Tylo, P.A. Nygren, S. Olofsson, P. Boulanger, L. Lindholm, and S.S. Hong. 2007. Decreased immune reactivity towards a knobless, affibody-targeted adenovirus type 5 vector. *Gene Ther* 14:376-381.

13. Appledorn, D.M., S. Seregin, and A. Amalfitano. 2008. Adenovirus vectors for renal-targeted gene delivery. *Contrib Nephrol* 159:47-62.

14. Amalfitano, A., and R.J. Parks. 2002. Separating fact from fiction: assessing the potential of modified adenovirus vectors for use in human gene therapy. *Current gene therapy* 2:111-133.

15. Amalfitano, A., M.A. Hauser, H. Hu, D. Serra, C.R. Begy, and J.S. Chamberlain. 1998. Production and characterization of improved adenovirus vectors with the E1, E2b, and E3 genes deleted. *J Virol* 72:926-933.

16. Palmer, D.J., and P. Ng. 2005. Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16:1-16.

17. Hacein-Bey-Abina, S., C. Von Kalle, M. Schmidt, M.P. McCormack, N. Wulffraat, P. Leboulch, A. Lim, C.S. Osborne, R. Pawliuk, E. Morillon, R. Sorensen, A. Forster, P. Fraser, J.I. Cohen, G. de Saint Basile, I. Alexander, U. Wintergerst, T. Frebourg, A. Aurias, D. Stoppa-Lyonnet, S. Romana, I. Radford-Weiss, F. Gross, F. Valensi, E. Delabesse, E. Macintyre, F. Sigaux, J. Soulier, L.E. Leiva, M. Wissler, C. Prinz, T.H. Rabbitts, F. Le Deist, A. Fischer, and M. Cavazzana-Calvo. 2003. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302:415-419.

18. Seggewiss, R., and C.E. Dunbar. 2005. A new direction for gene therapy: intrathymic T cell-specific lentiviral gene transfer. *J Clin Invest* 115:2064-2067.

19. Puck, J.M., and H.L. Malech. 2006. Gene therapy for immune disorders: good news tempered by bad news. *The Journal of allergy and clinical immunology* 117:865-869.

20. Donsante, A., D.G. Miller, Y. Li, C. Vogler, E.M. Brunt, D.W. Russell, and M.S. Sands. 2007. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 317:477.

21. Dave, U.P., K. Akagi, R. Tripathi, S.M. Cleveland, M.A. Thompson, M. Yi, R. Stephens, J.R. Downing, N.A. Jenkins, and N.G. Copeland. 2009. Murine leukemias with retroviral insertions at Lmo2 are predictive of the leukemias induced in SCID-X1 patients following retroviral gene therapy. *PLoS Genet* 5:e1000491.

22. Harui, A., S. Suzuki, S. Kochanek, and K. Mitani. 1999. Frequency and stability of chromosomal integration of adenovirus vectors. *J Virol* 73:6141-6146.

23. Hartman, Z.C., A. Kiang, R.S. Everett, D. Serra, X.Y. Yang, T.M. Clay, and A. Amalfitano. 2007. Adenovirus infection triggers a rapid, MyD88-regulated transcriptome response critical to acute-phase and adaptive immune responses in vivo. *J Virol* 81:1796-1812.

24. Appledorn, D.M., A. McBride, S. Seregin, J.M. Scott, N. Schuldt, A. Kiang, S. Godbehere, and A. Amalfitano. 2008. Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors. *Gene Ther* 15:1606-1617.

25. Hartman, Z.C., D.M. Appledorn, and A. Amalfitano. 2008. Adenovirus vector induced innate immune responses: impact upon efficacy and toxicity in gene therapy and vaccine applications. *Virus research* 132:1-14.

26. Kiang, A., Z.C. Hartman, R.S. Everett, D. Serra, H. Jiang, M.M. Frank, and A. Amalfitano. 2006. Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. *Mol Ther* 14:588-598.

27. Seregin, S.S., Y.A. Aldhamen, D.M. Appledorn, N.J. Schuldt, A.J. McBride, M. Bujold, S.S. Godbehere, and A. Amalfitano. 2009. CR1/2 is an important suppressor of Adenovirus-induced innate immune responses and is required for induction of neutralizing antibodies. *Gene Ther* 16:1245-1259.

28. Seregin, S.S., D.M. Appledorn, A.J. McBride, N.J. Schuldt, Y.A. Aldhamen, T. Voss, J. Wei, M. Bujold, W. Nance, S. Godbehere, and A. Amalfitano. 2009. Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol Ther* 17:685-696.

29. Raper, S.E., N. Chirmule, F.S. Lee, N.A. Wivel, A. Bagg, G.P. Gao, J.M. Wilson, and M.L. Batshaw. 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 80:148-158.

30. Makower, D., A. Rozenblit, H. Kaufman, M. Edelman, M.E. Lane, J. Zwiebel, H. Haynes, and S. Wadler. 2003. Phase II clinical trial of intralesional administration of the oncolytic adenovirus ONYX-015 in patients with hepatobiliary tumors with correlative p53 studies. *Clin Cancer Res* 9:693-702.

31. Grines, C.L., M.W. Watkins, J.J. Mahmarian, A.E. Iskandrian, J.J. Rade, P. Marrott, C. Pratt, and N. Kleiman. 2003. A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on

myocardial perfusion in patients with stable angina. *Journal of the American College of Cardiology* 42:1339-1347.

32. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nature immunology* 5:987-995.

33. Akira, S., and K. Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol* 4:499-511.

34. Lemmers, B., L. Salmena, N. Bidere, H. Su, E. Matysiak-Zablocki, K. Murakami, P.S. Ohashi, A. Jurisicova, M. Lenardo, R. Hakem, and A. Hakem. 2007. Essential role for caspase-8 in Toll-like receptors and NFkappaB signaling. *J Biol Chem* 282:7416-7423.

35. Hartman, Z.C., E.P. Black, and A. Amalfitano. 2007. Adenoviral infection induces a multi-faceted innate cellular immune response that is mediated by the toll-like receptor pathway in A549 cells. *Virology* 358:357-372.

36. Appledorn, D.M., S. Patial, A. McBride, S. Godbehere, N. Van Rooijen, N. Parameswaran, and A. Amalfitano. 2008. Adenovirus vector-induced innate inflammatory mediators, MAPK signaling, as well as adaptive immune responses are dependent upon both TLR2 and TLR9 in vivo. *J Immunol* 181:2134-2144.

37. Nociari, M., O. Ocheretina, J.W. Schoggins, and E. Falck-Pedersen. 2007. Sensing infection by adenovirus: Toll-like receptor-independent viral DNA recognition signals activation of the interferon regulatory factor 3 master regulator. *J Virol* 81:4145-4157.

38. Cerullo, V., M.P. Seiler, V. Mane, N. Brunetti-Pierri, C. Clarke, T.K. Bertin, J.R. Rodgers, and B. Lee. 2007. Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. *Mol Ther* 15:378-385.

39. Shayakhmetov, D.M., A. Gaggar, S. Ni, Z.Y. Li, and A. Lieber. 2005. Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol* 79:7478-7491.

40. DeWire, S.M., S. Ahn, R.J. Lefkowitz, and S.K. Shenoy. 2007. Beta-arrestins and cell signaling. *Annual review of physiology* 69:483-510.

41. Wang, Y., Y. Tang, L. Teng, Y. Wu, X. Zhao, and G. Pei. 2006. Association of beta-arrestin and TRAF6 negatively regulates Toll-like receptor-interleukin 1 receptor signaling. *Nature immunology* 7:139-147.

42. Parameswaran, N., C.S. Pao, K.S. Leonhard, D.S. Kang, M. Kratz, S.C. Ley, and J.L. Benovic. 2006. Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFkappaB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *J Biol Chem* 281:34159-34170.

43. Fan, H., L.M. Luttrell, G.E. Tempel, J.J. Senn, P.V. Halushka, and J.A. Cook. 2007. Beta-arrestins 1 and 2 differentially regulate LPS-induced signaling and pro-inflammatory gene expression. *Molecular immunology* 44:3092-3099.

44. Wolins, N., J. Lozier, T.L. Eggerman, E. Jones, E. Aguilar-Cordova, and J.G. Vostal. 2003. Intravenous administration of replication-incompetent adenovirus to rhesus monkeys induces thrombocytopenia by increasing in vivo platelet clearance. *British journal of haematology* 123:903-905.

45. Liu, Q., A.K. Zaiss, P. Colarusso, K. Patel, G. Haljan, T.J. Wickham, and D.A. Muruve. 2003. The role of capsid-endothelial interactions in the innate immune response to adenovirus vectors. *Hum Gene Ther* 14:627-643.

46. Appledorn, D.M., A. Kiang, A. McBride, H. Jiang, S. Seregin, J.M. Scott, R. Stringer, Y. Kousa, M. Hoban, M.M. Frank, and A. Amalfitano. 2008. Wild-type adenoviruses from groups A-F evoke unique innate immune responses, of which HAd3 and SAd23 are partially complement dependent. *Gene Ther* 15:885-901.

47. Cichon, G., S. Boeckh-Herwig, H.H. Schmidt, E. Wehnes, T. Muller, P. Pring-Akerblom, and R. Burger. 2001. Complement activation by recombinant adenoviruses. *Gene Ther* 8:1794-1800.

48. Kirschfink, M. 1997. Controlling the complement system in inflammation. *Immunopharmacology* 38:51-62.

49. Makrides, S.C. 1998. Therapeutic inhibition of the complement system. *Pharmacological reviews* 50:59-87.

50. Barrington, R., M. Zhang, M. Fischer, and M.C. Carroll. 2001. The role of complement in inflammation and adaptive immunity. *Immunol Rev* 180:5-15.

51. Wagner, E., and M.M. Frank. Therapeutic potential of complement modulation. *Nature reviews* 9:43-56.

52. Mollnes, T.E., W.C. Song, and J.D. Lambris. 2002. Complement in inflammatory tissue damage and disease. *Trends in immunology* 23:61-64.

53. Atkinson, J.P. 2006. C5a and Fcgamma receptors: a mutual admiration society. *J Clin Invest* 116:512-520.

54. Atkinson, J.P., and M.M. Frank. 2006. Bypassing complement: evolutionary lessons and future implications. *J Clin Invest* 116:1215-1218.

55. Jiang, H., Z. Wang, D. Serra, M.M. Frank, and A. Amalfitano. 2004. Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-ad antibodies. *Mol Ther* 10:1140-1142.

56. Zaiss, A.K., M.J. Cotter, L.R. White, S.A. Clark, N.C. Wong, V.M. Holers, J.S. Bartlett, and D.A. Muruve. 2008. Complement is an essential component of the immune response to adeno-associated virus vectors. *J Virol* 82:2727-2740.

57. Carlisle, R.C., Y. Di, A.M. Cerny, A.F. Sonnen, R.B. Sim, N.K. Green, V. Subr, K. Ulbrich, R.J. Gilbert, K.D. Fisher, R.W. Finberg, and L.W. Seymour. 2009. Human erythrocytes bind and inactivate type 5 adenovirus by presenting Coxsackie virus-adenovirus receptor and complement receptor 1. *Blood* 113:1909-1918.

58. Muruve, D.A., V. Petrilli, A.K. Zaiss, L.R. White, S.A. Clark, P.J. Ross, R.J. Parks, and J. Tschopp. 2008. The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* 452:103-107.

59. Xu, Z., J. Tian, J.S. Smith, and A.P. Byrnes. 2008. Clearance of adenovirus by Kupffer cells is mediated by scavenger receptors, natural antibodies, and complement. *J Virol* 82:11705-11713.

60. Gasque, P. 2004. Complement: a unique innate immune sensor for danger signals. *Molecular immunology* 41:1089-1098.

61. Kawano, M. 2000. Complement regulatory proteins and autoimmunity. *Archivum immunologiae et therapiae experimentalis* 48:367-372.

62. Morgan, B.P., K.J. Marchbank, M.P. Longhi, C.L. Harris, and A.M. Gallimore. 2005. Complement: central to innate immunity and bridging to adaptive responses. *Immunol Lett* 97:171-179.

63. Kemper, C., and J.P. Atkinson. 2007. T-cell regulation: with complements from innate immunity. *Nat Rev Immunol* 7:9-18.

64. Prechl, J., and A. Erdei. 2000. Immunomodulatory functions of murine CR1/2. *Immunopharmacology* 49:117-124.

65. Tedder, T.F., M. Inaoki, and S. Sato. 1997. The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* 6:107-118.

66. Tedder, T.F., K.M. Haas, and J.C. Poe. 2002. CD19-CD21 complex regulates an intrinsic Src family kinase amplification loop that links innate immunity with B-lymphocyte intracellular calcium responses. *Biochemical Society transactions* 30:807-811.

67. Carroll, M.C. 2004. The complement system in B cell regulation. *Molecular immunology* 41:141-146.

68. Boackle, S.A., V.M. Holers, and D.R. Karp. 1997. CD21 augments antigen presentation in immune individuals. *Eur J Immunol* 27:122-129.

69. Holers, V.M., and L. Kulik. 2007. Complement receptor 2, natural antibodies and innate immunity: Inter-relationships in B cell selection and activation. *Molecular immunology* 44:64-72.

70. Fairweather, D., S. Frisancho-Kiss, D.B. Njoku, J.F. Nyland, Z. Kaya, S.A. Yusung, S.E. Davis, J.A. Frisancho, M.A. Barrett, and N.R. Rose. 2006. Complement receptor 1 and 2 deficiency increases coxsackievirus B3-induced myocarditis, dilated cardiomyopathy, and heart failure by increasing macrophages, IL-1beta, and immune complex deposition in the heart. *J Immunol* 176:3516-3524.

71. Haas, K.M., M. Hasegawa, D.A. Steeber, J.C. Poe, M.D. Zabel, C.B. Bock, D.R. Karp, D.E. Briles, J.H. Weis, and T.F. Tedder. 2002. Complement receptors CD21/35 link innate and protective immunity during Streptococcus pneumoniae infection by regulating IgG3 antibody responses. *Immunity* 17:713-723.

72. Ding, E.Y., B.L. Hodges, H. Hu, A.J. McVie-Wylie, D. Serra, F.K. Migone, D. Pressley, Y.T. Chen, and A. Amalfitano. 2001. Long-term efficacy after [E1-, polymerase-] adenovirus-mediated transfer of human acid-alpha-glucosidase gene into glycogen storage disease type II knockout mice. *Hum Gene Ther* 12:955-965.

73. Svensson, E.C., H.B. Black, D.L. Dugger, S.K. Tripathy, E. Goldwasser, Z. Hao, L. Chu, and J.M. Leiden. 1997. Long-term erythropoietin expression in rodents and non-human primates following intramuscular injection of a replication-defective adenoviral vector. *Hum Gene Ther* 8:1797-1806.

74. Lasaro, M.O., and H.C. Ertl. 2009. New Insights on Adenovirus as Vaccine Vectors. *Mol Ther*

75. Abbink, P., A.A. Lemckert, B.A. Ewald, D.M. Lynch, M. Denholtz, S. Smits, L. Holterman, I. Damen, R. Vogels, A.R. Thorner, K.L. O'Brien, A. Carville, K.G. Mansfield, J. Goudsmit, M.J. Havenga, and D.H. Barouch. 2007. Comparative seroprevalence and immunogenicity of six rare serotype recombinant adenovirus vaccine vectors from subgroups B and D. *J Virol* 81:4654-4663.

76. Cohen, P. 2006. Immunity's yin and yang. A successful vaccine must first avoid being eliminated by pre-existing immunity before it can promote a protective immune response. *IAVI Rep* 10:1-5.

77. Molinier-Frenkel, V., H. Gahery-Segard, M. Mehtali, C. Le Boulaire, S. Ribault, P. Boulanger, T. Tursz, J.G. Guillet, and F. Farace. 2000. Immune response to recombinant adenovirus in humans: capsid components from viral input are targets for vector-specific cytotoxic T lymphocytes. *J Virol* 74:7678-7682.

78. Sumida, S.M., D.M. Truitt, M.G. Kishko, J.C. Arthur, S.S. Jackson, D.A. Gorgone, M.A. Lifton, W. Koudstaal, M.G. Pau, S. Kostense, M.J. Havenga, J. Goudsmit, N.L. Letvin, and D.H. Barouch. 2004. Neutralizing antibodies and CD8+ T lymphocytes both contribute to immunity to adenovirus serotype 5 vaccine vectors. *J Virol* 78:2666-2673.

79. Flomenberg, P., V. Piaskowski, R.L. Truitt, and J.T. Casper. 1995. Characterization of human proliferative T cell responses to adenovirus. *J Infect Dis* 171:1090-1096.

80. Tang, J., M. Olive, K. Champagne, N. Flomenberg, L. Eisenlohr, S. Hsu, and P. Flomenberg. 2004. Adenovirus hexon T-cell epitope is recognized by most adults and is restricted by HLA DP4, the most common class II allele. *Gene Ther* 11:1408-1415.

81. Tang, J., M. Olive, R. Pulmanausahakul, M. Schnell, N. Flomenberg, L. Eisenlohr, and P. Flomenberg. 2006. Human CD8+ cytotoxic T cell responses to adenovirus capsid proteins. *Virology* 350:312-322.

82. Hashimoto, M., J.L. Boyer, N.R. Hackett, J.M. Wilson, and R.G. Crystal. 2005. Induction of protective immunity to anthrax lethal toxin with a nonhuman primate adenovirus-based vaccine in the presence of preexisting anti-human adenovirus immunity. *Infect Immun* 73:6885-6891.

83. Hackett, N.R., S.M. Kaminsky, D. Sondhi, and R.G. Crystal. 2000. Antivector and antitransgene host responses in gene therapy. *Curr Opin Mol Ther* 2:376-382.

84. O'Riordan, C.R., A. Lachapelle, C. Delgado, V. Parkes, S.C. Wadsworth, A.E. Smith, and G.E. Francis. 1999. PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo. *Hum Gene Ther* 10:1349-1358.

85. Matthews, C., G. Jenkins, J. Hilfinger, and B. Davidson. 1999. Poly-L-lysine improves gene transfer with adenovirus formulated in PLGA microspheres. *Gene Ther* 6:1558-1564.

86. Lee, S.G., S.J. Yoon, C.D. Kim, K. Kim, D.S. Lim, Y.I. Yeom, M.W. Sung, D.S. Heo, and N.K. Kim. 2000. Enhancement of adenoviral transduction with polycationic liposomes in vivo. *Cancer Gene Ther* 7:1329-1335.

87. Croyle, M.A., H.T. Le, K.D. Linse, V. Cerullo, G. Toietta, A. Beaudet, and L. Pastore. 2005. PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. *Gene Ther* 12:579-587.

88. Croyle, M.A., N. Chirmule, Y. Zhang, and J.M. Wilson. 2002. PEGylation of E1-deleted adenovirus vectors allows significant gene expression on readministration to liver. *Hum Gene Ther* 13:1887-1900.

89. Wortmann, A., S. Vohringer, T. Engler, S. Corjon, R. Schirmbeck, J. Reimann, S. Kochanek, and F. Kreppel. 2008. Fully detargeted polyethylene glycol-coated adenovirus vectors are potent genetic vaccines and escape from pre-existing anti-adenovirus antibodies. *Mol Ther* 16:154-162.

90. Weaver, E.A., and M.A. Barry. 2008. Effects of Shielding Adenoviral Vectors with Polyethylene Glycol (PEG) on Vector-specific and Vaccine-mediated Immune Responses. *Hum Gene Ther*

91. Ogawara, K., M.G. Rots, R.J. Kok, H.E. Moorlag, A.M. Van Loenen, D.K. Meijer, H.J. Haisma, and G. Molema. 2004. A novel strategy to modify adenovirus tropism and enhance transgene delivery to activated vascular endothelial cells in vitro and in vivo. *Hum Gene Ther* 15:433-443.

92. Einfeld, D.A., D.E. Brough, P.W. Roelvink, I. Kovesdi, and T.J. Wickham. 1999. Construction of a pseudoreceptor that mediates transduction by adenoviruses expressing a ligand in fiber or penton base. *J Virol* 73:9130-9136.

93. Wickham, T.J., D.M. Segal, P.W. Roelvink, M.E. Carrion, A. Lizonova, G.M. Lee, and I. Kovesdi. 1996. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J Virol* 70:6831-6838.

94. McConnell, M.J., X. Danthinne, and M.J. Imperiale. 2006. Characterization of a permissive epitope insertion site in adenovirus hexon. *J Virol* 80:5361-5370.

95. McConnell, M.J., P.C. Hanna, and M.J. Imperiale. 2006. Cytokine response and survival of mice immunized with an adenovirus expressing Bacillus anthracis protective antigen domain 4. *Infect Immun* 74:1009-1015.

96. Belousova, N., V. Krendelchtchikova, D.T. Curiel, and V. Krasnykh. 2002. Modulation of adenovirus vector tropism via incorporation of polypeptide ligands into the fiber protein. *J Virol* 76:8621-8631.

97. Matthews, Q.L., D.A. Sibley, H. Wu, J. Li, M.A. Stoff-Khalili, R. Waehler, J.M. Mathis, and D.T. Curiel. 2006. Genetic incorporation of a herpes simplex virus type 1 thymidine kinase and firefly luciferase fusion into the adenovirus protein IX for functional display on the virion. *Mol Imaging* 5:510-519.

98. Worgall, S., A. Busch, M. Rivara, D. Bonnyay, P.L. Leopold, R. Merritt, N.R. Hackett, P.W. Rovelink, J.T. Bruder, T.J. Wickham, I. Kovesdi, and R.G. Crystal. 2004. Modification to the capsid of the adenovirus vector that enhances dendritic cell infection and transgene-specific cellular immune responses. *J Virol* 78:2572-2580.

99. Kurachi, S., N. Koizumi, F. Sakurai, K. Kawabata, H. Sakurai, S. Nakagawa, T. Hayakawa, and H. Mizuguchi. 2007. Characterization of capsid-modified adenovirus vectors containing heterologous peptides in the fiber knob, protein IX, or hexon. *Gene Ther* 14:266-274.

100. Waddington, S.N., J.H. McVey, D. Bhella, A.L. Parker, K. Barker, H. Atoda, R. Pink, S.M. Buckley, J.A. Greig, L. Denby, J. Custers, T. Morita, I.M. Francischetti, R.Q. Monteiro, D.H. Barouch, N. van Rooijen, C. Napoli, M.J. Havenga, S.A. Nicklin, and A.H. Baker. 2008. Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell* 132:397-409.

101. Dmitriev, I.P., E.A. Kashentseva, and D.T. Curiel. 2002. Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J Virol* 76:6893-6899.

102. Wickham, T.J., P.W. Roelvink, D.E. Brough, and I. Kovesdi. 1996. Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat Biotechnol* 14:1570-1573.

103. Koizumi, N., H. Mizuguchi, N. Utoguchi, Y. Watanabe, and T. Hayakawa. 2003. Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J Gene Med* 5:267-276.

104. Dmitriev, I., V. Krasnykh, C.R. Miller, M. Wang, E. Kashentseva, G. Mikheeva, N. Belousova, and D.T. Curiel. 1998. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol* 72:9706-9713.

105. Wu, H., T. Han, N. Belousova, V. Krasnykh, E. Kashentseva, I. Dmitriev, M. Kataram, P.J. Mahasreshti, and D.T. Curiel. 2005. Identification of sites in adenovirus hexon for foreign peptide incorporation. *J Virol* 79:3382-3390.

106. Vellinga, J., M.J. Rabelink, S.J. Cramer, D.J. van den Wollenberg, H. Van der Meulen, K.N. Leppard, F.J. Fallaux, and R.C. Hoeben. 2004. Spacers increase the accessibility of peptide ligands linked to the carboxyl terminus of adenovirus minor capsid protein IX. *J Virol* 78:3470-3479.

107. Heise, C., T. Hermiston, L. Johnson, G. Brooks, A. Sampson-Johannes, A. Williams, L. Hawkins, and D. Kirn. 2000. An adenovirus E1A mutant that demonstrates potent and selective systemic anti-tumoral efficacy. *Nat Med* 6:1134-1139.

108. Coughlan, L., S. Vallath, A. Saha, M. Flak, I.A. McNeish, G. Vassaux, J.F. Marshall, I.R. Hart, and G.J. Thomas. 2009. In vivo retargeting of adenovirus type 5 to alphavbeta6 integrin results in reduced hepatotoxicity and improved tumor uptake following systemic delivery. *J Virol* 83:6416-6428.

109. Rein, D.T., M. Breidenbach, and D.T. Curiel. 2006. Current developments in adenovirus-based cancer gene therapy. *Future oncology (London, England)* 2:137-143.

110. Kroot, E.J., A.M. Huisman, J. Van Zeben, J.M. Wouters, and H.C. Van Paassen. 2006. Oral pulsed dexamethasone therapy in early rheumatoid arthritis: a pilot study. *Ann N Y Acad Sci* 1069:300-306.

111. Kardash, K.J., F. Sarrazin, M.J. Tessler, and A.M. Velly. 2008. Single-dose dexamethasone reduces dynamic pain after total hip arthroplasty. *Anesthesia and analgesia* 106:1253-1257, table of contents.

112. Ebrahimi, S., and B. Sarkari. 2007. Comparative efficacy of dexamethasone versus hydrocortisone in severe acute pediatric asthma. *Iranian journal of allergy, asthma, and immunology* 6:159-160.

113. Pace, T.W., F. Hu, and A.H. Miller. 2007. Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. *Brain, behavior, and immunity* 21:9-19.

114. Chinenov, Y., and I. Rogatsky. 2007. Glucocorticoids and the innate immune system: crosstalk with the toll-like receptor signaling network. *Molecular and cellular endocrinology* 275:30-42.

115. Liberman, A.C., J. Druker, M.J. Perone, and E. Arzt. 2007. Glucocorticoids in the regulation of transcription factors that control cytokine synthesis. *Cytokine & growth factor reviews* 18:45-56.

116. Ma, W., K. Gee, W. Lim, K. Chambers, J.B. Angel, M. Kozlowski, and A. Kumar. 2004. Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytic cells by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF-kappa B transcription factors. *J Immunol* 172:318-330.

117. Mogensen, T.H., R.S. Berg, S.R. Paludan, and L. Ostergaard. 2008. Mechanisms of dexamethasone-mediated inhibition of Toll-like receptor signaling induced by Neisseria meningitidis and Streptococcus pneumoniae. *Infect Immun* 76:189-197.

118. Vital, A.L., M. Goncalo, M.T. Cruz, A. Figueiredo, C.B. Duarte, and M.C. Lopes. 2003. Dexamethasone prevents granulocyte-macrophage colony-stimulating factor-induced nuclear factor-kappaB activation, inducible nitric oxide synthase expression and nitric oxide production in a skin dendritic cell line. *Mediators of inflammation* 12:71-78.

119. Gessani, S., S. McCandless, and C. Baglioni. 1988. The glucocorticoid dexamethasone inhibits synthesis of interferon by decreasing the level of its mRNA. *J Biol Chem* 263:7454-7457.

120. Smith, S.J., A.M. Piliponsky, F. Rosenhead, U. Elchalal, A. Nagler, and F. Levi-Schaffer. 2002. Dexamethasone inhibits maturation, cytokine production and Fc epsilon RI expression of human cord blood-derived mast cells. *Clin Exp Allergy* 32:906-913.

121. Braun, S., C. Jenny, C. Thioudellet, F. Perraud, M.C. Claudepierre, F. Langle-Rouault, D. Ali-Hadji, K. Schughart, and A. Pavirani. 1999. In vitro and in vivo effects of glucocorticoids on gene transfer to skeletal muscle. *FEBS letters* 454:277-282.

122. Liu, Y., H.D. Liggitt, S. Dow, C. Handumrongkul, T.D. Heath, and R.J. Debs. 2002. Strain-based genetic differences regulate the efficiency of systemic gene delivery as well as expression. *J Biol Chem* 277:4966-4972.

123. Malone, R.W., M.A. Hickman, K. Lehmann-Bruinsma, T.R. Sih, R. Walzem, D.M. Carlson, and J.S. Powell. 1994. Dexamethasone enhancement of

gene expression after direct hepatic DNA injection. *J Biol Chem* 269:29903-29907.

124. Boulis, N.M., D.E. Turner, J.A. Dice, V. Bhatia, and E.L. Feldman. 1999. Characterization of adenoviral gene expression in spinal cord after remote vector delivery. *Neurosurgery* 45:131-137; discussion 137-138.

125. Ishimoto, S., K. Kawamoto, T. Stover, S. Kanzaki, T. Yamasoba, and Y. Raphael. 2003. A glucocorticoid reduces adverse effects of adenovirus vectors in the cochlea. *Audiology & neuro-otology* 8:70-79.

126. Otake, K., D.L. Ennist, K. Harrod, and B.C. Trapnell. 1998. Nonspecific inflammation inhibits adenovirus-mediated pulmonary gene transfer and expression independent of specific acquired immune responses. *Hum Gene Ther* 9:2207-2222.

127. Kumahara, K., H. Nagata, K. Watanabe, N. Shimizu, Y. Arimoto, K. Isoyama, Y. Okamoto, and H. Shirasawa. 2005. Suppression of inflammation by dexamethasone prolongs adenoviral vector-mediated transgene expression in murine nasal mucosa. *Acta oto-laryngologica* 125:1301-1306.

128. Price, A.R., M.P. Limberis, J.M. Wilson, and S.L. Diamond. 2007. Pulmonary delivery of adenovirus vector formulated with dexamethasone-spermine facilitates homologous vector re-administration. *Gene Ther* 14:1594-1604.

129. Schiedner, G., W. Bloch, S. Hertel, M. Johnston, A. Molojavyi, V. Dries, G. Varga, N. Van Rooijen, and S. Kochanek. 2003. A hemodynamic response to intravenous adenovirus vector particles is caused by systemic Kupffer cell-mediated activation of endothelial cells. *Hum Gene Ther* 14:1631-1641.

130. Appledorn, D.M., S. Patial, S. Godbehere, N. Parameswaran, and A. Amalfitano. 2009. TRIF, and TRIF-Interacting TLRs Differentially Modulate Several Adenovirus Vector-Induced Immune Responses. *J Inn Imm* 1:376-388.

131. Zhu, J., X. Huang, and Y. Yang. 2007. Type I IFN signaling on both B and CD4 T cells is required for protective antibody response to adenovirus. *J Immunol* 178:3505-3510.

132. Li, Y., D.A. Muruve, R.G. Collins, S.S. Lee, and P. Kubes. 2002. The role of selectins and integrins in adenovirus vector-induced neutrophil recruitment to the liver. *Eur J Immunol* 32:3443-3452.

133. Schiedner, G., S. Hertel, and S. Kochanek. 2000. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum Gene Ther* 11:2105-2116.

134. Miwa, K., A. Igawa, and H. Inoue. 1997. Soluble E-selectin, ICAM-1 and VCAM-1 levels in systemic and coronary circulation in patients with variant angina. *Cardiovasc Res* 36:37-44.

135. Manickan, E., J.S. Smith, J. Tian, T.L. Eggerman, J.N. Lozier, J. Muller, and A.P. Byrnes. 2006. Rapid Kupffer cell death after intravenous injection of adenovirus vectors. *Mol Ther* 13:108-117.

136. Cotter, M.J., A.K. Zaiss, and D.A. Muruve. 2005. Neutrophils interact with adenovirus vectors via Fc receptors and complement receptor 1. *J Virol* 79:14622-14631.

137. Hu, H., D. Serra, and A. Amalfitano. 1999. Persistence of an [E1-, polymerase-] adenovirus vector despite transduction of a neoantigen into immune-competent mice. *Hum Gene Ther* 10:355-364.

138. Everett, R.S., B.L. Hodges, E.Y. Ding, F. Xu, D. Serra, and A. Amalfitano. 2003. Liver toxicities typically induced by first-generation adenoviral vectors can be reduced by use of E1, E2b-deleted adenoviral vectors. *Hum Gene Ther* 14:1715-1726.

139. Zinn, K.R., A.J. Szalai, A. Stargel, V. Krasnykh, and T.R. Chaudhuri. 2004. Bioluminescence imaging reveals a significant role for complement in liver transduction following intravenous delivery of adenovirus. *Gene Ther* 11:1482-1486.

140. Hawlisch, H., and J. Kohl. 2006. Complement and Toll-like receptors: key regulators of adaptive immune responses. *Molecular immunology* 43:13-21.

141. Carroll, M. 1999. Role of complement receptors CD21/CD35 in B lymphocyte activation and survival. *Current topics in microbiology and immunology* 246:63-68; discussion 69.

142. Sato, S., A.S. Miller, M.C. Howard, and T.F. Tedder. 1997. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol* 159:3278-3287.

143. Miwa, T., and W.C. Song. 2001. Membrane complement regulatory proteins: insight from animal studies and relevance to human diseases. *International immunopharmacology* 1:445-459.

144. Miwa, T., L. Zhou, R. Tudoran, J.D. Lambris, M.P. Madaio, M. Nangaku, H. Molina, and W.C. Song. 2007. DAF/Crry double deficiency in mice exacerbates nephrotoxic serum-induced proteinuria despite markedly reduced systemic complement activity. *Molecular immunology* 44:139-146.

145. Turnberg, D., and M. Botto. 2003. The regulation of the complement system: insights from genetically-engineered mice. *Molecular immunology* 40:145-153.

146. Tian, J., Z. Xu, J.S. Smith, S.E. Hofherr, M.A. Barry, and A.P. Byrnes. 2009. Adenovirus activates complement by distinctly different mechanisms in vitro and in vivo: indirect complement activation by virions in vivo. *J Virol*

147. Croyle, M.A., N. Chirmule, Y. Zhang, and J.M. Wilson. 2001. "Stealth" adenoviruses blunt cell-mediated and humoral immune responses against the virus and allow for significant gene expression upon readministration in the lung. *J Virol* 75:4792-4801.

148. Ricklin, D., and J.D. Lambris. 2008. Compstatin: a complement inhibitor on its way to clinical application. *Advances in experimental medicine and biology* 632:273-292.

149. Green, T.D., B.R. Newton, P.A. Rota, Y. Xu, H.L. Robinson, and T.M. Ross. 2001. C3d enhancement of neutralizing antibodies to measles hemagglutinin. *Vaccine* 20:242-248.

150. Ross, T.M., Y. Xu, R.A. Bright, and H.L. Robinson. 2000. C3d enhancement of antibodies to hemagglutinin accelerates protection against influenza virus challenge. *Nature immunology* 1:127-131.

151. Draper, S.J., A.C. Moore, A.L. Goodman, C.A. Long, A.A. Holder, S.C. Gilbert, F. Hill, and A.V. Hill. 2008. Effective induction of high-titer antibodies by viral vector vaccines. *Nat Med* 14:819-821.

152. Ogun, S.A., L. Dumon-Seignovert, J.B. Marchand, A.A. Holder, and F. Hill. 2008. The oligomerization domain of C4-binding protein (C4bp) acts as an adjuvant, and the fusion protein comprised of the 19-kilodalton merozoite surface protein 1 fused with the murine C4bp domain protects mice against malaria. *Infect Immun* 76:3817-3823.

153. Ricklin, D., and J.D. Lambris. 2007. Complement-targeted therapeutics. *Nat Biotechnol* 25:1265-1275.

154. Muruve, D.A. 2004. The innate immune response to adenovirus vectors. *Hum Gene Ther* 15:1157-1166.

155. Sahu, A., B.K. Kay, and J.D. Lambris. 1996. Inhibition of human complement by a C3-binding peptide isolated from a phage-displayed random peptide library. *J Immunol* 157:884-891.

156. Mallik, B., M. Katragadda, L.A. Spruce, C. Carafides, C.G. Tsokos, D. Morikis, and J.D. Lambris. 2005. Design and NMR characterization of active analogues of compstatin containing non-natural amino acids. *Journal of medicinal chemistry* 48:274-286.

157. Sahu, A., D. Morikis, and J.D. Lambris. 2003. Compstatin, a peptide inhibitor of complement, exhibits species-specific binding to complement component C3. *Molecular immunology* 39:557-566.

158. Yeatman, M., C.W. Daggett, C.L. Lau, G.W. Byrne, J.S. Logan, J.L. Platt, and R.D. Davis. 1999. Human complement regulatory proteins protect swine lungs from xenogeneic injury. *Ann Thorac Surg* 67:769-775.

159. Maelandsmo, G.M., P.J. Ross, M. Pavliv, R.A. Meulenbroek, C. Evelegh, D.A. Muruve, F.L. Graham, and R.J. Parks. 2005. Use of a murine secreted alkaline phosphatase as a non-immunogenic reporter gene in mice. *J Gene Med* 7:307-315.

160. Van Linthout, S., M. Lusky, D. Collen, and B. De Geest. 2002. Persistent hepatic expression of human apo A-I after transfer with a helper-virus independent adenoviral vector. *Gene Ther* 9:1520-1528.

161. Mathis, J.M., M.A. Stoff-Khalili, and D.T. Curiel. 2005. Oncolytic adenoviruses - selective retargeting to tumor cells. *Oncogene* 24:7775-7791.

162. Stone, D., Y. Liu, Z.Y. Li, S. Tuve, R. Strauss, and A. Lieber. 2007. Comparison of adenoviruses from species B, C, e, and f after intravenous delivery. *Mol Ther* 15:2146-2153.

163. Hartman, Z.C., D.M. Appledorn, D. Serra, O. Glass, T.B. Mendelson, T.M. Clay, and A. Amalfitano. 2008. Replication-attenuated Human Adenoviral Type 4 vectors elicit capsid dependent enhanced innate immune responses that are partially dependent upon interactions with the complement system. *Virology* 374:453-467.

164. Kuzmin, A.I., M.J. Finegold, and R.C. Eisensmith. 1997. Macrophage depletion increases the safety, efficacy and persistence of adenovirus-mediated gene transfer in vivo. *Gene Ther* 4:309-316.

165. Kay, M.A., L. Meuse, A.M. Gown, P. Linsley, D. Hollenbaugh, A. Aruffo, H.D. Ochs, and C.B. Wilson. 1997. Transient immunomodulation with anti-CD40 ligand antibody and CTLA4Ig enhances persistence and secondary

adenovirus-mediated gene transfer into mouse liver. *Proc Natl Acad Sci U S A* 94:4686-4691.

166. Kuriyama, S., K. Tominaga, A. Mitoro, H. Tsujinoue, T. Nakatani, M. Yamazaki, S. Nagao, Y. Toyokawa, S. Okamoto, and H. Fukui. 2000. Immunomodulation with FK506 around the time of intravenous re-administration of an adenoviral vector facilitates gene transfer into primed rat liver. *International journal of cancer* 85:839-844.

167. Song, M.Y., S.K. Park, C.S. Kim, T.H. Yoo, B. Kim, M.S. Kim, Y.S. Kim, W.J. Kwag, B.K. Lee, and K. Baek. 2008. Characterization of a novel anti-human TNF-alpha murine monoclonal antibody with high binding affinity and neutralizing activity. *Experimental & molecular medicine* 40:35-42.

168. Ho, P.P., P. Fontoura, M. Platten, R.A. Sobel, J.J. DeVoss, L.Y. Lee, B.A. Kidd, B.H. Tomooka, J. Capers, A. Agrawal, R. Gupta, J. Zernik, M.K. Yee, B.J. Lee, H. Garren, W.H. Robinson, and L. Steinman. 2005. A suppressive oligodeoxynucleotide enhances the efficacy of myelin cocktail/IL-4-tolerizing DNA vaccination and treats autoimmune disease. *J Immunol* 175:6226-6234.

169. Ren, L., J.B. Blanchette, L.R. White, S.A. Clark, D.J. Heffner, L.A. Tibbles, and D.A. Muruve. 2005. Soluble fibronectin induces chemokine gene expression in renal tubular epithelial cells. *Kidney international* 68:2111-2120.

170. Brunetti-Pierri, N., G.E. Stapleton, D.J. Palmer, Y. Zuo, V.P. Mane, M.J. Finegold, A.L. Beaudet, M.M. Leland, C.E. Mullins, and P. Ng. 2007. Pseudo-hydrodynamic delivery of helper-dependent adenoviral vectors into non-human primates for liver-directed gene therapy. *Mol Ther* 15:732-740.

171. Shayakhmetov, D.M., Z.Y. Li, S. Ni, and A. Lieber. 2004. Analysis of adenovirus sequestration in the liver, transduction of hepatic cells, and innate toxicity after injection of fiber-modified vectors. *J Virol* 78:5368-5381.

172. Shayakhmetov, D.M., A.M. Eberly, Z.Y. Li, and A. Lieber. 2005. Deletion of penton RGD motifs affects the efficiency of both the internalization and the endosome escape of viral particles containing adenovirus serotype 5 or 35 fiber knobs. *J Virol* 79:1053-1061.

173. Barnes, P.J. 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* 94:557-572.

174. Huang, H.W., W. Bi, G.N. Jenkins, and J.L. Alcorn. 2008. Glucocorticoid regulation of human pulmonary surfactant protein-B mRNA stability involves the 3'-untranslated region. *American journal of respiratory cell and molecular biology* 38:473-482.

175. Schnell, M.A., Y. Zhang, J. Tazelaar, G.P. Gao, Q.C. Yu, R. Qian, S.J. Chen, A.N. Varnavski, C. LeClair, S.E. Raper, and J.M. Wilson. 2001. Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol Ther* 3:708-722.

176. Miller, T.A., and F.W. Schaefer, 3rd. 2007. Changes in mouse circulating leukocyte numbers in C57BL/6 mice immunosuppressed with dexamethasone for Cryptosporidium parvum oocyst production. *Veterinary parasitology* 149:147-157.

177. Jeklova, E., L. Leva, Z. Jaglic, and M. Faldyna. 2008. Dexamethasone-induced immunosuppression: a rabbit model. *Veterinary immunology and immunopathology* 122:231-240.

178. Anderson, B.H., D.L. Watson, and I.G. Colditz. 1999. The effect of dexamethasone on some immunological parameters in cattle. *Veterinary research communications* 23:399-413.

179. Lieber, A., C.Y. He, L. Meuse, D. Schowalter, I. Kirillova, B. Winther, and M.A. Kay. 1997. The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J Virol* 71:8798-8807.

180. Stone, D., Y. Liu, D. Shayakhmetov, Z.Y. Li, S. Ni, and A. Lieber. 2007. Adenovirus-platelet interaction in blood causes virus sequestration to the reticuloendothelial system of the liver. *J Virol* 81:4866-4871.

181. Franchimont, D., J. Galon, M. Gadina, R. Visconti, Y. Zhou, M. Aringer, D.M. Frucht, G.P. Chrousos, and J.J. O'Shea. 2000. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *J Immunol* 164:1768-1774.

182. Ramirez, F., D.J. Fowell, M. Puklavec, S. Simmonds, and D. Mason. 1996. Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. *J Immunol* 156:2406-2412.

183. Olive, M., L. Eisenlohr, N. Flomenberg, S. Hsu, and P. Flomenberg. 2002. The adenovirus capsid protein hexon contains a highly conserved human CD4+ T-cell epitope. *Hum Gene Ther* 13:1167-1178.

184. Sumida, S.M., D.M. Truitt, A.A. Lemckert, R. Vogels, J.H. Custers, M.M. Addo, S. Lockman, T. Peter, F.W. Peyerl, M.G. Kishko, S.S. Jackson, D.A. Gorgone, M.A. Lifton, M. Essex, B.D. Walker, J. Goudsmit, M.J. Havenga, and D.H. Barouch. 2005. Neutralizing antibodies to adenovirus serotype 5 vaccine vectors are directed primarily against the adenovirus hexon protein. *J Immunol* 174:7179-7185.

185. Hodges, B.L., H.K. Evans, R.S. Everett, E.Y. Ding, D. Serra, and A. Amalfitano. 2001. Adenovirus vectors with the 100K gene deleted and their potential for multiple gene therapy applications. *J Virol* 75:5913-5920.

186. Hodges, B.L., D. Serra, H. Hu, C.A. Begy, J.S. Chamberlain, and A. Amalfitano. 2000. Multiply deleted [E1, polymerase-, and pTP-] adenovirus vector persists despite deletion of the preterminal protein. *J Gene Med* 2:250-259.

187. Dietzman, R.H., R.A. Ersek, J.M. Bloch, and R.C. Lillehei. 1969. High-output, low-resistance gram-negative septic shock in man. *Angiology* 20:691-700.

188. Lefering, R., and E.A. Neugebauer. 1995. Steroid controversy in sepsis and septic shock: a meta-analysis. *Critical care medicine* 23:1294-1303.

189. Oaks, W.W., and H.E. Cohen. 1967. Endotoxin shock in the geriatric patient. *Geriatrics* 22:120-130.

190. Wang, X.Q., X. Zhou, Y. Zhou, L. Rong, L. Gao, and W. Xu. 2008. Low-dose dexamethasone alleviates lipopolysaccharide-induced acute lung injury in rats and upregulates pulmonary glucocorticoid receptors. *Respirology (Carlton, Vic*

191. Zhou, Y., E.A. Ling, and S.T. Dheen. 2007. Dexamethasone suppresses monocyte chemoattractant protein-1 production via mitogen activated protein kinase phosphatase-1 dependent inhibition of Jun N-terminal kinase and p38 mitogen-activated protein kinase in activated rat microglia. *Journal of neurochemistry* 102:667-678.

192. Lee, C.W., J.H. Chuang, P.W. Wang, N.K. Chang, H.C. Wang, C.C. Huang, M.M. Tiao, and S.K. Lo. 2006. Effect of glucocorticoid pretreatment on oxidative liver injury and survival in jaundiced rats with endotoxin cholangitis. *World journal of surgery* 30:2217-2226.

193. Hafner, D., and P.G. Germann. 1999. Dexamethasone enhances the activity of rSP-C surfactant but not of exosurf in a rat model of the acute lung injury. *Journal of pharmacological and toxicological methods* 42:39-48.

194. Schiedner, G., S. Hertel, M. Johnston, V. Dries, N. van Rooijen, and S. Kochanek. 2003. Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther* 7:35-43.

195. Zsengeller, Z., K. Otake, S.A. Hossain, P.Y. Berclaz, and B.C. Trapnell. 2000. Internalization of adenovirus by alveolar macrophages initiates early proinflammatory signaling during acute respiratory tract infection. *J Virol* 74:9655-9667.

196. Tao, N., G.P. Gao, M. Parr, J. Johnston, T. Baradet, J.M. Wilson, J. Barsoum, and S.E. Fawell. 2001. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* 3:28-35.

197. Everett, R.S., H.K. Evans, B.L. Hodges, E.Y. Ding, D.M. Serra, and A. Amalfitano. 2004. Strain-specific rate of shutdown of CMV enhancer activity in murine liver confirmed by use of persistent [E1(-), E2b(-)] adenoviral vectors. *Virology* 325:96-105.

198. Cross, S.L., N.F. Halden, M.J. Lenardo, and W.J. Leonard. 1989. Functionally distinct NF-kappa B binding sites in the immunoglobulin kappa and IL-2 receptor alpha chain genes. *Science* 244:466-469.

199. Yang, T.C., J.B. Millar, N. Grinshtein, J. Bassett, J. Finn, and J.L. Bramson. 2007. T-cell immunity generated by recombinant adenovirus vaccines. *Expert review of vaccines* 6:347-356.

200. He, T.C., S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 95:2509-2514.

201. Fontana, L., M. Nuzzo, L. Urbanelli, and P. Monaci. 2003. General strategy for broadening adenovirus tropism. *J Virol* 77:11094-11104.

202. Ng, P., and F.L. Graham. 2002. Construction of first-generation adenoviral vectors. *Methods in molecular medicine* 69:389-414.

203. Sargent, K.L., P. Ng, C. Evelegh, F.L. Graham, and R.J. Parks. 2004. Development of a size-restricted pIX-deleted helper virus for amplification of helper-dependent adenovirus vectors. *Gene Ther* 11:504-511.

204. Colby, W.W., and T. Shenk. 1981. Adenovirus type 5 virions can be assembled in vivo in the absence of detectable polypeptide IX. *J Virol* 39:977-980.

205. Gommerman, J.L., D.Y. Oh, X. Zhou, T.F. Tedder, M. Maurer, S.J. Galli, and M.C. Carroll. 2000. A role for CD21/CD35 and CD19 in responses to acute septic peritonitis: a potential mechanism for mast cell activation. *J Immunol* 165:6915-6921.

206. Rajagopal, K., E.J. Whalen, J.D. Violin, J.A. Stiber, P.B. Rosenberg, R.T. Premont, T.M. Coffman, H.A. Rockman, and R.J. Lefkowitz. 2006. Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc Natl Acad Sci U S A* 103:16284-16289.

207. Higginbotham, J.N., P. Seth, R.M. Blaese, and W.J. Ramsey. 2002. The release of inflammatory cytokines from human peripheral blood mononuclear cells in vitro following exposure to adenovirus variants and capsid. *Hum Gene Ther* 13:129-141.

208. Hofstetter, J.R., A. Zhang, A.R. Mayeda, T. Guscar, J.I. Nurnberger, Jr., and D.K. Lahiri. 1997. Genomic DNA from mice: a comparison of recovery methods and tissue sources. *Biochemical and molecular medicine* 62:197-202.

209. Hensley, S.E., A.S. Cun, W. Giles-Davis, Y. Li, Z. Xiang, M.O. Lasaro, B.R. Williams, R.H. Silverman, and H.C. Ertl. 2007. Type I interferon inhibits antibody responses induced by a chimpanzee adenovirus vector. *Mol Ther* 15:393-403.

210. Loniewski, K.J., S. Patial, and N. Parameswaran. 2007. Sensitivity of TLR4- and -7-induced NF kappa B1 p105-TPL2-ERK pathway to TNF-receptor-associated-factor-6 revealed by RNAi in mouse macrophages. *Molecular immunology* 44:3715-3723.

211. Jia, Q., H.R. Zhou, Y. Shi, and J.J. Pestka. 2006. Docosahexaenoic acid consumption inhibits deoxynivalenol-induced CREB/ATF1 activation and IL-6 gene transcription in mouse macrophages. *The Journal of nutrition* 136:366-372.

212. Loniewski, K., Y. Shi, J. Pestka, and N. Parameswaran. 2008. Toll-like receptors differentially regulate GPCR kinases and arrestins in primary macrophages. *Molecular immunology* 45:2312-2322.

213. Farina, S.F., G.P. Gao, Z.Q. Xiang, J.J. Rux, R.M. Burnett, M.R. Alvira, J. Marsh, H.C. Ertl, and J.M. Wilson. 2001. Replication-defective vector based on a chimpanzee adenovirus. *J Virol* 75:11603-11613.

214. Lemckert, A.A., S.M. Sumida, L. Holterman, R. Vogels, D.M. Truitt, D.M. Lynch, A. Nanda, B.A. Ewald, D.A. Gorgone, M.A. Lifton, J. Goudsmit, M.J. Havenga, and D.H. Barouch. 2005. Immunogenicity of heterologous

prime-boost regimens involving recombinant adenovirus serotype 11 (Ad11) and Ad35 vaccine vectors in the presence of anti-ad5 immunity. *J Virol* 79:9694-9701.

215. Liu, J., K.L. O'Brien, D.M. Lynch, N.L. Simmons, A. La Porte, A.M. Riggs, P. Abbink, R.T. Coffey, L.E. Grandpre, M.S. Seaman, G. Landucci, D.N. Forthal, D.C. Montefiori, A. Carville, K.G. Mansfield, M.J. Havenga, M.G. Pau, J. Goudsmit, and D.H. Barouch. 2009. Immune control of an SIV challenge by a T-cell-based vaccine in rhesus monkeys. *Nature* 457:87-91.

216. Gao, H., Y. Sun, Y. Wu, B. Luan, Y. Wang, B. Qu, and G. Pei. 2004. Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. *Molecular cell* 14:303-317.

217. Di Paolo, N.C., E.A. Miao, Y. Iwakura, K. Murali-Krishna, A. Aderem, R.A. Flavell, T. Papayannopoulou, and D.M. Shayakhmetov. 2009. Virus binding to a plasma membrane receptor triggers interleukin-1 alpha-mediated proinflammatory macrophage response in vivo. *Immunity* 31:110-121.

218. Smith, J.G., and G.R. Nemerow. 2008. Mechanism of adenovirus neutralization by Human alpha-defensins. *Cell Host Microbe* 3:11-19.

219. Moser, B., and K. Willimann. 2004. Chemokines: role in inflammation and immune surveillance. *Annals of the rheumatic diseases* 63 Suppl 2:ii84-ii89.