THE MALARIA VACCINE DILEMMA:
NOVEL APPROCHES TO ADENOVIRAL VECTORED MALARIA VACCINES

By

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Despite the discovery more than 30 years ago that artificial or “unnatural” protection against malaria is achievable, a practical protective malaria vaccine has yet to be realized. Recent developments in sub-unit malaria vaccine platforms are bridging the gap between high levels of protection and feasibility. However, the current leading sub-unit vaccine, RTS,S, has only demonstrated the ability to induce protection from malaria infection in up 56% of RTS,S vaccinees. Though encouraging, these results may fall short of protection levels generally considered to be required to achieve eradication of malaria. The uses of viral vectored vaccine platforms have recently been pursued to further improve the efficacy of malaria vaccines. Adenovirus serotype 5 (Ad5) based vaccine platforms have demonstrated potent anti-malaria immune responses, although it is clear more potent Ad5-induced immune responses are required if Ad5-based malaria vaccines are to confer protection. Through explication of Ad interactions with the innate immune system we have uncovered multiple targets that could be exploited to improve the immunogenicity of Ad-based malaria vaccines. We have also attempted to overcome an oft cited difficulty with use of Ad5 in malaria vaccine platforms, namely the high seroprevalence of Ad5. We sought to improve Ad-induced immunogenicity in Ad5 immune patients by the use of an alternative serotype of Ad (Adenovirus serotype 4 (Ad4)) in heterologous prime boost regimens with Ad5. Instead, we uncovered a previously unknown cross-reactivity between these two Ad serotypes that resulted in severely ablated immunogenicity. We then tested the utility of various immunomodulators expressed from Ad

vectors to stimulate innate immune system responses and ultimately improve adaptive responses to Ad-expressed malaria antigens. We found a promising immunomodulator in a SLAM receptor adaptor protein (EAT-2). Co-injection of an Ad5 vaccine expressing EAT-2 with an Ad5 vaccine expressing a malaria antigen (Circumsporozoite protein (CSP)) improved CSP specific CD8+ T cell responses and *in vivo* cytotoxicity. Currently, we are testing the ability of this immunomodulator to improve protection against a mouse malaria challenge model. Our research has unearthed multiple valuable advancements in Ad-based vaccine technology that can be utilized in malaria and non-malaria vaccine platforms alike.

I would like to dedicate this dissertation to beloved parents Marcia and Jerry Schuldt. It is because of them that I am where I am today. With my fulfillment of the requirements to earn a Doctorate of Philosophy in Genetics my parents will have nurtured the first and second PhDs in my extended family. It is by no coincidence that my older brother and I have both achieved this goal. Rather, it is due to the excellent examples of hard work and determination that my parents have set for us. I am proud to share this honor with my parents and the rest of my family for it is only thanks to them that I am here.

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Key Symbols or Abbreviations

µL	microliters
µM	micromoles
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AAV	Adeno-associated virus
ABSL-2	Animal Biosafety level 2
Ad	Adenovirus
Ad26	Adenovirus Serotype 26
Ad26CSP	Adenovirus Serotype 26 expressing circumsporozoite protein
Ad35	Adenovirus Serotype 35
Ad35CSP	Adenovirus Serotype 35 expressing circumsporozoite protein
Ad35PyCS	Adenovirus Serotype 35 expressing *Plasmodium yoelli* Circumsporozoite protein
Ad4	Adenovirus serotype 4
Ad4-CSP	Adenovirus serotype 4 expressing circumsporozoite protein
Ad5	Adenovirus serotype 5
Ad5-CSP	Adenovirus serotype 5 expressing circumsporozoite protein
Ad5-GFP-IX-dDAF_REO	Adenovirus serotype 5 with protein IX capsid fusion to human decay accelerating factor in the retro-orientation expressing GFP
Ad5-IX-GFP	Adenovirus serotype 5 with protein IX capsid fusion to green fluorescent protein
Ad5*Pf*CS	Adenovirus serotype 5 expressing *Plasmodium falciparum* circumsporozoite protein
Ad5*Py*CS	Adenovirus serotype 5 expressing *Plasmodium yoelli* circumsporozoite protein

ADAR	Adenosine deaminase, ribonucleic acid specific
AdC9	Chimpanzee adenovirus serotype 9
Ad-GFP	Adenovirus expressing green fluorescent protein
Ad-GFP/rEA	Adenovirus expressing green fluorescent protein and recombinant *eimeria tenella* antigen
AMA-1	Apical membrane antigen 1
ANOVA	Analysis of variance
AP	Alternative pathway
AP-C3	Acute respiratory disease
AS01B	Adjuvant series 01B
AS02A	Adjuvant series 02A
BALB/cJ	Albino laboratory mouse strain
CAR	Coxsackievirue and adenovirus receptor
C1	Complement component 1
C1q-KO	Complement component 1q knock out mouse
C1qrs	Complement complex made up of complement components 1, q, r, and s
C1r	Complement component 1r
C1s	Complement component C1s
C2	Complement component 2
C3	Complement component 3
C3-C5	Complement component 3/complement component 5 convertase
C3d	Complement component 3d
C4	Complement component 4

C4-KO	Complement component 4 knock out mouse
C57BL/6	Black laboratory mouse strain
C6	Complement component 6
C7	Complement component 7
C8	Complement component 8
C9	Complement component 9
CAR	Coxsackie adenovirus receptor
CCL7	Chemokine (C-C motif) ligand 7
CCPR	Complement control protein repeats
CD107a	Cluster of differentiation 107a also known as Lysosomal-associated membrane protein
CD127	Cluster of differentiation 127 also known as Interleukin-7 receptor subunit alpha
CD14	Cluster of differentiation 14
CD19	B lymphocyte antigen CD19 Cluster of differentiation 19
CD2	Cluster of differentiation 2
CD22	Cluster of differentiation 22
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD46	Cluster of differentiation also known as membrane cofactor protein
CD62L	Cluster of differentiation 62 ligand also known as L-selectin
CD69	Cluster of differentiation 69
CD8	Cluster of differentiation 8
CFSE	Carboxyfluorescein diacetate

ChAd63	Chimpanzee adenovirus serotype 63
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CP	Classical pathway of complement
CP-C3	Classical pathway of complement component 3 convertase
CR	Complement receptor
CR1	Complement receptor 1
CR1/2	Complement receptor 1/2
CR2	Complement receptor 2
CRACC	CD2-like receptor activating cytotoxic cells
Crry	Rodent specific complement receptor 1 related gene/protein-y
CS	Circumsporozoite
CSP	Circumsporozoite protein
CTL	Cytotoxic T lymphocyte
CXCL9	Chemokine (C-X-C motif) ligand 9
DAF	Decay accelerating factor
DMEM	Dulbecco/vogt modified eagle's minimal essential medium
DNA	Deoxyribonucleic acid
Dpi	Days post injection
E1	E1 region of Adenovirus genome encodes proteins trans-acting transcription factor
E2b	E2b region of Adenovirus genome encodes Adenovirus polymerase
E3	E3 region of Adenovirus genome encodes multiple immune defense proteins

E4	E4 region of Adenovirus genome encodes genes for lytic growth
EAT-2	Ewing's sarcoma related transcript 2
ELISA	Enzyme-linked immunosorbant Assay
ELISpot	Enzyme-linked immunosorbant spot assay
E-selectin	Also known as cluster of differentiation 62 (CD62)
FB	Factor B
FB-KO	Factor B knock-out
FcγRIIB	Fcγ receptor IIB also known as cluster of differentiation 32 (CD32)
Gag	Human immunodeficiency virus viral core protein
GATA-3	Trans-acting T cell specific transcription factor that recognizes the deoxyribonucleic acid sequence GATA
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GSK	Glaxo-Smith-Kline
hAd	Human Adenovirus
hAd5	Human Adenovirus serotype 5
HBsAg	Hepatitis B surface antigen
HEK 293	Human embryonic kidney 293 cells
HIV	Human immunodeficiency virus
hpi	Hours post injection
ICAM	Intracellular cell adhesion protein
ICS	Intracellular staining
IFNα	Interferon alpha

IFNβ	Interferon beta
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG1	Immunoglobulin G1
IgG2a	Immunoglobulin G2a
IgG2b	Immunoglobulin G2b
IgG2c	Immunoglobulin G2c
IgG3	Immunoglobulin G3
IgM	Immunoglobulin M
IL-12	Interleukin 12
Il-12 (p40)	Interleukin 12 subunit protein 40
IM	Intramuscular
IRF-1	Interferon regulatory protein 1
IRF-7	Interferon regulatory protein 7
IRF-8	Interferon regulatory protein 8
ITSM	immune-receptor tyrosine-based switch motif
IV	Intravenous
JAK-1	Janus kinase 1
JAK-3	Janus kinase 3
kb	kilobase
KC	Also known as chemokine (C-X-C motif) ligand 1
kD	Kilodalton
KO	Knock out

LAG-3	Lymphocyte-activation gene 3
MASP	Mannose-binding lectin-associated serine protease
MBL	Mannose-binding lectin
MCP	Monocyte chemotactic protein
MCP-1	Monocyte chemotactic protein 1
mCR	Murine complement receptor
mCR1/2	Murine complement receptor 1/2
ME.TRAP	multi-epitope thrombospondin related adhesive protein
MIP-1β	Macrophage inflammatory protein -1β
mL	milliliter
MPL	Monophosphoryl lipid A
MSP-1	Merozoite specific protein 1
MVA	Modified vaccinia Ankara
MyD88	Myeloid differentiation primary response gene 88
NALP	A Nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-κB-RelA	Nuclear factor kappa-light-chain-enhancer of activated B cells subunit Rel A also known as protein 65 (p65)
ng	Nanograms
NIH	National institute of health
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain-containing protein (NOD)-like receptors
NOD-1	Nucleotide-binding oligomerization domain-containing protein 1

NOD-2	Nucleotide-binding oligomerization domain-containing protein 2
O.D.	Optical density
OAS1a	2’-5’ oligoadenylate synthetase 1 a
ORF	Open reading frame
P. berhei	*Plasmodium berghei*
P. falciparum	*Plasmodium falciparum*
P. yoelli	*Plasmodium yoelli*
P. vivax	*Plasmodium vivax*
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PD-1	Programmed death 1
Pfs25	*Plasmodium falciparum* surface protein 25
pg	picagrams
PHS	Public health services
pIX	Adenovirus surface protein IX
polyA	Polyadenylation
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
QS21	*Quillaja saponaria* extract 21
rAd	Recombinant adenovirus
rAd35	Recombinant adenovirus serotype 35
rAd5	Recombinant adenovirus serotype 5
RANTES	Regulated upon activation, normal T cell expressed, and secreted

rEA	*Eimeria tenella* antigen
RIG	Retinoic acid-inducible gene protein
RLR	Retinoic acid-inducible gene protein (RIG)-like receptor
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTS,S	Hepatitis B surface antigen-circumsporozoite protein fusion based malaria vaccine developed by Glaxo-Smith-Kline
RTS,S/AS01B	Hepatitis B surface antigen-circumsporozoite protein fusion based malaria vaccine developed by Glaxo-Smith-Kline adjuvanted with adjuvant series 01B
SFC	Spot forming cells
SLAM	Signaling lymphocytic activation molecule
SOCS-1	Suppressor of cytokine signaling1
SOCS-3	Suppressor of cytokine signaling 3
TBK-1	TANK-binding kinase 1
Tcm	Central memory T cells
Tem	Effector memory T cells
Tet	tetramer
Th1	T helper cell 1
Th2	T helper cell 2
TK	Thymidine kinase
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR6	Toll-like receptor 6

TLR9	Toll-like receptor 9
TMB	3,3’,5,5’ tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
TRAF2-bp	Tumor necrosis factor receptor associated factor 2-bp
TRAP	Tumor necrosis factor receptor associated protein
TRIM30	Tripartite-motif protein 30
TSR	Thrombospondin-like type I repeat region
VCAM	Vascular cell adhesion molecule
vp	Viral particles
WT	Wildtype

Chapter 1:

Introduction: Malaria Vaccines: Focus on Adenovirus based vectors

1.1 Pathogenesis:

Five protozoan parasites are known to cause malaria in humans, *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium vivax*, and *Plasmodium knowlesi*, with *P. falciparum* being the most deadly accounting for 80% of all malaria cases, and 90% of all malaria deaths.[1] The parasites are transferred from human to human by the bite of the *Anopholes* mosquito. As a consequence of the intermediate host, the parasites have both a complex mosquito, and human, life cycle. The human stage begins when an infected mosquito takes a blood meal from a human. The parasites traverse the mosquito's proboscis from the salivary gland and enter the humans in the form of sporozoites. The sporozoites rapidly travel to the liver through the blood stream where they infect hepatocytes in what is known as the exo-erythrocytic phase. Here the parasites multiply into thousands of merozoites. Merozoites then leave the liver and enter the blood stream where they infect red blood cells, this begins the erythrocytic phase. Once inside the erythrocyte the merozoite transforms into a trophozoite, this stage is sometimes referred to as the feeding stage. The trophozoite nucleus then divides asexually to produce a multi-nucleated schizont. The schizont divides into many mononucleated merozoites which are then released back into the blood stream to infect more erythrocytes. Occasionally, when merozoites enter erythrocytes they transform into male or female gametocytes and do not rupture the erythrocyte. Gametocyte infected red blood cells can then be picked up by another mosquito taking a blood meal beginning the mosquito stage of the lifecycle.

In the mosquito gut the male and female gametocytes merge creating a diploid zygote that forms an oocyst in the intestinal wall of the mosquito. Inside the oocyst multiple cell divisions take place resulting in the production of many sporozoites. Sporozoites then travel to

the salivary gland of the mosquito reinitiating the human stage of the life cycle when the mosquito bites another human.

1.2 Epidemiology:

Recently the numbers of malaria cases and malaria deaths have decreased worldwide in large part due to use of pesticides and bed nets that together kill or prevent mosquitoes from biting susceptible humans. In 2009, 225 million people were infected with malaria, down from 244 million in 2005.[2] While this is an encouraging trend, there were still 781,000 malaria deaths worldwide, indicating new preventions must be developed and employed if malaria is to be eradicated. Malaria has been considered "eliminated" in the United States of America since 1970 and there were no locally acquired cases of *P.falciparum* reported in the European region in 2009.[2] However, malaria still remains a prominent threat in areas of South America, Sub-Saharan Africa and Southeast Asia placing roughly one third of the world's population at risk of contracting malaria.[2]

1.3 Prevention:

Malaria prevention focuses on three main targets; vector control, prophylactics, and vaccination. The use of long lasting insecticide treated bed nets and indoor residual spraying have proven to be successful and relatively economical strategies to reduce the risk of malaria transmission, provided high coverage is achieved and sustained.[3,4] However, mosquito pesticide resistance and behavioral adaptations (like biting immediately after sunset and before sunrise) that allow the mosquito to circumvent these preventions have already been detected.[5,6]

Chemical prophylactics are used to prevent parasite replication in the human host. They are often used successfully, however, no prophylactic is 100% effective and most prophylactics

are strain specific. The current preferred chemoprophylactics include chloroquine, proguanil, doxycycline, and mefloquine.[7,8] Unfortunately, uncomfortable gastrointestinal, hematological, and/or neurological side effects often accompany utilization of these medications.[7,9] In addition the prophylactics must be taken regularly to be effective, that and the uncomfortable side effects can make it difficult for some people to remain compliant on the treatment. Overuse of the prophylactic chloroquine in eastern Africa has lead to chloroquine resistant *P. falciparum* parasites in those regions. As a result, close monitoring of parasite resistance is required in order to rapidly change the prophylactic in use should resistance occur.[10]

1.4 Natural Infection and hopes for a malaria vaccine:

Sadly, the majority of malaria related deaths occur in children, since many adults have acquired immunity to malaria "naturally" over time as a result of surviving repeated malaria infections.[11] While it appears that natural immunity to human malaria is largely IgG antibody mediated, it has proven difficult to pinpoint the specific antigens these antibodies target.[11,12,13] Antibody responses to malaria antigens are generally short-lived, possibly because natural malaria infections hinder the development of B cell memory responses.[11,12,13,14,15] For example, *P. falciparum* infection can induce expression of a T cell inhibitory receptor called Programmed Death-1 (PD-1), leading to poor CD4+ T cell responses. Simultaneous blockade of both PD-1 ligand and Lymphocyte Activation Gene - 3 (LAG-3: a negative regulator of T cell function) together can allow for more rapid clearance of blood stage infections in mouse models, and has been recently targeted as a strategy to treat active malaria infection in humans.[16]

1.5 Previous examples of putative malaria vaccines:

Alternative malaria prevention methods attempt to proactively vaccinate individuals from malaria infection (i.e.: "unnatural" immunity). Potent humoral and/or CD8+ T cell responses against multiple malaria antigens have been identified to be partially responsible for protection from malaria infection. Based upon these findings, it has been postulated that pre-emptive induction of adaptive immune responses to malaria derived antigens may be of benefit in preventing the symptoms of subsequent malaria infection, if not protection from malaria infections in general.

We know that artificial or "unnatural" inductions of immunity to malaria are achievable. In 1975 mosquitoes infected with *P. vivax* or *P. falciparum* were irradiated (preventing live parasite transmission) and then used to bite a human volunteer, as a result the volunteer was protected from natural malaria infections for a short period of time.[17] Although the experiment was subsequently validated in larger groups of human volunteers, the approach was not practical as many hundreds of bites were required, and the resulting protection was no-less short lived.[18,19] Despite this disadvantage, the approach still remains one of the most protective malaria vaccine platforms to date, as protection rates approached over 90%.

Another non-irradiated mosquito bite based vaccine platform utilizes chloroquine to control parasite infection. In this platform *P. falciparum* infected mosquitoes are allowed to bite volunteers while chloroquine is administered to prevent blood stage infection by the live parasites. Chloroquine controlled infection has shown high rates of effector memory mediated protection upon parasite rechallenge that lasted for up to 2 years, a significant increase over the few months observed with use of irradiated mosquito based vaccine platforms.[20,21] Chloroquine controlled infection also decreased the amount of mosquito bites required for protection, from many hundreds of bites to only 10-15.[20] Notably, chloroquine controlled

infection also decreases the number of PD-1 expressing CD4+ T cells improving the CD4+ T cell exhaustion phenotype.[16] However, due to extensive use of chloroquine in East Africa, some strains of *P. falciparum* have developed resistance to the prophylactic.[10] Since non-chloroqine resistant strains must be used in this method, further research must be conducted to ensure that chloroqine controlled vaccination with non-chloroquine resistant parasites can protect against challenge with chloroquine resistant parasites.

The use of purified, radiation attenuated sporozoite's as a prophylactic malaria vaccine is another method attempted for use in humans. In this method sporozoites are harvested from the salivary glands of irradiated mosquitoes and injected with a needle rather than via bites from the irradiated mosquitoes. Analysis of immune correlates of protection performed after vaccinations with irradiated sporozoites suggest that CD8+ T cell responses against the liver stage of the parasite appear to be more important for achieving protection, relative to antibody responses.[22,23,24,25] Further supporting this correlation, studies where CD8+ T cells specific for liver stage malaria antigens were passively transferred into naive mice demonstrated protection from intravenous (IV) sporozoite challenge.[26]

Unfortunately the method requires higher doses of sporozoites and was demonstrated to be poorly immunogenic in human trials.[23,27] More recent animal studies confirmed that protection was improved by IV injection of irradiated sporozoites, and future human trials will be required to assess the immunogenicity of IV injected irradiated sporozoites.[23] The complexities of harvesting, storing, and transporting purified irradiated sporozoites is a concern as well, as sporozoites are very fragile outside of their mosquito host. Attempts to cryopreserve irradiated sporozoites demonstrated that they do not survive the freeze thaw process well.

Inoculations with cryopreserved irradiated sporozoites also required fourfold more sporozoites than fresh sporozoite preparations to achieve the same effectiveness in animal models.[23]

Use of live (non-irradiated) but genetically attenuated sporozoites to increase the immunogenicity and therefore decrease the number sporozoites required to achieve protection has also been recently described. In a mouse model of malaria, attenuated *P. yoelli* sporozoites that have been genetically engineered to arrest late in the liver stage were capable of stimulating broader and more potent CD8+ T cell responses to malaria antigens (including blood stage antigens) than what was observed using irradiated *P. yoelli* sporozoites as the vaccinating antigen.[28] Mice vaccinated with lower numbers of the genetically attenuated sporozoites displayed a wider range of antigen responses and were also protected against blood stage challenge as compared to purified radiation attenuated sporozoites injected IV.[28] Whether these results translate to *P. falciparum* and human malaria infections remains to be seen.

The use of attenuated sporozoites, whether purified and injected, or administered through mosquito bites, has shown promising results in the laboratory. However, the necessity for multiple bites from infected mosquitoes and the inability to mass produce and preserve purified sporozoites according to regulatory standards for human use, has prompted the development of alternative, subunit based vaccines targeting specific malaria parasite antigens.

1.6 RTS,S:

Circumsporozoite protein (CSP) is the most abundantly expressed protein during the sporozoite stage and is found both on the surface of sporozoites and in the plasma membrane and cytoplasm of infected hepatocytes during early liver infection.[29] CSP has been repeatedly shown to be an immunodominant protective antigen.[22,26,30] In fact, when transgenic mice were altered to express and therefore tolerate CSP, the absence of a CSP specific B and T cell

response dramatically decreased the ability of irradiated sporozoites to protect the transgenic mice from malaria challenge.[30] Conversely, mice vaccinated with irradiated sporozoites engineered to express CSP of a different malaria parasite species were still capable of stimulating protective immunity when challenged with parasites of the same species as the irradiates sporozoites, indicating that species specific B and T cell responses to CSP are not required for protection.[31,32] Despite these conflicting findings CSP remains the most commonly utilized malaria antigen in malaria vaccines and there is a wealth of evidence demonstrating that CSP, if not required, is protective on some level.

CSP is a 58 kD protein composed of a C-terminus containing the thrombospondin-like type I repeat region ((TSR) involved in liver sinusoid attachment), a central region of [NANP] repeats, and a N-terminal site that when in contact with the liver sinusoid is cleaved exposing the TSR.[33,34,35] The most successful malaria subunit vaccine candidate to date is composed of a novel fusion between the immunogenic hepatitis B surface antigen (HBsAg), and amino acids 207-395 of CSP from *P. falciparum* strain NF54, clone 3D7.[36] Developed by Glaxo-Smith-Kline (GSK), the fusion protein is thought to spontaneously form a pseudo virion structure that displays CSP on its surface.

Initial tests proved the fusion to be poorly immunogenic, so potent adjuvants were added to increase overall immunogenicity.[37,38,39,40,41] These adjuvants include AS02A (squalene-in-water emulsion containing monophosphoryl lipid A (MPL)) and AS01B (liposome preparation of MPL) and a plant extract known as QS21). Inclusion of these adjuvants with the HBsAg-CSP fusion protein (referred to as RTS,S) has achieved up to 50% protection from malaria infection in several human trials.[36,37,38,39,42,43] Most recently, a phase 3 study was conducted with RTS,S/AS01B on 6000 infants aged 5 to 17 months in 7 African countries, and

managed to achieve 56% protection against naturally occurring clinical malaria infections.[44] While encouraging, these results are well below that achieved by most vaccines currently used for other infectious diseases, higher protection rates are likely required to eradicate malaria from the endemic regions.

The efficacy of early forms of the fusion vaccine was attributed to induction of high CSP specific antibody titers.[45] Inclusion of AS01B maintained anti-CSP antibody titers while also significantly increasing T cell responses to CSP as well, a finding that positively correlated with improved protection of vaccinated volunteers from experimental malaria challenges via bites from *P. falciparum* infected mosquitoes.[46] The result suggests that further improvement of T cell responses to malaria antigens such as CSP is desirable of a putative malaria vaccine.

Vaccination regimens that utilize a priming vaccination with one type of vaccine platform followed by boosting vaccinations with a different (heterologous) vaccine platform, each of which includes the same antigenic target may provide increased T cell immune responses to the desired antigen relative to sole use of one vaccine platform (homologous prime-boosting). Heterologous prime-boost vaccinations combining the use of the RTS,S/AS01B platform with virus vector based malaria vaccine platforms that also express CSP induced more potent and longer lasting CSP specific CD8+ T cell responses in rhesus macaques, relative to use of either vaccine platform alone.[47] Such studies suggest that the use of virus based vaccine platforms may improve malaria antigen specific adaptive immune responses, and/or increase potency when used in heterologous prime-boost vaccination regimens in malaria targeted vaccine formulations, as summarized below.

1.7 Viral vectors as malaria vaccines:

Viral based vaccines provide a means by which to rapidly activate the host innate immune system (due to the presence of pathogen associated molecular patterns (PAMPs)) simultaneous with delivery malaria antigen expressing genes without the requirement of additional adjuvants *per se*. Viral vectors can also be used to overcome the manufacturing hurdles that accompany mosquito and/or sporozoite based vaccine formulations, as some viral vectors are relatively easy to produce to high titer. Many viral vectors have been utilized as potential malaria vaccines inclusive of alphavirus, flavivirus, morbillivirus, adeno-associated virus (AAV), modified vaccinia virus Ankara (MVA), and adenovirus (Ad).[48] MVA based vaccines targeting malaria antigen have been shown to induce both CD4+ and CD8+ T cells responses, and have even been easily administered via a microneedle array transdermal patch.[49] However, MVA based malaria vaccines have been more commonly successful as a boosting vaccination used in heterologous prime-boost vaccination combinations, especially when paired with Ad based malaria vaccines.[50]

1.8 Adenovirus based vaccines:

The Ad family of viruses have an icosahedral capsid that protects the non-enveloped linear double stranded DNA genome.[51] There are at least 52 human serotypes divided into subgroups A-F primarily based on lack of cross-neutralization by antisera. Of these serotypes, human adenovirus serotype 5 of subgroup C (Ad5) is the most studied and well understood. Wildtype Ad5 enters cells via interactions with the coxsackievirus and adenovirus receptor (CAR), as well secondary interactions with integrins. Ad5 has a 36 kb genome that is functionally divided into early and late genes based on temporal expression relative to the initial infection event. Early gene transcription is initiated by the E1 gene products which function in *trans* to augment expression of the other Ad encoded transcription units. Deletion of the E1

region of the Ad genome partially renders the virus replication incompetent and provides space to incorporate a gene encoding an antigen of interest for expression by the recombinant virus upon infection.[52,53] E1 deleted hAd5s ([E1-]Ad5s) are cultivated in special HEK 293 cells that have the E1 region incorporated into their genome and can therefore supply E1 in *trans* to initiate replication.[52,53] Recombinant viruses are then purified to high titers by use of cesium chloride gradients, or when large scale applications are required, column chromatography.[52,53,54] High titer Ad vectors can then be easily administered intravenously, intramuscularly, subcutaneously, intranasally, and even orally.[55,56,57,58,59] Further attenuation by removing different combinations (or all) of the Ad5 E2b, E3, and E4 genes can provide a cloning capacity of over 33kb have also been described, with some having unique abilities as a vaccine platform.[51,60,61,62]

1.9 Innate/adaptive immune systems:

The innate immune system heavily influences and augments the development of an antigen specific adaptive response.[63] Innate immune responses are stimulated through multiple receptors and sensors specially suited for detecting the aforementioned PAMPs, that include foreign protein, DNA, RNA, and polysaccharides. These receptors include membrane and endosome bound receptors called toll-like receptors (TLRs), intracellular receptors like NOD-like receptors (NLRs) and RIG-like receptors (RLRs), the inflammasome/NALP pathway, and the complement pathway. These receptors and sensors recognize multiple pathogen associated signals and activate innate immunity (which includes multiple cytokine and chemokines) in response to the detected infection. Without an associated danger signal many antigens would otherwise be well tolerated by the immune system. Deliberate stimulation of an innate immune response by adjuvants can provide the danger signals necessary to signify the antigen provided

by a vaccine as a pathogen, resulting in a more robust adaptive response against the antigen of choice. TLRs, NLRs and RLRs, inflamasome/NALP pathway, and complement mediated induction of innate cytokines have all been shown to greatly augment adaptive responses against the pathogens from which the PAMPs originate from.[64,65,66,67,68,69,70]

rAds have shown great promise as a vaccine platform for several reasons. In general they are highly regarded for their ability to stimulate potent cellular (CD8+ T cells) and humoral adaptive responses against expressed antigens, a feature thought to be due in part by the Ad capsid and genome simultaneously stimulating several arms of the innate immune response, including the NLRs, TLRs, complement system, and the inflammasome/NALP pathways.[65,69,71,72,73] Stimulation of the innate immune system in this multi-faceted fashion likely promotes the development of robust adaptive responses against a specific antigen, responses that would otherwise be less immunogenic without these associated "danger signals".[63] Ad5 based vectors have proven very successful at stimulating adaptive responses against several antigens, including those targeting rabies, cancer (P815 tumor), and HIV, as well as malaria.[60,74,75,76] My research has elucidated Ad vector interactions with complement that could lead to improvements in Ad vector design and immunogenicity.

1.10 rAd5 use in malaria vaccines:

Ad5 based malaria targeted vaccines have repeatedly matched or surpassed the ability of other vaccine platforms to induce beneficial, malaria antigen specific adaptive immune responses.[59,75,77,78,79] For example, a rAd5 expressing *P. falciparum* CSP (Ad5*Pf*CS) provoked equivalent CSP specific lymphocyte activation and antibody titers as the leading adjuvanted malaria subunit vaccine, RTS,S/AS01B, without the use of additional adjuvants.[75] In mouse models of malaria, rAd5s expressing *P. yoelii* derived CSP (Ad5*Py*CS) provided CSP

specific CD8+ T cell mediated protection from malaria challenge with live, intravenously injected *P. yoelii* sporozoites.[59,77] Ad5*Py*CS induced CSP specific T cell responses greater than what was observed in mice similarly vaccinated with irradiated sporozoites.[78] While potent CD8+ T cell responses have been implicated as being important for protection against pre-erythrocytic antigens, Ad5 based vaccines are also capable of inducing humoral responses against erythrocytic stage antigens. rAd5s expressing blood stage antigen candidates such as the apical membrane antigen-1 (AMA-1) or the merozoite surface protein-1 (MSP-1), induced anti-antigen antibody titers equivalent to adjuvant enhanced protein based vaccines. The Ad based malaria vaccines were also able to successfully induce malaria antigen specific IgG in vaccinated rabbits to levels that inhibited *P. falciparum* in erythrocytes growth assays.[79]

A rAd5 vaccine formulation combining two Ad vectors, with one expressing the CSP and the other expressing the erythrocytic stage antigen AMA-1, has recently moved forward to safety and efficacy studies in humans. The vaccine formulation was found to be well tolerated; however, prime-boost vaccinations with the vaccine were not able to provide sterile protection from malaria challenge (vaccinated volunteers exposed to mosquitoes infected with *P. falciparum*). Additionally, the second vaccine dose appeared less immunogenic than the first, suggesting that anti-Ad5 immune responses induced by the priming vaccination may have prevented boosting of malaria antigen specific immune responses.[80,81,82] This notion has been supported recently, as the repeated use of DNA based priming vaccinations (encoding CSP and AMA-1) followed by boosting with rAd based malaria vaccines expressing the same antigens provided for up to 27% protection from malaria challenge (i.e.: exposure to mosquitoes infected with *P. falciparum*).[81]

1.11 Pre-existing Ad immunity:

In Africa and Southeast Asia over 50% of the population has significantly elevated anti-hAd5 neutralizing antibody titers.[83,84] Pre-existing immunity to wildtype hAd5 is common in the sub-Saharan regions of Africa and correlates with weakened hAd5 vaccine vector induced immune responses to rAd5 expressed antigens.[85] Pre-existing Ad5 immunity encompasses not only hAd5 neutralizing antibodies, but cytotoxic T cell responses against hAd5 infected cells as well.[86,87,88] For example, advanced generation E1 deleted Ad5 based vectors that are additionally deleted for the Ad polymerase gene can allow for induction of robust antigen specific immune responses in Ad5 immune animals, including non-human primates, a property that may be due to avoidance of pre-existing cellular immune responses to the Ad polymerase protein.[60,61,89]

1.12 Alternative rAd serotypes for use in malaria vaccines:

Although it is important to note that malaria typically affects young children and infants in Africa, which rarely possess neutralizing antibody against hAd5, the use of alternative Ad serotypes (other than Ad5) may afford increased levels of efficacy in an Ad5 immune population.[48] As a result of the seroprevalence of hAd5, alternative serotypes of Ad are being studied more frequently for use in HIV and malaria vaccines.

Use of subgroup B derived recombinant Adenovirus serotype 35 (rAd35) has become a very popular alternative to rAd5 in vaccinations designed for use in areas of high Ad5 seroprevalence. Ad35 has a neutralizing antibody seroprevalence of less than 20% in malaria endemic regions (Africa and Southeast Asia).[83,84] Much like Ad5, Ad35 can be made replication defective by deletion of the Ad35 E1 genes, and E1 deleted Ad35 based vaccines have been shown to be capable of stimulating potent malaria antigen specific adaptive immune responses. For example, an Ad35 vaccine expressing the CSP (Ad35*Pf*CS) stimulates potent CSP

specific B and T cell responses in mice equivalent to those induced by either Ad5*Pf*CS or RTS,S/ASO1B.[75] Comparison of Ad35 vaccines expressing *P. yoelii* CSP (Ad35*Py*CS) and Ad5*Py*CS showed that both vaccines were capable of stimulating B and T cell responses. These responses positively correlated with the vaccines reducing parasite infection in the liver of vaccinated animals after challenge (injection) with purified live *P. yoelii* sporozoites, in this case as measured by parasite rRNA levels present in the liver of challenged animals.[90] Furthermore, Ad35*Py*CS induced similarly high responses in hAd5 immune mice; whereas Ad5*Py*CS could not, as CSP responses were severely ablated in Ad5 immune mice treated with Ad5*Py*CS.[90]

Ad35 utilizes CD46 to gain entrance into human cells, which is ubiquitously expressed in humans but is only expressed in the testis of mice. Therefore, results of research performed in mice may be a poor predictor of efficacy or safety in humans.[91] However, Ad35*Pf*CSP has also been shown to be effective when used as a priming vaccine in rhesus macaques prior to boosting with either RTS,S/ASO1B or Ad5*Pf*CS.[47,92] Based upon these promising results, several clinical trials are currently underway to assess the safety and efficacy of rAd35 based malaria vaccine platforms in humans, inclusive of an Ad35*Pf*CSP phase I trial also using Ad26*Pf*CSP in a heterologous prime boost based regimen.Ad serotype 26 (Ad26) is another rare Ad serotype with seroprevalence below 20% in Africa and Southeast Asia.[83,84] Ad26 is a member of Ad subgroup D, which uses a combination of CAR, CD46, and sialic acid to gain entry into cells. Use of malaria targeted rAd26 vaccines in the context of heterologous prime boost vaccinations with CSP, or Ad35 expressing CSP were able to induce potent, and long lasting IFNγ+, TNFα+, CD8+ T cells specific for CSP in mice.[93]

In a similar vein to the use of alternative human Ad serotypes, rAd based malaria vaccines derived from Simian derived Ad serotypes have also been utilized as alternatives to

Ad5 malaria vaccines, as there should theoretically be no pre-existing immunity to simian Ads in the human population. For example, a single dose of a simian Ad (AdC9) expressing an enhanced form of the *P. berghei* thrombospondin-related anonymous protein (ME.TRAP) (a protein found on the surface of sporozoites) provided a potent Tem cell response and protection from purified *P. berghei* sporozoite challenge in hAd5 seropositive mice.[94] Simian Ad based vaccines also can serve as potent priming vaccinations in heterologous prime boost studies with MVA, much like hAds.[95,96,97,98] For example, following priming vaccination with a Simian Ad expressing ME.TRAP, boosting with an MVA expressing ME.TRAP, increased the polyfunctionality of the resultant TRAP specific T cell responses, and increased protection from purified *P. berghei* sporozoite challenge in a mouse model.[98] In vaccinations aimed at inhibiting transmission of malaria parasites from human to mosquito host, simian Ads (ChAd63) expressing the ookinete surface protein (Pfs25) were used as a priming vaccination, which were then boosted with an MVA vaccine expressing the same antigen in a *P. berghei* mouse model of malaria. Specifically, the ChAd63 and MVA based immunizations were just as effective at eliciting anti-Pfs25 IgG, and at reducing oocyst intensity in a standard feeding assay as was similar use of hAd5 and the MVA vaccines expressing the same antigen.[99] These results held true in rhesus monkey studies where priming vaccinations with a simian Ad vaccine, followed by boosting vaccinations with an MVA vaccine also expressing AMA-1, resulted in long lasting multifunctional CD8+ T cell responses to AMA-1.[97]

In general, alternative serotype Ads have not proven particularly more immunogenic or safer than Ad5 in Ad5 naïve subjects.[75,90] It should also be noted that alternative serotype Ads can vary dramatically not only in their abilities to infect certain types of cells, but also in their differential abilities to stimulate the innate immune system.[84,100,101] Therefore, extensive

biodistribution and safety studies for each serotype should be undertaken, as baseline data from widespread use of rAd based on the Ad5 serotype may not be applicable.

1.13 Summary:

It has been over 30 years since the discovery that immunity to malaria can be obtained prophylactically, yet development of a viable, highly efficacious malaria vaccine has not been achieved to date. Though some in the malaria field may be disheartened by this drought of progress, the studies and efforts to date confirm that an effective malaria vaccine can likely be produced. As it currently stands, sporozoite based vaccine platforms may be an impractical method by which to vaccinate the many millions of people in need of malaria protection. The RTS,S platform can overcome some of these shortcomings, and though laudable, contemporary RTS,S based vaccine regimens have only reached a 56% level of protection.[44]

Viral vector based malaria vaccine platforms provide an alternative means by which to produce large amounts of potentially efficacious malaria vaccines. This dissertation describes my contribution to important works performed in our lab focused on elucidation of Ad interactions with the innate immune system, and then capitalizes upon these insights by development of advanced, Ad based malaria vaccines that utilize rare Ad serotypes or express potent and novel immunomodulators. These studies have contributed to improvements in Ad based malaria vaccines that have the potential to close the gap between protective efficacy and ease of production and inoculation, which together could one day help to achieve global malaria eradication.

Chapter 2:

Adenovirus induced innate immune responses

Portions of this chapter are derived from the following three research articles that have been previously published:

Complex interactions with several arms of the complement system dictate innate and humoral immunity to adenoviral vectors. Gene Therapy (2008); 15: 1606-1617

Authors: Appledorn DM, McBride A, Seregin S, Scott, JM, **Schuldt N**, Kiang A, Godbehere S, Amalfitano A: **Schuldt NJ** – assisted with qRT-PCR measuring liver transcriptome dysregulation and performed ELISAs measuring Total IgG and subisotypes

CR1/2 is an important suppressor of Adenovirus-induced innate immune responses and is required for induction of neutralizing antibodies. Gene Therapy (2009); 16: 1245-1259

Authors: Seregin SS, Aldhamen YA, Appledorn DM, **Schuldt NJ**, McBride AJ, Buhold M, Godbehere SS, Amalfitano A: **Schuldt NJ** – assisted with qRT-PCR measuring liver transcriptome dysregulation and performed ELISAs measuring Total IgG and subisotypes

Adenovirus capsid-display of the retro-oriented human complement inhibitor DAF reduces Ad-vector triggered immune responses *in vitro* and *in vivo*. Blood (2010); 116(10): 1669-1677

Authors: Seregin SS, Aldhamen YA, Appledorn DM, Hartman ZC**, Schuldt NJ**, Scott J, Godbehere S, Jiang H, Frank M, Amalfitano A: **Schuldt NJ** – assisted with construction of Ad5-GFP-IX-dDAF-REO virus

2.1 Introduction:

rAd vectors have been used extensively as both gene transfer and vaccine vectors. While Ad induction of the innate system is a major obstacle in its efficacy as a gene transfer vector, it can be exploited to intensify an adaptive immune reaction to a transgene for the purpose of directing immunity to that transgene. Despite the popularity of Ad based vectors very little is actually known about specific interaction between Ad and the innate immune system or how Ad induction of the innate immune systems leads to adaptive responses. Understanding the complex interaction between Ads and the innate immune system is relevant to both gene transfer and vaccine functions of Ad vectors. With this knowledge we can develop less immunogenic Ad based gene transfer vectors and/or Ad based vaccine vectors that induce more immunogenic responses against transgenes.

We have previously demonstrated the involvement of complement system and uncovered the importance of TLR intracellular signaling in forming adaptive responses subsequent to Ad exposures.[67,68,69,102,103] Here we focus on further elucidating interactions between the complement system and rAds. The complement system serves to recognize various pathogens through detection of PAMPs and then initiate a cascade of immune responses aimed at clearing the pathogen. The complement system can be divided into three activation pathways; the classical pathway (CP), the alternative pathway (AP), and the mannan-binding lectin pathway. The classical complement pathway begins with the binding of antibody to pathogenic invaders. Antibody bound to the surface of a pathogen forms a complex that is recognized by the C1qrs protein complex. The C1r and C1s subunits of the complex become activated by cross-proteolysis and recruit C4. C4 is then cleaved by C1s and binds the surface of the pathogen. C2 binds to C4 and is also cleaved by the activated C1s to form CP-C3 convertase. C3 convertases

then cleave C3 to assemble the C3-C5 convertase which leads to the cleavage of multiple C3 and C5 molecules. Cleaved C5 binds with C6 and C7 and begins to form the membrane attack complex. C8 binds to this complex and inserts itself into the surface of the pathogen and assists in the assembly of multiple C9 proteins to form a pore on the pathogen surface. The membrane attack complex disrupts the ionic and osmotic equilibrium leading to destruction of the pathogen. Conversely, the alternative pathway recognizes pathogen motifs and results in direct binding and activation of C3 independent of antibody binding. In the alternative pathway, C3 bound to the pathogen membrane recruits Factor B which is then cleaved by Factor D resulting in the active AP-C3 convertase. This complex then binds properdin, which stably links the complex to the pathogen surface. Then, much like the classical pathway, the AP-C3 convertase cleaves multiple C3 molecules, which can then bind the AP-C3 convertase and form the alternative pathway C5 convertase. From here the alternative pathway functions identically to the classical pathway. The mannan-binding lectin pathway differs only in that carbohydrate motifs on bacterial pathogens are recognized by mannose-binding lectin (MBL) and mannose associated serine protease (MASP) molecules which directly activate C4 and C2 to create the C3 convertase in a similar manner to the classical pathway. Unchecked complement activation can result in severe tissue damage. Therefore, the complement system has multiple proteins (complement receptors (CR), decay accelerating factor (DAF), membrane co-factor protein (MCP)) for the purpose of down-regulating the complement system preventing damage and ultimately returning the system to a monitoring mode.[104]

Activation of the complement system can induce a pathogen specific humoral response.[64,105,106] Following opsonization of the pathogen by C3, B cells and dendritic cells can bind to the pathogen through interactions with complement receptors CR1 and CR2. CR1 is

also involved in clearance of immune complexes and B cell maturation. In humans, CR2 is found only on the surface of B cells, follicular dendritic cells, and thymocytes. Human CR2 binding to C3d-opsonized pathogens has been shown to lower B cell activation 1000 fold.

Murine complement receptors (mCRs) 1 and 2 are products of alternative splicing from the same gene and are known to be expressed on B cells and dendritic cells similar to human CR2.[107] Although murine CR1/2 protein's role in adaptive immunity is well studied, its role in inhibiting/regulating murine complement has not been described, possibly because the parallel acting protein, Crry, was suggested to play the predominant role in controlling complement activation in most mouse models. We feel that the role of murine CR1/2 protein in complement dependent innate immune responses may be more important than previously considered.

Our previous studies demonstrate that rAds activate the complement system in a C3-dependent manner.[67,102] Here we attempt to determine what roles CP and AP of complement have in creating humoral responses. We also analyze mCR1/2's role in regulating innate and humoral responses to rAd and to transgenes. Knowledge of anti-Ad humoral responses could be used to improve upon current generations of Ad based vectors and may even result in the development of rAds that can circumvent previous Ad immunity. This knowledge can also be used to ablate the Ad-induced innate immune response to Ad-based gene therapy vectors making them less immunogenic and therefore extending expression of the therapeutic transgene.

Our results led us to hypothesize that genetic engineering of the native Ad capsid in a manner that minimized its capacity to activate the complement system would mitigate Ad capsid induced, complement dependent, immune responses. To test this hypothesis we engineered a rAd vector to display complement inhibitor DAF on the surface of the Ad capsid by fusing DAF to pIX in the retro-oriented form of the human DAF protein (thereby displaying the primary DAF

amino acid sequence in a more native conformation relative to the Ad capsid surface). Human DAF contains four complement control protein repeats (CCPRs) that decreases complement activation by increasing the rate of decay of the classical and alternative C3 convertases generated during pathogen mediated complement activation.[108] DAF-fusion to viral capsid/envelope proteins has been shown to retain functionality of the CCPRs. For example, baculovirus, retrovirus, and lentivirus have all been shown to successfully evade complement mediated lysis when DAF was incorporated onto their surface.[109,110,111,112] Additionally it has been shown that proteins displayed at the C-terminus of Ad5 protein IX can retain their functionality (GFP, TK).[113,114] Here we created a novel cDNA encoding a retro-form of human DAF in order to better simulate natural DAF orientation of human cell surfaces. This "retro-DAF" was incorporated into an adenoviral capsid by fusion to pIX. Multiple studies have previously demonstrated retention of functionality in retro-oriented proteins.[115,116,117,118] We confirm that the native Ad capsid can "capsid-display" the natural complement inhibitor decay accelerating factor DAF as a C-terminal fusion protein with the Ad capsid protein, pIX. Ad capsid-display of DAF can minimize the induction of the complement system, and complement dependent innate immune responses.

2.2 Results:

Ad-induced transcriptome dysregulation is modulated by factors of both classical and alternative pathway and is regulated by CR1/2 in murine livers

We have previously described C3-dependent changes to the transcriptome of murine livers after administration of rAd.[67] Here we further elucidate the role of complement in transcriptome dysregulation by using CP-deficient mice (C4-KO and C1q-KO) and AP deficient mice (FB-KO). Additionally, we investigate C3-dependence of other complement regulated Ad-induced gene inductions previously untested. Six hours after systemic injection of $7.5x10^{10}$ vp per mouse of adenovirus, liver lysates were collected and tested by qRT-PCR for transcript levels of TLR related proteins (*MyD88*, *TRAF2-bp*, *TRIM30*, *CD14*, and *TLRs 2, 3, 6,* and *9*), endothelial cell activation markers (intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (*VCAM* and *E-selectin*), interferon response markers (*IRF-1* and *OAS1a*), and negative regulators of cytokine signaling (*SOCS-1* and *SOCS-3*) (Table 1). All of the above have previously been shown to be dysregulated by rAd in a complement dependent manner.[67] While we did find that dysregulation in all genes except *E-selectin* and *IRF-7* was dependent upon presence of a functional C3 protein, we were unable to determine if these changes were dependent on AP or CP. Surprisingly, we observed significantly higher levels of *ADAR, ICAM, IRF-7, TLR2, and TRIM30* gene inductions in FB-KO mice and higher levels of *CXCL9* and *E-selectin* in C1q-KO mice ($P<0.05$). Suggesting that FB and C1q are not only required to activate transcription of multiple genes, but also negatively regulate genes. Lower levels of *TLR2* were observed in C4-KO mice while there was no change in C1q-KO mice indicating functional C4, but not C1q, is required for complete induction of *TLR2*.

Table 1: Ad5-LacZ induced gene expression in liver at 6hpi (fold over Mock)					
Gene	**C57BL6**	**C3-KO**	**FB-KO**	**C4-KO**	**C1q-KO**
ADAR	7.2 ± 1.1	4.5 ± 0.6	**11.7 ± 1.0**	5.6 ± 1.2	6.3 ± 1.7
CCL7	23.5 ± 5.7	8.1 ± 0.6	27.3 ± 8.6	24.0 ± 8.3	47.0 ± 23.4
CXCL9	18.8 ± 7.4	9.3 ± 1.3	24.1 ± 10.2	21.5 ± 2.8	**59.9 ± 37.7**
CD14	12.6 ± 3.4	1.9 ± 1.0	9.1 ± 5.6	10.1 ± 3.3	22.7 ± 16.0
E-Selectin	9.2 ± 4.2	6.5 ± 2.2	11.4 ± 8.4	11.8 ± 5.3	**27.2 ± 15.0**
VCAM	5.02 ± 1.6	2.8 ± 0.4	6.8 ± 2.1	3.5 ± 1.0	7.9 ± 3.6
ICAM	7.8 ± 2.5	4.3 ± 0.5	9.4 ± 2.5	7.0 ± 2.0	8.5 ± 3.6
IRF-7	20.5 ± 8.6	15.8 ± 2.9	**39.7 ± 6.7**	24.0 ± 5.1	28.3 ± 8.1
OAS-1a	16.5 ± 2.5	11.6 ± 2.7	**24.0 ± 4.2**	20.8 ± 4.6	21.1 ± 8.3
SOCS-1	22.3 ± 6.9	8.2 ± 2.4	17.0 ± 5.6	21.7 ± 7.8	20.4 ± 9.6
SOCS-3	13.4 ± 3.8	1.5 ± 0.5	16.0 ± 2.4	22.2 ± 9.2	10.2 ± 4.5
MyD88	8.6 ± 2.8	3.7 ± 0.5	11.5 ± 2.0	7.8 ± 2.0	11.5 ± 5.4
TLR2	106.0 ± 36.8	22.9 ± 5.0	**195.6 ± 51.4**	54.8 ± 17.7	107.7 ± 73.6
TLR3	28.4 ± 6.6	11.7 ± 2.6	30.2 ± 6.8	28.7 ± 9.4	28.0 ± 11.3
TLR6	5.4 ± 1.0	2.2 ± 0.7	4.1 ± 1.4	4.6 ± 0.9	5.7 ± 1.6
TLR9	3.1 ± 0.5	2.1 ± 0.3	3.3 ± 0.7	2.9 ± 0.7	2.9 ± 0.7
TRAF2bp	33.1 ± 12.5	5.2 ± 0.9	31.7 ± 9.1	20.7 ± 9.4	39.5 ± 20.0
TRIM30	24.7 ± 7.4	13.5 ± 2.8	**41.1 ± 9.0**	29.9 ± 7.4	32.3 ± 7.8

Table 1: Ad5-LacZ-induced gene expression in liver at 6 hpi (fold over mock). *Student's t-tests were completed between C57BL/6 and each respective genotype. Statistically significant decreases are highlighted in light gray boxes. Statistically significant increases are highlighted in dark gray boxes with bold numerals.

When gene induction was similarly tested using mCR1/2-KO mice in a larger panel of genes we observed further induction of 13 genes when compared to Ad-injected wildtype mice at 6hpi. Indicating these genes (*ADAR, GATA-3, ICAM1, JAK3, MYD88, NOD-1, OAS-1, SOCS-1, TBK-1, TLR2, TLR3, TRAF2bp,* and *VCAM1*) are Ad-induce, but mCR1/2 suppressed (Table 2). Taken together with data from C3-KO experiments we can determine mCR1/2 plays a significant role in down-regulating Ad-induced C3-dependent proinflammatory gene expression.

Ad-induced cytokine and chemokine responses are C3 dependent and are suppressed by the presence of functional CR1/2

To describe the role of several complement proteins in Ad-induced innate and adaptive responses we injected AP deficient mice (FB-KO), CP deficient mice (C4-KO and C1q-KO) CR1/2-KO, and C3-KO mice intravenously with $7.5x10^{10}$ vp per mouse. We then measured the amounts of cytokines known to be induced by Ad at 6 hpi by multiplex bead-based enzyme linked immunosorbant assay. Under these conditions only C3-KO mice demonstrated significantly decreased amounts of Ad-induced RANTES, MCP-1, IL-12p40, G-CSF, and KC, indicating Ad-induced cytokine and chemokine production are C3-dependent, but may only be partially dependent on C4, C1q, and FB if at all (Figure 1). Conversely, CR1/2-KO mice demonstrated increased levels of Ad-induced RANTES, MCP-1, and G-CSF, supporting a role for CR1/2 in suppressing compliment activation after systemic Ad administration (Figure 2).

Inductions of rAd specific humoral responses are dependent on functioning AP, CP, and CR1/2.

We assessed the role of complement in shaping neutralizing antibody responses *in vivo* by measuring plasma borne anti-Ad neutralizing antibody in C3-KO, FB-KO, and C1q-KO mice 30 dpi after intravenous injection of $7.5x10^{10}$ vp per mouse. We observed dramatically decrease

Table 2: 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	1.1 ± 0.1	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**

Table 2: Ad5-LacZ-induced gene expression in a liver (fold over C57BL/6_WT_Mock) The numbers represent mean ± s.d. 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 no significant differences were detected between mock-injected WT and CR1/2-KO mice.

Figure 1

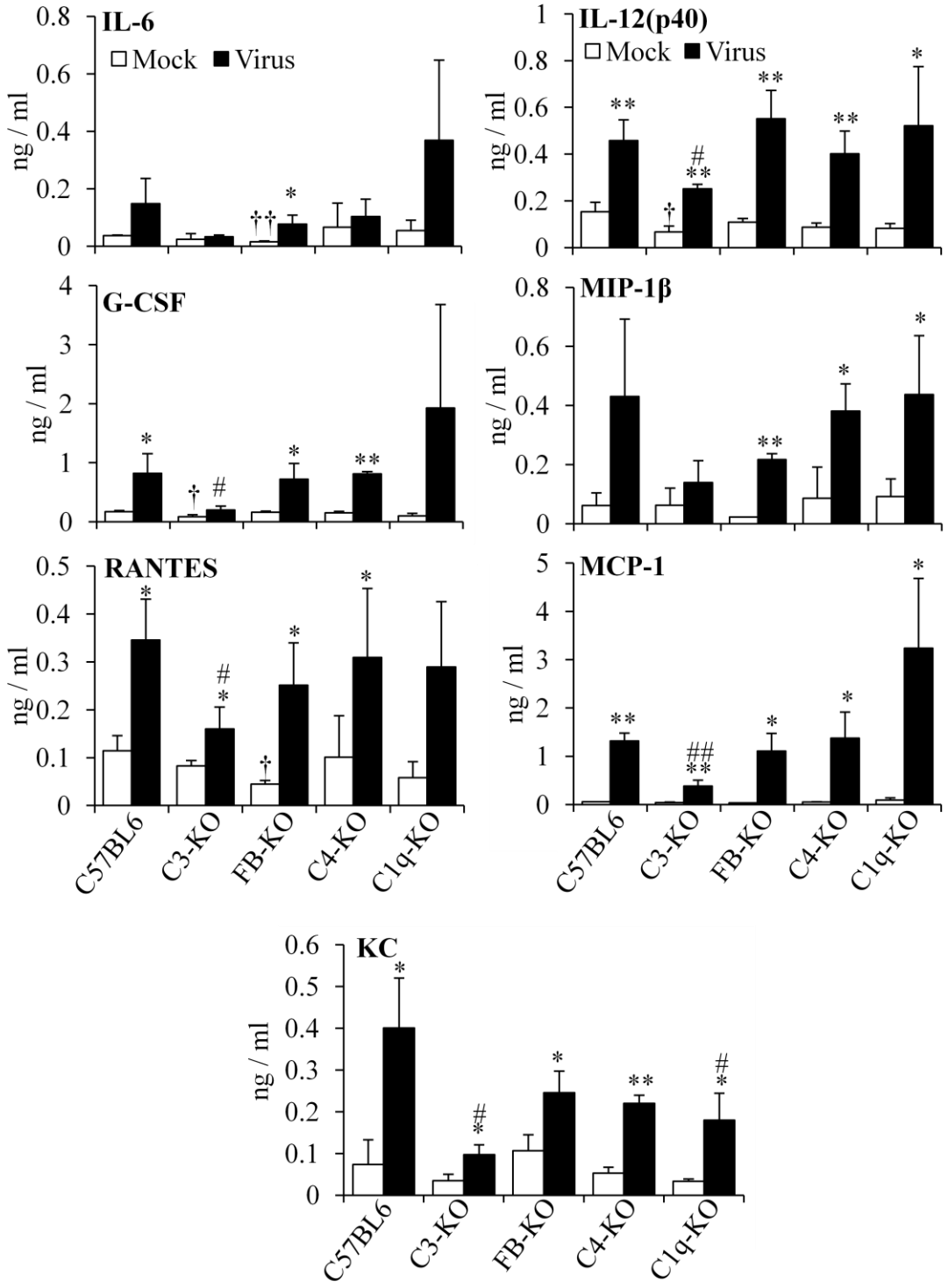

IL-6
Mock
Virus
ng / ml
IL-12(p40)
G-CSF
MIP-1β
RANTES
MCP-1
KC
C57BL6
C3-KO
FB-KO
C4-KO
C1q-KO

Figure 1 cont'd: Plasma cytokine and chemokine elevations after intravenous adenovirus (Ad) injection. C57BL/6, C3-KO, Factor B knockout (FB-KO), C4-KO and C1q-KO mice were injected with $7.5x10^{10}$ viral particle (vp) per mouse of rAd5-LacZ. Plasma samples were isolated at 6 h.p.i. and analyzed using a Bio-Plex bead-based enzyme-linked immunosorbent assay (ELISA) assay (see Materials and methods). Bars represent mean±s.d. Student's t-tests were completed between mock-injected samples (†P<0.05 and ††P<0.01), virus-injected samples (#P<0.05 and ##P<0.01) and between mock and virus-injected samples within the same genotype (*P<0.05 and **P<0.01). N=3 for all samples tested.

Figure 2

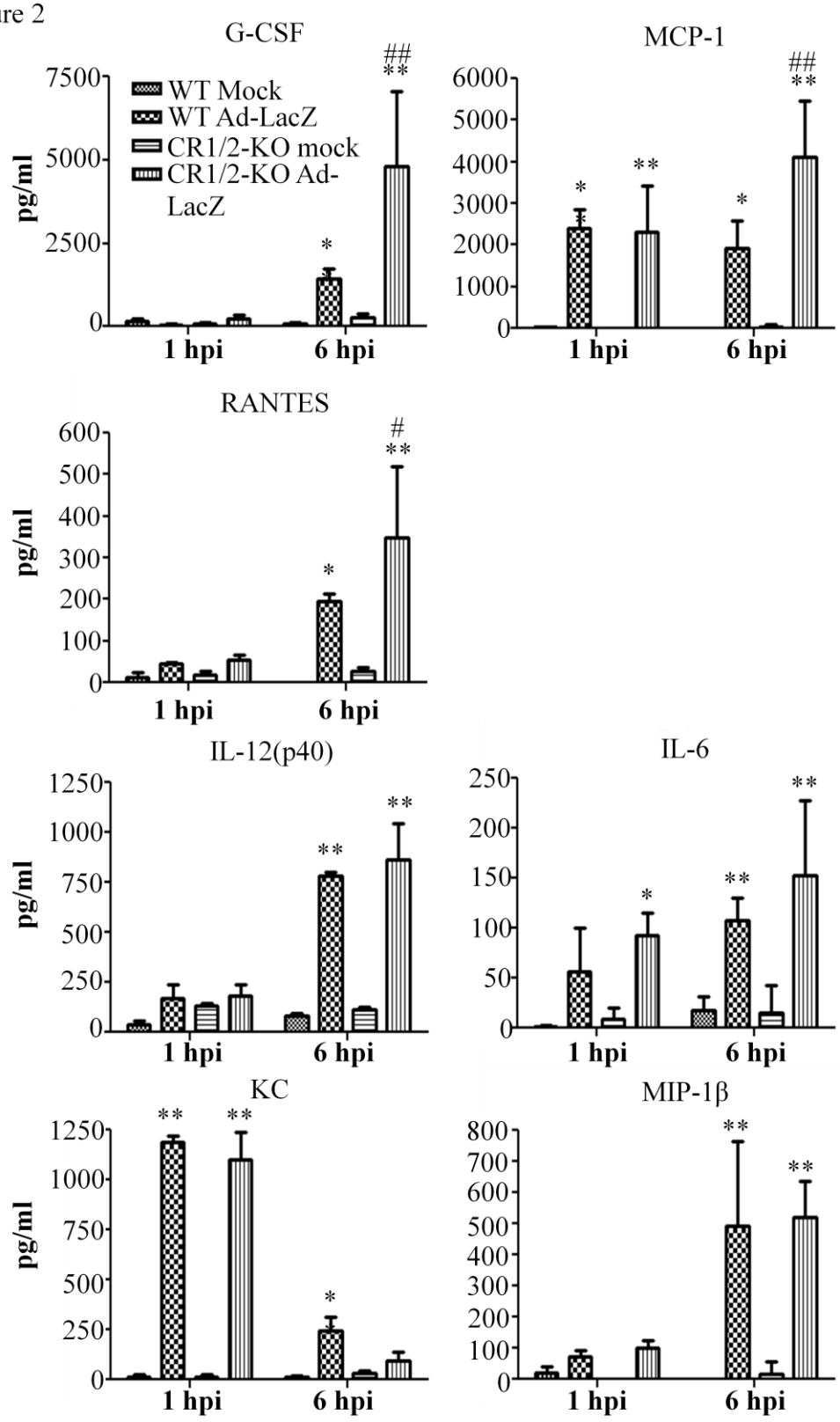

G-CSF
MCP-1
RANTES
IL-12(p40)
IL-6
KC
MIP-1β
WT Mock
WT Ad-LacZ
CR1/2-KO mock
CR1/2-KO Ad-LacZ
pg/ml
1 hpi
6 hpi

Figure 2 cont'd: Murine complement receptor 1/2 (mCR1/2) mitigates Adenovirus (Ad)-mediated cytokine and chemokine release in C57BL/6 mice. C57BL/6 wild-type (WT) and CR1/2-KO mice were intravenously injected with $0.75x10^{11}$ vp per mouse of Ad5-LacZ vector. Plasma samples were collected at 1 and 6 h post-virus injection (hpi). Plasma samples were analyzed using a multiplexed bead array-based system. Statistical analysis was completed using two-way analysis of variance (ANOVA) with a Bonferroni post hoc test. The N=4 for mock (phosphate-based saline; PBS)-injected animals, N=6 for virus-injected mice at 1 hpi and N=12 for virus-injected mice at 6 hpi. The bars represent mean ± s.d. *,**Indicate plasma cytokine values that are statistically different from those in mock-injected animals of the same treatment at the same time point (that is, CR1/2-KO_Ad5-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.

anti-rAd neutralizing antibody in plasma of C3-KO mice. High variability or lack of assay sensitivity did not allow us to determine if the observed decrease of rAd-specific neutralizing antibody in C3-KO was due to AP, CP, or a combined result of both (Figure 3).

To determine if the observed decrease in rAd-specific neutralizing antibody was a result of overall decreases in titers of IgG, we analyzed plasma for titers of total IgG (Figure 4). We observed that total anti-Ad IgG was significantly decreased in both C3-KO and FB-KO mice. We further characterized this response by analyzing the same plasma for sub-isotypes of IgG (Figure 4). IgG 1 and IgG3 were both significantly lower in C3-KO mice, while IgG2c was significantly higher in C3-KO mice. C1q-KO had significantly decreased levels of plasma borne IgG1 and IgG2c. FB-KO mice only showed decreased titers of IgG3. IgG2c/IgG1 ratio is believed to indicate relative contribution of the Th1/Th2 response. Based on the remarkably different IgG2c and IgG1 tiers in C3-KO mice we hypothesized there may be a shift in the Th1/Th2 balance. When we analyzed the IgG2c/IgG1 ratio we found an eight fold increase in Th1 type antibody compared to Th2 antibody in C3-KO mice as compared to wildtype.

When we similarly analyzed mCR1/2 role in Ad-specific neutralizing antibody we found mCR1/2-KO mice had significantly diminished capacity to make anti-Ad neutralizing antibody that was nearly identical to what was observed in C3-KO mice. These data indicate C3-dependent induction of neutralizing antibody could be mediated by interactions involving mCR1/2 (Figure 5). Further analysis of total IgG and sub-isotypes also showed decreased titers of total anti-Ad IgG, IgG1 and IgG3 similar to C3-KO mice. However, mCR1/2-KO mice also showed a diminished capacity to induce titers of IgG2a, IgM, IgA, and IgG2b (Figure 6).

When we analyzed the same KO animal's ability to induce humoral responses against Ad expressed transgenes we found C3-KO mice actually had increased titers of total anti-transgene

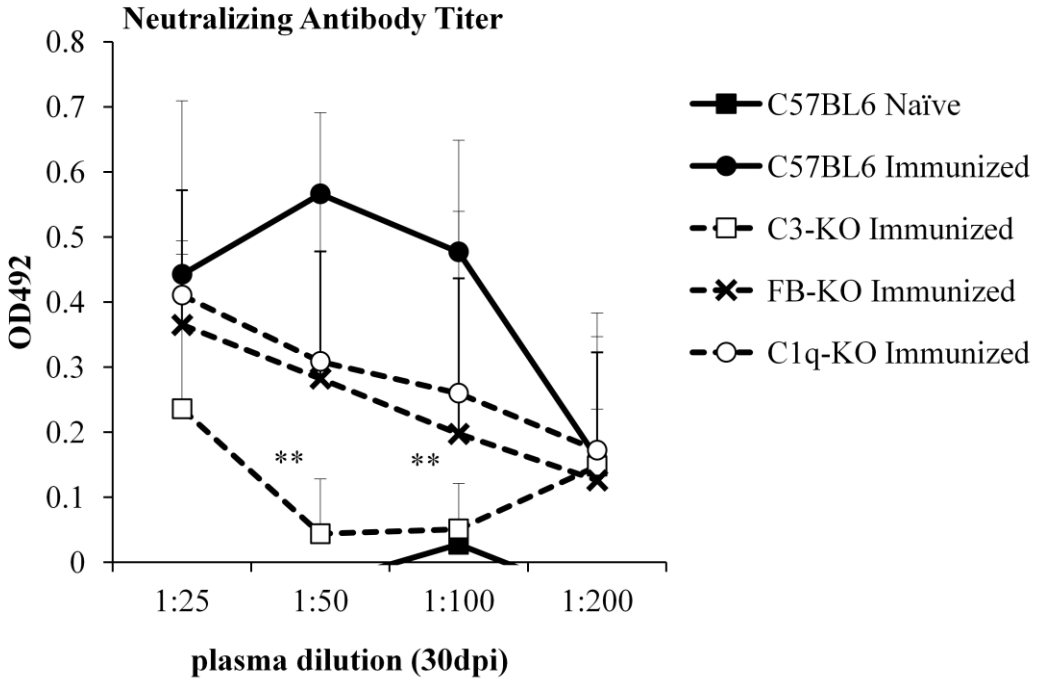
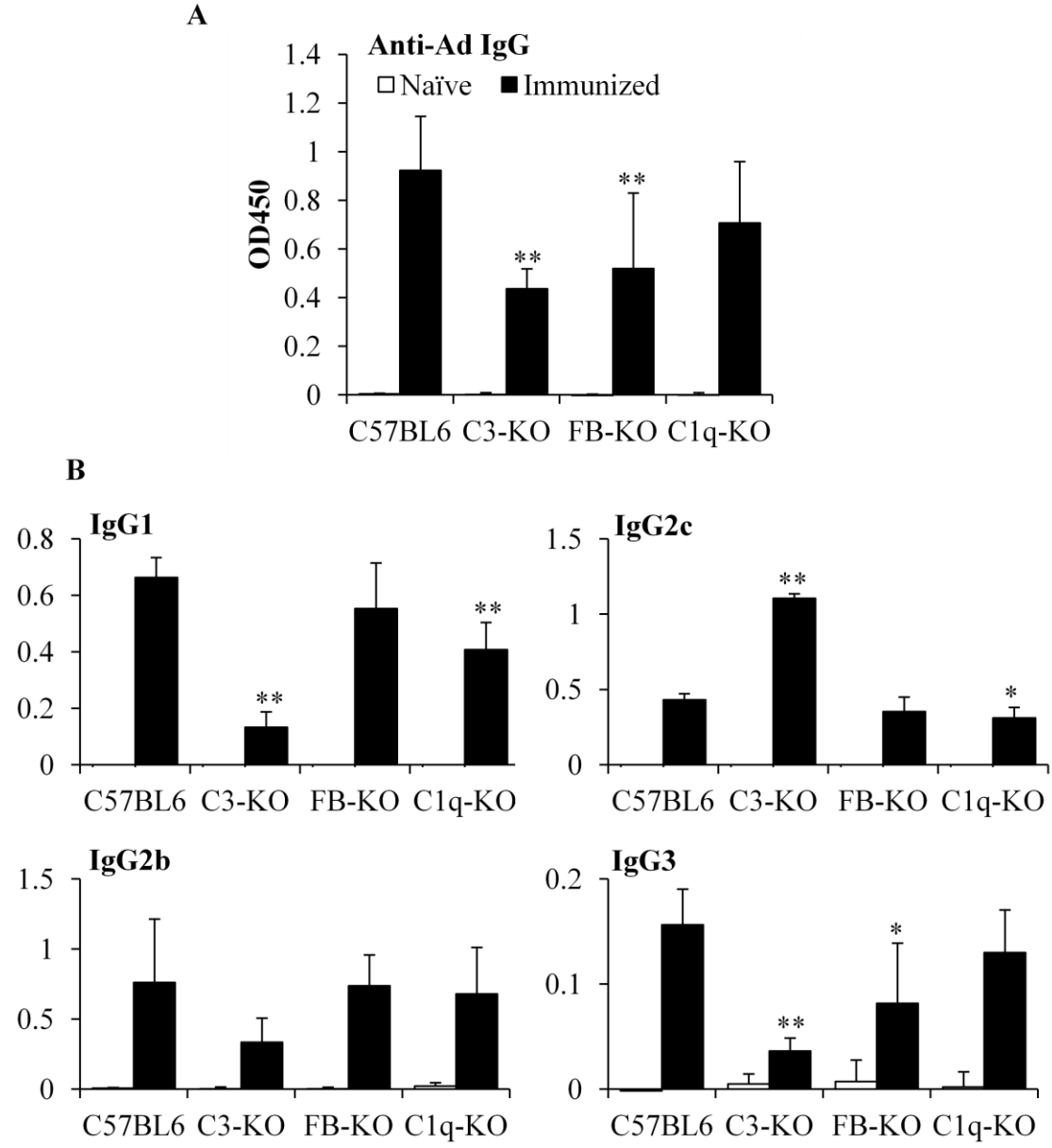


Figure 3: Ad neutralizing antibody titers are C3-dependent. C57BL/6, C3-KO, Factor B knockout (FB-KO) and C1q-KO mice were injected with $7.5x10^{10}$ vp per mouse of rAd5-GFP (N=3 for recombinant adenovirus (rAd)-naïve animals, N=4 for all immunized animals). Plasma samples were isolated at 30 d.p.i. and analyzed for neutralizing antibodies using four successive dilutions of plasma (see Materials and methods). Bars represent mean ± s.d. '**' represents a statistical difference, as measured by a homoscedastic t-test, in neutralizing antibody titers found in plasma derived from C57BL/6 animals versus C3-KO animals ($P<0.01$).

Figure 4



C

	IgG2c/IgG1 (Th1/Th2)
C57BL6	0.65
C3-KO	8.33
FB-KO	0.64
C1q-KO	0.76

Figure 4 cont'd: Anti-recombinant adenovirus (rAd)-specific antibodies are dependent on both alternative pathway (AP) and classical pathway (CP) of complement. C57BL/6 (N=3 naive and N=7 immunized), C3-KO (N=4 naive and N=9 immunized), Factor B knockout (FB-KO) (N=4 naive and N=11 immunized) and C1q-KO (N=3 naive and N=5 immunized) mice were injected with $7.5x10^{10}$ vp per mouse of rAd5-GFP. (a) At 30 d.p.i., plasma was analyzed for anti-rAd capsid-specific total IgG antibodies at the appropriate dilution (1:800). Bars represent mean ± s.d. (b) Plasma was also analyzed for various IgG subclasses. Data points represent mean ± s.d. *$P<0.05$ and **$P<0.01$ indicate a statistical difference between C57BL/6 and each respective genotype. (c) The IgG2c/IgG1 ratio, indicative of a Th1/Th2 response, was calculated based on subclass titering.

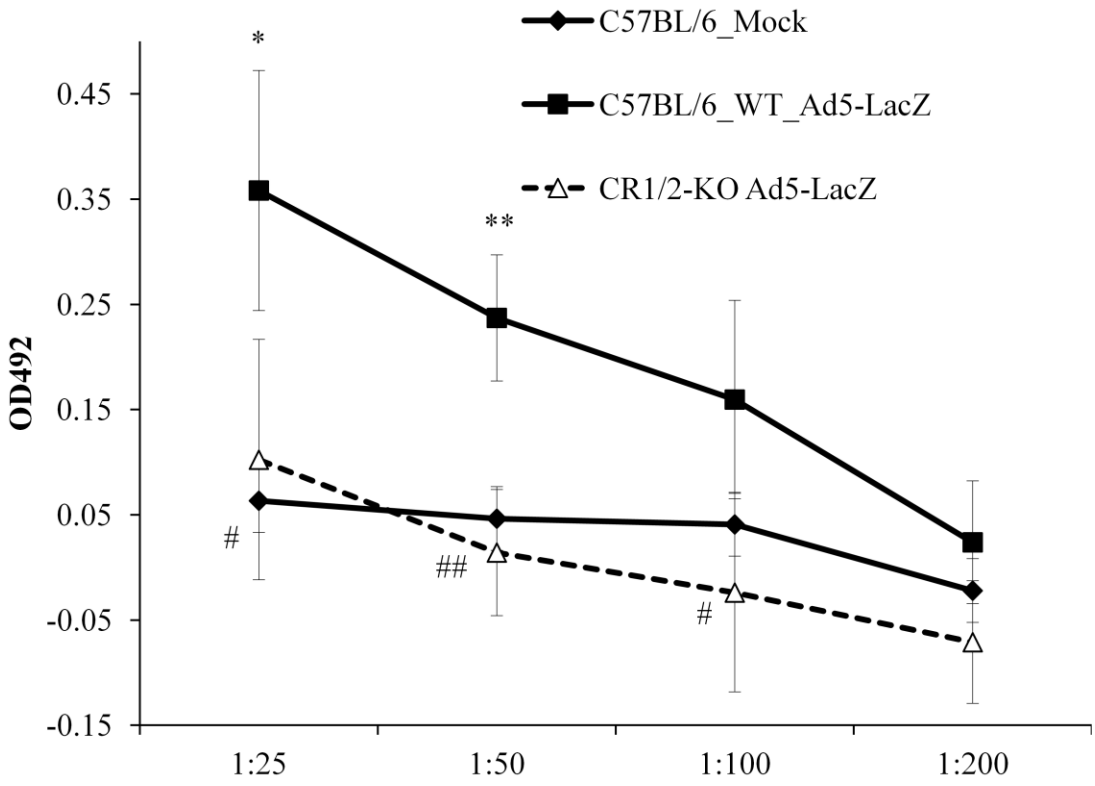


Figure 5: mCR1/2-KO mice exhibit significantly reduced Adenovirus (Ad) capsid-specific neutralizing antibodies titer. Three groups of mice were treated as described in Materials and methods: Wild-type (WT)_mock (N=4), WT_Ad5-LacZ (N=5), CR1/2-KO_Ad5-LacZ (N=5). Plasma samples were collected at 28 dpi and assayed for neutralizing antibodies using successive dilutions (see Materials and methods). The error bars represent ± s.d. Statistical analysis was completed using one-way analysis of variance (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.

Figure 6

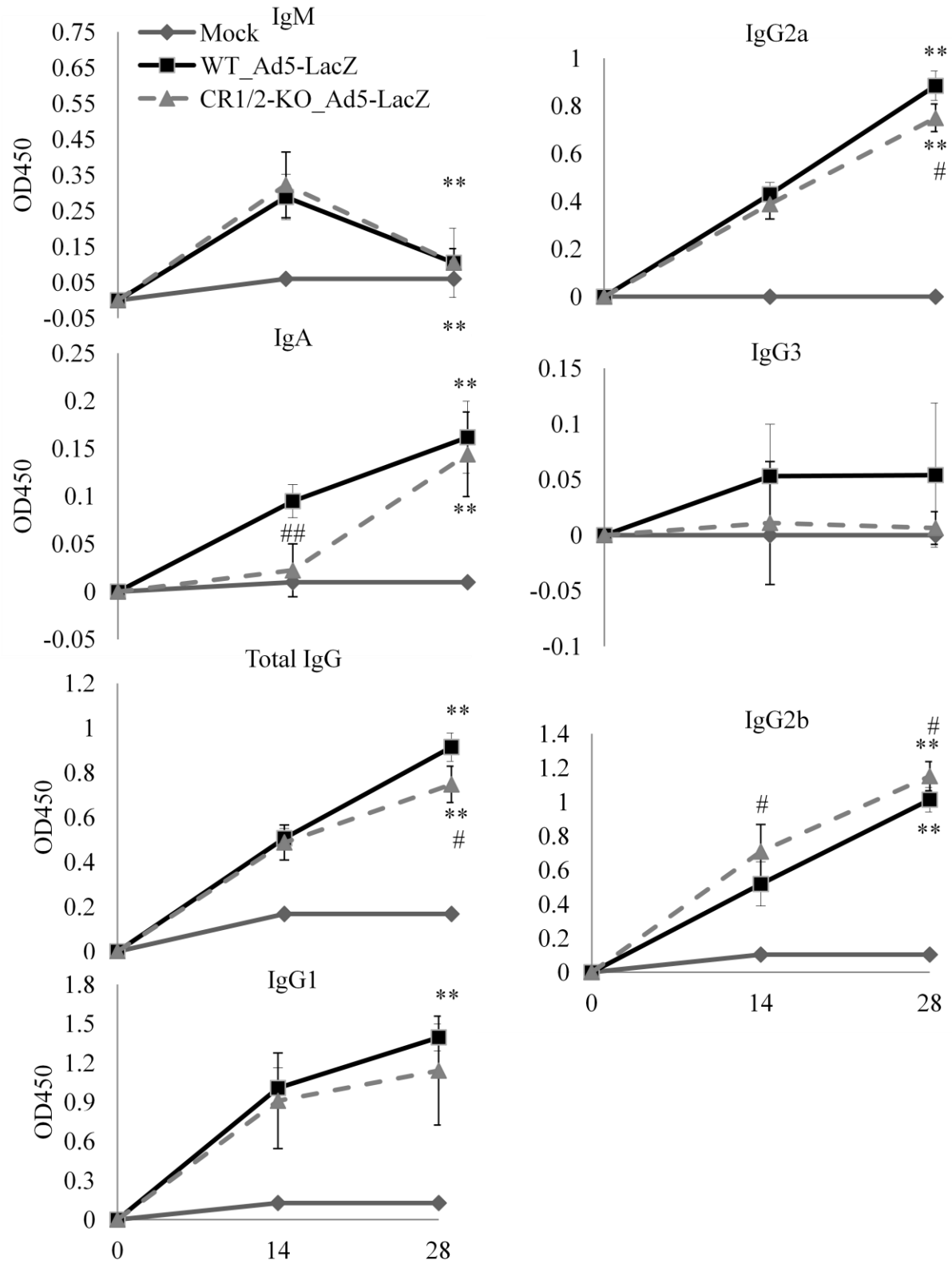

IgM
Mock
WT_Ad5-LacZ
CR1/2-KO_Ad5-LacZ
OD450
IgA
Total IgG
IgG1
IgG2a
IgG3
IgG2b
0
14
28

Figure 6 cont'd: mCR1/2-KO mice exhibit significantly reduced Adenovirus (Ad) vector capsid-specific humoral immune responses. Three groups of mice were treated as described in Materials and methods: Wild-type (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 immunoglobulin M (IgM), IgA and IgG antibodies and various IgG subclasses. The error bars represent±s.d. Statistical analysis was completed using two-tailed Student's t-test to compare two groups of virus-injected animals. ##Indicate statistically different values in CR1/2-KO_Ad5-LacZ group compared to WT_Ad5-LacZ group, $P<0.001$. *,**Indicate values, statistically different from animals of the same group at different time point, $P<0.05$, $P<0.001$, respectively.

IgG with a strong bias of the Th1/Th2 response toward Th1 (Figure 7). mCR1/2 mice levels of anti-transgene IgG were relatively identical to wildtype mice (data not shown).

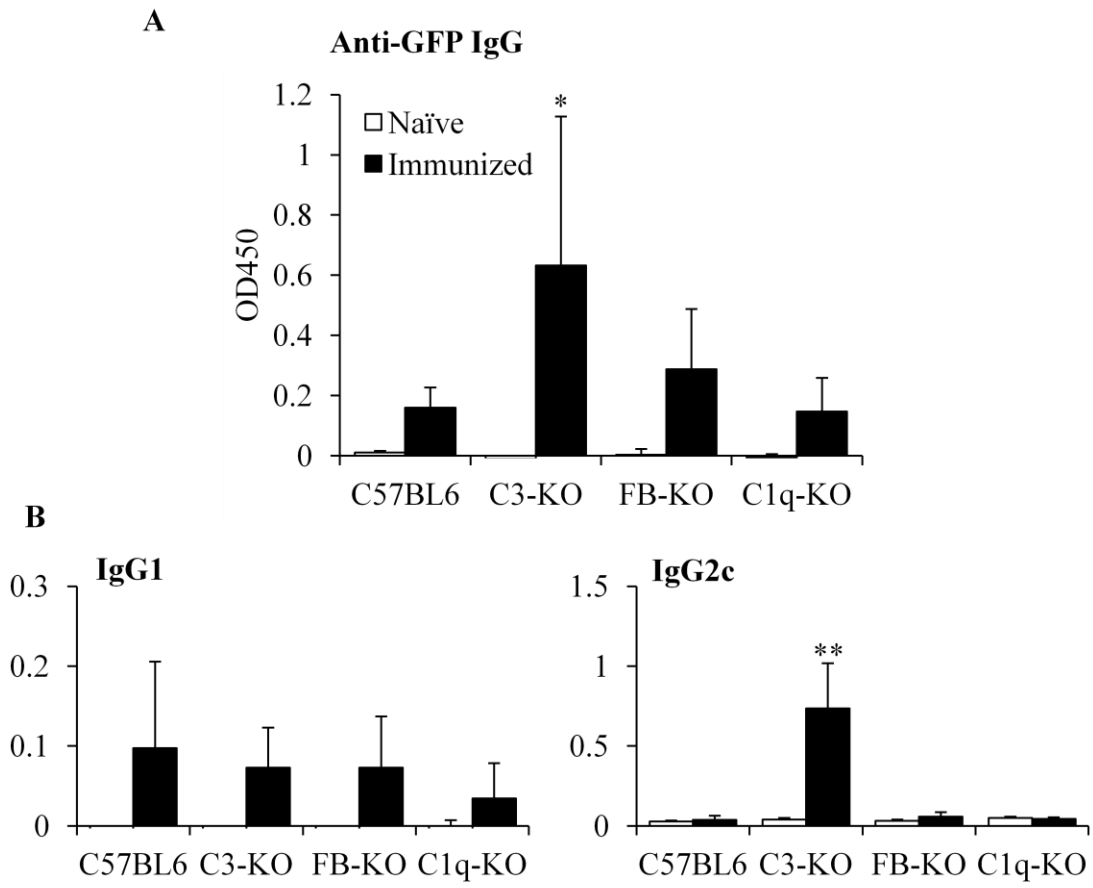
Capsid display of DAF minimizes Ad dependent complement activation *in vivo*

Ad capsid mediated complement activation has been indirectly associated with toxicity, as many of these toxicities can be avoided when Ad vectors are injected into C3-KO mice.[66,67,101] Based on our results we hypothesized that genetic engineering of a rAd to display a natural complement inhibitor (DAF) as a C-terminal fusion on an Ad-capsid protein (pIX) would mitigate Ad capsid induced complement.

We constructed Ad viruses displaying DAF in a native (N terminus exposed to the extracellular milieu) conformation by producing a synthetic cDNA encoding the primary amino acid sequence of DAF in reverse order relative to the native DAF gene sequence. This was then sub-cloned into the C-terminus of the pIX open reading frame resulting in a re-orientation of the human DAF relative to the Ad capsid (Ad5-GFP-IX-dDAF_REO).

Animals were either mock injected or intravenously injected with $7.5x10^{10}$ vp per mouse with Ad5 expressing GFP (Ad5-GFP), Ad displaying GFP on pIX (Ad5-IX-GFP), or Ad5-GFP-IX-dDAF_REO. 6hpi liver dysregulation was measured as performed previously. Multiple transcripts were found to be significantly decreased in Ad5-GFP-IX-dDAF_REO treated wildtype mice as compared to Ad5-GFP treated wildtype mice (*CXCL-9, ICAM, IRF-7, IRF-8, JAK-1, JAK-3, MyD88, NF-κB-RelA, OAS-1a, TBK-1, TLR-6,* and *TRAF2bp*) (Table 3).

When we analyzed levels of cytokines in the plasma at 6hpi we found that mice treated with Ad5-GFP-IX-dDAF_REO had significantly less induction of IL-12(p40) and MCP-1 (at low dose) and MIP-1β (at high dose) as compared to conventional Ad5 vectors (Figure 8).



C

	IgG2c/IgG1 (Th1/Th2)
C57BL6	0.39
C3-KO	10.08
FB-KO	0.79
C1q-KO	1.28

Figure 7: Anti transgene (GFP)-specific antibodies are C3-dependent. C57BL/6 (N=3 naive and N=7 immunized), C3-KO (N=4 naive and N=9 immunized), Factor B knockout (FB-KO) (N=4 naive and N=11 immunized) and C1q-KO (N=3 naïve and N=5 immunized) mice were injected with $7.5x10^{10}$ vp per mouse of rAd5-GFP. (a) At 30 d.p.i., plasma was analyzed for anti-GFP-specific total IgG antibodies at the appropriate dilution (1:800). Bars represent mean ± s.d. (b) Plasma was also analyzed for various IgG subclasses. Data points represent mean ± s.d. *P<0.05 and **P<0.01 indicate a statistical difference between C57BL/6 and each respective genotype. (c) The IgG2c/ IgG1 ratio, indicative of a Th1/Th2 response, was calculated based on subclass titering.

Table 3: Ad5 induced gene expression in a liver of C57BL/6_WT mice (fold over Mock, 6 hpi)

	Mock	Ad5-GFP	Ad5-IX-dGFP	Ad5-GFP-IX-dDAF4	Ad5-GFP-IX-dDAF4-REO
ADAR	1.0 ± 0.3	2.2 ± 0.4	2.6 ± 0.2	1.9 ± 0.5	2.4 ± 0.8
CD14	1.0 ± 0.2	6.0 ± 1.5	10.0 ± 5.4	6.2 ± 3.2	3.4 ± 2.4
CXCL-9	1.0 ± 0.2	10.7 ± 3.0	17.4 ± 5.7	10.6 ± 1.2	**5.1 ± 1.3**
DAF	1.0 ± 0.1	1.5 ± 0.5	1.2 ± 0.2	1.5 ± 0.3	1.1 ± 0.2
e-Selectin	1.0 ± 0.4	4.0 ± 0.6	5.7 ± 1.0	3.7 ± 1.3	3.5 ± 0.8
ICAM	1.0 ± 0.3	4.2 ± 0.4	3.8 ± 0.8	3.6 ± 0.7	**2.2 ± 0.3**
IFNα	1.0 ± 0.4	1.3 ± 0.4	1.4 ± 0.1	0.8 ± 0.5	1.5 ± 0.5
IFNβ	1.0 ± 0.4	1.2 ± 0.4	1.5 ± 0.3	0.8 ± 0.5	1.7 ± 0.7
IRF-7	1.0 ± 0.4	16.4 ± 3.6	19.6 ± 2.7	16.5 ± 5.0	**10.9 ± 2.2**
IRF-8	1.0 ± 0.2	3.7 ± 1.1	3.6 ± 0.8	3.2 ± 1.1	**1.9 ± 0.4**
Jak-1	1.0 ± 0.3	2.3 ± 0.6	1.6 ± 0.3	1.8 ± 0.6	**1.3 ± 0.3**
Jak-3	1.0 ± 0.2	2.8 ± 0.8	1.7 ± 0.2	1.9 ± 1.0	**1.2 ± 0.4**
MyD88	1.0 ± 0.3	7.0 ± 1.4	8.9 ± 1.3	6.9 ± 1.9	**4.0 ± 0.9**
NFkB-RelA	1.0 ± 0.2	2.1 ± 0.4	2.6 ± 0.6	1.7 ± 0.4	**1.2 ± 0.2**
NOD-1	1.0 ± 0.2	2.4 ± 0.4	2.6 ± 0.3	2.7 ± 0.5	1.9 ± 0.3
NOD-2	1.0 ± 0.4	2.0 ± 0.4	2.3 ± 0.2	1.5 ± 0.4	2.4 ± 0.7
OAS-1a	1.0 ± 0.5	12.1 ± 1.0	14.5 ± 1.4	12.9 ± 4.8	**5.9 ± 1.6**
SOCS-1	1.1 ± 0.7	12.6 ± 4.0	12.7 ± 4.1	11.7 ± 4.7	6.7 ± 2.3
SOCS-3	1.1 ± 0.6	5.4 ± 1.8	5.8 ± 1.4	4.6 ± 0.9	4.0 ± 0.9
TBK-1	1.0 ± 0.2	6.5 ± 0.8	6.1 ± 1.7	6.7 ± 1.9	**3.5 ± 0.7**
TLR-2	1.0 ± 0.4	45.2 ± 14.3	86.7 ± 30.0	48.4 ± 16.0	29.3 ± 7.6
TLR-3	1.0 ± 0.2	19.2 ± 2.9	29.7 ± 4.0	25.3 ± 7.0	15.0 ± 6.0
TLR-6	1.0 ± 0.3	6.0 ± 1.6	4.2 ± 0.6	6.0 ± 2.6	**3.0 ± 0.9**
TLR-9	1.0 ± 0.2	2.0 ± 0.4	2.5 ± 0.3	1.9 ± 0.4	2.5 ± 0.8
TRAF2bp	1.1 ± 0.5	16.3 ± 7.8	36.2 ± 6.3	19.7 ± 6.3	**8.2 ± 1.9**
VCAM	1.0 ± 0.4	2.1 ± 0.8	1.9 ± 0.1	1.5 ± 0.7	1.7 ± 0.6

Table 3: Medium dose of virus used for injection: $0.75x10^{11}$ vp/mouse. 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. N=4 for mock injected groups, N=5 for virus injected groups. Significant differences compared to WT_Mock are highlighted in light grey color. Significant reductions of transcriptional activation compared to WT_Ad5-GFP group are indicated in table with black frame and boldface font; significant induction of transcriptional activation compared to WT_Ad5-GFP group are indicated by dark grey color. Gene names highlighted in bold were shown to be induced in C3 dependent manner after systemic Ad injection.

Figure 8

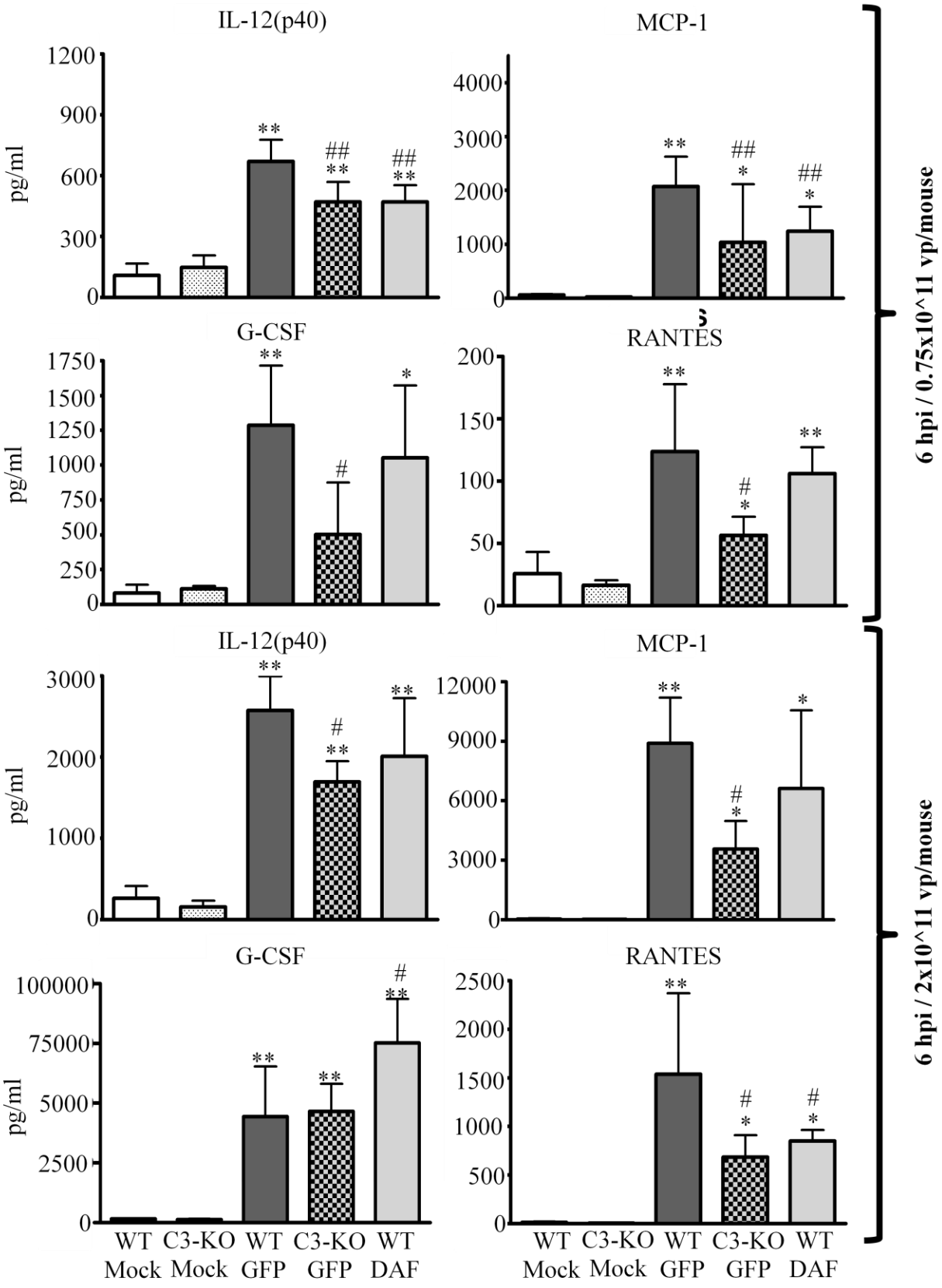

IL-12(p40)
MCP-1
G-CSF
RANTES
IL-12(p40)
MCP-1
G-CSF
RANTES
pg/ml
6 hpi / 0.75x10^11 vp/mouse
6 hpi / 2x10^11 vp/mouse
WT Mock
C3-KO Mock
WT GFP
C3-KO GFP
WT DAF

Figure 8 cont'd: Ad5 vectors "capsid-displaying" retro-DAF complement inhibitor significantly reduce Ad dependent activation of endothelial cells in C57BL/6 mice. C57BL/6 WT and C3-KO mice were intravenously injected with **(A)** $0.75x10^{11}$ vp/mouse (medium dose) or **(B)** $2x10^{11}$ vp/mouse (high dose) of Ad5 based control and experimental vectors. 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. *, ** - Indicate values, statistically different from those in Mock injected animals of both genotypes, 30 $p<0.05$, $p<0.001$ respectively. # - Indicate significant reduction of EC activation as compared to both WT_Ad5-GFP and WT_Ad5-IX-dGFP injected mice, $p<0.05$. Note that levels of EC activation triggered by Ad5-GFP-IX-dDAF_REO novel Ad closely parallel the levels observed in C3-KO mice treated with conventional Ad5-GFP vector (shaded bars).

However, multiple cytokines and chemokines known to be induced by rAds were not reduced by display of DAF on the Ad capsid (G-CSF, RANTES, KC, and IL-6) (Figure 8).

2.3 Discussion:

In these experiments we focused upon elucidating Ad interactions with the complement system, and how these interactions shape important Ad-induced immune responses. These discoveries have implications in gene transfer protocols as well as Ad vaccines, as evidenced by our successful display of DAF on the surface of an Ad capsid resulting in reduced Ad-induced immune responses. We and others have demonstrated Ad opsonization by complement.[66,119] Here we attempted to tease out which complement pathways are involved in multiple aspects of Ad-induced innate immune responses.

We show that intravenous injection of rAd induces the transcription of multiple genes in the liver in a C3-dependent manner inclusing *MyD88,* many *TLR*s, and endothelial activation markers like *VCAM* and *ICAM*. However, we were unable to delineate the specific roles of CP and AP in these responses. We did however detect a negative role for FB in regulation of *ADAR, IRF-7, OAS-1a, TLR2,* and *TRIM30* as well as a negative role for C1q in regulation of *CXCL9* and *E-selectin*. These data suggest that CP and AP of complement actually modulate rather than synergistically amplify these responses. Furthermore, we found that many of these responses are suppressed by the presence of functional mCR1/2 inclusing *ADAR, ICAM, MyD88, OAS-1a, SOCS-1, TLR2, TLR3, TRAF2bp,* and *VCAM*.

We and others have shown that many cytokines and chemokines are induced as a result of intravenous Ad administration.[67,103,120,121] We show here that at 6 hpi, Ad inductions of G-CSF, MCP-1, and RANTES are dependent on the presence of functional C3. We were however unable to find a distinct role for CP or AP in these responses. While the variability of the mice and sensitivity of the assay played roles in our inability to delineate specific roles for CP or AP in mediating these responses, the results may suggest redundancy in the CP and AP

pathways when sensing Ads, a hypothesis that can only be confirmed in future, more expansive studies. However, we were able to show that mCR1/2 is responsible for down-regulating G-CSF, MCP-1, and RANTES.

Given that cytokine and chemokine secretion modulates T and B cell proliferation and activation and, as we have shown, many Ad-induced chemokines and cytokines are complement dependent, we sought to elucidate the role of complement in initiating humoral immune responses. Here we show that induction of Ad specific neutralizing antibody is dependent on functional C3. While we could not determine the impact of CP (C1q) or AP (FB) on induction of Ad specific neutralizing antibody due to assay insensitivity, there are indications that both CP and AP are required for maximal antibody induction. For example, further analysis revealed that induction of total anti-Ad IgG and IgG3 was dependent upon functional C3 and FB. Additionally, analysis of immunoglobulin sub-isotypes revealed that CP (C1q-KO)-deficient mice were unable to stimulate titers of IgG1 or IgG2c equivalent to wildtype mice, indicating a role for CP in stimulating the maximal anti-Ad antibody response. We found striking differences in Th1/Th2 antibody ratios in C3-KO animals. These data suggest that complement plays a significant role in modulating the balance between the Th1 and Th2 antibody responses to Ad vectors. This dramatic skewing of the IgG2a/IgG1 ratio could be responsible for the observed decrease in anti-Ad neutralizing antibody titers in these animals, as it has been shown that a balanced IgG2a/IgG1 ratio is associated with improved protection.[122] However, it is possible that C3-KO mice produce more IgG2c compensatory to the lack of C3, as IgG2c (analogous to IgG2a in function) is known to assist in the binding of C3 to a pathogen. While functionally C3 was required for an anti-Ad IgG response, complement does not interfere with anti-transgene humoral inductions. In fact, transgene specific antibody titers were actually higher in C3-KO

mice. On the other hand, similar Ad treatments of mCR1/2-KO mice induced lower titers of transgene specific antibody. Decreased antigen expression by Ads in these latter animals is likely responsible for mCR1/2-KO animal's low titers of anti-transgene antibody as we observed equivalent transduction but lower levels of transgene expression at 24 hpi and 28 dpi (data not shown). It is possible that the altered cytokoine and chemokine responses indirectly resulted in decreased CMV-driven expression of the transgene from the Ad vector, a phenomenon previously noted by our group in other studies.[100]

Unexpectedly, mCR1/2-KO mice had Ad specific neutralizing antibody titers equivalent to mock injection despite the presence of stronger Ad-induced innate responses. Lack of anti-Ad neutralizing antibody titers in both mCR1/2-KO and C3-KO mice positively correlated with lack of B cell activation as measured by analyzing percentages of splenocytes that are CD19+ CD69+ (data not shown). Since Ad treated C3-KO exhibited decreased levels of proinflammatory cytokines while mCR1/2-KO demonstrated increased levels in many of the same cytokines when compared to wildtype mice, cytokine levels *per se* cannot be the reason for the lack of anti-Ad neutralizing antibody. Instead, we propose a mechanism where C3-opsonization of Adenovirus allows for interaction with mCR1/2 on B cells activating the B cell to produce anti-Ad neutralizing antibody. Therefore, blockade of C3 opsonized Ad from interaction with CR1/2 (like in C3-KO mice) could be preventing activation of B cells that would otherwise produce anti-Ad capsid antibody while still allowing induction of transgene specific antibody responses by T cell dependent B cell activation.

Despite the necessity of complement to have been activated before DAF can "decay" C3 convertases, we were able to provide evidence that recombinant DAF fusion to the Ad-capsid can prevent complement activation. As evidenced by intravenous injection of "DAF-displaying"

Ads inhibition of complement mediated responses inclusive of observed reductions in pro-inflammatory gene expression and decreased cytokine release when compared to intravenous injection of control Ads.

These data cumulatively describe rAd interactions with multiple components of the complement system and their involvement in generating humoral responses. We have confirmed the roles of C3, C4, FB and mCR1/2 in regulating multiple Ad-induced innate immune responses. Furthermore, we describe a novel Ad successfully engineered to actively reduce Ad-trigged innate immune responses by modulating interactions of the Ad capsid with the complement system. It is important to understand these multiple interactions, as this knowledge will foster safer and more efficacious use of Ad-based vectors in the same vein as our "DAF-displaying" Ad vector.

Chapter 3:

Efficacy when utilizing Adenovirus serotype 4 and 5 vaccines expressing Circumsporozoite protein in naïve and Ad5 immune mice

3.1 Introduction:

Despite use of prophylactic medications and vector control, malaria continues to be one of the world's most deadly health concerns claiming the lives of almost 1 million people annually. The protozoan parasite, *P. falciparum*, accounts for about 90% of these deaths.[1] Numerous *P. falciparum* targeted vaccine studies are currently underway in efforts to eliminate this dangerous killer. The *P. falciparum* derived CSP is the most studied and commonly used antigen for the purpose of developing a vaccine against malaria.[30,123,124,125,126] CSP is abundant on the surface of the sporozoite, and is also present in the plasma membrane and cytosol of plasmodium infected hepatocytes.

Of the several malaria vaccine vectors that target CSP, the most successful to date is a vaccine formulation that consists of a novel fusion protein between the Hepatitis B surface protein (HBsAg) and CSP, and additional adjuvants. This formulation, referred to as RTS,S/AS01B, is currently in a phase 3 clinical trial.[127] This vaccine has been able to confer protection to 56% of vaccinated individuals.[36,37,38,39,44,46,124] Although promising, the results also suggest that more potent immune responses may be required to achieve higher levels of protection. For this reason other vectors and immunogenic strategies incorporating CSP are being pursued in efforts to develop a highly efficacious, malaria specific vaccine.

Recombinant adenovirus serotype 5 (rAd5) based vaccines are important in this regard as they have been confirmed to elicit potent adaptive responses against expressed transgenes.[71,128,129] Multiple studies have utilized rAd5s genetically engineered to express CSP in human and mouse models of malaria.[75,90,126] However, pre-existing Ad5 immunity is common in regions where malaria is endemic, and the presence of neutralizing antibodies against Ad5 has been shown to hinder Ad5 based vaccine efficacy.[83,84,85] We and others have

hypothesized that the use of alternative serotype based rAds may induce improved immunogenic responses to antigens irrespective of pre-existing Ad5 immunity, for example in HIV vaccine development.[130,131] Use of alternative serotype based Ad vectors can serve other important purposes aside from stimulating immune responses in Ad5 immune patients. Heterologous prime boost regimens where the prime and boost vaccinations are derived from two different Ad serotypes based vaccines can provide greater inductions of immunity than homologous prime boosting with a single Ad serotype based vaccine.[47,92,97]

In this context, Ad4 based vectors may be promising for use in malaria specific applications. The efficacy and safety of Ad4 vaccine platforms has been established. For instance, as the principal serotype causing Acute Respiratory Disease (ARD) in military recruits, an orally administered, live Ad4 virus was utilized for decades in vaccinations of recruits against ARD.[132,133,134,135] More recently, Ad4 based vaccines have been successfully utilized in HIV vaccine strategies in dog and chimpanzee models.[130,131] Here we analyze the ability of an Ad4 based malaria specific vaccine expressing CSP to stimulate potent immune responses when used in homologous or heterologus prime boost regimens with an Ad5 vaccine also expressing CSP, both in the context of Ad5 naïve and Ad5 immune animals.

3.2 Results:

rAds of serotype 4 and serotype 5 were engineered to express a codon optimized form of CSP using methods previously described.[100,136] Four vaccination regimens were utilized; 1. Ad5-CSP/Ad5-CSP, 2. Ad5-CSP/Ad4-CSP, 3. Ad4-CSP/Ad4-CSP, and 4. Ad4-CSP/Ad5-CSP, where the Ad serotype used in the priming vaccination is immediately followed by the serotype of the boosting vaccination in each vaccine regimen or group. Initially, (day 0) Ad naïve BALB/cJ mice were injected IM with either Ad4-CSP or Ad5-CSP ($1x10^{10}$ vp/mouse) (n=10). 14 days later 5 mice from each treatment group received a homologous boost (same Ad-CSP serotype vaccine) of $1x10^{10}$ vp/mouse, the other 5 mice from the same group received a heterologous boosting vaccination of $1x10^{10}$ vp/mouse with the alternative Ad-CSP serotype vaccine. 28 days after the priming vaccinations, splenocytes were harvested from the animals and stimulated with the CSP derived peptide (NYDNAGTNL) and the number of IFNγ secreting splenocytes were quantified by ELISpot. While every vaccine treatment resulted in a significant increase in the numbers of CSP responsive INFγ secreting splenocytes when compared to non-vaccinated animals, the Ad4-CSP/Ad5-CSP heterologous prime boosting vaccine treatment group induced significantly higher numbers of IFNγ secreting splenocytes than any other treatment group (Figure 9A). These results were further supported by intracellular staining with antibodies against CD3, CD8, and IFNγ, as the percentage of CSP responsive CD3+ CD8+ IFNγ+ cells present in splenocytes derived from mice vaccinated with the Ad4-CSP/Ad5-CSP regimen were significantly higher when compared to splenocytes from animals treated with the other vaccination strategies (Figure 9B). Intracellular staining was also performed to enumerate

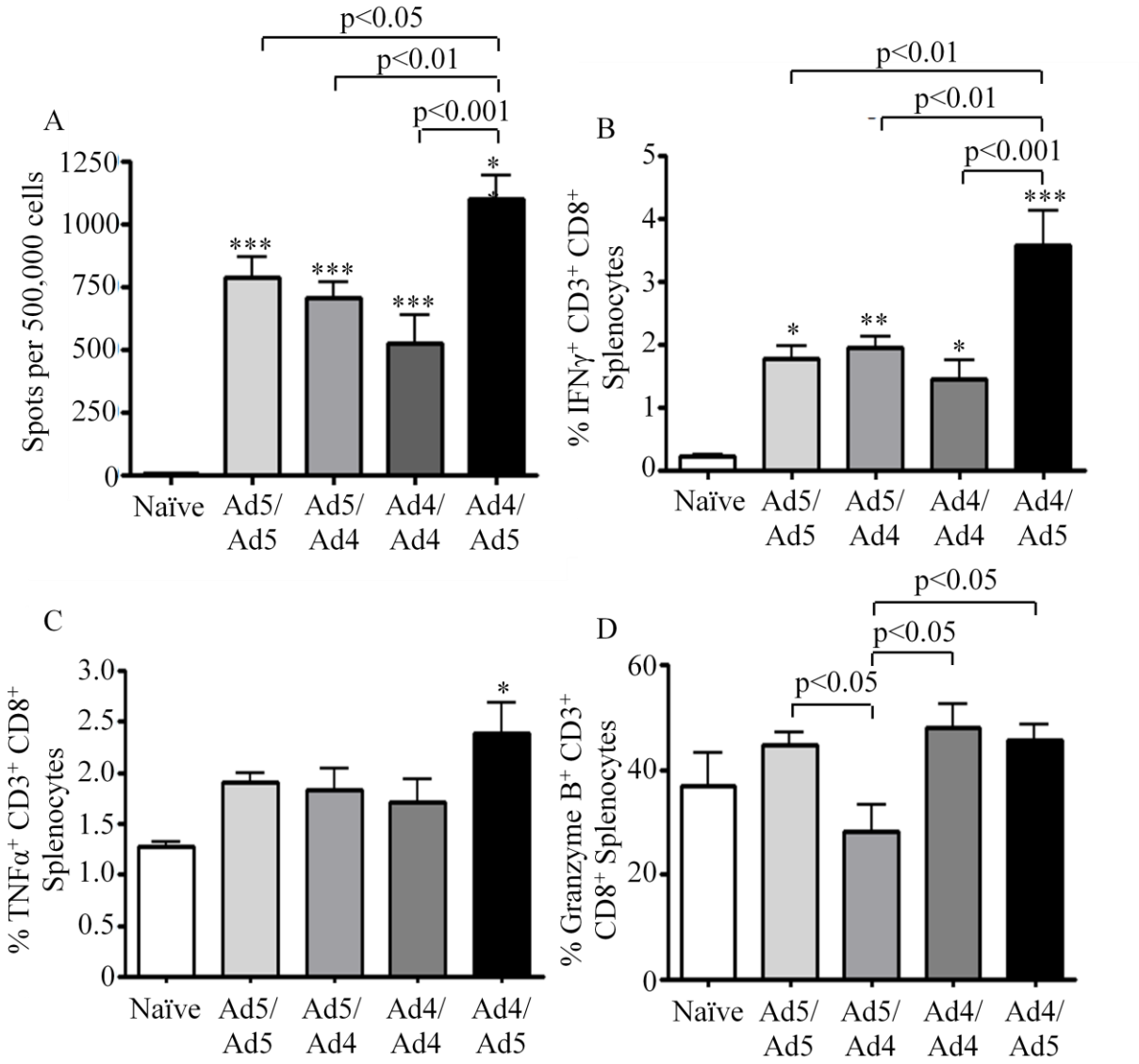


Figure 9: Ad4-CSP/Ad5-CSP heterologous prime boost results in improved quality of T cell response. A prime injection of $1x10^{10}$ vp/mouse Ad4-CSP followed by a boost of $1x10^{10}$ vp/mouse of Ad5-CSP resulted in significantly more IFNγ secretion by splenocytes measured by ELISpot (A) and CD3+ CD8+ T cells measured by flow cytometry (B). Ad4-CSP/Ad5-CSP was the only treatment to stimulate a significantly higher percentage of TNFα production as compared to unvaccinated animals (C). Cells were stained with CD3-APC-Cy7, CD8-Alexa flour700, TNFα-PE-Cy7, IFNγ-FITC, and Granzyme B-APC. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, P<0.05, P<0.01, P<0.001.

the frequency of TNFα and Granzyme B producing CD8+ T cells present in the spleens of the variously vaccinated animals. Again, the Ad4-CSP/Ad5-CSP experimental vaccination regimen appeared to confer the most robust immune responses against CSP, as it was the only treatment to induce significantly higher percentages of CSP responsive CD3+ CD8+ TNFα+ cells as compared to non-vaccinated animals (Figure 9C). Interestingly, none of the vaccination strategies induced significantly higher percentages of CSP responsive CD3+ CD8+ Granzyme B+ cells as compared to non-vaccinated animals; however, animals from the Ad5-CSP/Ad4-CSP vaccination group had significantly lower percentages of CD3+, CD8+, Granzyme B+ T cells as compared to all other treatment groups (Figure 9D).

As detected by use of the NYDNAGTNL tetramer, each of the vaccination regimens induced significantly higher percentages of CSP specific CD3+ CD8+ T cells in the spleen as compared with non-vaccinated control animals ($p<0.001$) (Figure 10A). Of the four groups, the Ad4-CSP/Ad5-CSP heterologous prime boosting regimen induced the lowest percentage of CD3+ CD8+ tet+ T cells, a decrease that was statistically significant as compared to both the Ad5-CSP/Ad5CSP and the Ad5-CSP/Ad4-CSP treatment groups ($p<0.01$; $p<0.05$ respectively). When peripheral blood mononuclear cells (PBMCs) from the vaccinated mice were similarly analyzed, again all groups of vaccinated mice had significantly increased numbers of CD3+ CD8+ tet+ T cells present as compared to non-vaccinated mice. However, the Ad4-CSP/Ad4-CSP treated animals elicited the lowest percentages of CD3+ CD8+ tet+ T cells of the four groups, this decrease reaching statistical significance when this group was compared to both the Ad5-CSP/Ad5-CSP and Ad5-CSP/Ad4-CSP treatment groups ($p<0.05$ for each group) (Figure 10B).

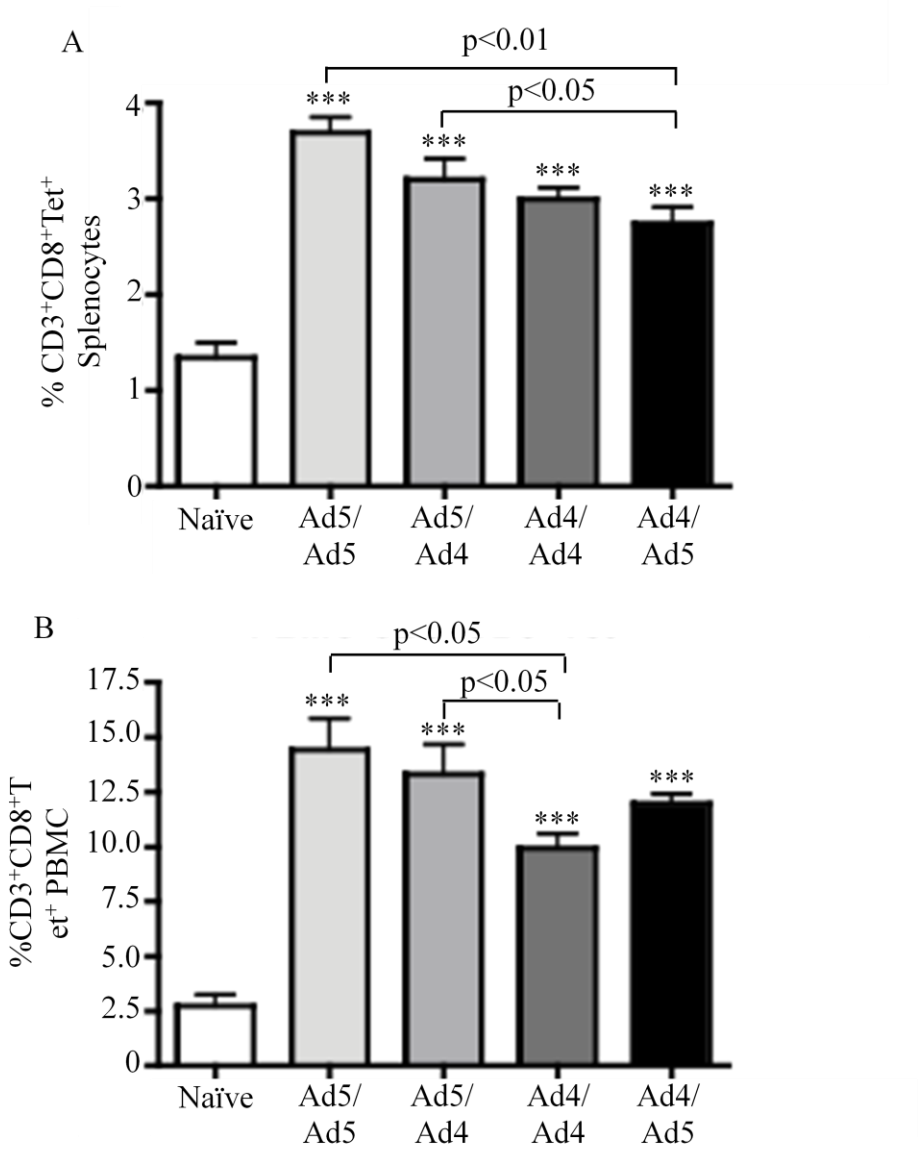


Figure 10: Ad5-CSP/Ad5-CSP vaccination resulted in higher percentage of tetramer positive CD8+ T cells than Ad4-CSP/Ad5-CSP in the spleen. Splenocytes and PBMCs were collected two weeks after final vaccination. All vaccination regimens resulted in significantly higher percentage of CD3+ CD8+ NYDNAGTNL tetramer positive T cells in the spleen (A) and circulating blood (B) as measured by flow cytometry, cells were stained with CD8-Alexa flour700, CD3-APC-Cy7, and CSP (NYD)-Tetramer-PE. Ad5-CSP/Ad5-CSP stimulated a higher percentage of CD3+ CD8+ tet+ than Ad4-CSP/Ad5-CSP treated animals in the spleen (A) and higher percentage of CD3+ CD8+ tet+ than Ad4-CSP/Ad4-CSP in the circulating blood (B). Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$.

Ad vectors are known to elicit strong Tem cell responses thought to be due to more persistent antigen production. This is important in the context of a malaria vaccine as Tem cell responses have been shown to be beneficial in protecting against liver stage malaria.[25] We compared the magnitude of CSP-specific central memory and effector memory CD8+ T cell responses that each of the various prime boost regimens induced in splenocytes and PBMCs harvested 14 days after the boosting vaccinations. All prime boost regimens demonstrated much higher percentages of CSP specific Tcm and Tem cells than was observed in non-vaccinated animals as indicated by the percent of CD127+ CD62L+ and CD127+ CD62L- tet+ T cells present in the splenocytes (Figure 11B-C). We also analyzed the percentage of CSP specific Tcm and Tem cells circulating in the blood and found the Ad5-CSP/Ad4-CSP vaccination group was the only group that had a significantly higher percentage of CSP specific Tcm cells in circulating blood when compared to non-vaccinated animals, while all vaccinated animals had higher percentages of CSP specific Tem cells present in this compartment as compared to non-vaccinated animals (Figure 11D-E). When we analyzed memory phenotypes by gating on tetramer positive cells first and then gating for CD127 and CD62L we found that the tetramer positive cells of all groups had similar memory phenotypes as defined by comparison of the percentages of tet+ cells that were Tem and those that were Tcm cell (data not shown).

Splenocytes from all treatments were analyzed for the presence of anti-Ad4 and/or anti-Ad5 antigen specific IFNγ secreting T cells by ELISpot. There was no significant cross stimulation between the two serotypes detected by this assay, as animals that received Ad4-CSP/Ad4-CSP treatment had significantly less Ad5 specific IFNγ secreting cells than all other vaccination regimens, and were not significantly different than naïve animals. Likewise, animals that were vaccinated with Ad5-CSP/Ad5-CSP had significantly less Ad4 specific IFNγ secreting

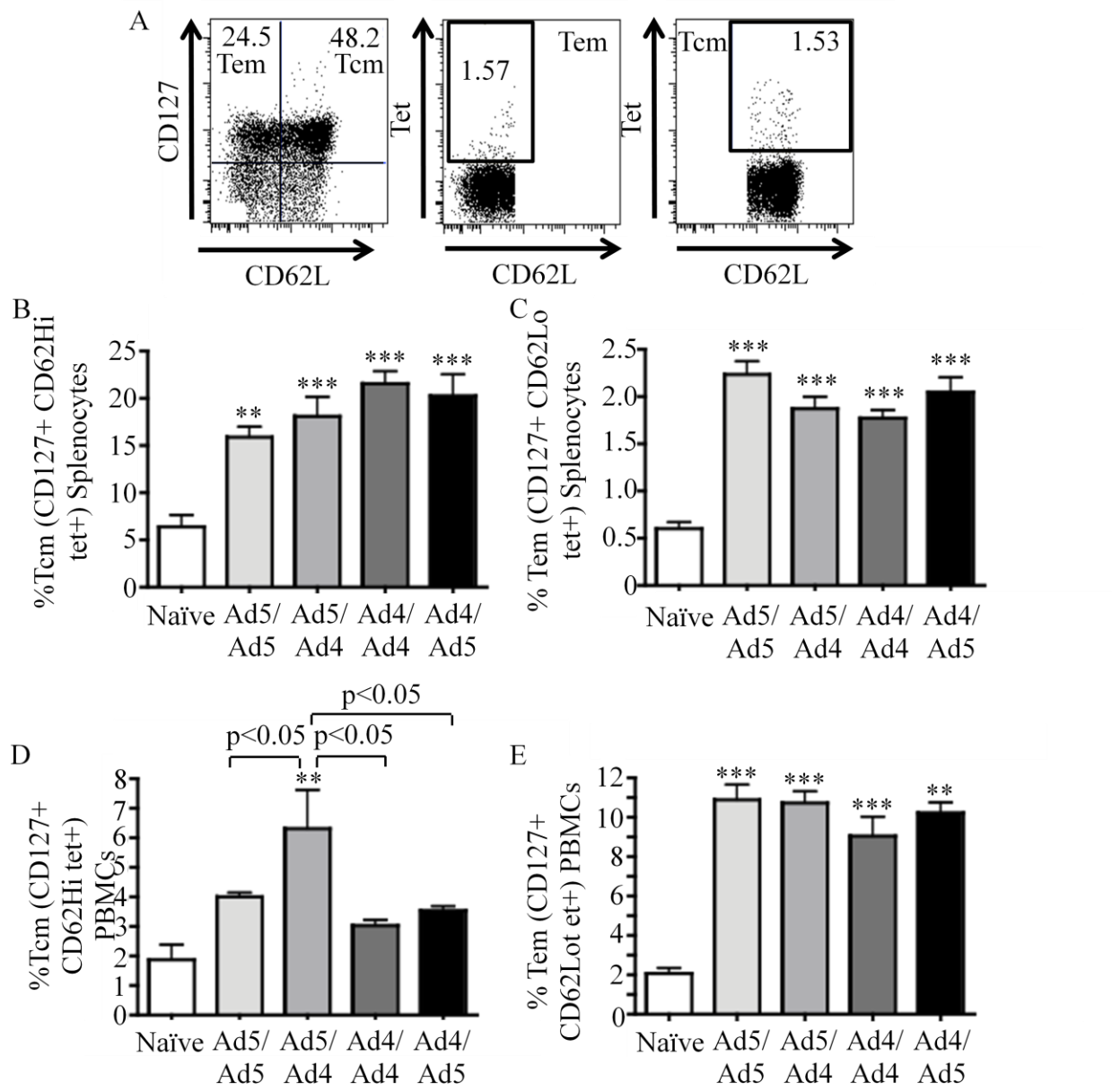


Figure 11: Memory responses triggered by vaccination with homologous and heterologous prime boost regimens utilizing Ad4-CSP and Ad5-CSP in Ad naïve mice. Splenocytes (B-C) and PBMCs (D-E) were collected two weeks after final vaccination. Cells were stained for CD62L-V450, CD127-PerCP Cy5.5, and CSP (NYD) tet-PE. CSP specific central memory T cells were determined as CD62L+ CD127+ cells that are tet+ and effector memory cells are CD62Llo CD127+ cells that are tet+. Provided above is an example of gating (A). Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, **, *** denotes significance over naïve, P<0.01, P<0.001.

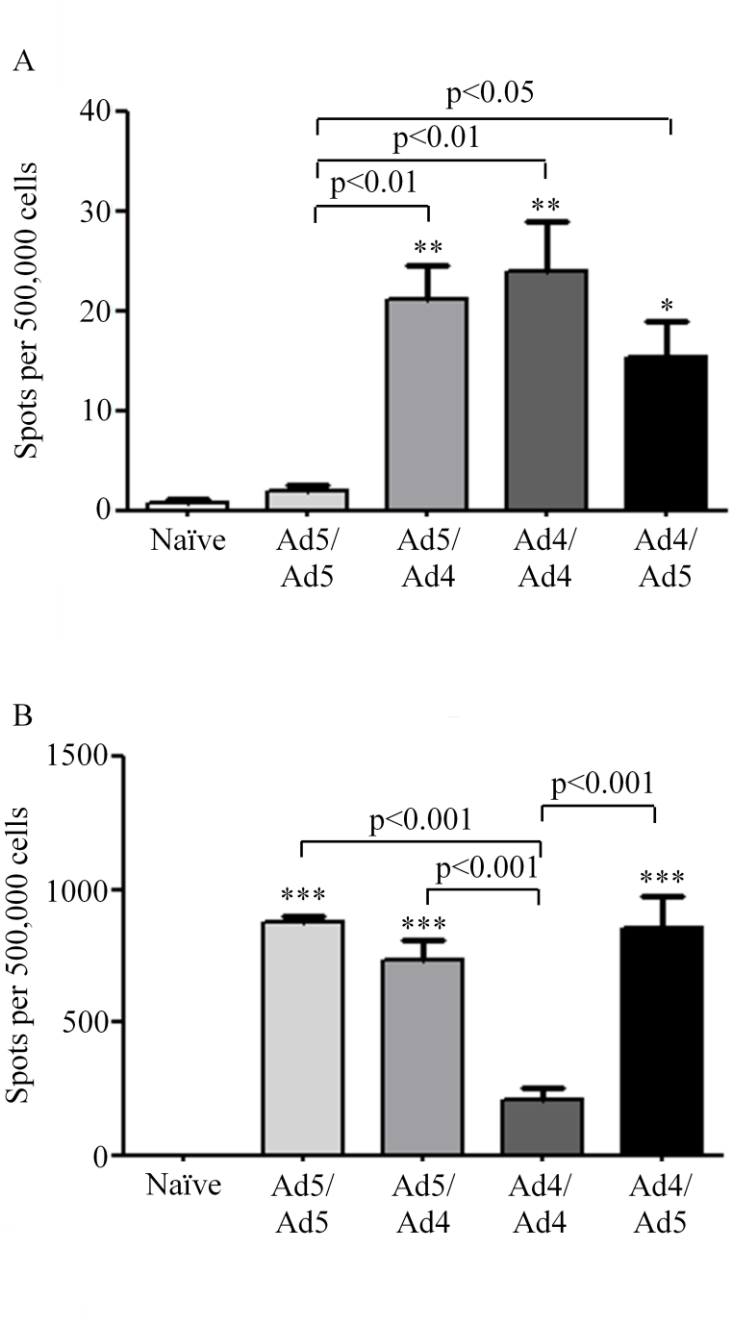


Figure 12: Ad4-CSP/Ad4-CSP and Ad5-CSP/Ad5-CSP vaccinated animals have no significant cross stimulation of splenocytes. Splenocytes were collected 14 days post final vaccination and were stimulated with either heat inactivated Ad4-Null or heat inactivated Ad5-Null. Animals treated with Ad5-CSP/Ad5-CSP were not significantly different from naïve animals when stimulated with heat inactivated Ad4-CSP as measured by IFNγ secretion by ELISpot. Likewise, animals treated with Ad4-CSP/Ad4-CSP were not significantly different from naïve animals when stimulated with heat inactivated Ad5-CSP as measured by IFNγ secretion by ELISpot. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$.

cells than animals that received Ad4-CSP injections and were also not significantly different than naïve animals (Figure 12).

We measured how prime boost vaccinations combining Ad4-CSP and Ad5-CSP might affect CSP specific antibody production as compared to homologous prime boosts using the same vectors. Plasma was collected from BALB/cJ mice injected with the four prime boost regimens and 28 days post initial injection was tested by ELISA for total anti-CSP IgG antibody levels. Mice from the Ad4-CSP/Ad4-CSP vaccination group demonstrated significantly lower plasma levels of IgG anti-CSP relative to unvaccinated animals at the 1:100 dilutions ($p<0.05$) (Figure 13). All other vaccination regimens induced significantly higher levels of anti-CSP IgG as compared to both the non-vaccinated animals and animals receiving the Ad4-CSP/Ad4-CSP regimen ($p<0.001$) (Figure 13). Similar trends were observed when sub-isotyping analysis was performed for anti-CSP IgG1, IgG2a, IgG2b, and IgG3 levels (Figure 14). We also analyzed the IgG2a/IgG1 ratio as an indirect assessment of Th1 vs. Th2 immune responses in animals treated with the vaccine regimens; however the Th1/Th2 ratio was not significantly different with use of any of the vaccination regimens (Figure 15).

To assess the efficacy of Ad4 based vaccination regimens to induce functional, CSP specific cytolytic T cell responses, we measured CSP specific cytotoxic T lymphocyte killing *in vivo*. BALB/cJ mice were vaccinated with the homologous and heterologous prime boost regimes as described above. 28 days after the initial vaccination, splenocytes from naïve mice were collected and incubated with either a high concentration of CFSE (10μM) and NYDNAGTNL peptide or a low concentration of CFSE (1μM) and a non-specific peptide. Stained and peptide pulsed splenocytes were then mixed at equal quantities and injected intravenously into vaccinated or non-vaccinated animals. After 18 hours, CSP specific cell

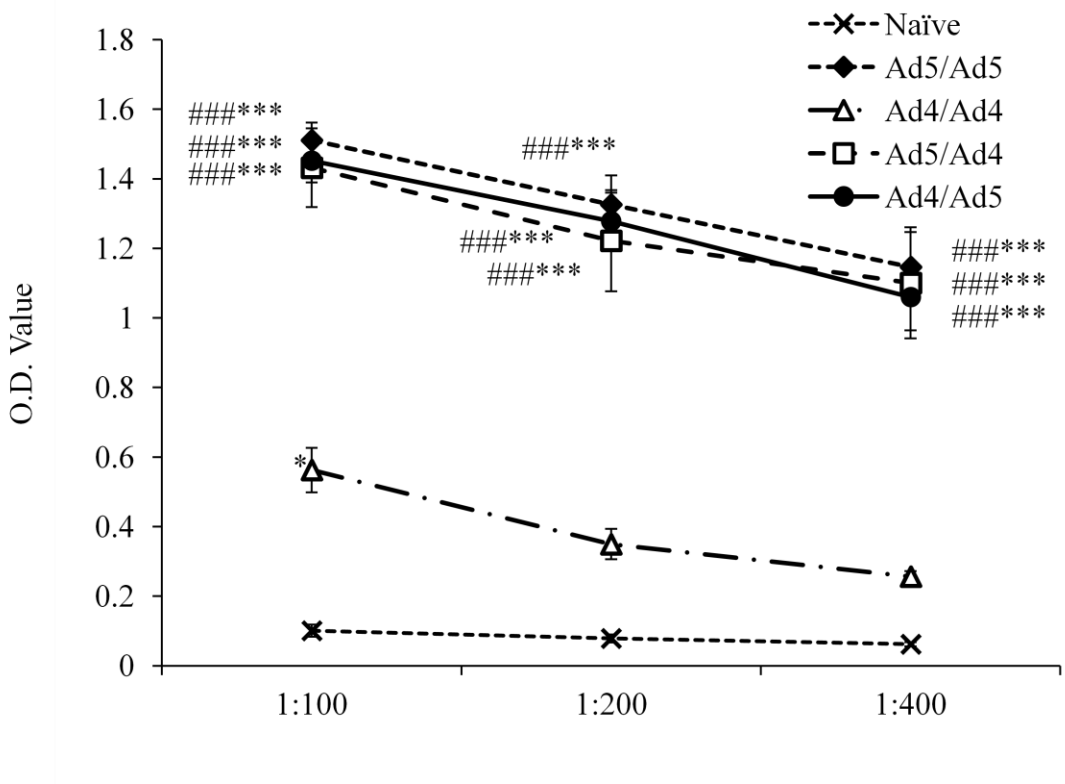


Figure 13: All vaccinations stimulated significantly higher anti-CSP total IgG than unvaccinated and AD4-CSP/Ad4-CSP vaccination in Ad naïve animals. Plasma was collected 14 days post the final vaccination. Plasma was diluted 1:100, 1:200, and 1:400 and measured for total IgG against CSP by ELISA. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$. ### denotes significance over Ad4-CSP/Ad4CSP treatment, $P<0.001$.

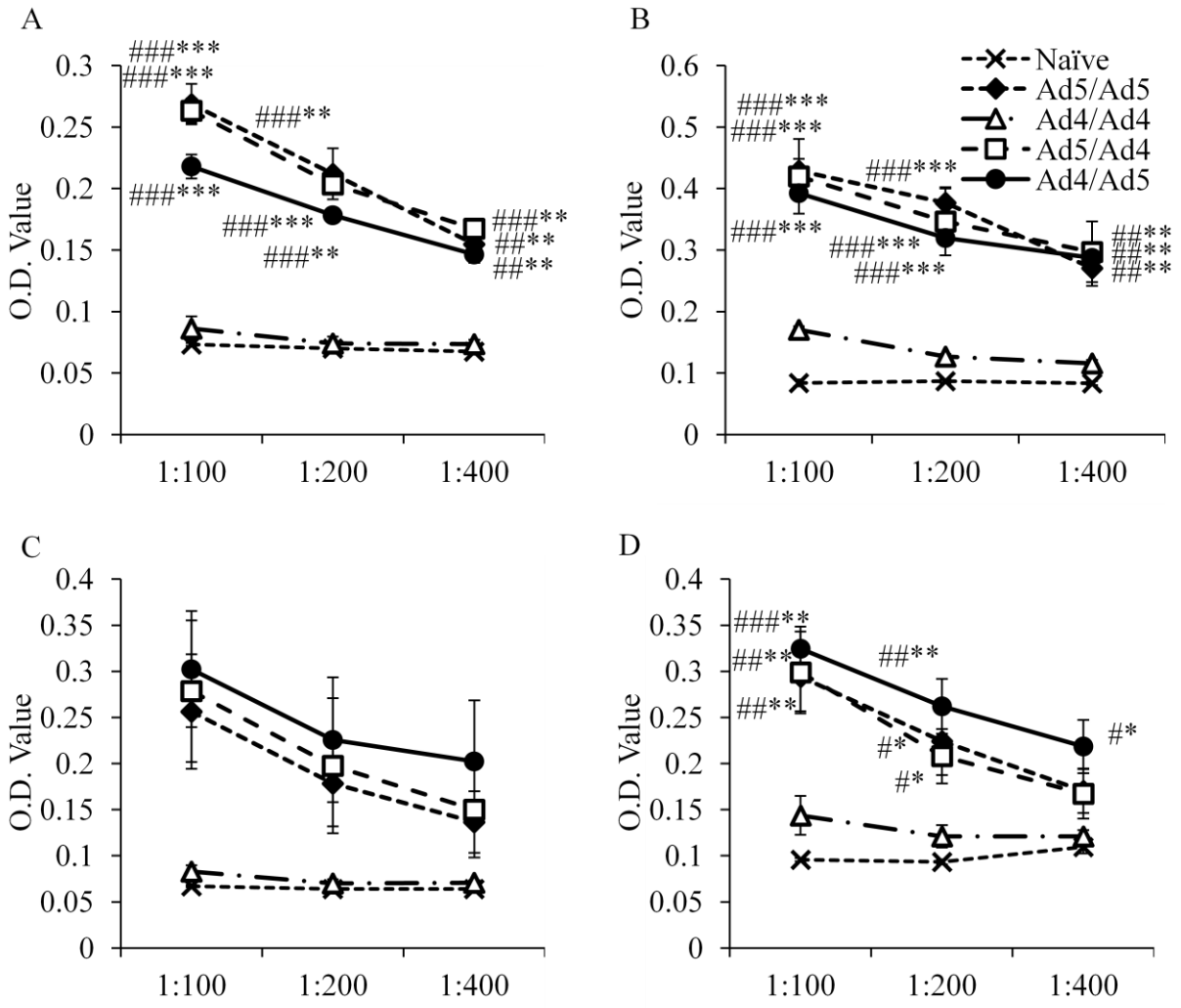


Figure 14: Sub-isotype analysis of IgG antibody from plasma of mice vaccinated with heterologous and homologous prime boost regimens utilizing Ad4-CSP and Ad5-CSP. Plasma was collected 14 days post final vaccination. The amount of CSP specific subisotype IgG1 (A), IgG2a (B), IgG2b (C), and IgG3 (D) were analyzed by ELISA. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$.

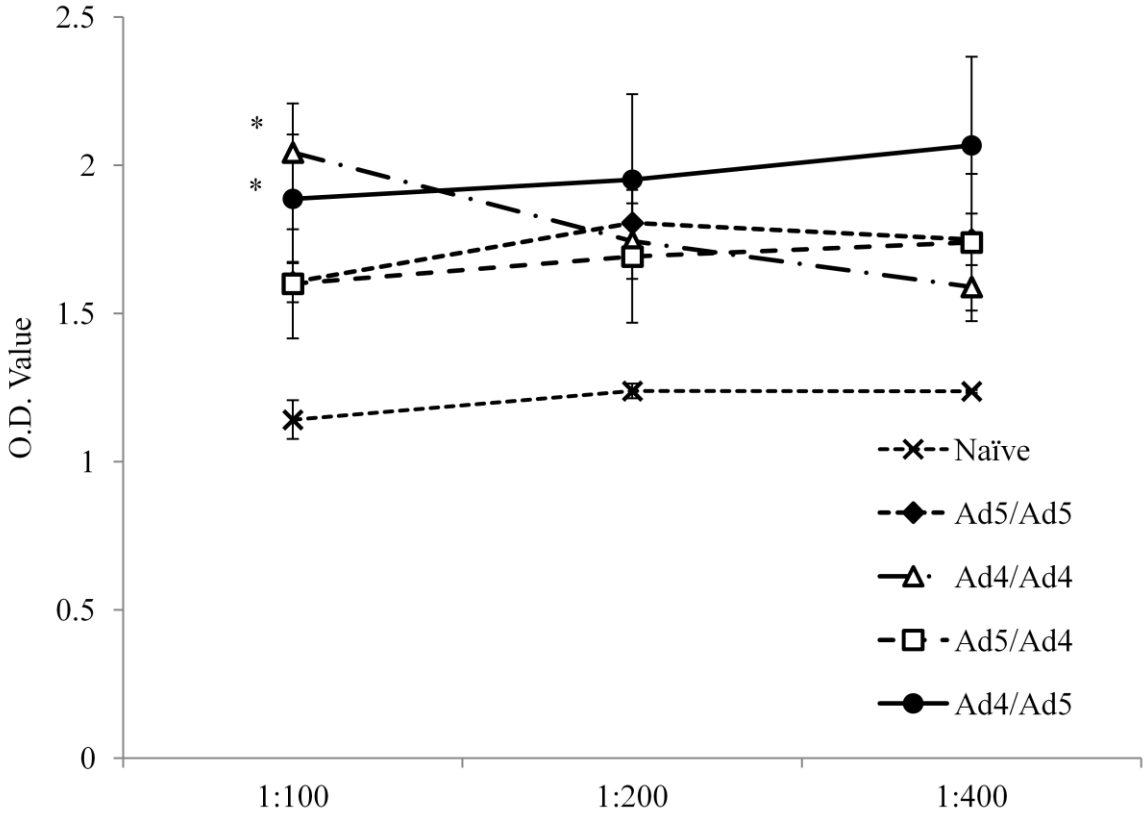


Figure 15: Th1 to Th2 ratio (IgG2a/IgG1) of plasma from vaccinated Ad naïve animals. Plasma was collected 14 days post final vaccination. The amount of CSP specific IgG subisotypes was measured by ELISA. Th1 to Th2 ratio was determined by dividing O.D. values from IgG2a and IgG1. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, * denotes significance over naïve, $P<0.05$.

killing was measured in the spleens of the vaccinated animals by flow cytometry. Only animals that received the Ad5-CSP/Ad5-CSP and Ad4-CSP/Ad5-CSP vaccination regimens achieved significantly elevated levels of CSP specific cell killing as compared to non-vaccinated animals ($p<0.01$) (Figure 16).

Given the high seroprevalence of wildtype Ad5 in adults living in malaria endemic regions, we also analyzed the ability of these homologous and heterologous prime boost vaccine regimens to elicit potent CSP specific adaptive responses in animals that were made Ad5 immune prior to receipt of the various vaccine regimens. BALB/cJ mice received two injections 14 days apart of $1x10^{10}$ vp/mouse of an Ad5 vector that does not express a transgene (Ad5-Null). It has been previously demonstrated that two immunizations with $1x10^{10}$ vps of rAd5-Null vector induced Ad5 neutralizing antibodies titers that were >1:200, a level that closely parallels levels of pre-existing Ad5 immunity noted in human populations.[60] 14 days after the last injection of Ad5-Null, Ad5-immune animals received $1x10^{10}$ vp/mouse prime injection of either Ad4-CSP or Ad5-CSP followed by either a heterologous or homologous boost 14 days after the initial priming vaccination. 28 days after the prime vaccination plasma, PBMCs, and splenocytes were collected. Splenocytes were stimulated as before with NYDNAGTNL and were analyzed for CSP specific IFNγ secreting cells by ELISpot. Ad5-CSP/Ad4-CSP, Ad4-CSP/Ad4-CSP, and Ad4-CSP/Ad5-CSP vaccinated Ad5 immune animals all had significantly higher numbers of NYDNAGTNL responsive IFNγ secreting cells present when compared to the Ad5-CSP/Ad5-CSP cohort or the non-vaccinated animals (Figure 17). However, as compared to Ad5 naive animals, overall induction of NYDNAGTNL responsive, IFNγ secreting splenocytes was notably diminished in Ad5 immune animals despite use of Ad4-CSP in some of the regimens

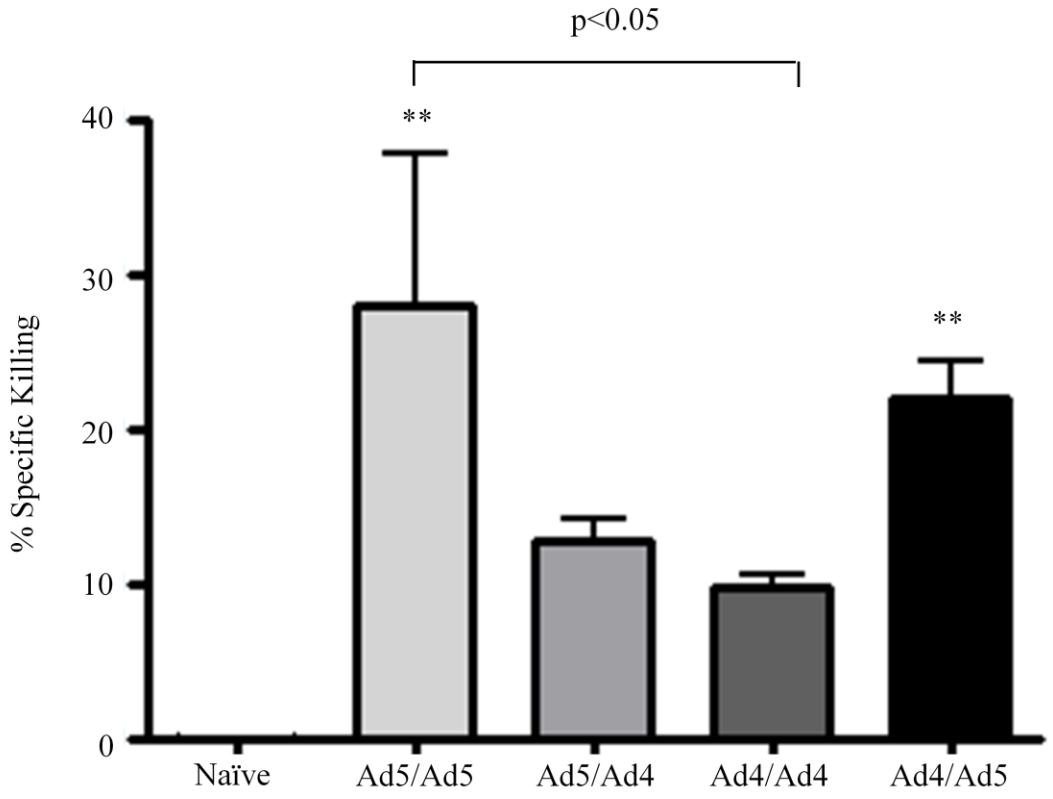


Figure 16: Ad5-CSP/Ad5-CSP and Ad4-CSP/Ad5-CSP both stimulated more percent specific killing than unvaccinated animals. 14 days post vaccination splenocytes from naïve animals were pulsed with either NYDNAGTNL and high concentration of CFSE or non-specific peptide and low concentration of CFSE. Stained splenocytes were combined in equal amounts and roughly 8 million cells were injected into vaccinated animals IV. After 20 hours splenocytes from vaccinated mice were collected and analyzed by flow cytometry to assess the amount of NYDNAGTNL specific killing. % Specific killing = 1-((%CFSEhigh/%CFSElow)immunized/(%CFSEhigh/CFSElow)non-immunized. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, **denotes significance over naïve, P<0.01.

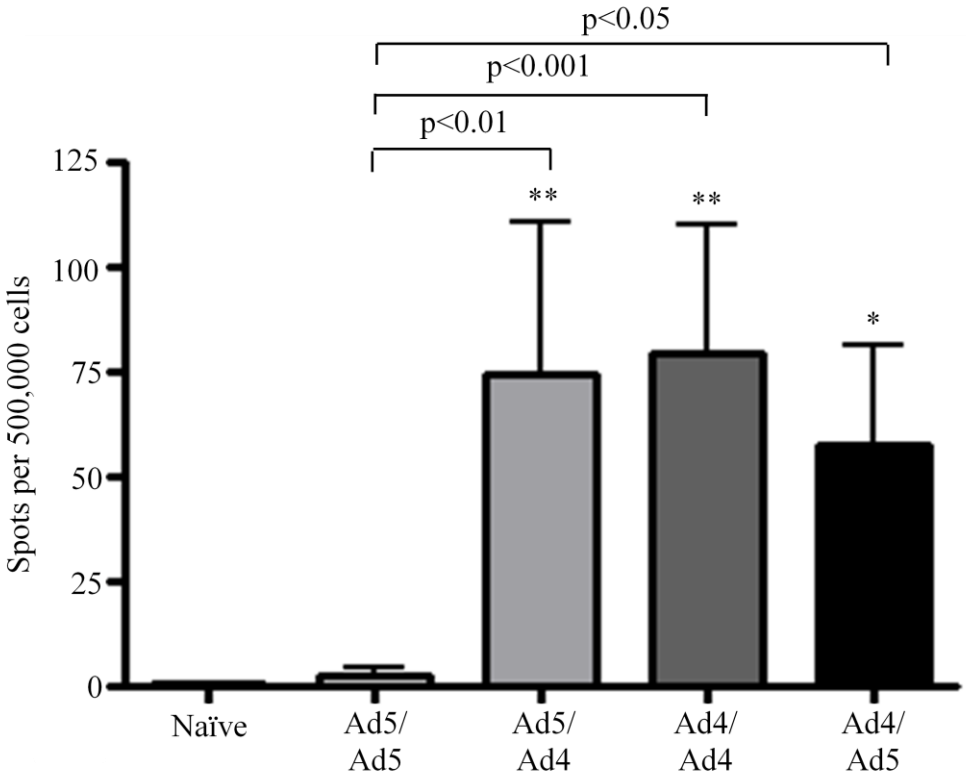


Figure 17: IFNγ secretion of cells from Ad5 immune mice vaccinated with heterologous and homologous prime boost regimens utilizing Ad4-CSP and Ad5-CSP. All vaccinations were capable of stimulating significantly more IFNγ secreting cells than unvaccinated and Ad5-CSP/Ad5-CSP vaccinated Ad5 immune animals. Splenocytes were collected 14 days after final vaccination. Splenocytes were then stimulated with CSP dominant antigen NYDNAGTNL and IFNγ secretion was measure by ELISpot. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, ** denotes significance over naïve, $P<0.05$, $P<0.01$.

(Table 4). The reductions prevented detection of significant differences between the treatments when ICS of the splenocytes for IFNγ, TNFα, and Granzyme B was undertaken (Figure 18A-C).

We analyzed PBMCs and splenocytes for CD3+ CD8+ T cells that were CSP peptide tetramer binding by flow cytometry and found that all vaccinated Ad5 immune animals, including Ad5-CSP/Ad5-CSP vaccinated animals, had a significantly higher percentage of CSP specific CD3+ CD8+ tet+ T cells present in both the spleen and peripheral blood (Figure 19A-B). All treatments including Ad5-CSP/Ad5-CSP also had significantly higher percentages of tetramer positive Tcm cells when compared to the non-vaccinated animals in both the spleen and in the peripheral blood (Figure 20B, D). Only mice from the Ad5-CSP/Ad-5CSP treatment group had higher frequencies of CSP specific Tem cells in their spleens as compared to non vaccinated mice (Figure 20C). Ad5-CSP/Ad5-CSP, Ad5-CSP/Ad4-CSP, and Ad4-CSP/Ad4-CSP vaccination groups all stimulated significantly more Tem cells in the peripheral blood than non-vaccinated and Ad4-CSP/Ad-5CSP vaccinated, Ad5 immune-animals (Figure 20E). When we analyzed T cells for memory phenotypes we found that homologous prime boost vaccinations biased the T cell responses toward Tcm rather than Tem cell phenotype memory in Ad5 immune mice (Figure 21). We also evaluated the *in vivo* cytolytic activity of CD8+ T cells in Ad5 pre-immune mice. No significant increase in percent specific killing was observed in any treatment groups when compared to unvaccinated Ad5 immune animals (data not shown).We analyzed undiluted plasma collected from unvaccinated animals and Ad5 immune animals from each vaccination regimen for anti-CSP total IgG by ELISA. From the undiluted plasma we found that Ad5-CSP/Ad4-CSP, Ad4-CSP/Ad4-CSP, and Ad4-CSP/A5-CSP vaccinated animals all had significantly more CSP specific total IgG than non-vaccinated animals ($p<0.001$) and the Ad5 immune animals homologously vaccinated with Ad5-CSP ($p<0.001$) (Figure 22).

Table 4. Decreased Mean Spot Forming Cells in Ad5 Immune animals		
Vaccination	**Ad5 Naive**	**Ad5 Immune**
Naive	2.71	2.71
Ad5-CSP/Ad5-CSP	791.25	2.80
Ad5-CSP/Ad4-CSP	708.00	74.50
Ad4-CSP/Ad4-CSP	527.20	79.60
Ad4-CSP/Ad5-CSP	1100	57.60

Table 4: Decreased Mean Spot Forming Cells in Ad5 Immune animals. All vaccinations, inclusive of homologous Ad4-CSP prime boost, elicited fewer IFNγ secreting splenocytes in Ad5 immune animals as measured by ELISpot. The table displays the mean numbers of spot forming cells per 500,000 splenocytes from spleens of Ad5 naïve and Ad5 immune mice treated with each prime boost regimen.

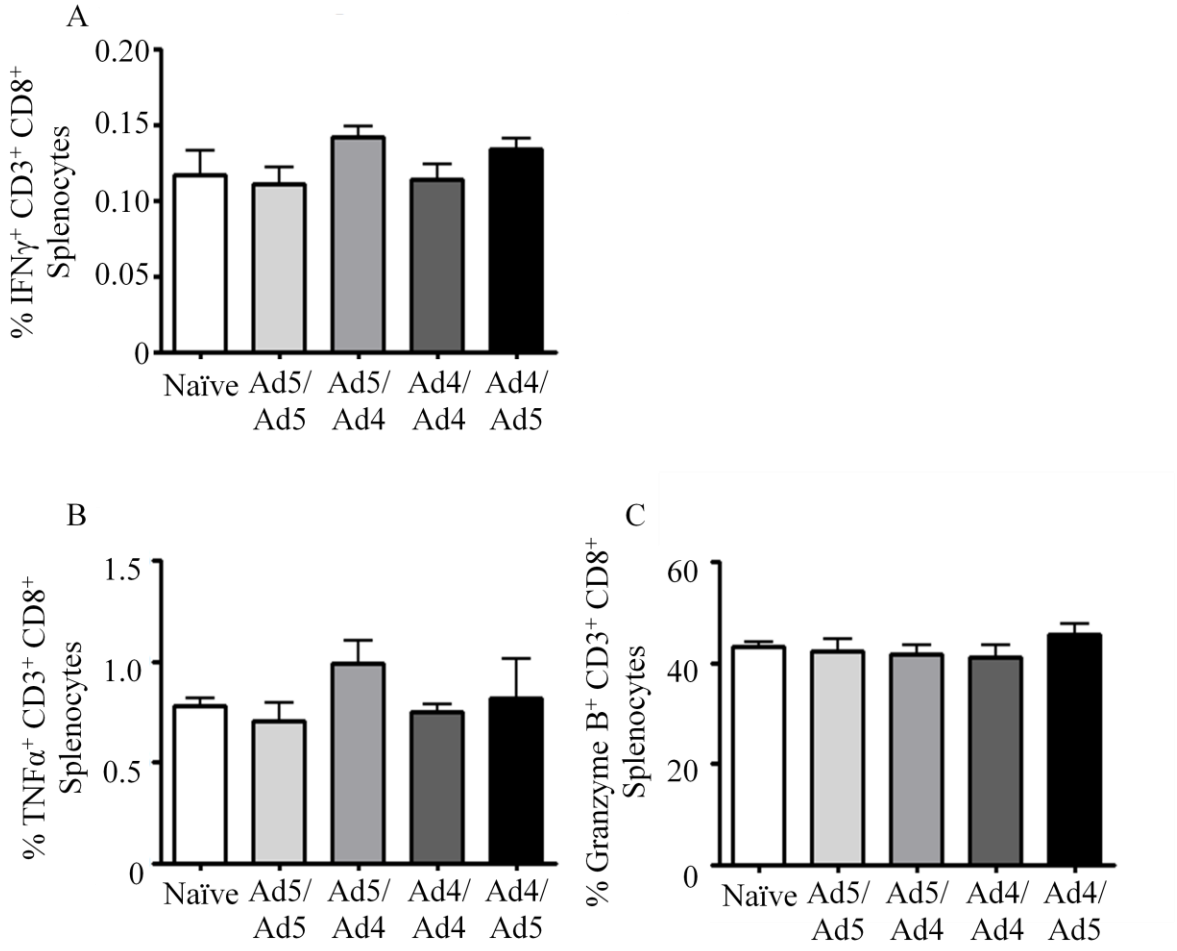


Figure 18: $CD8^+$ T cell activation in Ad5 immune animals vaccinated with heterologous or homologous prime boost regimens utilizing Ad4-CSP and Ad5-CSP. Splenocytes were collected from vaccinated animals 14 days post the final vaccination. Cells were stained with CD8-Alexa flour700, CD3-APC-Cy7, TNFα-PE-Cy7, IFNγ-FITC, and Granzyme B-APC and analyzed by flow cytometry for INFγ secreting $CD3^+$ $CD8^+$ T cells (A), TNFα secreting $CD3^+$ $CD8^+$ T cells (B), and granzyme B^+ $CD3^+$ $CD8^+$ T cells (C). Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$.

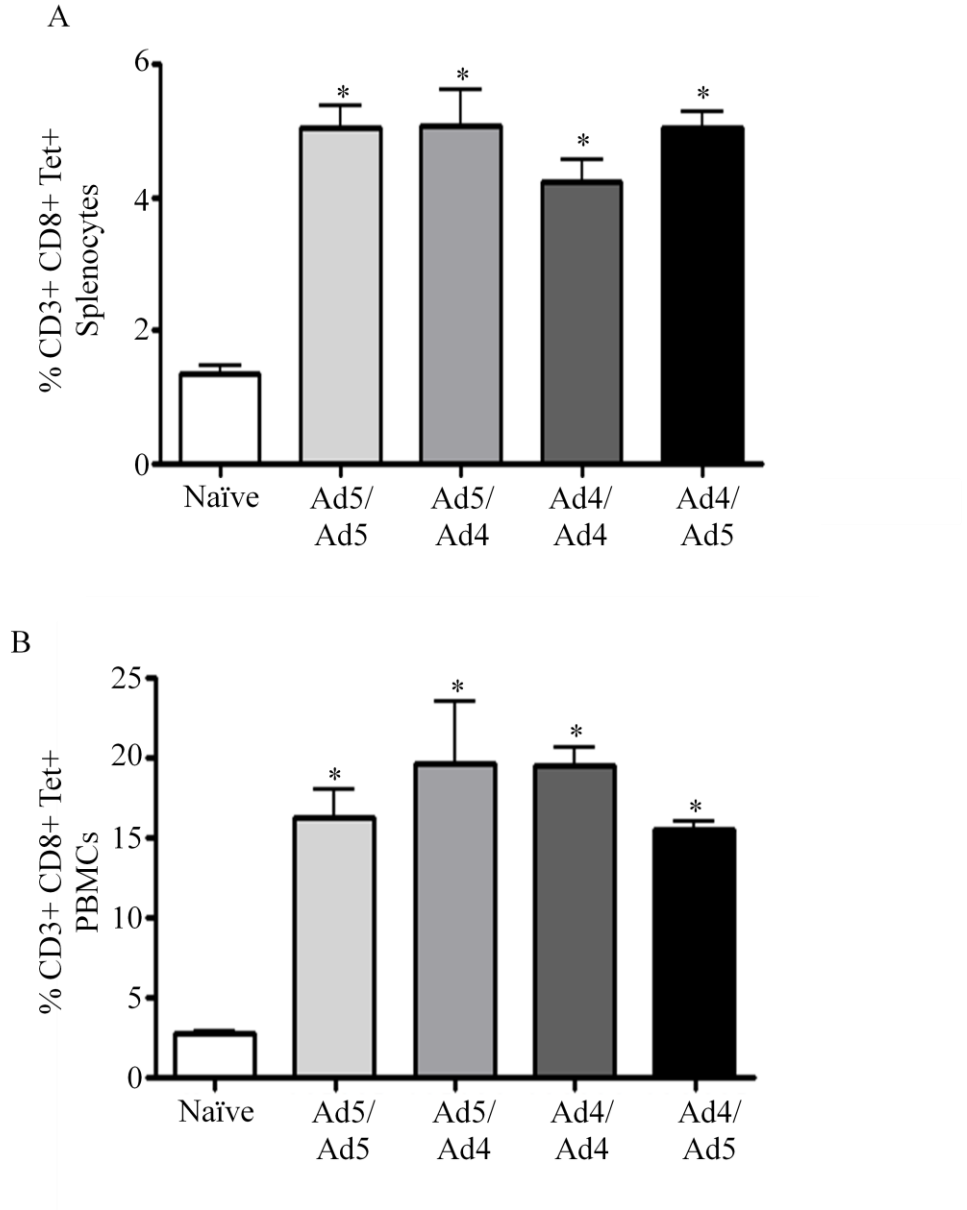


Figure 19: All vaccinations in Ad5 immune animals resulted in significantly higher percentages of CD8+ CSP tetramer positive cells than unvaccinated Ad5 immune animals. Splenocytes and PBMCs were collected two weeks after final vaccination. All vaccination regimens resulted in significantly higher percentage of CD3+ CD8+ NYDNAGTNL tetramer positive T cells in the spleen (A) and circulating blood (B) as measured by flow cytometry, cells were stained with CD8-Alexa flour700, CD3-APC-Cy7, and CSP (NYD)-Tetramer-PE. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, * denotes significance over naïve, $P<0.05$.

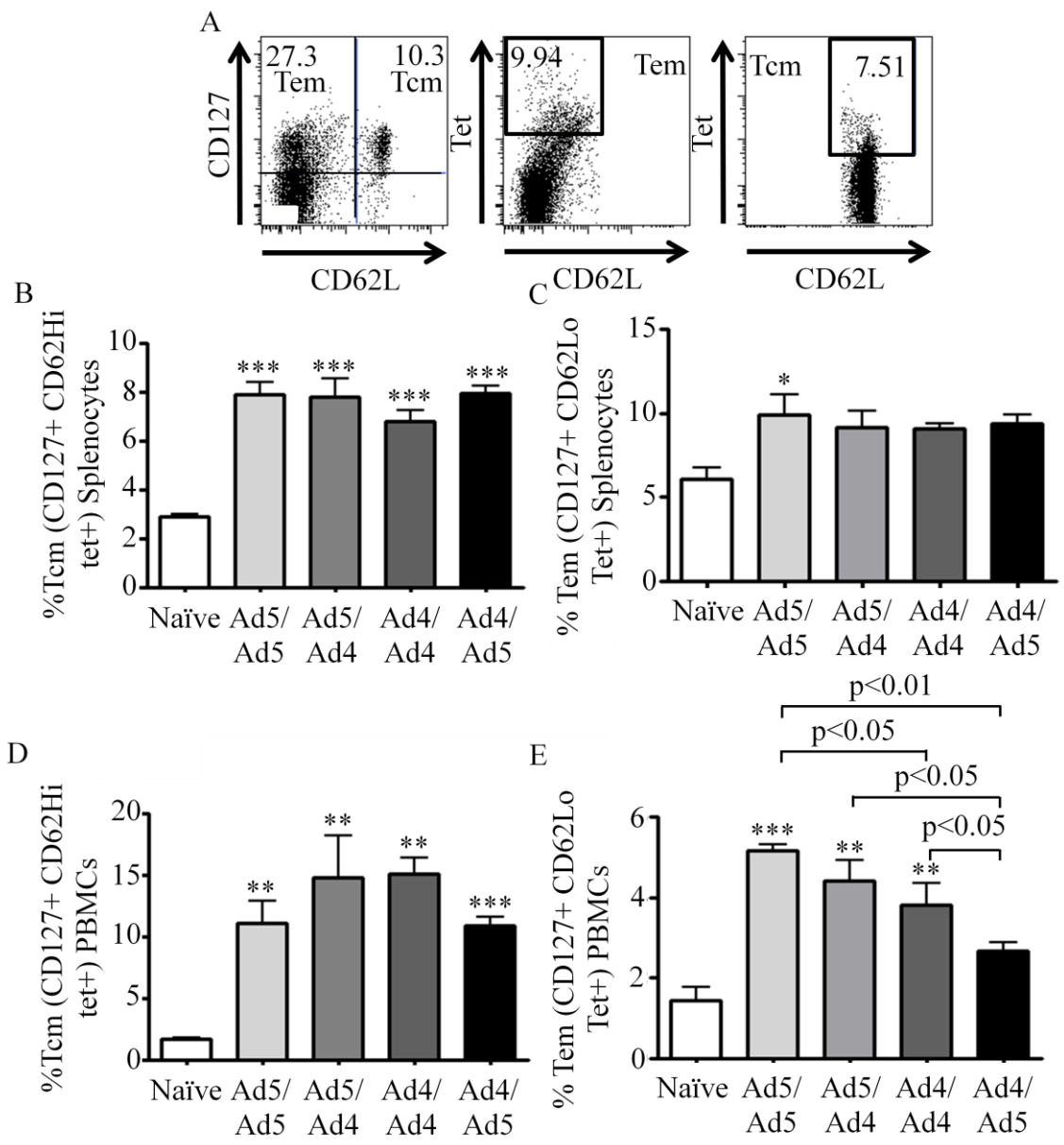


Figure 20: Memory responses triggered by vaccination with homologous and heterologous prime boost regimens utilizing Ad4-CSP and Ad5-CSP in Ad5 immune animals. Splenocytes (B-C) and PBMCs (D-E) were collected two weeks after final vaccination. Cells were stained for CD62L-V450, CD127-PerCP Cy5.5, and CSP (NYD) tet-PE. CSP specific central memory T cells were determined as CD62L+ CD127+ cells that are tet+ and effector memory cells are CD62Llo CD127+ cells that are tet+. Provided above is an example of gating (A). Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve, $P<0.05$, $P<0.01$, $P<0.001$.

Figure 21

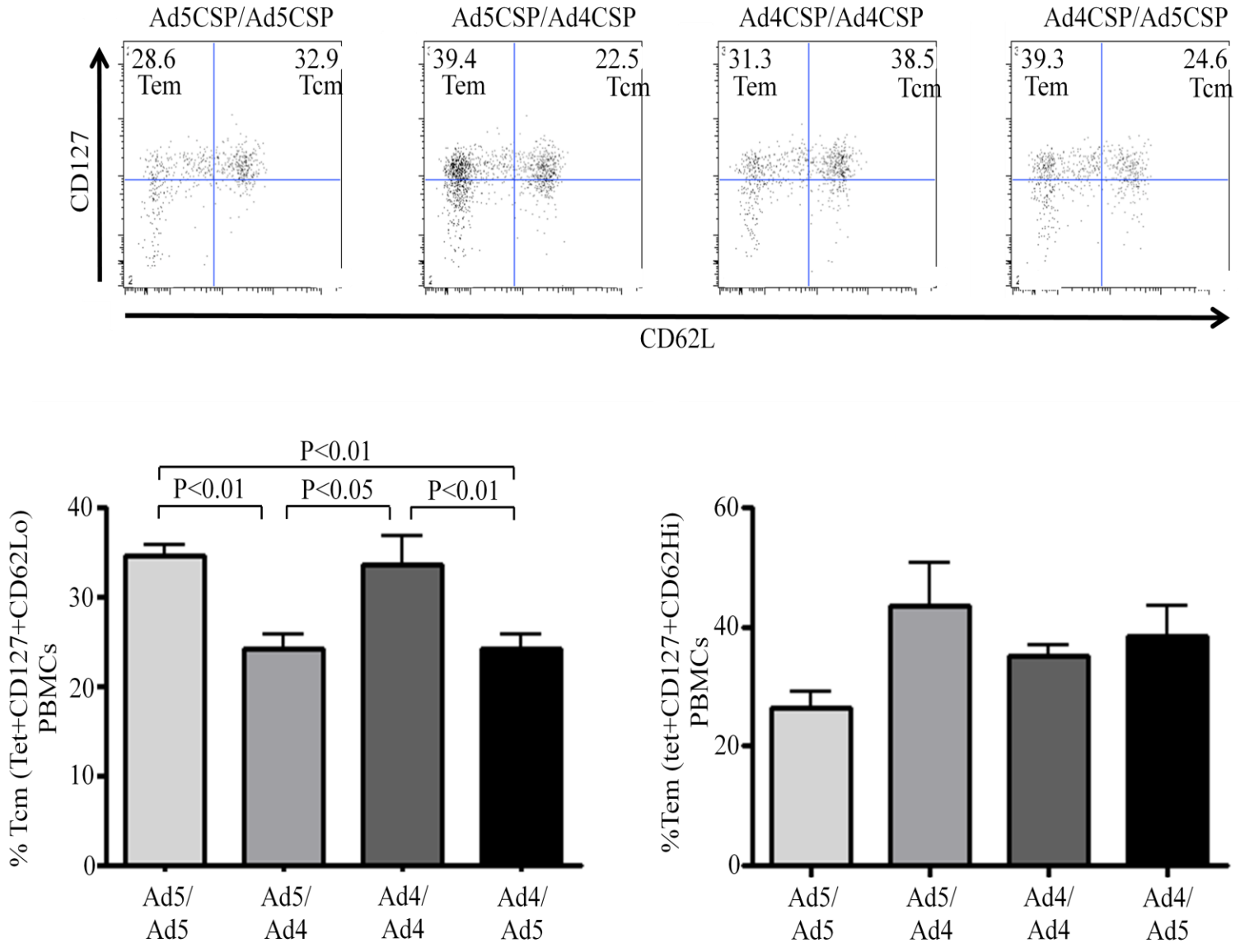

Ad5CSP/Ad5CSP
Ad5CSP/Ad4CSP
Ad4CSP/Ad4CSP
Ad4CSP/Ad5CSP
28.6 Tem
32.9 Tcm
39.4 Tem
22.5 Tcm
31.3 Tem
38.5 Tcm
39.3 Tem
24.6 Tcm
CD127
CD62L
P<0.01
P<0.01
P<0.05
P<0.01
% Tcm (Tet+CD127+CD62Lo) PBMCs
%Tem (tet+CD127+CD62Hi) PBMCs
Ad5/ Ad5
Ad5/ Ad4
Ad4/ Ad4
Ad4/ Ad5

Figure 21 cont'd: Memory Phenotype. Homologous prime boost regimens favor a Tcm cell phenotype in the peripheral blood of Ad5 immmune mice. Memory phenotype was defined as percent of CSP (NYD) tetramer positive cells that are Tem cells (CD62Llo CD127+) and percentage that are Tcm cells (CD62L+ CD127+) as opposed to percent of Tem and Tcm cells that are Tet+. PBMCs were collected on day 14 post final injection and stained according to the above defined memory phenpotype. Example of gating appears above the graphs (A). Percentage of Tcm Cells was significantly higher PBMCs from homologously boosted animals in Ad5 immune mice (B). There was no significant difference in the percentage of Tem cells present between any of the groups (C). Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc Test.

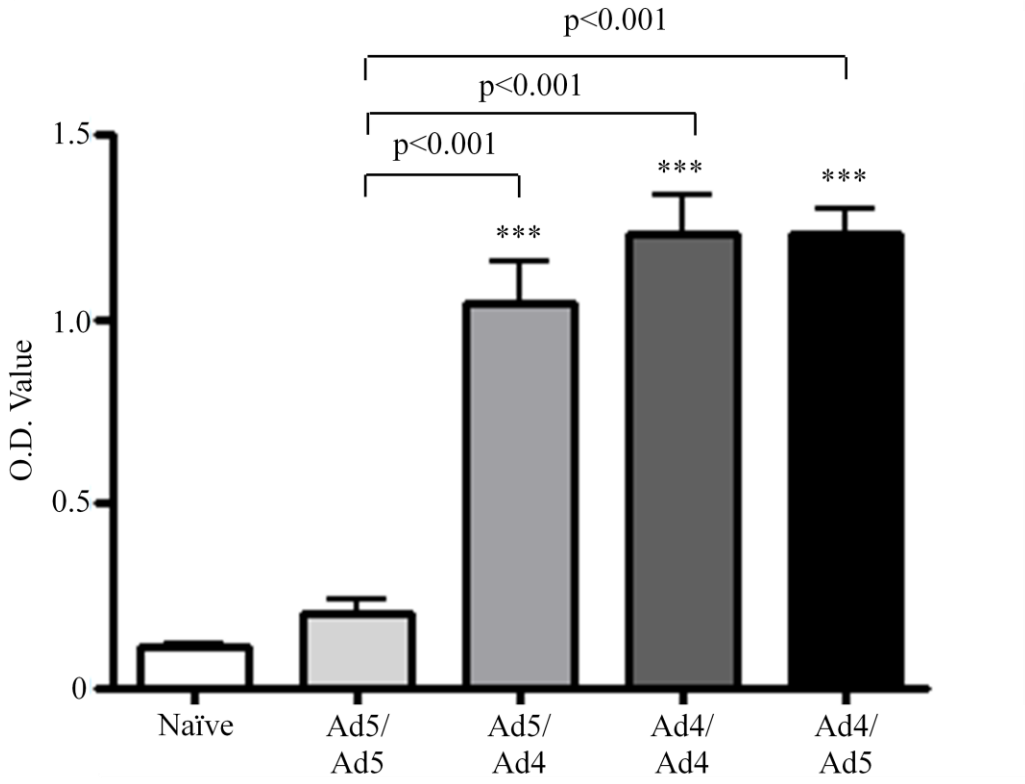


Figure 22: All vaccinations stimulated significantly higher anti-CSP total IgG than unvaccinated and Ad5-CSP/Ad5-CSP vaccination in Ad5 immune animals. Plasma was collected 14 days post the final vaccination. Plasma was measured undiluted for total IgG against CSP by ELISA. Serial dilutions were not possible as the undiluted plasma data point required the majority of the plasma collected from an animal. Bars represent ± standard error. Statistical analysis was completed using One Way ANOVA with Student-Newman-Keuls post-hoc test, *** denotes significance over naïve, P<0.001.

3.3 Discussion:

Ad4 has many qualities that make it a desirable choice as a vaccine platform, inclusing an ability to induce robust early innate responses and a high rate of infectivity.[100] Ad4 also has a long history of use as a vaccine vector, dating back to 1971 when Ad4 was used as an enteric live Ad4 vaccine by the military to vaccinate recruits against acute respiratory disease.[132,133,134,135] For these reasons Ad4 has already been utilized as a potential HIV vaccine vector in several large animal HIV models.[130,131] In this study, we chose to investigate how Ad4 based vaccines targeting malaria might be incorporated into malaria vaccine regimens, either in isolation, or in combination with a first generation Ad5 vaccine platform.

The combination of a priming vaccination of Ad4-CSP boosted by Ad5-CSP in Ad5 naïve animals results in induction of higher levels of activated CD8+ T cells than any other vaccination regimen used in this study. The activated T cells induced by an Ad4-CSP priming vaccination boosted by Ad5-CSP were also capable of potent CSP specific killing to levels that are equivalent to use of Ad5-CSP homologous vaccinations, despite the fact that animals homologously vaccinated with Ad5-CSP had higher levels of CSP specific CD8+ T cells detectable by staining with antibodies for CD3, CD8, and tetramer specific for CSP. These data suggest that combined use of Ad4-CSP priming followed by an Ad5-CSP boosting vaccination induced more efficient cytotoxic T cell killers than those induced by homologous prime boost of Ad5-CSP. If the aim is to provide a large quantity of CSP reactive T cells, a homologous prime boost vaccination of Ad5-CSP should be utilized. However, if one wishes to elicit IFNγ and TNFα secreting T cells specific for CSP a priming vaccination of Ad4-CSP followed by boosting vaccination of Ad5-CSP should be selected.

While Ad4-CSP provided benefit when utilized as a priming vaccination prior to boosting with Ad5-CSP, Ad4-CSP was not as capable as Ad5-CSP at stimulating potent CSP specific immune responses when utilized in a homologous prime boost regimen. Additionally, boosting a prime of Ad5-CSP with Ad4-CSP induced very poor CSP specific immune responses in general. Diminished induction by Ad4 based vaccines of transgene-specific IgG has been previously observed by us, and the effect was suggested to be a result of the Ad4 capsid inducing high levels of IFN-β, interfering with the CMV promoter used to drive expression of the transgene. Interference with the CMV promoter may ultimately reduce the length of time the CSP antigen is expressed from Ad4 vaccine platforms, and may explain the decrease in efficacy when Ad4-CSP is utilized in isolation or as a boosting vaccination in our current studies.[100]

To obtain protection from liver stage malaria, the presence of Tem cells are thought to be an essential element and a significant correlate to predicting vaccine efficacy.[25] Among our vaccination regimens, the induction of CSP specific Tem cell and Tcm cells were grossly similar when Ad4 or Ad5 based CSP vaccine treatments were conducted in Ad naïve animals. However, vaccination regimens did not perform equally when we tested *in vivo* cytotoxicity, as only the animals receiving an Ad4-CSP priming vaccination boosted by Ad5-CSP, or animals receiving the homologous Ad5-CSP prime-boost vaccination regimens resulted in detection of significantly improved levels of CSP specific cell killing, as compared to non-vaccinated animals. Since 14 days post vaccination is within the time frame when peak of CD8+ effector T cell responses may be present, and CD8+ T cell contraction usually does not take place until after three weeks post vaccination, it is likely that the observed CSP specific killing is a result of the lingering presence of CD8+ effector T cells, rather than induction of Tem cells.

Another reason we undertook these studies was to determine if the use of serologically distinct Ad4 based malaria targeted vaccines might allow for improved induction of CSP immune responses, relative to repeated use of Ad5 in Ad5 immune animals. Indeed, Ad4-CSP was capable of stimulating the induction of significantly more CSP antigen specific IFNγ secreting splenocytes, as well as higher levels of anti-CSP antibodies when incorporated into prime boost regimens in Ad5 immune mice (as compared to use of the Ad5-CSP vaccine), albeit to levels well below what was observed when the Ad4-CSP vaccine was utilized in Ad5 naïve animals. Furthermore, a more stringent functional analysis suggested that use of Ad4 in Ad5 immune animals also did not result in improved induction of CSP specific cytotoxic activity as compared to non-vaccinated animals. Although Ads are segregated into subgroups based on anti-sera neutralization there is evidence that T cell responses can react across subgroups.[137] These cross reactive T cells could be responsible for the decrease of immunogenicity observed in Ad5 immune animals homologously vaccinated with Ad4-CSP. Therefore, *a priori* assumptions that different subgroups of adenovirus are not cross reactive must be reconsidered in light of data demonstrating diminished immunogenicity when using some alternative serotypes in Ad5 immune animals. Likely, mild cross reactivity not measured by conventional means (such as the neutralizing antibody and ELISpot based assays used in our study) is still capable of diminishing immunogenicity of two very distinct serotypes on the bases of perhaps only a few cross reactive epitopes.[138]

Prior immunity to Ad5 did not appear to affect the ability of any of the Ad4-CSP or Ad5-CSP vaccination regimens to induce CSP specific CD8+ T cells. The percentages of CSP specific tetramer positive CD8+ T cells observed in Ad5 immune animals were similar to percentages observed in Ad naive animals. All vaccinations appeared to increase the percentage

of CD8+ T cells specific to the CSP epitope NYDNAGTNL in Ad5 immune animals in spite of the observed ablation in cytokine production by these same cells. Similarly, all vaccinations except an Ad4-CSP prime boosted by Ad5-CSP resulted in high percentages of CSP specific Tem cells in the circulating blood. Heterologous prime boost vaccinations even appear to trend toward a Tem cell phenotype while homologous vaccinations biased toward a Tcm cell phenotype. However, none of these responses correlated with evidence of improved *in vivo* CSP specific cytotoxic T cell killing when either of these vectors was deployed into Ad5 immune animals. Ad5 cross reactivity with Ad4 appears to result in the ablation of IFNγ and TNFα secreting CSP specific cytotoxic T cells induction by Ad4-CSP based vaccines, despite allowing for the induction of high percentages of CSP specific T cells.

Our data shows that there exists a complex interaction between immune responses triggered by a rAd4 (subgroup E) and those triggered by rAd5 (subgroup C), each expressing the same malaria antigen, in this instance, CSP. While combined use of Ad4-CSP priming vaccinations with Ad5-CSP boosting vaccinations results in the induction of greater numbers of CSP responsive cytokine secreting, cytotoxic T cells in Ad5 naïve animals, there appears to be interference between the two seemingly distinct Ad subgroups, resulting in diminished inductions of transgene specific immune responses in Ad5 immune animals despite the use of the Ad4 platform. Future studies need to be performed to further elucidate the mechanism behind Ad4's decreased ability to stimulate immune responses in an Ad5 immune background. Based on our results it is important that future use of other alternative serotypes be scrutinized under similarly stringent assay conditions to ascertain their true effectiveness in overcoming pre-existing Ad5 immunity.

Acknowledgments: We wish to thank Michigan State University Laboratory Animal support facilities for their assistance in the humane care and maintenance of the animals utilized in this work, the NIH Core Tetramer Facility at Emory University for manufacturing the NYDNAGTNL tetramer, and the Michigan State University flow cytometry facility for their assistance with the multiple experiments.

Chapter 4:

Vaccine platforms combining Circumsporozoite protein and potent immune modulators, rEA or EAT-2, paradoxically result in opposing immune responses

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Nathaniel J. Schuldt: Engineered Ad5-CSP virus, performed all injections, ELISA, ELISpot assays, *in vivo* CTL assay, and wrote the manuscript.

4.1 Introduction:

Some of the most successful malaria vaccine studies to date have attempted to induce adaptive immune responses to the *P. falciparum* CSP. Induction of potent cellular immune responses to CSP by a prophylactic malaria vaccine could potentially eradicate both sporozoites and infected hepatocytes, potentially stopping the infection before clinical symptoms occur. The leading malaria subunit vaccine, RTS,S/AS01B, stimulates both anti-CSP antibody as well as a CSP specific cytotoxic T cell response, although initial formulations without the adjuvant AS01B stimulated less cytotoxic T cells and were less effective. These results underscore the importance of potent CSP specific CD8+ T cell responses. Multiple studies have also demonstrated the importance of CD8+ T cell responses in combating murine malaria infections.[22,26,59,139] It has even been shown that passive transfer of CD8+ T cells specific for murine malaria CSP antigen resulted in 100% survival upon sporozoite challenge.[26] Furthermore, Ads expressing murine malaria derived CSP have been shown to be capable of providing cytotoxic T cell mediated inhibition of parasite liver stage development up to 93%.[59]

rAds are particularly good at stimulating CD8+ T cell responses against a transgene. Ad induction of potent innate responses through TLR signaling can be exploited to drive robust adaptive responses against a transgene.[71,72] Many studies, outlined previously in this dissertation, have demonstrated Ads abilities to induce potent CMI responses. Although Ad induced responses have not yet provided significant protection from malaria in humans.[81] The high seroprevalence of Ad5 throughout Africa has been implicated as a reason for poor Ad5 based malaria vaccine protection, which has led many to pursue alternative Ad serotypes. This reaction may be imprudent given recently published data showing low seroprevalence of Ad5 in children of Africa, which make up the primary group at risk for death from malaria.[48]

Alternative serotypes could still be utilized to create potent boosting vaccinations and should continue to be studied for this purpose. However, as our studies and those of others have confirmed, the utilization of alternative serotype rAds or chimp derived rAds as a vaccine platform may be dangerous due to the increased innate toxicity of non-Ad5 rAds and cross-reactivity between sub-groups.[101] Therefore, improving the capability of rAd5 vaccines to induce more potent antigen specific adaptive immune responses is a high priority in the drive to find an efficacious malaria vaccine. In this study we sought to improve CSP specific CMI responses induced by CSP-expressing rAd5s by co-expression of innate immune response modulating proteins by the vaccine platform.

The innate immune system plays an integral role in augmenting and/or shaping the induction of antigen specific adaptive immune responses.[63] A group of cellular receptors that recognize a variety of pathogen derived antigens, known as the TLRs, play a crucial role in identifying PAMPs, and then augmenting adaptive responses to those PAMPs. We have previously confirmed that rAds ability to induce innate and adaptive responses are dependent upon several TLR's, and that many of these responses are primarily dependent upon MyD88 functionality.[69,140] Given data demonstrating TLR adaptor molecules can enhance vaccine induced adaptive responses to viral and tumor antigens, we hypothesized that further stimulation of TLR pathways by inclusion of TLR agonists could potentially improve adaptive responses to a rAd expressed antigen.[141] To this end we demonstrated that when rAd5 vaccines engineered to express a novel TLR agonist derived from *Eimeria tenella*, rEA, are co-administered with rAd5 vaccines expressing a target antigen there was significant improvement in the ability of the vaccine to induce antigen specific cellular immune responses.[142]

rEA was discovered while searching bovine small intestine for anti-cancer agents. They uncovered a non-bovine protein capable of stimulating the secretion of large amounts of IL-12 from murine dendritic cells *in vitro*, as well as increased systemic levels of IL-12 and other Th1 cytokines *in vivo* after intra-peritoneal injection of the protein in mice.[143] The protein was later found to be homologous to a highly conserved *Eimeria* surface protein with unknown function, although it has been suggested the protein's purpose may be to modulate the immune system.[143] Uses of rEA in murine *in vivo* models of disease have been shown to increase protective immunity and human clinical trials have demonstrated rEA induced innate immune responses.[144,145] Given our previous research demonstrating improved adaptive responses to an Ad expressed antigen following co-injection of an Ad expressing rEA, we hypothesized that use of this platform could likewise induce adaptive responses to CSP.[142]

Similarly, we recently discovered that augmentation of the adaptive immune response can also be achieved by the expression of an adaptor protein found in dendritic cells called Ewing's sarcoma-related transcript-2 (EAT-2) that signals through signaling lymphocyte activation molecule (SLAM) receptors. When Ad-EAT-2 is co-injected with a rAd5 expressing HIV antigen (Gag) improved antigen specific T cell responses are observed including, increased antigen specific proliferation, IFNγ secretion, and improved cytotoxic activity.[136,146] SLAM family of receptors are expressed in hemopoietic cells and signal through homotypic self-sensing by their extracellular domains to activate immune cells.[147,148] EAT-2 is a member of the SLAM associated adaptor family of protein and is only found in dendritic cells, macrophages, and natural killer cells.[147,148] EAT-2 signaling has yet to be fully elucidated; however, it is known that EAT-2 possesses a src homology 2 domain (SH2) that interacts with the immunoreceptor tyrosine-based switch motif (ITSM) of the SLAM receptor as well as CD2-like

receptor activating cytotoxic cells (CRACC) to ultimately activate the cell.[149] It has been shown that expression of EAT-2 from a rAd5 results in more mature and activated dendritic cells that lead to an enhanced T cell response.[146] The observed enhanced T cell responses are likely due to improved synapses between dendritic cells and T cells mediated by the presence of more co-stimulatory cell surface receptors present on the dendritic cell surface. Further studies will need to be performed to assess this platforms ability to confer protection.

In this study, we determined what the impact of modulation of innate immune responses during CSP presentation would have upon induction of subsequent CSP specific immune responses *in vivo*. Unexpectedly, use of a TLR agonist uncovered a potent immunosuppressive activity inherent to the combined use of rEA and CSP, an activity that mitigated induction of any CSP specific adaptive immune responses. Fortunately, expression of the SLAM receptors adaptor protein EAT-2 overcame and enlightened possible mechanisms underlying the paradoxical CSP immunosuppressive activity we uncovered when stimulating TLR pathways.

4.2 Results:

CSP expressed from rAd5 based vaccines can induce CSP specific B and T cell responses

A rAd5 based vaccine expressing a codon optimized form of the CSP (Ad5-CSP) was constructed (Figure 23), and a dose study was initially performed to assess at what dose optimal CS specific B and T cell responses could be detected. BALB/cJ mice were intra-muscularly (IM) injected with varying doses of Ad5-CSP ranging from $1x10^7$ to $1x10^9$ vps per animal. At 14 dpi splenocytes derived from the vaccinated animals were harvested, and exposed to an immunodominant CSP derived peptide (NYDNAGTNL). Significantly increased numbers of IFNγ secreting splenocytes were noted in Ad5-CSP vaccinated mice treated with $5.0x10^7$ to $1.0x10^9$ vps, with peak numbers achieved at a dose of $1.0x10^8$ vps/mouse. Higher Ad5-CSP doses resulted in a trend of decreasing, though not significantly less, numbers of spot forming cells (SFCs) (Figure 24A). This phenomenon has also been observed by other groups, however an explanation for this phenomenon has yet to be forwarded.[77,150] These finding were further supported in individual splenocytes derived from the vaccinated animals, where CD8+ T cell IFNγ, TNFα, and IL-2 levels were measured by intracellular staining (ICS) using flow cytometry. IFNγ and TNFα production peaked at the $5.0x10^8$ vps/mouse with similar decreasing trend occurring at $1.0x10^9$ vps/mouse. IL-2 producing cells were much lower in percentage, with the greatest numbers being observed as the $5.0x10^7$ vps/mouse dose (Figure 24B). We will further discuss the importance of these findings in the Discussion section.

To determine if Ad5-CSP is also capable of stimulating B cell responses specific to the CSP, plasma was collected from the vaccinated mice and assayed by a IgG CSP specific ELISA

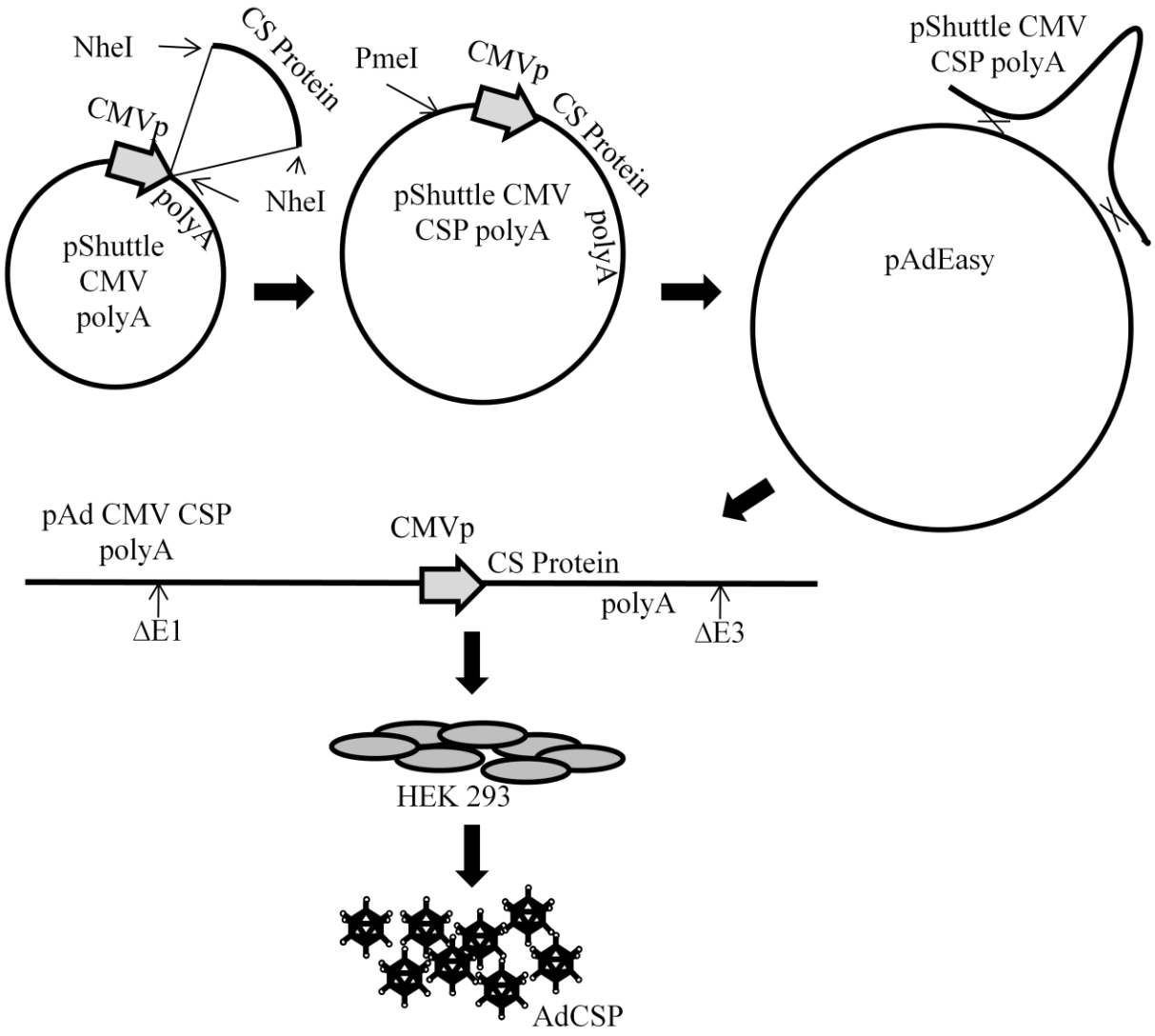

Figure 23: Ad5-CSP construction. Recombinant Ad5-CSP was constructed by creating a codon optimized CSP sequence flanked by NheI sites in a pGA4 plasmid. The sequence was excised with the *Nhe1* and cloned into a pShuttle containing a CMV expression cassette. The resulting plasmid was linearized with *PmeI* and recombined with pAdeasyI Ad5 vector in BJ 5183 cells. pAd5-CSP was then purified and linearized with *PacI* enzyme and transfected into HEK 293 cells from which Ad5-CSP was purified using cesium gradients.

Figure 24

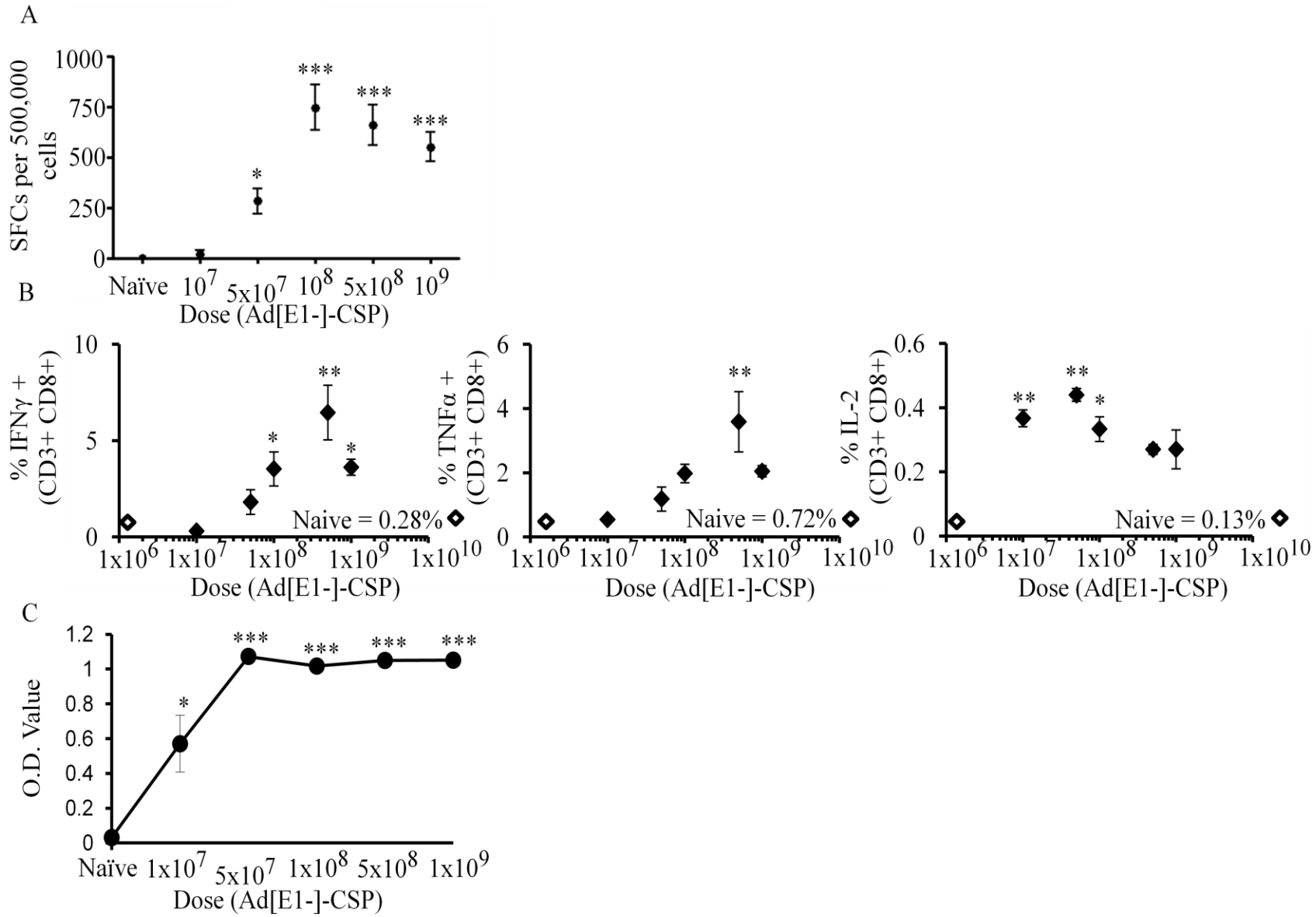
A
SFCs per 500,000 cells
1000
750
500
250
0
***
***
***
*
Naïve
10^7
5x10^7
10^8
5x10^8
10^9
Dose (Ad[E1-]-CSP)
B
% IFNγ + (CD3+ CD8+)
10
5
0
**
*
*
Naive = 0.28%
1x10^6
1x10^7
1x10^8
1x10^9
1x10^10
Dose (Ad[E1-]-CSP)
% TNFα + (CD3+ CD8+)
6
4
2
0
**
Naive = 0.72%
Dose (Ad[E1-]-CSP)
% IL-2 (CD3+ CD8+)
0.6
0.4
0.2
0
**
**
*
Naive = 0.13%
Dose (Ad[E1-]-CSP)
C
O.D. Value
1.2
1
0.8
0.6
0.4
0.2
0
*
***
***
***
***
Naïve
1x10^7
5x10^7
1x10^8
5x10^8
1x10^9
Dose (Ad[E1-]-CSP)

Figure 24 cont'd: Ad5-CSP Stimulates CSP specific T and B cell responses. CSP specific immune responses increase in an Ad5-CSP dose dependent manner. BALB/cJ mice (n=3) were injected IM with Ad5-CSP ranging from $1x10^7$ to $1x10^9$ vps/mouse, increasing by half logs. 14 days post injection splenocytes and plasma were collected. (A) ELISpot assays were performed to quantify IFNγ secreting cells from splenocytes stimulated with CSP peptide, NYDNAGTNL, *ex vivo*. (B) IFNγ, TNFα, and IL-2 expression by splenocyte derived CD3+ CD8+ T cells was analyzed by flow cytometry following *ex vivo* stimulation with NYDNAGTNL. (C) Total IgG against CSP was assessed by ELISA. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, *,**,*** denotes significance over naïve, $p<0.05$, $p<0.01$, $p<0.001$.

at 14 dpi. Significant increases in CS specific IgG were detected in all mice treated with Ad5-CSP with a peak response occurring at $5.0x10^7$ vps/mouse, demonstrating Ad5-CSP is capable of stimulating a B cell response against CSP even at the lowest dose used in the study (Figure 24C).

The use of a TLR agonist unexpectedly reduces CSP specific cellular immune responses

Previous experiments confirm that expressing the TLR agonist rEA from an Ad vector stimulates innate immune responses during Ad mediated vaccination, responses that positively correlated with improved induction of antigen specific adaptive immune responses against several antigens, such as the HIV antigen, Gag.[142] In this study, we sought to utilize rEA to improve induction of CSP specific immune responses. We first confirmed that expression of rEA along with CSP facilitated induction of pro-inflammatory innate immune responses, responses we had noted in our previous studies of rEA.[142] Plasma cytokine levels at 6 hours post injection (hpi) in mice co-injected intravenously (IV) with either $3.75x10^{10}$ vps of Ad5-CSP and $3.75x10^{10}$ vps Ad-GFP/rEA were compared to responses measured after identical co-injections utilizing an Ad-GFP expressing vector (that does not express rEA) as a control (Figure 25) . We observed significantly higher levels of IL-6, IL-12(p40), G-CSF, MCP-1, MIP-1β, RANTES, KC, and TNFα in mice treated with Ad5-CSP+Ad-GFP/rEA as compared to control virus treated animals, as well as, mock infected animals (Figure 25).

To assess the impact that these early increases in cytokine and chemokine responses had on cell mediated immune (CMI) responses to CSP we IM co-injected $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP/rEA and compared the induction of CS specific adaptive immune responses to those noted in our control animals receiving $5x10^7$ vps/mouse of Ad5-CSP

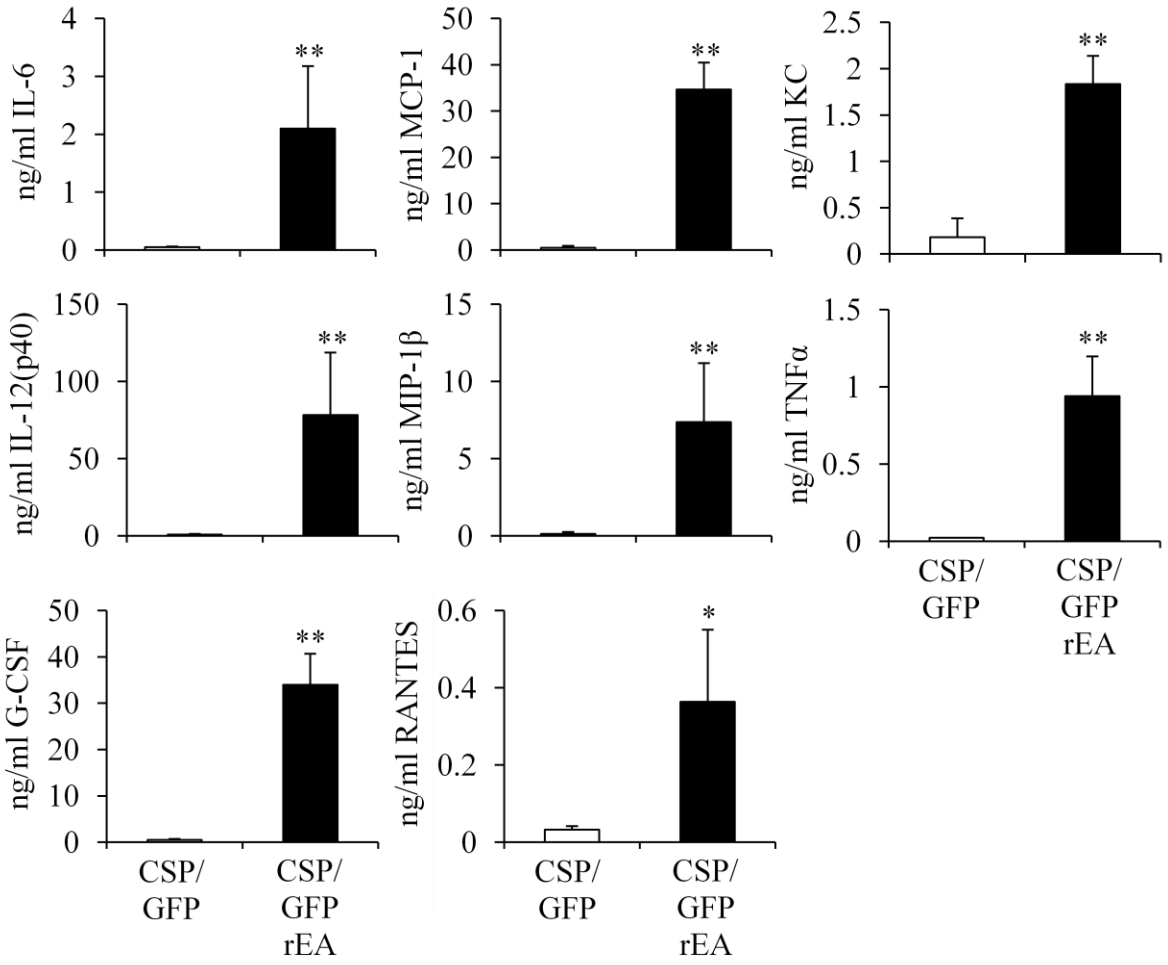

Figure 25: TLR agonist, rEA, induced innate cytokines 6 hours post injection. Co-injection of Ad-GFP/rEA and Ad5-CSP stimulated robust expression of innate cytokines and chemokines as compared to the control vaccine. BALB/cJ mice were injected IV with either $3.75x10^{10}$ vps/mouse of Ad5-CSP+Ad-GFP or $3.75x10^{10}$ vps/mouse Ad-GFPrEA+Ad5-CSP. Plasma was collected at 6 hours post injection. Plasma cytokine/chemokine levels were measured with a mouse 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, *,** denotes significance between treatments, $p<0.05$, $p<0.01$.

and $5x10^7$ vps/mouse of Ad-GFP IM, or mock infected mice. Splenocytes derived from mock vaccinated animals did not show the presence of CSP specific CMI responses while Ad5-CSP+Ad-GFP confirmed induction of CSP specific CMI responses using ELISpot analysis ($p<0.05$) (Figure 26A). However, despite the rEA enhanced activation of the innate immune responses noted in Figure 25, ELISpot analysis of splenocytes derived from Ad5-CSP+Ad-GFP/rEA vaccinated animals confirmed a profound lack of induction of average CSP specific CMI responses, responses that were essentially identical to CS responses measured in naïve mice ($p>0.05$) (Figure 26A). Previously we have not observed an ablation of CMI responses when CSP was co-administered with Ads expressing other antigens at these low doses, further suggesting that this effect may be specific to simultaneous TLR stimulation (Figure 27). Despite there being no significant differences between CSP responses in Ad5-CSP+Ad-GFP/rEA treated animals and naïve animals, we did note that in one Ad5-CSP+Ad-GFP/rEA animal there was some evidence of an elevated CSP specific response, independently verifying that this group did in fact receive viable Ad5-CSP vector (Figure 26A).

Augmentation of the innate immune responses via SLAM adaptor EAT-2, improves CSP specific T-Cell responses

Based upon the loss of CSP responsiveness after utilizing TLR mediated augmentation along with CSP antigen vaccination we hypothesized that the CSP may have an ability to mitigate induction of beneficial innate immune responses in the context of excessive, TLR pathway mediated activation as the ablated immune responses were only observed after Ad-GFP/rEA doses exceeded $5x10^6$ vp/mouse (Figure 28). To attempt to test this hypothesis, we made use of a recently described, alternative method for augmenting induction of antigen

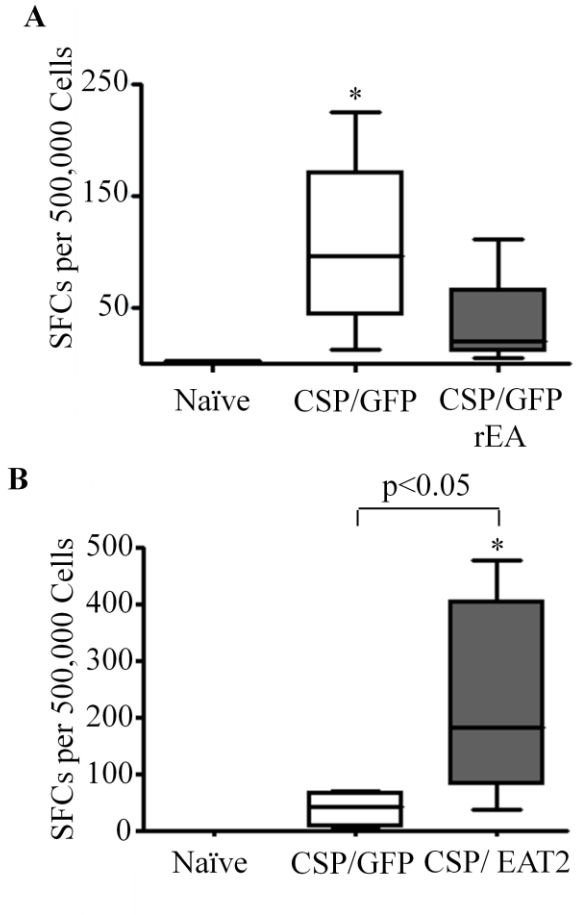

Figure 26: Immuno-modulating proteins conversely affect IFNγ secreting splenocytes. Co-vaccination with Ad5-CSP and Ad-EAT2 dramatically increases IFNγ secreting splenocytes in response to stimulation with CSP epitope, NYDNAGTNL. BALB/cJ mice were injected IM with either $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse Ad-GFP or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of either (A) Ad-GFP/rEA (n=5) or (B) Ad-EAT2 (n=6). Splenocytes were collected 14 days post co-injection. ELISpot were performed on the splenocytes of these mice stimulated with NYDNAGTNL peptide to assess the amount of IFNγ secreting cells. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test,* Denotes significance over naïve animals, $p<0.05$. Representative figures of two independent experiments.

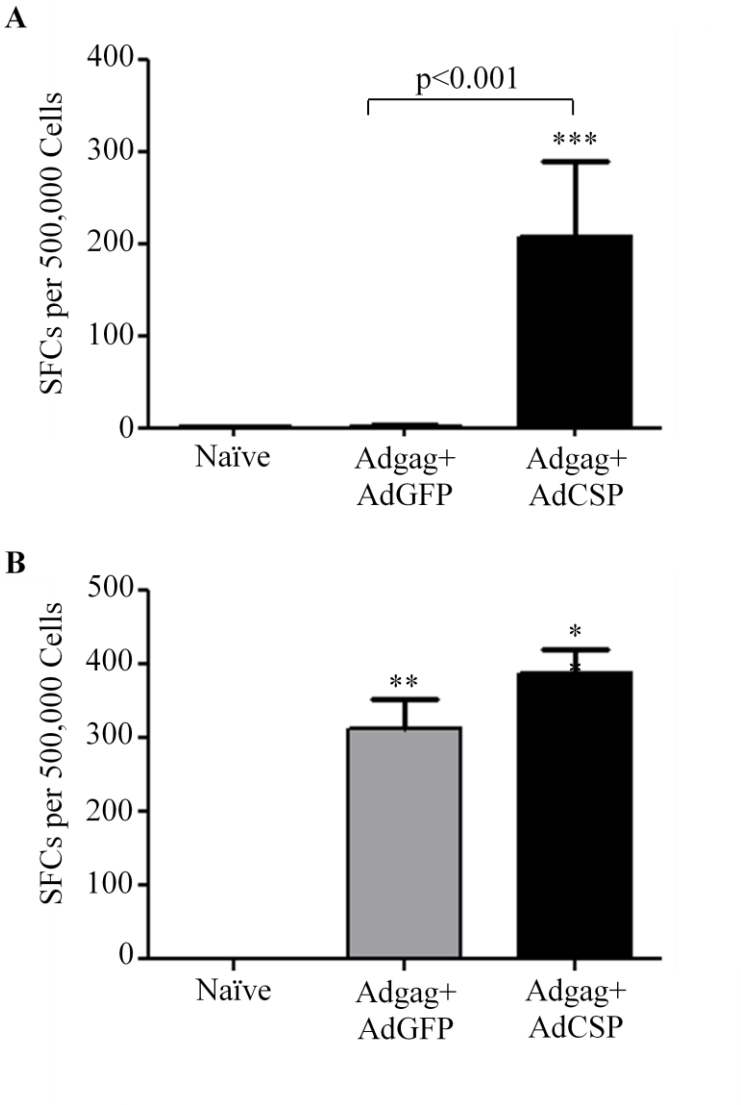

Figure 27: CSP expression does not interfere with antigen specific immune responses against other transgenes at low doses. Co-vaccination with Ad-gag+Ad5-CSP did not result in decreased gag specific immune responses.BALB/cJ mice were injected with $5x10^5$ vp/mouse of Ad-gag and $5x10^7$ vp/mouse of Ad5-CSP or $5x10^5$ vp/mouse of Ad-gag and $5x10^7$ vp/mouse of Ad-GFP. Splenocytes were collected 14 dpi and assayed by ELISpot for CSP peptide (NYDNAGTNL) specific IFNγ secretion (A) or gag peptide (AMQMLKETI) specific IFNγ secretion (B). The bars represent mean ± SD. Statistical analysis for Supplemental Figure 3A included other peptides tested from the peptide library that are not displayed in the graph. Two Way ANOVA with Student-Newman-Keuls post-hoc test (A) or One Way ANOVA with a Student-Newman-Keuls post-hoc test (B) were utilized for statistical analysis. **,*** denotes significance between treatments, $p<0.01$, $p<0.001$.

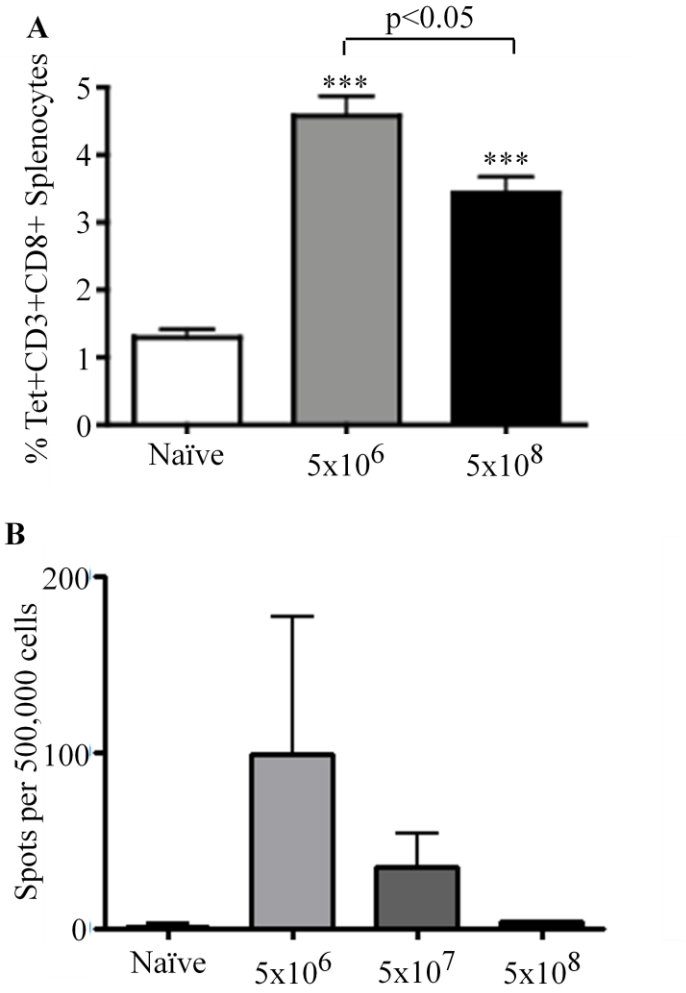

Figure 28: Ad-GFP/rEA combined with $5x10^7$ vp/mouse of Ad5-CSP begins to display a diminished CSP specific CMI response after a dose of $5x10^6$ vp/mouse. Only after the dose of Ad-GFP/rEA exceeds $5x10^6$ vp/mouse do we observe a diminished CS specific CMI response when combined with $5x10^7$ vp/mouse of Ad5-CSP. BALB/cJ mice were injected with doses ranging from $5x10^6$ to $5x10^8$ vp/mouse of Ad-GFP/rEA combined with $5x10^7$ vp/mouse of Ad5-CSP. Splenocytes were collected 14 dpi and were analyzed by flow cytometry for NYDNAGTNL tetramer+ CD3+ and CD8+ cells (A) or ELISpot for CSP specific IFNγ secretion (B). Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, *** denotes significance between treatments, $p<0.01$, $p<0.001$.

specific adaptive immune responses, utilizing Ad mediated co-expression of a SLAM receptor signaling pathway adaptor, EAT-2, along with a targeted antigen.[146] To accomplish this we co-injected $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-EAT2, and compared the induction of CS specific adaptive immune responses to those noted in the control mice receiving $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP IM, as well as mock vaccinated mice. Again, splenocytes were collected at 14 dpi and stimulated with the CS derived peptide NYDNAGTNL *ex vivo*. In dramatic contrast to our previous results utilizing the Ad-GFP/rEA and Ad5-CSP vaccination strategy, splenocytes from mice co-treated with Ad5-CSP and Ad-EAT2 had significantly more IFNγ secreting cells than splenocytes from both mock injected mice as well as mice co-treated with the control vaccine ($p<0.05$) (Figure 26B). Given these results, we sought to further characterize the EAT-2 dependent improvement in CS specific immune responses by flow cytometry. Peripheral blood mononuclear cells (PBMC) derived from the vaccinated animals were stained with CD3 and CD8 fluorescent antibodies, as well as a NYDNAGTNL peptide loaded tetramer. Ad5-CSP+Ad-EAT2 treated mice had significantly higher percentages of CSP specific tetramer positive CD8+cells present in their PBMCs than the percentage noted in the Ad5-CSP+Ad-GFP control group ($p<0.001$) (Figure 29A). CD3+ CD8+ splenocytes were additionally analyzed for IFNγ and perforin by ICS using flow cytometry. The percent of CD3+ CD8+ cells that secreted IFNγ was significantly higher in Ad5-CSP+Ad-EAT2 treated mice as compared to Ad5-CSP+Ad-GFP treated control ($p<0.05$) (Figure 29B). The percent of CSP peptide specific CD3+ CD8+ perforin+ cells also tended to be higher in animals given the Ad-EAT2+Ad5-CSP vaccination cocktails however this did not reach statistical significance (Figure 29C). To confirm that the differences in the responses observed are not a

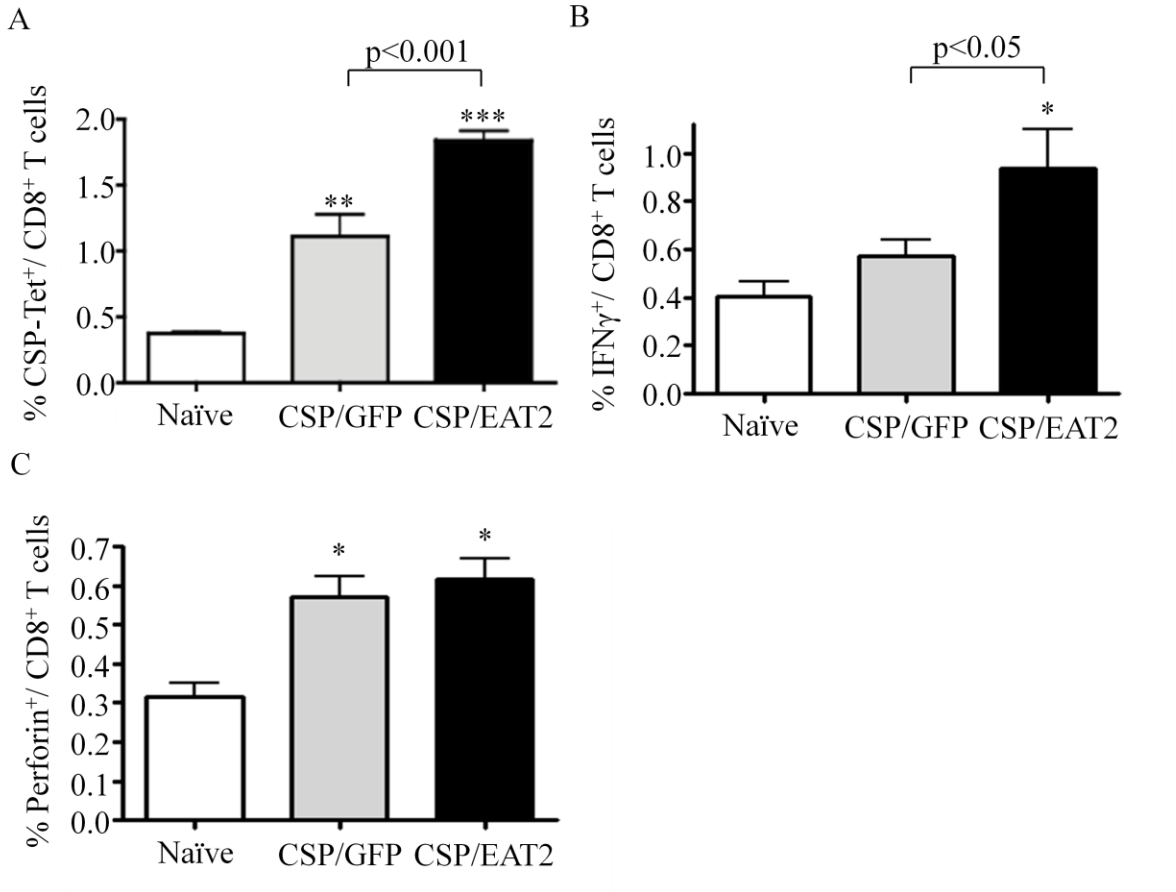

Figure 29: Co-expression of CSP and EAT-2 stimulates more potent CSP specific CMI responses. Co-vaccination with Ad5-CSP and Ad-EAT2 resulted in increased NYDNAGTNL tetramer positive CD8+ T cells as well as improved IFNγ secretion from CD8+ T cells. BALB/cJ mice (n=6) were co-injected IM with $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-EAT2 or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP. (A) Peripheral Blood Mononuclear Cells (PBMCs) were stained with CD8-Alexa Flour700, CD3-APC-Cy7, and CSP (NYD)-Tetramer. (B-C) Intracellular staining was performed on splenocytes after stimulation with NYDNAGTNL peptide. Cells were stained with CD8-Alexa Flour700, CD3-APC-Cy7, ViViD, IFNg-APC, and Perforin-PE antibodies. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, *, **, *** denotes significance over naïve animals, $p<0.05$, $p<0.01$, $p<0.001$.

result of GFP antigens competing with CSP antigens, but are in fact a direct result of the expression of EAT-2 we injected mice with either Ad5-CSP+Ad-GFP or Ad5-CSP + an empty Ad vector (Ad-Null). We observed no differences between the treatments, indicating GFP does not interfere with induction of CSP specific CMI responses (Figure 30).

Increased breadth of CMI responses to a pathogen derived protein has been shown to be beneficial relative to eventual protection against actual pathogen challenge.[151,152,153] To detect CMI responses against other peptides present within the CSP (and therefore to gauge the breadth of response against the whole CSP) we generated a CSP specific peptide library. This library consists of 15 mer peptides that overlap each other by 5 amino acids and spans the non-repeating regions of the full length CSP. At 14 dpi, pooled splenocytes derived from the control or experimental groups of vaccinated animals were stimulated *ex vivo* with one 15mer peptide per well. Mice co-vaccinated with Ad5-CSP and Ad-GFP/rEA had an overall lower breadth of response as is evident by the number of wells with more than 15 spots (Figure 31A). In contrast to the response seen in rEA treated animals, animals co-vaccinated with Ad5-CSP and Ad-EAT2 demonstrated a dramatic increase in breadth of response to CS derived peptides when similarly analyzed (Figure 31B).

Co-injection of Ad5-CSP and Ad-EAT2 improves the cytolytic activity of CSP specific T cells *in vivo*

To better assess the functional consequence of the improved CS specific CMI responses noted by expression of EAT-2, we stimulated splenocytes from naïve mice, mice vaccinated with the control vaccine, and mice vaccinated with Ad5-CSP+Ad-EAT2 with NYDNAGTNL *ex vivo*, then analyzed them by flow cytometry for CD3+, CD8+ T cells that were also positive for a degranulation marker, CD107a. Both control treated and Ad5-CSP+Ad-EAT2 treated mice

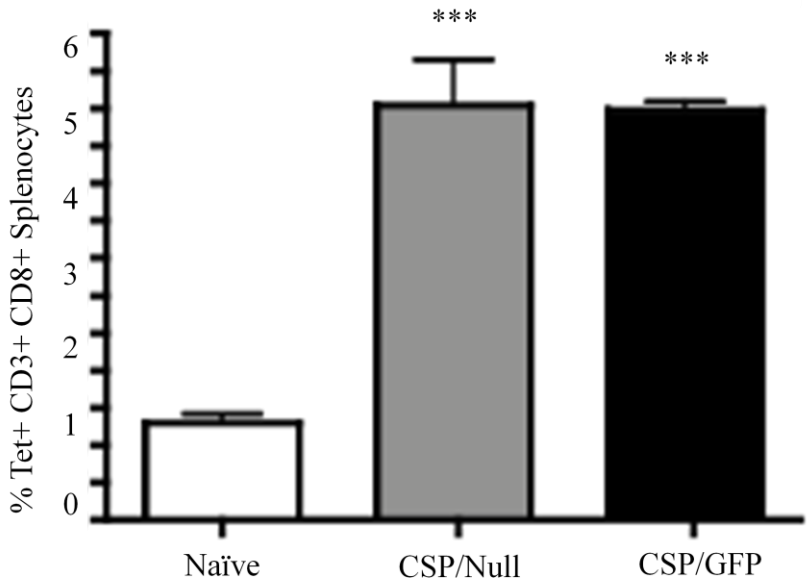

Figure 30: Expression of GFP does not interfere with CSP specific CMI responses. Co-injection of Ad-GFP does not interfere with Ad5-CSP initiated CSP specific CMI responses. BALB/cJ mice were co-injected with $5x10^7$ vp/mouse of Ad-GFP and 5x107 vp/mouse of Ad5-CSP or 5x107 vp/mouse of Ad-Null and $5x10^7$ vp/mouse of Ad5-CSP. Splenocytes were collected 14 dpi and cells were measured for NYDNAGTNL tet+, CD3+, CD8+ T-cells. Both treatments had a higher percentage of CSP specific tet+, CD3+, CD8+ T-cells than Naïve with no difference observed between Ad5-CSP+Ad-Null and Ad5-CSP+Ad-GFP. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, *** denotes significance between treatments, $p<0.01$, $p<0.001$.

Figure 31

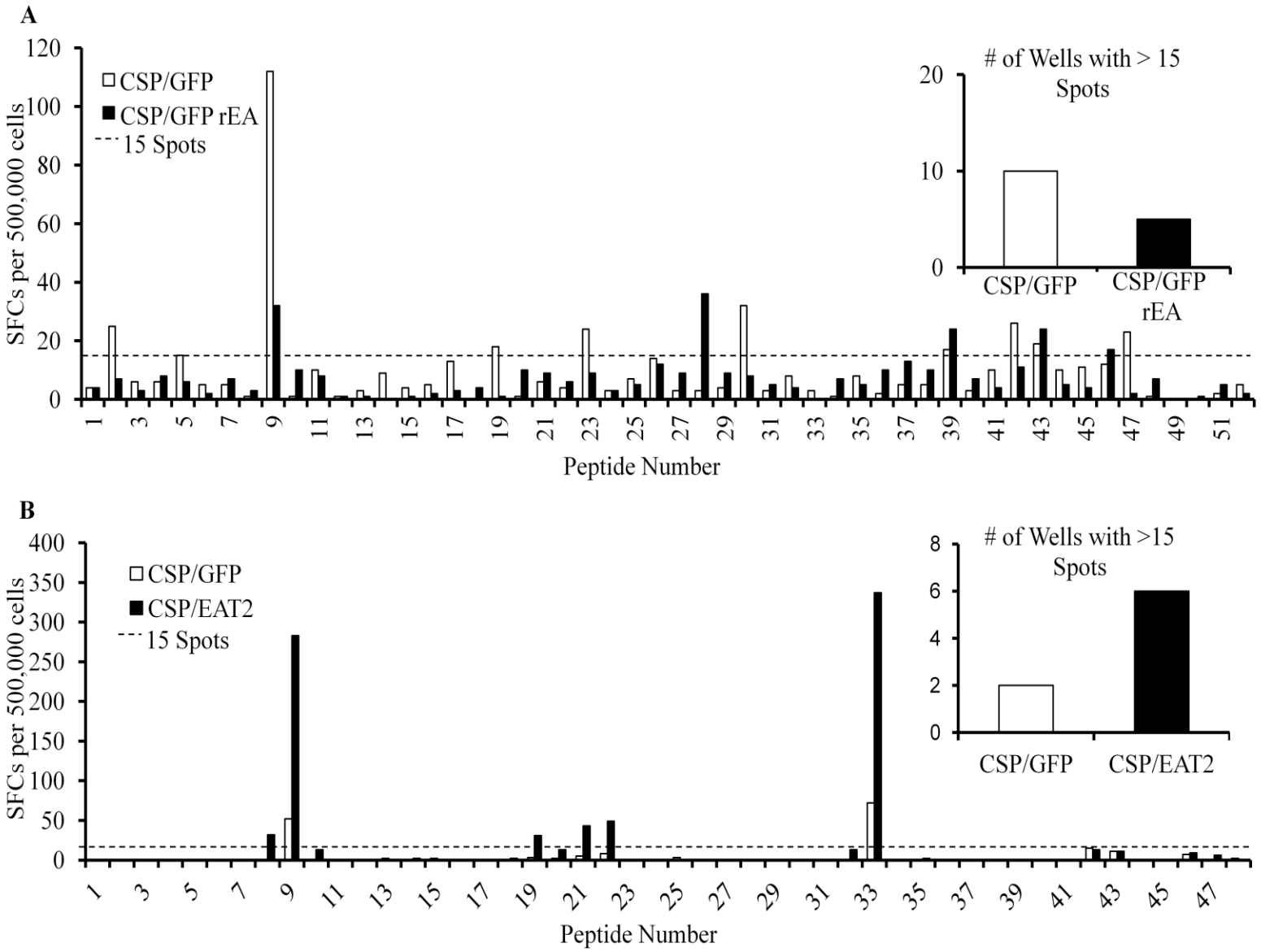
A
120
100
80
60
40
20
0
SFCs per 500,000 cells
CSP/GFP
CSP/GFP rEA
15 Spots
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51
Peptide Number
# of Wells with > 15 Spots
20
10
0
CSP/GFP
CSP/GFP rEA
B
400
350
300
250
200
150
100
50
0
SFCs per 500,000 cells
CSP/GFP
CSP/EAT2
15 Spots
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47
Peptide Number
# of Wells with >15 Spots
8
6
4
2
0
CSP/GFP
CSP/EAT2

Figure 31 cont'd: Co-expression of CSP and EAT-2 increases the breadth of response against CSP. Increased breadth of response against CSP epitopes was observed in mice co-vaccinated with Ad5-CSP and Ad-EAT2 as compared to the control vaccine. Splenocytes from groups of five BALB/cJ mice were collected and pooled together from 14 days post injection with either innate modulating treatments or control. ELISpots were performed to measure IFNγ secreting cells when stimulated with a CSP peptide library made up of 52 15mers that overlap by 5 a.a. on either side. Wells that contained more than 15 spots were counted and compared between treatment groups (inset). (A) Mice were co-injected IM with $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP/rEA or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP. (B) Mice were co-injected IM with $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-EAT2 or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP. As a negative conrol, naïve splenocytes were also tested against paired peptides from the peptide pool, with an average background of spots per paired peptides being only 2.2 spots.

demonstrated significantly higher number of CD8+, CD107a+ T cells than those quantified in naïve mice, indicating increased ability of CD8+ T cells to express granules when stimulated with a CSP epitope (Figure 32). However, the assay was not sensitive enough to measure a difference between the control vaccinated mice and Ad5-CSP+Ad-EAT2 vaccinated mice. We then conducted a more sensitive *in vivo* CTL assay.[154] Mice were co-vaccinated with either $1x10^8$ vp/mouse of Ad5-CSP and $1x10^8$ vps/mouse of Ad-GFP or $1x10^8$ vp/mouse of Ad5-CSP and $1x10^8$ vps/mouse of Ad-EAT2. 14 days later vaccinated mice were treated with CFSE labeled splenocytes that had been incubated with either the NYDNAGTNL peptide, or a non-specific control peptide, and the elimination of NYDNAGTNL pulsed cells (CFSEhigh cells) was measured by flow cytometry. Based on the calculated percent specific killing, animals vaccinated with Ad5-CSP+Ad-EAT2 were more effective at killing cells exposed to the NYDNAGTNL peptide than animals vaccinated with the control Ad5-CSP vaccine (Figure 33).

Induction of CSP specific antibody responses by Ad5-CSP vaccines augmented by rEA or EAT-2 expressing rAds

CSP antibody specific ELISAs were also performed on plasma derived from Ad5-CSP+Ad-GFP/rEA and Ad5-CSP+Ad-GFP treated animals. CSP specific total IgG antibody levels in control vaccine treated animals were significantly elevated ($p<0.05$) as compared to naïve animals. However, there was again no significant difference observed in Ad5-CSP+Ad-GFP/rEA treated animals when compared to naïve animals ($p<0.05$) (Figure 34A). Conversely, plasma collected from Ad5-CSP+Ad-EAT2 treated animals had significantly higher levels of CSP specific IgG as compared to levels detected in naïve mice ($p<0.05$) (Figure 34B). However, the mice receiving the control vaccine treatment had higher total CSP specific IgG levels than naïve and Ad5-CSP+Ad-EAT2 treated animals ($p<0.05$) (Figure 34B). Further isotyping of IgG

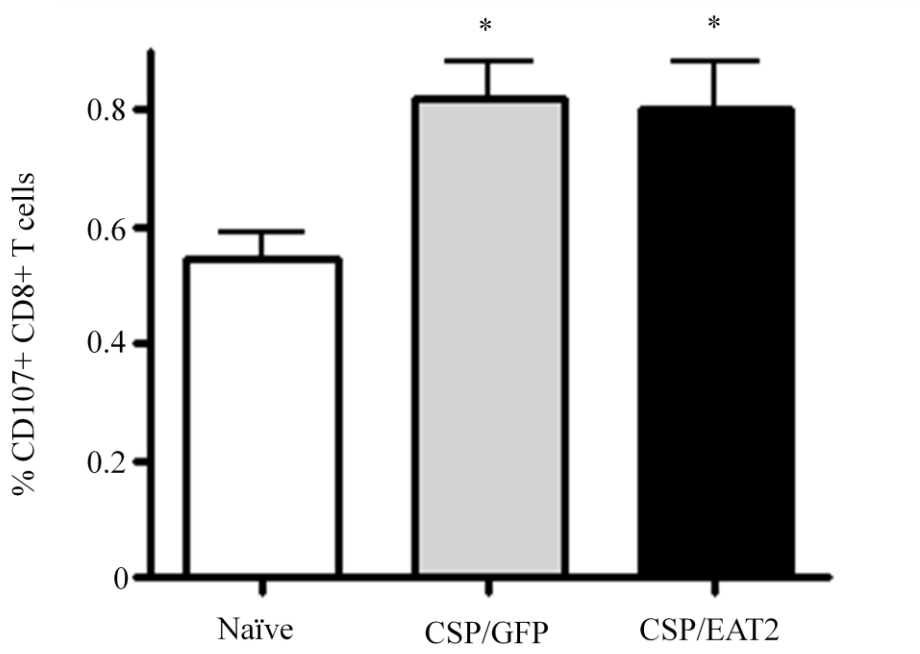

Figure 32: Improved degranulation of CD8+ T cells in mice co-vaccinated with Ad5-CSP and Ad-EAT2. Degranulation marker, CD107a, expression in CD8+ T cell from mice co-vaccinated with Ad5-CSP+Ad-EAT2 or Ad5-CSP+Ad-GFP. Splenocytes were collected from BALB/cJ mice 14 days post co-injection of either $5x10^7$ vps of Ad5-CSP and $5x10^7$ vps of Ad-GFP or $5x10^7$ vps of Ad5-CSP and $5x10^7$ vps of Ad-EAT2. 2×10^6 splenocytes from naive or mice co-vaccinated with either treatment were stimulated with 2ug NYD-peptide at 37°C for 3 days. Cells were then washed with FACS buffer and stained with CD8-Alexa700, CD107-FITC antibodies and viability dye (ViViD) and ran on LSR-II. % of live CD107+ CD3+ T cells is shown. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test,* Indicates significance over naïve $p<0.05$

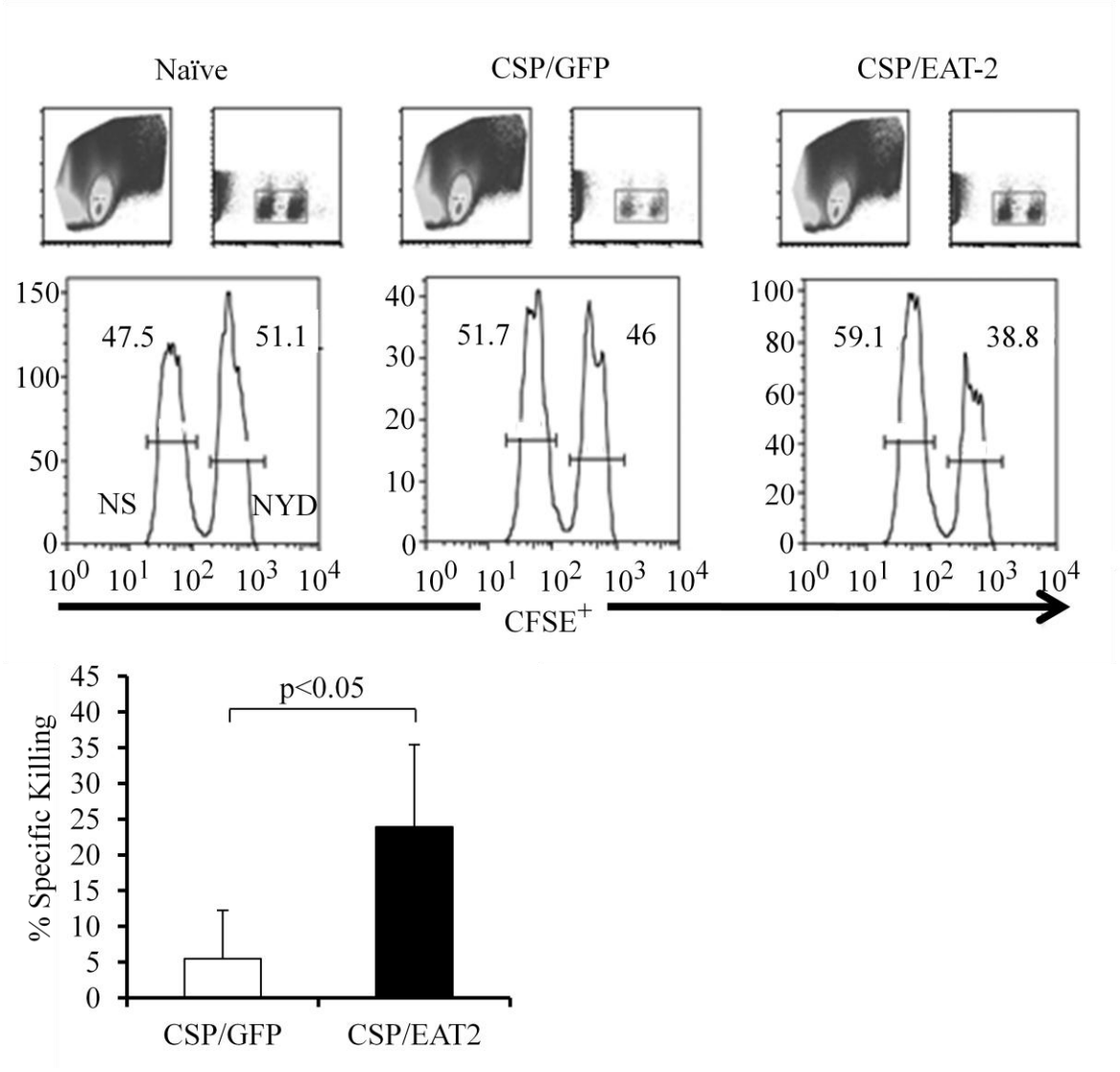

Figure 33: Co-expression of CSP and EAT-2 increases cytolytic activity of CSP specific T cells. Co-vaccination of mice with Ad5-CSP and Ad-EAT2 increased specific killing cells pulsed with CSP peptides. BALB/cJ mice (n=4) were co-injected IM with either $1x10^8$ vps/mouse Ad5-CSP and $1x10^8$ vps/mouse Ad-GFP or $1x10^8$ vps/mouse Ad5-CSP and $1x10^8$ vps/mouse Ad-EAT2 on Day 0. Day 14 splenocytes were collected from naïve mice and pulsed with either NYDNAGTNL peptide or an irrelevant peptide. NYDNAGTNL pulsed splenocytes were stained with a high concentration of CFSE while splenocytes pulsed with irrelevant peptide were stained with a low concentration of CFSE. Stained splenocytes were then combined in equivalent doses. 8 million cells were then injected IV into naïve, Ad5-CSP+Ad-GFP co-vaccinated, or Ad5-CSP+Ad-EAT2 co-vaccinated mice. After 18 hrs splenocytes from these mice were collected and analyzed by flow cytometry to assess the amount of NYDNAGTNL specific killing. % Specific killing = 1-((%CFSEHigh/%CFSELow)immunized/(%CFSEHigh/CFSELow)non-immunized). * denotes significant difference between treatments $p<0.05$.

was performed, the ratios of Th1 to Th2 antibody (IgG2a/IgG1) in mice treated with Ad-EAT2+Ad5-CSP were similar to the ratio of Th1 to Th2 antibody in control treated mice in all dilution except 1:400, indicating that expression of EAT-2 did not induce a Th1 or Th2 bias in these mice at 14 dpi as measured by this assay (Figure 35). In addition, when measured by ICS, there was no significant difference in the number of likely CD4+ IFNγ expressing T-cells, as the number of CD8- CD3+ T cells in Ad5-CSP+Ad-EAT2 treated animals and were similar to the numbers of these cells noted in Ad5-CSP+Ad-GFP treated animals (Figure 36).

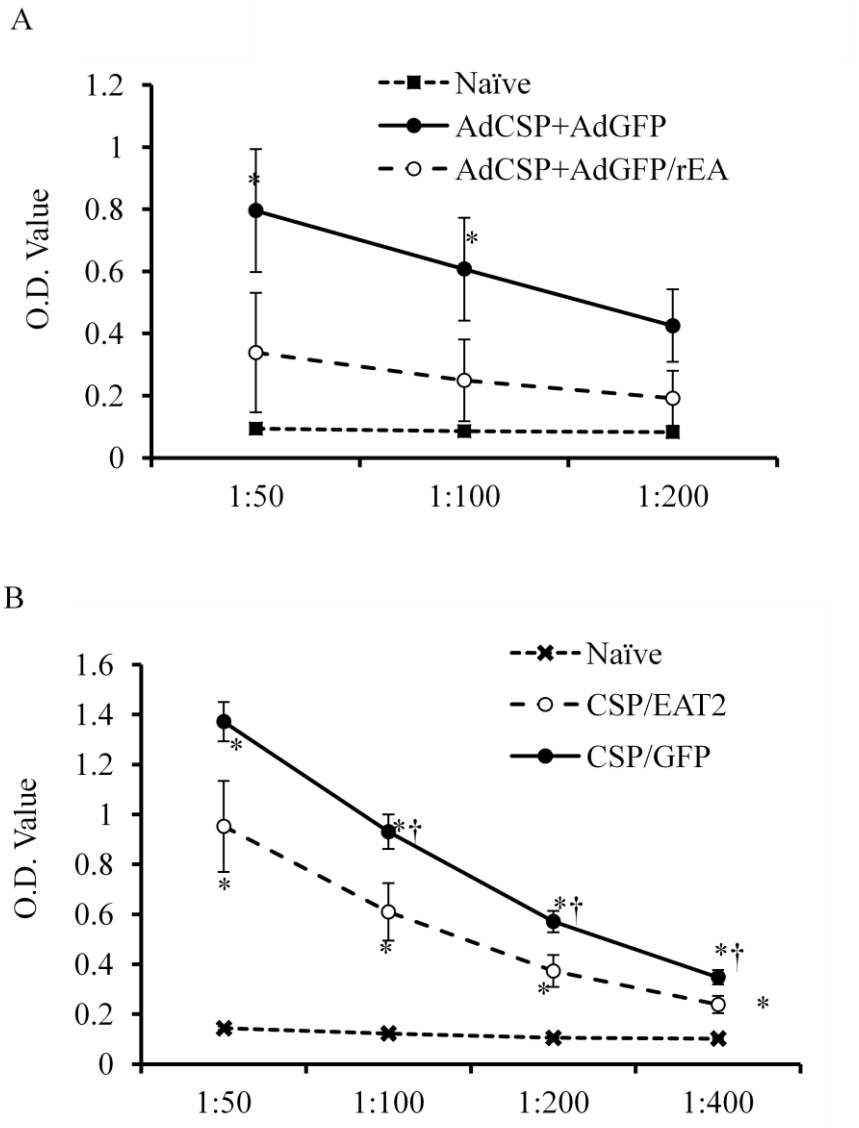

Figure 34: Induction of CSP specific antibody responses by Ad5-CSP vaccines augmented by rEA or EAT-2. Total IgG antibody against CSP is ablated in Ad5-CSP+Ad-GFP/rEA co-vaccinated mice while Ad5-CSP+Ad-EAT2 co-vaccinated mice demonstrated significantly more CSP specific IgG than naïve animals. BALB/cJ mice (n=5) were co-injected IM with $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of either (A) Ad-GFP/rEA or (B) Ad-EAT2. Plasma was collected at day 14. Total IgG against CSP in the plasma was measured by ELISA. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test, * Denotes significance over naïve $p<0.05$. † Denotes significant difference between treatments $p<0.05$.

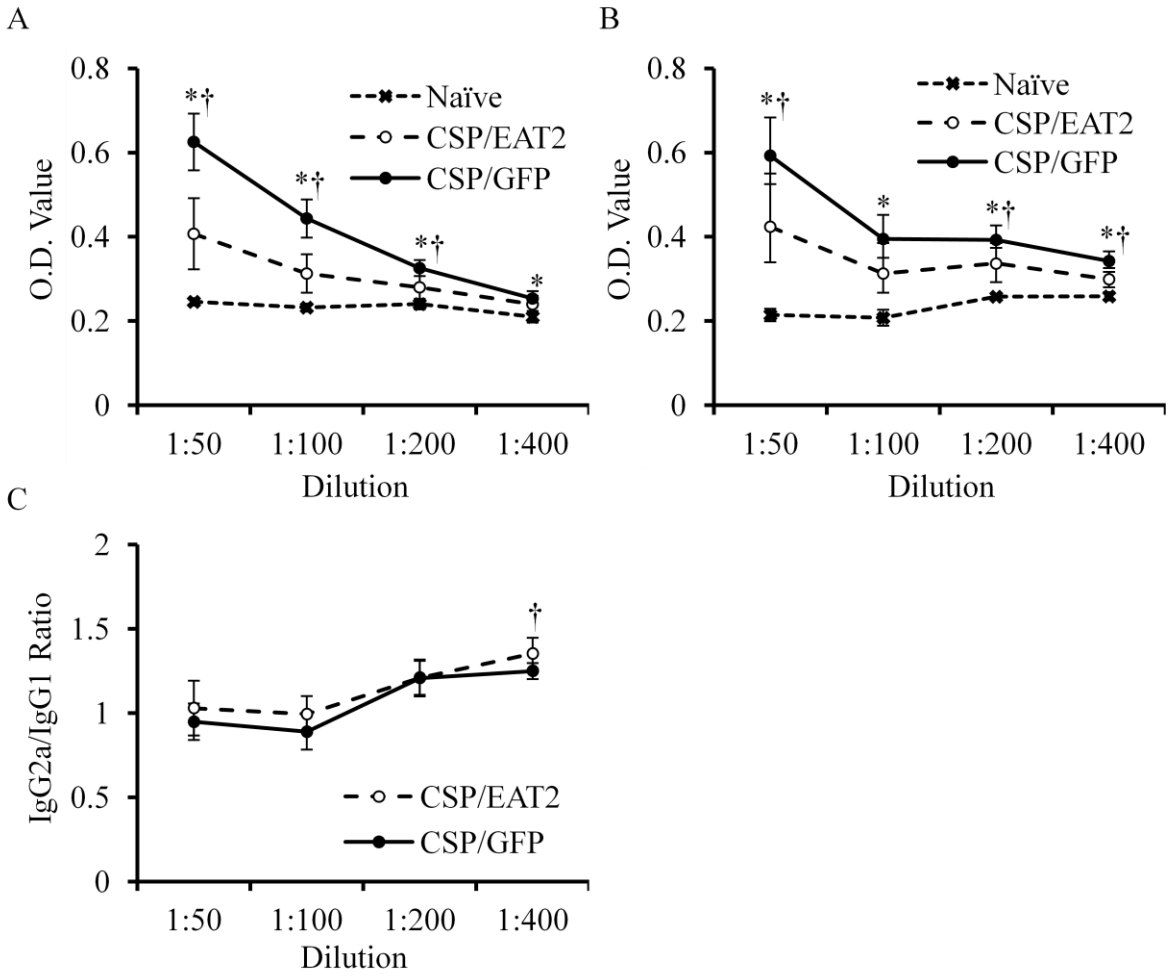

Figure 35: Sub-isotype analysis of IgG antibody from plasma of mice co-vaccinated with Ad5-CSP and Ad-EAT2. BALB/cJ mice (n=6) were co-injected i.m. with $5x10^7$ vps of Ad5-CSP and $5x10^7$ vps of Ad-GFP or $5x10^7$ vps of Ad5-CSP and $5x10^7$ vps of Ad-EAT2. Plasma was collected at day 14. The amount of CSP specific IgG subisotypes, IgG1 (A) and IgG2a (B) was measured by ELISA. The ratio of IgG2a/IgG1 was calculated to indicate the Th1 to Th2 response ratio (C). The bars represent mean ± SD. Statistical analysis for sub-isotyping (A,B) was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test and standard t-test was performed for Th1 to Th2 ratios (C),* indicates significance over naïve $p<0.05$. † Indicates significance between treatments $p<0.05$.

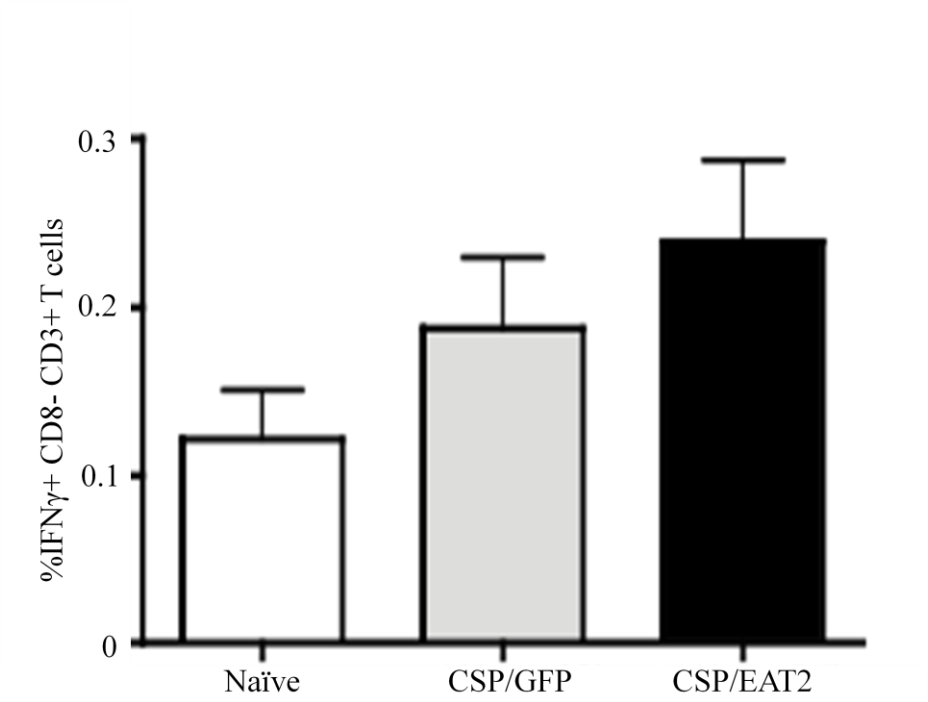

Figure 36: CD3+ CD8- IFNγ+ cells respond similarly to both vaccine regimens. Co-vaccination with Ad5-CSP and Ad-EAT2 resulted in similar IFNγ secretion from CD3+ CD8- T cells. BALB/cJ mice (n=6) were co-injected IM with $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-EAT2 or $5x10^7$ vps/mouse of Ad5-CSP and $5x10^7$ vps/mouse of Ad-GFP. Splenocytes were stimulation with NYDNAGTNL peptide. Cells were stained with CD8-Alexa Flour700, CD3-APC-Cy7, ViViD, and IFNg-APC. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test.

4.3 Discussion:

Our earlier works and those of others suggest that activation of the innate immune system can play an important role in beneficially augmenting subsequent antigen specific adaptive immune responses.[68,69,72,142,155] For example, we previously augmented CMI responses against HIV-Gag by co-injecting a rAd5 vector expressing HIV-Gag with a rAd5 vector expressing a TLR agonist, rEA.[142] Similarly, co-injecting a rAd5 vector expressing HIV-Gag with a rAd5 vector expressing the SLAM receptors adaptor protein EAT-2 also augmented induction of innate immune responses, and improved the induction of HIV-Gag specific T cell responses.[146] As a new approach to increasing the potency of malaria specific vaccines, we now describe the use of adenoviral based vaccines engineered to express malaria derived proteins, simultaneously administered with rAds expressing proteins known to modulate the innate immune system. Most importantly, we have confirmed that rAd mediated expression of a SLAM pathway derived adaptor (EAT-2) can significantly augment the induction of malaria antigen (CSP) specific CMI responses. This was verified based upon ELISpost analysis of splenocytes (both as to their responsiveness to immunodominant peptides, as well the breadth of these responses to the full length CSP), ICS staining of cells for IFNγ, and most importantly by a CSP specific *in vivo* CTL functional assay. EAT-2 expressing vaccines should be considered for use in future malaria vaccine trials attempting to boost malaria antigen specific CMI responses. Furthermore, EAT-2 co-expression allowed for the induction of CS specific antibody responses as well.

In contrast, co-vaccination of mice with a rAd vaccine expressing a TLR agonist simultaneously with a rAd expressing CSP, actually had the opposite effect, and completely mitigated induction of CSP specific adaptive humoral and cellular immune responses, as

compared to responses typically induced by the rAd vaccines expressing CSP alone. There could be numerous reasons for these unexpected, paradoxical and potentially disturbing results. A simple reason could be that the increase in pro-inflammatory cytokines caused by rAd mediated expression of the TLR agonist, rEA, could be influencing expression of CSP from the rAd5 vector. However, this effect would have likely been observed in our previous studies utilizing the same vector combinations, as well as the same TLR or SLAM receptors derived adaptors, but a different target antigen (HIV-Gag). Those studies also confirmed induction of similar innate immune responses to those noted in this study.[142,146] It is more logical that the CSP somehow negatively interacts with immune pathways excessively activated by TLR agonists such as rEA, resulting in a complete ablation of CSP specific CMI responses. This immunosuppressive activity of CSP appears to only be unveiled after excessive stimulation of TLR pathways, as our use of EAT-2 demonstrated not only avoidance of CS immunosuppressive activity, but also allowed for enhanced induction of CS specific adaptive immune responses.

The CSP has been specifically confirmed to be capable of outcompeting the transcription factor NF-κB for binding to the nuclear transport protein, importan α, resulting in the downregulation of at least forty NF-κB controlled genes.[156] CSP was also shown to inhibit NF-κB entry into the nucleus by 75%.[156] As NF-κB is known to control numerous genes involved in pro-inflammatory immune responses, one hypothesis may be that the CSP can downregulate excessive (TLR-driven) NF-κB transcriptome responses, and result in a dramatically diminished acute inflammatory response, thereby blunting subsequent CSP antigen specific adaptive immune responses.[157] This may make biological sense, as infection of hepatocytes by malaria sporozoites has been shown to induce the activation of NF-κB in a MyD88 specific manner.[158] Expression of CSP by the parasite may have evolved to counteract

this inflammatory response and prevent excessive induction of malaria specific adaptive immune responses in the infected host. In support of our hypothesis that CSP mediated NF-κB interference ablates adaptive responses, recent studies on an immunosuppressive drug (dehydroxymethylepoxyquinomicin) that specifically interferes with the NF-κB-importan α interaction was shown at lower doses to only modestly affect IL-6 and TNFα levels, while dramatically affecting Th1 expansion, results paralleling those noted in our experiments.[159]

These notions may also explain our findings, as well results previously reported by others.[77,150] Those studies and ours verify that at very high doses, rAd vaccines expressing CSP also show a trend toward diminished induction of CSP specific CMI responses (Figure 24).[77,150] Multiple studies have shown that Ad vectors can also induce NF-κB.[102,103] Quite possibly, the CSP immunosuppressive effects are not uncovered until an "NF-κB activation threshold" has been broached, in this instance by use of excessively high doses of rAd vaccines expressing CSP, or by using more modest doses of the Ad vaccine coupled with potent TLR activation. Further studies will need to be performed to elucidate whether this or other mechanisms may be responsible for our results. Regardless, our data demonstrate the need to consider the impact the inclusion of CSP derived peptides, or the entire protein along with other immunostimulatory compounds may have upon present and future malaria specific vaccines. Taken together with recent data demonstrating that protection from malaria challenge can be independent of CSP suggests that the use of CSP in certain malaria vaccine formulations will have to be carefully considered.[30,31]

In contrast to co-expression of the TLR agonist, co-expression of EAT-2 and CSP eventuated in the enhanced induction of CMI responses to the CSP, relative to the use of the Ad5-CSP vaccine alone. We have also previously observed a potent CMI response against HIV

derived Gag in mice treated with Ad-EAT2+Ad-Gag.[146] Like TLRs, activation of the SLAM receptor pathway in DCs and macrophages can also enhance the production of pro-inflammatory cytokines.[160]

The biochemical mechanism and intracellular signaling pathway behind EAT-2's ability to function as a T cell (and possibly a B cell) stimulator in the face of CSP over expression is not fully elucidated, but is a question that has been unveiled by our studies. SLAM associated proteins like EAT-2 are known to play a role in several novel immunomodulatory pathways, including the SLAM, CD22, and FcγRIIB.[161,162,163] These pathways may not be subject to the immune suppressive actions of CSP possibly by virtue of its specific mode of action relative to NF-κB and/or TLR activation pathways described earlier.

It has been established that greater numbers of CD8+ T cells are required to police infected hepatocytes and achieve long term protective immunity against malaria, emphasizing the importance of inducing a large population of CD8+ T cells capable of killing.[24] There is some evidence that improved protection is also related to increased breadth of the CMI response in addition to the potency of the CMI response.[151,152,153] Here, as an accessory to the increased CMI response, we have demonstrated Ad-EAT2s ability to stimulate increased T cell responses against multiple CSP epitopes. We not only observed an increase in the percentage of CSP specific CD8+ T cells, but also improved *in vivo* CTL killing of CS pulsed splenocytes from mice treated with Ad5-CSP+Ad-EAT2. The use of EAT-2 to augment CSP specific functional CD8+ T cells may be of greatest importance in killing *Plasmodium* infected hepatocytes, as these types of responses are not only positively correlated with protective capability, but also may outweigh the need for induction of malaria antigen specific antibody responses.[22,24,26,164]

Improvements over sole use of Ad5-CSP to induce CSP antigen specific B cell responses were not achieved in mice treated with either Ad5-CSP vaccine cocktail. However, co-vaccination with the Ad5-CSP and Ad-EAT2 vectors at least prevented the loss of induction of CS specific antibody responses noted after use of the Ad-GFP/rEA and Ad5-CSP vaccine combination. These results did not appear to be due to a skewing from Th2 to Th1 type antibody response, as measured by IgG1/IgG2a ratios, there were also no observed differences in IFNγ secreting CD8- CD3+ T cells between treatment groups. Further research will need to be performed to elucidate the reasons behind the observed antibody responses.

The importance of stimulating a strong cytotoxic T cell response against *P.falciparum* infected hepatocytes is vital in creating a subunit based vaccine that is protective against malaria. With this study we have successfully stimulated a CMI response to CSP that can overcome CSP related adaptive immune response ablation and is even more potent than the previous generation of rAd5s expressing CSP. Incorporation of this new vaccine platform into ongoing or future malaria vaccine trials could potentially achieve the levels of prophylaxis needed to protect vulnerable populations against natural malaria infections. Future studies will need to be performed to assess this platforms ability to protect larger animals challenged with malaria.

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Chapter 5:

Capability of advanced generation, Adenovirus based malaria vaccines to prevent malaria infection.

5.1 Introduction

Previous attempts at putative malaria vaccines have all come up short in either their ability to generate long lasting protection to malaria infections, or in their inability to be practically manufactured and distributed. Use of irradiated sporozoite based vaccine formulations has proven that protection is achievable, reaching levels of protection greater than 90%.[19] However, irradiated sporozoites cannot survive long outside of the mosquito salivary gland, and they do not endure freeze thaw cycles well.[23] Delivery of sporozoites via bites from irradiated infected mosquitoes can prolong the lifespan of the sporozoite, but it is not a practical vaccination method as many hundreds of bites are required to achieve protection.[18,19,21] On the other hand, subunit vaccines (like the leading subunit malaria vaccine candidate (RTS,S) consisting of a pseudo virion made up of HBsAg-CSP) can be easily manufactured and administered, although no subunit vaccine has reached the levels of protection that irradiated sporozoites confer. In fact, early formulations of RTS,S were poorly immunogenic and stimulated a predominantly humoral response against CSP.[45] It was only after potent adjuvants aimed at improving CD8+ T cell responses were incorporated that the RTS,S platform was capable achieving 56% protection from naturally occurring malaria.[44]

While many disease vaccine platforms require potent antibody responses to achieve protection, protection from malaria appears to be mediated by a potent CD8+ T cell response against the liver stage. In fact, irradiated sporozoite mediated protection from malaria challenge has repeatedly been shown to be primarily due to CD8+ T cell responses against the liver stage.[22,24,26] Initially, Ad based vaccine vectors seemed like an excellent candidate to stimulate the potent CD8+ T cell responses against liver stage antigens that are required to achieve protection from malaria. Mice vaccinated with a single injection of Ad expressing *P.*

yoelii CSP even exhibited 93% inhibition of liver stage development following sporozoite challenge with *P. yoelii*.[59] However, despite Ad vectors natural ability to induce potent CD8+ T cell responses against a trangene, no Ad-based malaria vaccine has achieved improved protection over the leading malaria vaccine candidate, RTS,S.

Our previous works have demonstrated that Ad mediated induction of CD8+ T cells can be further augmented with the incorporation of a potent immunomodulator.[136,146] The expression of SLAM receptor adaptor protein, EAT-2, from an Ad co-injected with an Ad expressing CSP resulted in augmented induction of innate immune responses, which ultimately led to improved cytotoxic T cell responses against CSP.[136] Importantly, we have undertaken functional analysis to prove that this platform creates a cytotoxic T cell response that is more effective at killing cells displaying epitopes of CSP *in vivo* than the control vaccine.[136] Here, we assess this platforms ability to confer protection from live parasite malaria challenge in a mouse model. In doing so, we also assess the validity of our *in vivo* cytotoxic T lymphocyte assay to predict efficacy of a putative malaria vaccine.

5.2 Results:

Construction and validation of Ad5-*Pb*CSP

We constructed an Ad5 vector expressing *Plasmodium berghei* CSP from a CMV promoter (Ad5-*Pb*CSP) in the same manner as previously described for Ad5 expressing *P. falciparum* (Ad5-*Pf*CSP).[136] We then confirmed that this construct responds similarly in BALB/cJ mice as our previous experiments with Ad5-*Pf*CSP. BALB/cJ mice were co-vaccinated with $5x10^7$ vp/mouse of Ad5-EAT2 and $5x10^7$ vp/mouse of Ad5-*Pb*CSP (Ad5-EAT2+Ad5-*Pb*CSP) or $5x10^7$ vp/mouse of Ad5-Null and $5x10^7$ vp/mouse Ad5-*Pb*CSP (Ad5-Null+Ad5-*Pb*CSP) (n=5). 14 dpi splenocytes were harvested and stimulated with a *P. berghei* CSP dominant epitope (SYIPSAEKI) overnight. ELISpots were then performed measuring IFNγ secreting splenocytes. Ad5-EAT2+Ad5-*Pb*CSP co-vaccinated animals had significantly more IFNγ secreting splenocytes than both unvaccinated and Ad5-Null+Ad5-*Pb*CSP treated mice ($p<0.001$ and $p<0.01$ respectively) (Figure 37A). Likewise, when we analyzed CD8+ T cells specific for *P. berghei* CSP by staining with a SYIPSAEKI tetramer and antibodies for CD3 and CD8, we found Ad5-EAT2+Ad5-*Pb*CSP co-vaccinated animals had significantly higher percentages of tetramer positive CD8+ T cells than both unvaccinated and Ad5-Null+Ad5-*Pb*CSP treated mice ($p<0.05$) (Figure 37B).

P. berghei Challenge:

We next sought to determine our vaccine's ability to confer protection from malaria challenge in mice bitten with *P. berghei* ANKA strain infected mosquitoes. We obtained *P. berghei* ANKA strain engineered to express GFP from New York University Insectory. Although we were able to detect increased numbers of *P. berghei* CSP specific IFNγ secreting

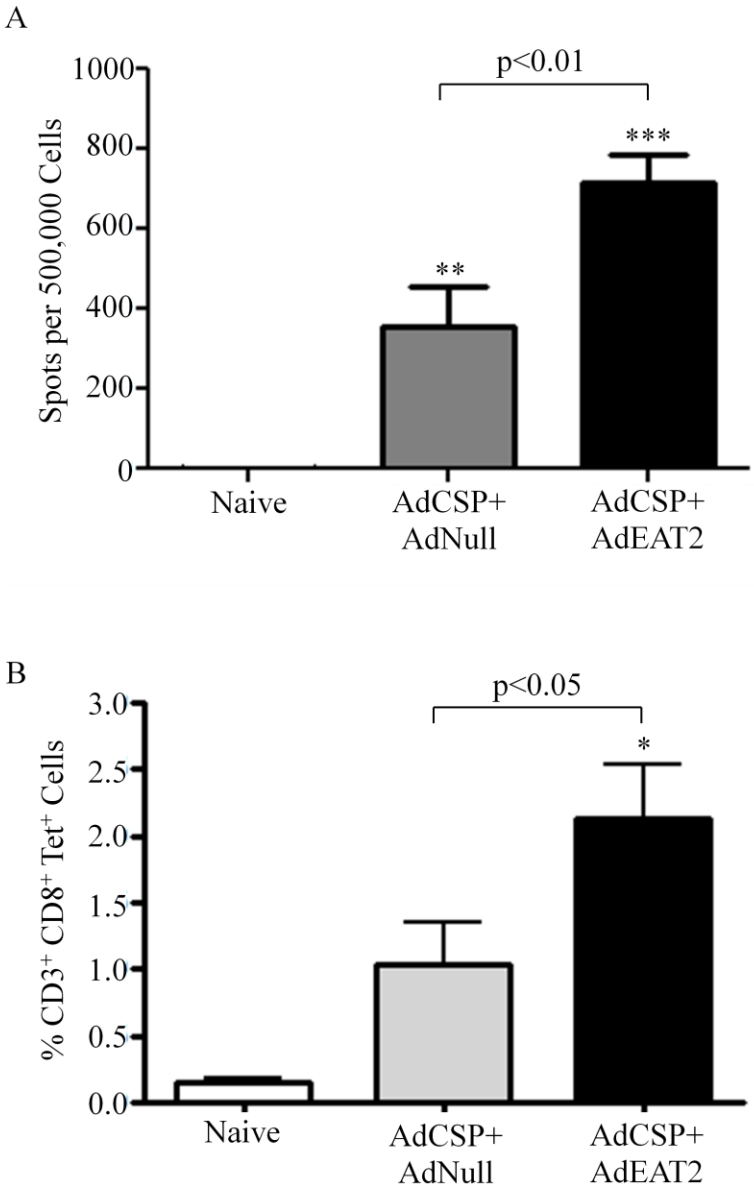

Figure 37: Co-vaccination with Ad5-*Pb*CSP and Ad5-EAT2 drastically increases *Pb*CSP specific CMI responses. BALB/cJ mice were injected IM with either $5x10^7$ vps/mouse of Ad5-*Pb*CSP and $5x10^7$ vps/mouse of Ad5-Null or $5x10^7$ vps/mouse of Ad5-*Pb*CSP and $5x10^7$ vps/mouse Ad5-EAT2 (n=5). Splenocytes were collected 14 days post co-injection. (A) ELISpot was performed on the splenocytes of these mice stimulated with SYIPSAEKI peptide to assess the amount of IFNγ secreting cells. (B) Splenocytes were also stained with a SYIPSAEKI tetramer and antibodies for CD3 and CD8 to determine CD8+ T cells specific for *Pb*CSP. The bars represent mean ± SD. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test,*,**,*** Denotes significance over naïve animals, $p<0.05$, $p<0.01$, and $p<0.001$.

splenocytes and increased percentages of CD8+ tetramer positive cells in animals co-vaccinated with Ad5-EAT2+Ad5-*Pb*CSP as compared to unvaccinated and Ad5-Null+Ad5-*Pb*CSP co-vaccinated animals, we were unable to detect differences in protective efficacy when BALB/cJ mice were co-vaccinated with $1x10^8$ vp/mouse of Ad5-EAT2 and $1x10^8$ vp/mouse of Ad5-*Pb*CSP (Ad5-EAT2+Ad5-*Pb*CSP) or $1x10^8$ vp/mouse of Ad5-Null and $1x10^8$ vp/mouse Ad5-*Pb*CSP (Ad5-Null+Ad5-*Pb*CSP) (n=10). We selected a slightly higher dose of vaccine to better optimize CSP specific CMI responses based on our previously published Ad5-*Pf*CSP dose curve (Figure 24). 14 dpi mice were anesthetized and placed over a net covered cup containing 12-15 infected mosquitoes. Mosquitoes were allowed to bite for 9 minutes repositioning the mice every 3 minutes. 7 and 14 days post challenge mice were bled via tail snip. Two drops of blood were collected from each mouse; one was used to make a thin blood smear and the other drop was collected in 500 µL of Alsever's solution. Blood smears were photographed under confocal microscopy filtered for GFP expressing cells and were subsequently stained with Giemsa stain and analyzed under microscopy for percent infected red blood cells. Blood collected in Alsever's solution was analyzed by flow cytometry for FITC fluorescing cells.

Our data supports previously published data demonstrating that Giemsa stain assessed percent parisitemia tightly correlates with flow cytomery measured percent parasitemia following challenge with GFP fluorescing *P.berghei* (Figure 38).[165] We observed no differences in percent parasitemia between non-vaccinated, Ad5-Null+Ad5-*Pb*CSP vaccinated, or Ad5-EAT2+Ad5-*Pb*CSP vaccinated animals by any assay at either time point (Figure 39). BALB/cJ mice are particularly susceptible to *P. berghei* infection.[166] It appears that allowing 12-15 mosquitoes per mouse to bite for 9 minutes results in an unrealistically high dose of parasite.

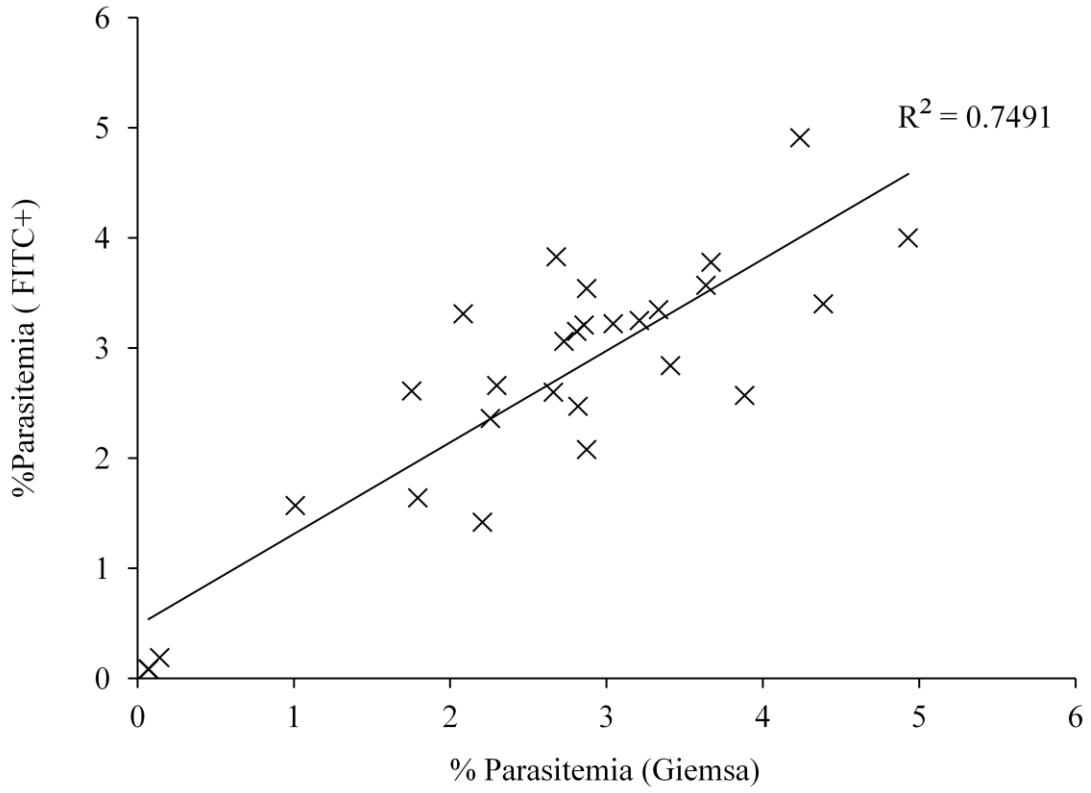

Figure 38: Giemsa stain strongly correlates with % FITC+ red blood cells. 7 and 14 days post challenge with *P. berghei* ANKA expressing GFP blood was collected via tail snip. Thin blood smears were stained with Giemsa stain. Infected red blood cells were counted on each frame and divided by average red blood cells per frame to determine % parasitemia (y-axis). Red blood cells were analyzed by flow cytometry for % FITC+ cells (x-axis). Giemsa stain was founf to strongly correlate with % FITC+ red blood cells (r=).

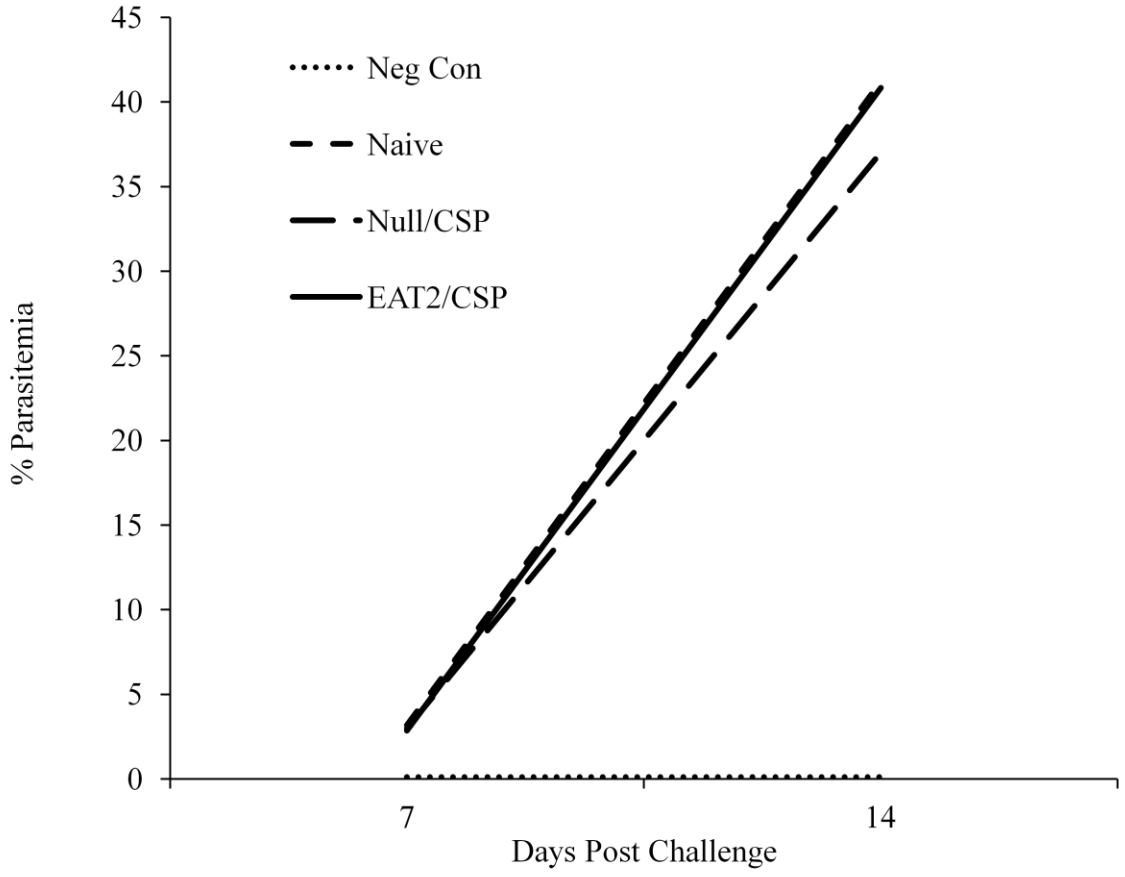

Figure 39: Parasitemia was similar between treatments at 7 and 14 days post challenge. Blood was collected via tail snip at 7 and 14 days post challenge with *P. berghei* ANKA expressing GFP and analyzed by flow cytometry for FITC+ red blood cells. Statistical analysis was completed using One Way ANOVA with a Student-Newman-Keuls post-hoc test.

This high dose of parasite challenged overwhelmed the ability of all vaccinations tested to prevent parasite growth.

5.3 Discussion:

We were able to detect increased numbers of *P. berghei* CSP specific IFNγ secreting splenocytes and increased percentages of CD8+ tetramer positive cells in animals co-vaccinated with Ad5-EAT2+Ad5-*Pb*CSP as compared to non-vaccinated and Ad5-Null+Ad5-*Pb*CSP co-vaccinated animals. However, we were unable to detect differences in protective efficacy between any of the vaccinated or unvaccinated groups after parasite challenge. Likely the unrealistically high dose of parasites used in these challenge experiments overwhelmed the protective capability of both vaccination strategies. BALB/cJ mice are particularly susceptible to *P. berghei* infection and it has been demonstrated that a single bite can result in over 80% infection of naïve BALB/cJ mice.[166] It is also possible that the vaccine may not be able to protect against live infections despite the induction of potent CSP specific cytotoxic T cell responses. The decreased humoral responses associated with the Ad5-EAT2+Ad5-CSP platform could be limiting the vaccines efficacy. While multiple studies demonstrate the need for potent CD8+ T cell responses, strong humoral responses may still be required for an effective CSP based malaria vaccine. Based on these results it is evident that functional, real world assays should be undertaken to determine putative malaria vaccine efficacy. Future experiments will be conducted repeating the experiments outlined above using fewer *P. berghei* infected mosquitoes per mouse in an effort to more realistically simulate real world infections. Alternatively, sporozoites can be harvested and injected IV in order to more closely regulate the amount of sporozoites administered per mouse. We could also switch mouse strains to a less susceptible strain of mice like Swiss Webster outbred mice to further our ability to discern if use of immune modulation via Ad mediated EAT-2 expression can prevent malaria parasite growth in vaccinated animals. Should the use of Ad5-EAT2+Ad5-CSP prove not to be effective as a single

injection it might still be utilized to improve CSP CD8+ T cells responses in heterologous prime boost regimens with more antibody biased malaria vaccines like RTS,S.

Use of GFP expressing *P. berghei* parasites proved to be a consistent and easy method for measuring percent parasitemia of challenged mice by analyzing FITC+ red blood cells by flow cytometry. Measuring percent FITC+ red blood cells by flow cytometry eliminates some human error and objectivity in measuring percent parasitemia. This method could be an important tool in future parasite challenge experiments.

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Chapter 6:

Summary and future directions

6.1 Ad interactions with the innate immune system:

Adenovirus is commonly used as a vector for two very disparate purposes. As a gene delivery vector, Ad interactions with the innate immune system is a detriment to its efficacy, while Ad mediated induction of innate immune responses can be exploited for stimulation of more potent adaptive immune responses to a targeted antigen in vaccine platforms. My research has helped to elucidate multiple complex interactions between Adenovirus and the innate immune system. These realizations can be used to potentially improve both gene delivery and vaccine vector functions of rAds. For example, we have uncovered potential targets that can be used to suppress or augment immune responses.

We have experimentally proven that Ad induced liver transcriptome dysregulation is complement dependent, but we were unable to delineate specific roles of either the classical or alternative complement pathways in transcriptome responses aside from a role for C4 in TLR2 transcription.[71] We did however find that the dysregulation of some genes are actually negatively regulated by CP and AP components (C1q and FB respectively). We also confirmed that many of the observed Ad-induced transcriptome changes are negatively regulated by mCR1/2. Similarly, inductions of some cytokines and chemokines by systemic delivery of adenovirus were also found to be complement dependent and are negatively regulated by mCR1/2.[167]

Primarily via the use of complement protein knockout mice, when we analyzed induction of anti-Ad neutralizing antibodies we found functional C3 is required for production of anti-Ad neutralizing antibody. Further, inductions of total anti-Ad IgG and IgG3 were both found to be dependent on FB and C3.[71] We also uncovered a substantial role for complement in modulating the balance between Th1 Th2 antibody responses. However, while complement is

required for maximal anti-Ad humoral responses, we did not detect a role for complement in anti-transgene humoral responses.[71] When we looked at the role of mCR1/2 in stimulation of anti-Ad humoral responses we found that despite mCR1/2's negative role in controlling multiple genes involved in inflammation and cytokines, mCR1/2 is actually required for induction of maximal anti-Ad antibody responses, inclusing anti-Ad neutralizing antibody responses. Unlike what we observed in C3-KO, anti-transgene humoral responses are decreased in mCR1/2-KO mice.[167]

We hypothesize that lack of functional mCR1/2 allows for unchecked secretion of cytokines, which interferes with trangene expression and might explain the decreased anti-transgene humoral responses. As for mCR1/2's involvement in anti-Ad humoral responses we determined mCR1/2 could be playing an important role in activating B cells opsonized with C3 (data not shown).[167]

Given our findings we sought to modulate Ad-induced innate immune responses by incorporating a negative regulator of complement (DAF) similar to mCR1/2 into the Ad-caspid. We fused a reoriented DAF to adenovirus capsid protein IX in order to display DAF in a more natural conformation. Use of this novel Ad vector successfully inhibited multiple Ad-induced complement mediated innate immune responses validating our previously discussed data. This novel Ad vector could prove to be a valuable tool in gene therapy applications.[168]

6.2 Studies of alternative Ad serotypes:

Our explication of Ad interactions with the innate immune system and its affect on adaptive responses can also be exploited for vaccine platforms. Our research further explores Ads for use as a vaccine vector using malaria as a model platform. First, we sought to overcome a commonly cited problem with utilizing Ad5 as a malaria vaccine vector, namely the high

seroprevalence of pre-existing Ad5 immunity of humans residing in malaria endemic regions. We attempted to utilize an alternative serotype to Ad5 that has been shown to be more immunogenic and has a history of use as a vaccine vector, Ad4. We engineered Ad4 and Ad5 vectors to express the most commonly used malaria antigen for vaccine applications (CSP) and examined how their distinctive stimulation of the innate immune system could be used in homologous and heterologous prime boost regimens in both Ad5 naïve and Ad5 immune backgrounds.

We found that the combined use of an Ad4-CSP prime followed by a boost with Ad5-CSP resulted in more efficient inductions of CSP specific cytotoxic T cells in Ad5 naïve animals, requiring fewer CSP specific CD8+ T cells to achieve the same level of CSP specific killing *in vivo* as homologous prime boost of Ad5-CSP. This knowledge could be used to design more effective heterologous prime boost vaccination regimens. However, when we looked at the efficacy of the Ad5 based malaria vaccine platform in Ad5 immune animals we observed dramatically ablated responses despite these viruses residing in different subgroups, based upon classical anti-sera neutralization categorization strategies.[100] This data suggests that separation of Ads based on anti-sera neutralization properties alone may not be sufficient to determine their levels of cross-reactivity when utilized in these types of applications. This critical finding unearths a serious concern with using alternative serotypes as replacements for Ad5 in Ad5 immune patients. Future studies should similarly and stringently analyze use of alternative serotype Ads to properly evaluate their potential for efficacy in Ad5-immune patients. Future studies comparing T cell antigens of Ads from different subgroups might result in the discovery of more immunologically distinct Ads that can be utilized to improve heterologous prime boost vaccine responses, as well as Ad-induced antigen specific immune responses in Ad5 immune

patients. Knowledge of specific antigens that are cross-reactive between different Ad subgroups could also allow for the engineering of Ad vectors that have genetically eliminated or altered the cross-reactive antigens. Likewise, further elucidation of Ad4 induced innate immune responses could uncover the Ad4 antigens responsible for the stimulation of the early innate cytokines that interfere with CMV promoter expression, the elimination of which could potentially improve Ad4-based vaccine vector efficacy.

6.3 Immunomodulation:

We then sought to improve upon Ad5 based malaria vaccine inductions of adaptive responses against CSP by use of innate immune modulators. Since Ad5 has been shown to activate the innate immune system through TLRs and has the ability to stimulates potent CD8+ T cell responses against a transgene, we hypothesized that further stimulation of TLRs would result in increased CD8+ T cell responses to CSP. To test this we engineered an Ad expressing a potent TLR agonist called rEA (Ad-GFP/rEA) and co-injected it with Ad5-CSP. Perplexingly, we found that despite large increases in secretion of multiple innate cytokines, adaptive responses to CSP were paradoxically ablated. It is possible that the increase in pro-inflammatory cytokines interfered with CSP expression. However, if this were the case we would have observed similar ablation in previous studies where we expressed an HIV derived protein instead of CSP as similar cytokine inductions were observed.[142] It is more logical that CSP negatively interacts with the specific immune pathway that is excessively activated by the TLR agonist (rEA).

CSP has been shown to interfere with NF-κB nuclear translocation by outcompeting NF-κB for important α, although how this interference can affect adaptive responses has not been shown.[169] We hypothesize that CSP mediated downregulation of the TLR-driven excessive NF-κB transcriptome response dramatically diminishes acute inflammatory responses therefore

resulting in ablated CSP specific adaptive immune responses. In support of our hypothesis, recent studies on an immunosuppressive drug (dehydroxymethylepoxyquinomicin) that specifically interferes with the NF-κB-importan α interaction was shown at lower doses to only modestly affect IL-6 and TNFα levels, while dramatically affecting Th1 expansion, results paralleling those noted in our experiments.[159] Here we uncover an important immunosuppressive function of CSP through inadvertent stimulation of the very pathway CSP suppresses. Future experiments could be undertaken to further elucidate CSP immunosuppression mechanisms by measuring CSP interference with NF-κB nuclear translocation and the resulting impact on a wider array of cytokines than was measured in our experiments. Deletion or mutation of CSP's nuclear localization site could also be undertaken in future CSP-based malaria vaccines to eliminate CSP immunosuppression. Additionally, TLR agonists, like rEA, might still have great benefit in non-CSP malaria vaccine platforms and should continue to be studied.

We then substituted a NF-κB independent immunomodulator, called EAT-2, in an attempt to bypass CSP mediated immunosuppression of the NF-κB pathway. We found simultaneous injection of Ads expressing EAT-2 and Ad5-CSP vectors successfully stimulated dramatic increases in CSP specific activation, percentages of CSP specific CD8+ T cells, and CSP specific cytotoxic activity *in vivo* despite CSP interference with NF-κB nuclear translocation. Like TLRs, activation of the SLAM receptor pathway in DCs and macrophages can also enhance the production of pro-inflammatory cytokines.[160] The biochemical mechanism and intracellular signaling pathway behind EAT-2 mediated activation of adaptive responses is not fully elucidated. SLAM associated proteins like EAT-2 are known to play a role in several novel immunomodulatory pathways, including the SLAM, CD22, and

FcγRIIB.[161,162,163] These pathways may be distinct from NF-κB and/or TLR activation pathways; therefore they may not be subject to the immune suppressive actions of CSP. Future studies could be performed elucidating EAT-2 signaling mechanisms and their role in dendritic cell and macrophage maturation.

6.4 Challenge study:

Based on our results we attempted to assess ability of the Ad5-EAT2 with Ad5-CSP platform to provide protection from parasite infection in a mouse model of malaria. We constructed and validated that the Ad5-*Pb*CSP virus performs just as Ad5-*Pf*CSP did when co-injected with Ad5-EAT2 as measured by ELISpot and tetramer staining. After co-vaccination with Ad5-EAT2+Ad5-*Pb*CSP or a control vaccine, mice were challenged via bites from mosquitoes infected with *P. berghei* ANKA expressing GFP (12-15 mosquitoes per mouse). Percent parasitemia was measured at 7 and 14 days post parasite challenge. Through this research we have proved that the use of flow cytometry to measure parasitemia after challenge with GFP expressing sporozoites is a valuable tool that eliminates some human error and objectivity. However, the dose of parasite used proved to be high, as we were unable to detect any differences between the two vaccination treatments or unvaccinated mice since both vaccinations were overwhelmed. It is also possible that the vaccines may not be able to protect against live infections despite the induction of potent T cell responses. This finding underlines the importance of including functional, real world assays in such studies. Future studies using lower doses of the parasite may allow for the vaccines to prevent infection, and/or confirm improved efficacy of Ad malaria vaccines expressing EAT-2. We might also consider the use of less susceptible animals, like Swiss Webster outbred mice, as an alternative to the highly susceptible BALB/cJ mice in future experiments. The potential of this vaccine to stimulate

potent CSP specific cytotoxic T cell responses when utilized in heterologous prime boost regimens with potent anti-CSP antibody stimulating vaccines, like RTS,S, should also be examined as combined use could result in improved responses over either vaccine when used alone. Should use of Ad5-EAT2+Ad5-CSP prove to be protective in these studies, we could move on to non-human primate models and ultimately human safety and efficacy clinical trials as has been done by GSK with their RTS,S malaria vaccine.

Chapter 7:

Material and methods

Materials and Methods:

Vector construction:

Ad-LacZ: The recombinant adenoviral vectors, rAd5-LacZand rAd5-GFP, are vectors carrying either CMV-LacZ or CMV-GFP transgene expression cassettes that replace the Ad E1 region of the [E3-]Ad genome, and were grown to high titers on human 293 cells as previously described.[170] Purification consisted of harvesting infected cell lysates, DNase and RNase treatment, and cesium chloride density gradient bandings as per the method of Ng and Graham.[171] The purified vector preparation was extensively dialyzed against 10 mM Tris (pH 8.0), and was stored in 1% sucrose/PBS at _80 1C. The vector preparation was determined to be free of replication competent Ad by PCR using E1-specific primers and titered by SDS disruption and by OD260 spectrophotometry essentially as previously described.10,12 The titer was further evaluated by in vitro transduction of 293 cells and by the enumeration of bacterial b-galactosidase staining cells as previously described, and the viral particle/bacterial b-galactosidase transducing unit titer was approximately 8:1 (data not shown).[172,173]

Ad5-GFP-IX-dDAF_REO: The N-terminal cDNA coding for the N-terminal domain (entire 320 amino acids: 35-354, DAF-CCPR1-4) of the human DAF gene was subcloned in-frame into the C-terminus of pIX. CCPR1-4 of DAF was PCR derived using following amplification primers tailed with *Nhe*I sites: (DAF-F 5'-gctagcgactgtggccttcccccagatgtacc-3', DAF-R 5'-gctagcacctgaagtggttccacttcctttatttgg-3'). The *Nhe*I tailed PCR product, amplified from a human DAF cDNA clone (ATCC# MGC-5192), was subcloned in-frame into the C-terminus of viral protein IX into pShuttle-IX/*Nhe*I, the latter constructed in our laboratory by introducing *Nhe*I recognition site at the C-terminus of capsid protein IX (just upstream of normal pIX stop codon)

as previously described.[174] The plasmid so obtained we refer to as pShuttle-IX-DAF, was linearised with *Pme*I restriction enzyme and homologously recombined with the rest of the Ad5 vector genome present in the plasmid pAdEasyI as previously described, yielding pAd-IX-DAF.[53]

We have also displayed DAF in a more native context (DAF_REO): N-terminuspIX-C-terminus-fusion/C-terminus-DAF-N-terminus. This required synthetic production (Geneart, Regensburg, Germany) of the DNA molecule encoding for the DAF amino acid sequence corresponding to 3'-5' DAF (i.e. aa sequence was reversed and displayed in the C-terminus of pIX) as described above. A GFP expression cassette was inserted into the MCS of the pShuttle-IX-DAF (or pShuttle-IX-DAF_REO) as previously described.[171] All viruses were found to be RCA free both by RCA PCR (E1 region amplification) and direct sequencing, methods as previously described.[175] All Ads have also been tested for the presence of bacterial endotoxin as previously described and were found to contain <0.15 EU per ml.

Ad5-CSP: The Open Reading Frame (ORF) of the *P. falciparum* CSP gene, composed of a codon optimized consensus of several *P. falciparum* CSP sequences (Figure 40), was incorporated into plasmid pGA4 (GENEART, Burlingame, CA) and excised from pGA4 using endonuclease *NheI* (NEB, Ipswich, MA). The excised portion was subcloned into the pAd Shuttle vector containing a CMV expression cassette. The resulting pAd5-CSP shuttle plasmid was linearized with *PmeI* restriction enzyme and homologously recombined with the pAdEasyI Ad5 vector genome as previously described yielding pAd5-CSP.[176] Virus was amplified in HEK293 cells. Ad5-CSP virus was purified using a CsCl2 gradient as previously described.[171] Direct sequencing and restriction enzyme mapping were carried out to confirm the fidelity of the

CS Protein Sequence Utilized for Constructing Ad-CSP

1 ASRSMMRKLA ILSVSSFLFV EALFQEYQCY GSSSNTRVLN ELNYDNAGTN
51 LYNELEMNYY GKQENWYSLK KNSRSLGEND DGNNNNGDNG REGKDEDKRD
101 GNNEDNEKLR KPKHKKLKQP ADGNPD**PNAN PNVDPNANPN VDPNANPNVD**
151 **PNANPNANPN ANPNANPNAN PNANPNANPN ANPNANPNAN PNANPNANPN**
201 **ANPNANPNAN PNVDPNANPN VDPNANPNAN PNANPNANPN ANPNANPNAN**
251 **PNANPNANPN ANPNANPNAN PNANPNANPN ANPNANPNAN PNAN**PNKNNQ
301 GNGQGHNMPN DPNRNVDENA NANNAVKNNN NEEPSDKHIE QYLKKIKN***SI***
351 ***STEWSPCSVT CGNGIQVRIK PGSANKPKDE LDYENDIEKK ICKMEKC***SSV
401 FNVVNS

Figure 40: CSP sequence. The CSP sequence utilized for constructing the Ad5-CSP vaccine was designed based on several known CSP sequences. The NYDNAGTNL peptide's location is underlined in the sequence. Bold font within the sequence indicates the repeat region of CSP. The location of the Thrombospondin-like Type 1 repeat region (TSR domain) is indicated by gray font.

CSP sequence. The same CSP consensus sequence was incorporated into an adenovirus serotype 4 vector. Ad4 vector construction was performed as previously described for other transgenes inserted into an Ad4 recombination based production system.[100] Construction of, Ad5-GFP, Ad5-GFP/rEA, and Ad5-EAT2 was performed as previously described.[71,142,146]

Animal procedures: All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC). Adult BALB/cJ mice, adult C57BL/6 mice, and B6.129S4-C3tmlCrr (C3-KO) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). FB-deficient mice, on the C57BL/6 background, were a generous gift of Dr Alex Szalai (University of Alabama). C4-KO and C1q-KO mice were a generous gift from Dr Garnett Kelsoe (Duke University Medical Center). mCR1/2-KO mice in C57BL/6 background were a kind gift from Dr. Tedder, (Duke University Medical Center).[177,178] Intravenous injections were performed via retro-orbital sinus in a volume of 200µL of PBS. Intramuscular (IM) injections were performed by injection into the tibialis anterior of the right hindlimb. Total injected volume of IM injections was 20 µl. When required, mice were anesthetized with a nose cone containing isoflurane. Splenocytes and plasma were collected. All procedures with rAds were performed under BSL-2, and all vector treated animals were maintained in ABSL-2 conditions. Care for mice was provided in accordance with PHS and AAALAC standards.

ELISA: ELISA-based antibody assays were completed as previously described.[71] High-binding flat bottom 96-well plates were coated with 0.2µg of purified CSP per well in a volume of 100µL and incubated overnight at 4℃. Plates were washed with PBS-Tween (0.05%) then blocked with blocking buffer (3% bovine serum albumin) for 1 hour at room temperature.

Plasma was diluted (1:50, 1:100, 1:200, 1:400) in blocking buffer.and added to the wells and incubated for 1 hour at room temperature. Wells were washed with PBS-Tween (0.05%) and HRP antibody (Bio-Rad) was added at 1:4000 dilution in PBS-Tween. Tetramethylbenzidine (TMB) (Sigma-Aldich) was added to each well and the reaction was stopped with 1N phosphoric acid. Plates are read at 450nm in a microplate spectrophotometer. Subisotyping tittering was completed with a hybridoma subisotyping kit (Calbiochem, La Jolla, CA) with plasma dilutions of 1:50, 1:100. 1:200. 1:400. Statistical analyses were performed using Student *t*-test.

Cytokine and chemokine analysis: Utilization of the 23-plex Bio-Rad cytokine assay system (Hercules, CA, USA) was complicated by lot-to-lot variations during the course of our studies. We therefore designed a 7-plex multiplex-based assay system to more accurately determine cytokine/chemokine plasma concentrations as per the manufacturer's instructions (Bio-Rad) by Luminex 100 technology (Luminex, Austin, TX, USA) essentially as previously described.[103] The presence of the following cytokine and chemokines were simultaneously queried in each plasma sample: IL-6, IL-12 (p40), G-CSF, KC, MIP-1b and RANTES. Statistics were completed using Student's t-test.

qRT-PCR analyses: To determine relative levels of a specific RNA transcript, tissues were snap-frozen in liquid nitrogen and RNA was harvested from E100 mg of frozen tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Following RNA isolation, reverse transcription was performed on 180 ng of total RNA using SuperScript II (Invitrogen) reverse transcriptase and random hexamers (Applied Biosystems, Foster

City, CA, USA) as per the manufacturer's protocol excluding RNaseOUT. Reverse transcription (RT) reactions were diluted to a total volume of 60 ml, and 2 ml 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/). Primers used for amplification have been previously described.[67,101] Quantitative polymerase chain reaction (qPCR) was carried out on an ABI 7900HT Fast Real-Time PCR System using SYBR Green PCR Mastermix (Applied Biosystems) in a 15 ml reaction. All PCRs were subjected to the following procedure: 95 1C for 10 min followed by 40 cycles of 95 1C for 15 s followed by 60 1C for 1 min. The comparative Ct method was used to determine relative gene expression using glyceraldehydes 3-phosphate dehydrogenase (GAPDH) to standardize expression levels across all samples. Relative expression increases were calculated based on levels of a respective transcript quantified in mock-injected animals of the same genotype. Statistical analyses were completed using Student's t-test comparing C57BL/6-treated animals with each respective genotype individually.

Neutralizing Antibody: Neutralizing antibody assays were performed as previously described.[71] $2x10^3$ HEK293 cells were plated in 125 µL of complete medie (Dulbecco's modified Eagle's medium (DMEM)), supplemented with 10% fetal bovine serum and penicillin/streptomycin/fungizone (PSF) and incubated overnight at 37°C, 5% CO2. Plasma was heat inactivated for 60 minutes at 56°C then brought to room temperature before dilutions were made in 100 µL and added to the appropriate wells. $1.3x10^6$ viral particals were then added to plasma dilutITER 96 AQueous One solution (Promega, Madison, WI, USA) was added to each well and incubated for 2 hours at 37°C, 5% CO2. 150 µL of media was removed into a clean microtiter plate and read at 492 nm in a spectrophotometer.

Isolation of lymphocytes: Splenocytes from individual mice were prepared by physical disruption of the spleen. The spleen was passed through a sterile 40μm nylon mesh cell strainer (Fisher Scientific, Pittsburgh, PA). Red blood cells were lysed using *ACK* lysis *buffer* (Invitrogen, Carlsbad, CA) remaining cells were resuspended in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin/fungizone.[142]

ELISPOT analysis: ELISpots were performed in accordance to manufacturer's protocol using the Ready-set Go IFNγ mouse ELISpot kit produced by eBiosciences (San Diego, CA). Splenocytes were stimulated *ex vivo* with 4μg/mL of the >98% pure CSP immunodominant peptide NYDNAGTNL (amino acids 43-51 of the CSP sequence) (GenScript Piscataway, NJ).[179] A library of 15mers overlapping by 5 amino acids spanning the entire CSP non-repeating region was constructed and also used to stimulate splenocytes *ex vivo* (Biosynthesis Inc., Lewisville, TX). Spots were counted and photographed by an automated ELISPOT reader system (Cellular Technology, Cleveland, OH). Ready-set Go IFNγ and IL-2 mouse ELISPOT kits purchased from eBioscience (San Diego, CA).

Cell staining and flow cytometry: Splenocytes were stained with various combinations of the following antibodies: PE-CD69, (3 μg/ml), FITC-CD8a, APC-CD3, APC-Cy7-CD3, Alexa Floure700-CD8a, PerCpCy5.5-CD19, PE-Cy7-NK1.1, PE-Cy7-TNFα, APC-IFNγ (4 μg/ml), PerCpCy5.5-CD127, PE-Cy7-CSP (NYD) tetramer, V450-CD62L, Granzyme B- (4 μg/ml) (All obtained from *BD Biosciences*, San Diego, CA) and PerCpCy5.5-IL-2 (4 μg/ml) (BioLegend, San Diego, CA). Cells were incubated on ice with the appropriate antibodies for 30 minutes,

washed, and sorted using an LSR II instrument and analyzed using FlowJo software. For intracellular cytokines staining, cells were surface stained, fixed with 2% formaldehyde (Polysciences, Warrington, PA), permeabilized with 0.2% Saponin (Sigma-Aldrich, St. Louis, MO), and stained for intracellular cytokines. Large cells and debris were excluded in the forward- and side-scatter plot, to minimize background levels of staining caused by nonspecific binding of antibodies; we initially stained the cells with CD16/32 FcR III/II antibody. In addition we included the violet fluorescent reactive dye (ViViD, Invitorgen) as a viability marker to exclude dead cells from the analysis.[180] Blood was isolated by retro-orbital bleeds and PBMCs were isolated using Lympholyte-Mammal (Cedarlane, Burlington NC).Tetramer staining of PBMCs was completed using a PE conjugated MHC-I (H2d) tetramer folded with the NYDNAGTNL peptide generated at the NIH Tetramer Core Facility.

In vivo CTL assay: BALB/cJ were co-vaccinated with equivalent doses of Ad5-CSP and either Ad-GFP or Ad-EAT2 (totaling 2×108 vps). At 14 days, syngeneic splenocytes were isolated and pulsed with either an irrelevant peptide or peptide specific to the *P. falciparum* circumsporozoite antigen (NYDNAGTNL) for 1 hour at 37ºC. Irrelevant peptide pulsed cells were stained with 1μM CFSE (CFSELow) while CSP-peptides pulsed cells were stained with 10μM CFSE (CFSEHigh). Naïve and immunized mice were injected with equivalent amount of both CFSELow and CFSEHigh stained cells via the retro-orbital sinus. After 18-24 hours splenocytes were harvested and sorted on an LSRII flow cytometer. FlowJo software was used to determine percentages of CFSE stained cells. % Specific killing = 1-((% CFSEHigh / % CFSELow) immunized / (% CFSEHigh / % CFSELow) non-immunized).

Statistical analysis: Statistically significant differences in ELISpot assays were determined using either Two Way ANOVA with a Bonferroni post-hoc test or a One Way ANOVA with a Student-Newman-Keuls post-hoc test (p value < 0.05). For ELISA analysis, a t-test was used to assess significance between treatments. For multiparameter flow cytometry, a One Way ANOVA with a Student-Newman-Keuls post-hoc test was used. For *in vivo* CTL assay, a One Way ANOVA with a Student-Newman-Keuls post-hoc test was used. All graphs in this paper are presented as Mean ± SD with the exception of graphs of ELISA data which use Mean ± SE. GraphPad Prism software was utilized for statistical analysis.

***P. berghei* challenge:** *P. berghei* ANKA GFP expressing parasite infected mosquitoes were purchased from New York University Insectory (New York, NY, USA) Mosquitoes were starved for 2 hours prior to be allowed to feed on BALB/cJ mice. BALB/cJ mice were anesthetized with 80 mg ketamine per kg weight and placed over a netted cup containing 12-15 mosquitoes in a dimly lit room. Mice were repositioned every 3 minutes for a total of 9 minutes.

Microscopy: Mice were anesthetized via isoflurane inhalation and 1mm was cut from the very end of the tail. One drop of blood was used to make a thin blood smear on a glass slide and was allowed to dry completely. Pictures of dried thin blood smears were then taken under 600X confocal microscopy filtered for green fluorescent protein. Three pictures were taken per slide. Sections were selected based on similar red blood cell confluence. Average red blood cells per frame were counted. Then the total green fluorescent cells per frame were counted. Percent parasitemia was calculated as number of green cells divided by average red blood cells per

frame. One Way ANOVA with a Student-Newman-Keuls post-hoc test was used to calculate statistical significance.

Geimsa Stain: Mice were anesthetized via isoflurane inhalation and 1mm was cut from the very end of the tail. One drop of blood was used to make a thin blood smear on a glass slide and was allowed to dry completely. Slides were fixed by submerging them in 100% methanol for 1 minute. Slides were allowed to dry and were then submerged in 10% Giemsa stain diluted in deionized water for 50 minutes (GIBCO, Grand Island, New York, USA). Slides were than rinsed with deionized water and allowed to dry. Pictures of slides were taken at 400X. Average red blood cells per frame were counted. Then the total stained cells per frame were counted. Percent parasitemia was calculated as number of stained cells divided by average red blood cells per frame. One Way ANOVA with a Student-Newman-Keuls post-hoc test was used to calculate statistical significance.

Flow cytometry measurement of parasitemia: Mice were anesthetized via isoflurane inhalation and 1mm was cut from the very end of the tail. One drop of blood was collected in 500 µL of Alsever's solution. Diluted blood was run on flow cytometry gating first by size then for FITC+ cells. One Way ANOVA with a Student-Newman-Keuls post-hoc test was used to calculate statistical significance.

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