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Adenovirus Vectors as Potent Adjuvants in Vaccine Development

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ADENOVIRUS VECTORS AS POTENT ADJUVANTS IN VACCINE DEVELOPMENT

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ABSTRACT

Due to their ability to activate the immune system, replication-defective Adenoviruses (Ad) are potential vaccine vectors for several pathogens. The rapid proinflammatory response generated by Ad is key to the ability of these vectors to generate a response to a target vaccine antigen. We found that reactive oxygen species (ROS) are an important signal in the proinflammatory response to Ad. We identified that serotype 5 adenovirus (Ad5) elicits ROS by inducing mitochondrial membrane damage, a process that is dependent on endosomal membrane rupture and Cathepsin release. Our studies demonstrated that mitochondrial damage contributes to NLRP3 inflammasome- and NFκB-dependent innate immune activation upon Ad infection. This ROS-dependent inflammatory response likely contributes to the adaptive immune response by supporting DC maturation and activation, lymphocyte recruitment to the site of vaccination, and cytokine production influencing B and T cell proliferation and differentiation.

We next exploited these immunostimulatory properties of Ad in vaccines to prevent transmission of malaria by *Plasmodium falciparum*, the most common and deadly form of the parasite. When combined with vaccines that protect against Malaria infection, transmission-blocking vaccines (TBVs) could limit the spread of *Plasmodium*. We generated novel Ad5-based TBVs incorporating the candidate transmission-blocking antigen Pfs25, which is expressed on the surface of *Plasmodium falciparum*. While several studies have designed Pfs25 protein, or subunit, vaccines with adjuvants, these vaccines do not efficiently elicit transmission-blocking antibodies to

Pfs25 despite employing multiple boost vaccinations. Since it can potently activate the immune response, we hypothesized Ad5 would serve as a vaccine delivery platform and adjuvant for Pfs25 to generate a robust and prolonged Pfs25-specific antibody response. We found that Ad5-pfs25, a vector expressing Pfs25, generated a robust Pfs25-specific antibody response characterized by a higher titer, higher relative affinity and broader IgG subclass switching as compared to alum-adjuvanted Pfs25 protein vaccination. Ad5-specific T cell activation correlated with the observed increase in Pfs25-specific antibody titer following Ad5-pfs25 vaccination.

To further improve the Pfs25-specific antibody response we combined Ad5-pfs25 primary vaccination and boosts with Ad vectors displaying relevant, transmission-blocking Pfs25 epitopes on the surface of the viral capsid within the major capsid protein, Hexon. Boost vaccination with these capsid-displayed Pfs25 vectors increased the Pfs25-specific antibody titer and further increased the relative affinities of Pfs25-specific antibodies as compared to homologous prime-boost with Pfs25-alum. These Ad-vectored prime-boosts also block transmission to the mosquito in vitro. Taken together, our data demonstrate that our Ad5-based vectors enhance the humoral immune response to Pfs25 to generate a Pfs25-specific antibody response capable of blocking transmission. These novel transmission-blocking vaccines can be further investigated as they achieve a more robust Pfs25-specific antibody response with fewer vaccinations than the antibody response elicited by an alum-adjuvanted Pfs25 protein vaccination.

CHAPTER I

INTRODUCTION

Vaccines

Since its discovery in the late 1700s, vaccination has made an important contribution to human health by eradicating or limiting infectious diseases worldwide (Andre 2003). Vaccines are a useful and relatively cost-effective way to prevent the incidence of infectious diseases worldwide. Vaccines against diseases such as polio, measles, mumps, rubella, and hepatitis A and B, lower the global burden and control the spread of infectious disease. In the case of the infectious disease, smallpox, vaccination led to the global eradication of the disease (Hinman 1999; Hsu 2013). While the small pox vaccine is an example of a successful vaccine effort, there is still a need to develop safe and effective vaccines against current diseases such as HIV and malaria.

For a vaccine to be effective, it must generate protective immunity within an individual against a particular disease. This protective immunity is achieved through immunization with a component of the disease-causing agent or a killed or live-attenuated version of the pathogen itself. Vaccination with a component of the pathogen is generally better received than live-attenuated pathogens, as immunization with a subunit alone eliminates the potential risk of an outbreak caused by an attenuated strain regaining virulence (Plotkin 2010). However, protein-based or subunit vaccines normally

require adjuvants to activate the innate immune response since proteins alone are often not very immunogenic (Coffman, Sher et al. 2010). Many proteins alone are thought to be poorly immunogenic since they do not generate protective immunity in the absence of an adjuvant. Traditional particulate adjuvants such as alum were thought to enhance the immune response by serving as an antigen depot, resulting in the persistent expression and efficient presentation of antigen. More recently, several studies suggest alum adjuvants activate the innate immune response, which is key to generating adaptive immunity and immunological memory to the vaccine antigen (De Gregorio, Tritto et al. 2008; Li, Willingham et al. 2008). Specifically, these studies demonstrate that the particulate alum activates a proinflammatory response mediated by the NLRP3 inflammasome, a pattern recognition receptor, which will be discussed in detail in a later section. This proinflammatory response is required to generate an antibody response to the target antigen (Eisenbarth, Colegio et al. 2008). It is interesting to consider that alum has been used for many years as an adjuvant in vaccine development prior to understanding how it enhances the immune response. These studies with alum shed light on the fact that, historically, vaccines have been designed with little insight into how they generate immunological memory and protection (Pulendran and Ahmed 2011). With the advent of molecular biology, much more is known about the nature of the innate and adaptive immune responses generated under different contexts. In turn, we can apply what we know about the immune response in the context of different adjuvants to develop vaccines rationally.

In an effort to design vaccines rationally, this study and others have used replication-defective adenoviruses (Ad) vectors as vaccine platforms. Adenovirus vectors are attractive vaccine platforms due to their ability to potently activate the immune system (Hartman, Appledorn et al. 2008). In addition to their immunostimulatory properties, adenoviral vaccine vectors have well-characterized physical properties. Ad has a genetically tractable genome that can accommodate exogenous transgenes. Additionally, the Ad genome does not integrate into the host cell DNA thus lowering the risk for insertional mutagenesis. Ad has a relatively stable viral capsid and its propagation generates a high titer of virus *in vitro*. Ad vectors can express exogenous antigens from a transgene or can display antigens within the viral capsid itself. Both of these types of Ad-based vectors can support the immune response to the vaccine antigen (Small and Ertl 2011). Since there is a great deal known about the physical characteristics and immunostimulatory properties of Ad vectors, we can better design Ad-based vectors as vaccine vehicles and adjuvants to generate the appropriate immune response to the target vaccine antigen. This rational vaccine design will lead to more effective vaccine vectors that generate the appropriate immune response depending on the vaccine antigen.

Adenoviruses

To rationally design vaccines employing adenovirus vectors, it is important to fully understand how the physical and biological properties of these vectors may influence the immune response to the vaccine antigen. Adenoviruses cause respiratory, gastrointestinal and ocular infections. While they can be pathogenic in elderly and immunocompromised individuals, they generally produce a mild, self-limiting infection

in healthy people. First identified over 50 years ago, the adenoviridae family contains over 57 human serotypes organized into 7 subgroups (A-G) based on their sequence homology and hemagglutination properties (Nemerow, Pache et al. 2009; Russell 2009). Adenovirus serotype 5 (Ad5), the focus of these studies, is a member of subgroup C.

Adenovirus is a non-enveloped virus with a double-stranded DNA genome. The double-stranded DNA genome of the virus is packaged into a viral capsid. The adenovirus capsid is 90 nm in diameter with 12 structural proteins making up the icosahedral capsid (Smith, Wiethoff et al. 2010). The major capsid protein, Hexon, present in 240 copies, comprises the majority of the viral capsid. At each vertex of the capsid, Penton base anchors trimers of the fiber protein. The minor capsid proteins eight and nine (pVIII and pIX) provide additional support to these major capsid proteins and stabilize the viral capsid. Several other viral proteins, such as protein six (pVI) and protein IIIa, are present on the interior of the viral capsid. Protein six (pVI) is responsible for endosomal membrane rupture during cell entry while protein IIIa assists in viral assembly late in infection (Nemerow, Pache et al. 2009). The remaining structural proteins are associated with the viral DNA (Figure 1).

Adenovirus initiates infection by attaching via its fiber to a cognate receptor expressed on the surface of a target cell. Different subgroups of adenovirus use different primary receptors. With the exception of subgroups B and D, most human adenoviruses, including Ad5, are thought to predominantly utilize the coxsackievirus and adenovirus receptor (CAR) as their primary receptor (Hidaka, Milano et al. 1999). Alternatively, subgroup C viruses, including Ad5, can enter cells in a CAR-independent manner by

using heparin sulfate proteoglycans, VCAM-1 and MHC class I for primary attachment (Russell 2009). Further, studies demonstrate that serum factors can facilitate Ad cell attachment, particularly to hepatocytes (Parker, Waddington et al. 2006; Waddington, McVey et al. 2008). Subgroup B and D viruses utilize CD46 and sialic acid, respectively, to attach to a target cell (Arnberg, Edlund et al. 2000; Gaggar, Shayakhmetov et al. 2003). Following fiber attachment, the RGD-containing penton base interacts with α_v integrins (Wickham, Mathias et al. 1993). This secondary interaction is required for the clathrin-mediated endocytosis of the virus into the cell (Meier and Greber 2004).

Following endocytosis and upon endosomal acidification, the viral capsid partially disassembles to release the internal capsid protein, protein VI. Protein VI lyses the endosomal membrane allowing the partially disassembled virion to escape into the cytoplasm of the cell (Wiethoff, Wodrich et al. 2005; Maier, Galan et al. 2010; Moyer, Wiethoff et al. 2011). Subgroup C viruses, such as Ad5, partially disassemble rather quickly and escape the early endosomal compartment 15 minutes post-infection. In contrast, other subgroups, such as subgroup B viruses, traffick to the late endosome prior to endosomal escape (Bremner, Scherer et al. 2009). These serotype-specific differences in endosomal trafficking are thought to be due to alternative receptor utilization between Ad serotypes. Once in the cytoplasm, the virus engages microtubule motor machinery and trafficks along microtubules to the nucleus where it docks at the nuclear pore complex (NPC) for nuclear import (Trotman, Mosberger et al. 2001; Meier and Greber 2004; Smith, Wiethoff et al. 2010).

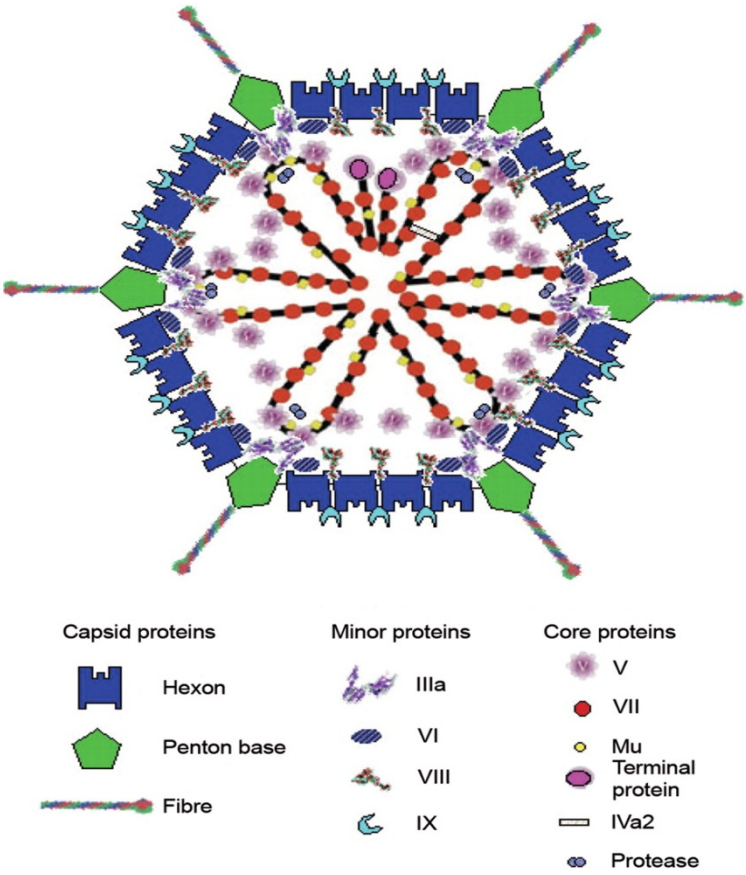

Figure 1. Depiction of the Adenovirus capsid. The viral capsid is made up of 7 capsid proteins with Hexon making up the majority of the capsid. Penton base anchors the fiber protein. Protein IIIa, protein VI, protein VIII and L3-23K protease reside in the interior of the capsid. Within the capsid, Terminal protein, Protein Mu, Protein VII, and Protein V, are associated with the viral DNA. Adapted from (Russell 2009).

Upon nuclear entry, viral gene expression and replication occurs. The viral genome encodes for early and late transcripts (Berk 2005). Some of the early transcripts are involved in the transcriptional activation of viral gene expression, particularly those associated with viral replication. For example, the E1 region of the genome is required for the activation of critical early genes. As a result, recombinant adenoviruses with the E1 region deleted, like the Ad5 vectors used in this study, are replication-defective (Berk 1986; Frisch and Mymryk 2002).

Innate Immune Response to Adenovirus

Adenovirus infection rapidly triggers the innate immune response suggesting that Ad cell entry triggers this response. This innate immune response is characterized by the release of numerous cytokines including IL-1, TNF-α, IL-6, and type I interferons (Liu and Muruve 2003). In mice, systemic release of IL-1β occurs within minutes of intravenous administration of Ad (Zsengeller, Otake et al. 2000). This same study demonstrated that the rapid release of IL-1β was followed by release of IL-6 and TNFα within hours. This rapid release of cytokines does not require viral replication since replication-defective Ads also elicit this proinflammatory response. Further supporting this notion, human adenoviruses generate a robust inflammatory response in mice despite being unable to replicate or express viral proteins in murine cells (Ginsberg and Prince 1994). These studies emphasize that the robust inflammatory response must come from recognition of incoming viral particles. Therefore, events early in the viral life cycle must activate the innate immune response. This section describes how events during adenoviral cell entry trigger the innate immune response. Furthermore, this section describes how

Ad rapidly activates multiple arms of the innate immune response. Together, these multiple arms of the innate response are critical in generating an antiviral state, recruiting immune cells and initiating the adaptive immune response.

To activate this robust and rapid innate response to Ad, cells must first sense the presence of Ad infection. Recognition of Ad occurs through sensors, called pattern recognition receptors (PRRs), which recognize motifs associated with infection. These motifs, called pathogen-associated molecular patterns (PAMPs) activate PRRs during Ad cell entry. PRR activation during Ad cell entry allows innate immune cells to rapidly respond to viral infection. PRRs such as Toll-like receptors (TLR), RIG-I like receptors (RLR), and Nod-like receptors (NLR) recognize important PAMPs and are key contributors to the innate immune response. The signals or PAMPs generated during viral cell entry and the PRRs they activate are described below.

The double-stranded DNA genome of the virus is one of the first PAMPs to activate a PRR during viral entry. This PRR, Toll-like receptor 9 (TLR9), which is present on the endosomal membrane, senses the viral genome when the virus partially uncoats following endosome acidification. (Yamaguchi, Kawabata et al. 2007). TLR9, in turn, activates NFκB, a key transcription factor involved in the upregulation of several proinflammatory cytokines (Cerullo, Seiler et al. 2007; Kawai and Akira 2008). TLR9 appears to play a role in the inflammatory response to Ad. *Yamaguchi et al.* found that inhibiting TLR9 activation following intravenous administration of Ad attenuates the production of proinflammatory cytokines in mice (Yamaguchi, Kawabata et al. 2007). However, the exact contribution of TLR9 alone to the inflammatory response to Ad is

unclear. TLR9 activation plays a role in plasmacytoid dendritic cells in that it is required to activate the type I interferon response in these cells (Basner-Tschakarjan, Gaffal et al. 2006). In contrast, TLR9 signaling appears to be dispensable for conventional macrophages and dendritic cells to activate NFκB. These cells require the TLR adaptor MyD88 to activate NFκB suggesting that multiple TLRs may play a redundant role in this process. Furthermore, in this case, MyD88, rather than any particular TLR, is required for NFκB activation and an effective immune response (Rhee, Blattman et al. 2011). These studies suggest that TLR signaling plays a cell type specific role in activating the innate immune system in response to adenovirus infection. TLR9 activation can still contribute to the innate response since TLR9-dependent signaling influences DC maturation, a key component to generating an effective innate and adaptive response (Yamaguchi, Kawabata et al. 2007). Further, studies with other viruses, such as the murine retrovirus, Friend virus, and virus-like particles, demonstrate that TLR7 or TLR9 signaling, respectively, on B cells is crucial in the germinal center reaction and the subsequent antibody response (Browne 2011; Hou, Saudan et al. 2011). Therefore, TLR9 signaling may not only contribute to the innate immune response but also may contribute to the adaptive immune response to Ad.

In addition to TLR-dependent recognition of the Ad genome during cell entry, cytosolic PRRs, such as Ddx41, AIM2 and RIG-I, activate IRF-3, which is a key transcription factor for the production of type I interferons. Type I interferons promote an antiviral environment that restricts viral infection. All three of these PRRs sense PAMPs following viral endosomal rupture. AIM2 and DDX41 are cytosolic DNA sensors that

recognize the viral genome when the virus ruptures and escapes from the endosome (Stein and Falck-Pedersen 2012). Recent studies demonstrate that the cytosolic RNA sensor RIG-I also contributes to IRF-3 activation during Ad infection. RIG-I recognizes virally-associated RNAs (VA-RNAs) expressed early after adenovirus infection. VA-RNAs are short RNAs of around 200 basepairs that form hairpins (Mathews 1995). During infection with replication-competent Ads these VA-RNAs are important for viral translation since they inhibit the Protein Kinase R (PKR) pathway. Activation of the PKR pathway upon viral infection leads to the phosphorylation, and inactivation, of eIF-2α, a crucial translation initiation factor. These dsRNAs activate RIG-I, which in turn activates the production of type I IFNs (IFN-I) through several transcription factors including IRF3 (Yamaguchi, Kawabata et al. 2010; Minamitani, Iwakiri et al. 2011). The extent of IFN-I production influences the adaptive immune response, as IFN-I is important for generating an antiviral state to restrict infection and viral gene expression. Additionally, type I interferon signaling on B and T cells is required for optimal production of neutralizing antibodies to adenovirus thus underscoring the importance of this innate response in activating the adaptive response (Zhu, Huang et al. 2007).

Another important PRR contributing to the innate immune response during Ad cell entry is the Nod-like receptor, NLRP3 (Barlan, Griffin et al. 2010; Barlan, Danthi et al. 2011). NLRP3 is a cytosolic receptor that becomes activated in response to several different PAMPs. These PAMPs are thought to be endogenous danger signals, called danger-associated molecular patterns (DAMPs), generated in response to many stressors including viral infection. DAMPS such as oxidative stress (reactive oxygen species),

exogenous ATP and particulates such as uric acid crystals are known to activate NLRP3 (Martinon, Burns et al. 2002; Martinon 2008; Latz 2010). Many different stimuli that activate the inflammasome generate ROS (Latz 2010). ROS are known to activate the innate immune system. The next section describes in detail how ROS contribute to the innate immune response. Following activation by these various stimuli, NLRP3 forms a complex, termed inflammasome, with the adaptor ASC and caspase-1. This oligomerization promotes the autocatalytic cleavage of caspase-1 to its active form. Active caspase-1 cleaves the key proinflammatory cytokines IL-1β and IL-18 to their mature form for secretion (Tschopp and Schroder 2010). While other viruses activate NLRP3 upon viral replication, Ad does not require replication to activate it (Allen, Scull et al. 2009). It is unclear what signals are involved in activating NLRP3 during Ad infection and this study helps to describe some of them. In particular, it is unknown whether ROS play a role in activating NLRP3 during Ad infection. This study describes how ROS are produced during Ad infection and explores the role for ROS in the innate immune response to Ad.

The NLRP3-dependent cytokines IL-1β and IL-18 are important cytokines determining the nature of Ad infection. IL-1R knockout mice or mice given neutralizing IL-1β antibodies prior to infection exhibit a less severe inflammatory response and reduced tissue toxicity (Shayakhmetov, Li et al. 2005). Thus IL-1β was shown not only to be a key mediator of the immune response to Ad but also a key molecule involved in the pathogenesis of Ad infection. Similar to IL- 1β, IL-18 is also important for the immune response to Ad infection (Berclaz, Shibata et al. 2002). *Berclaz et al.* found that mice

with impaired IL-18 production exhibited an attenuated Th1 response. This diminished Th1 response correlates with reduced uptake of Ad virions by alveolar macrophages due to altered receptor expression (Zsengeller, Otake et al. 2000). Thus, the proinflammatory cytokines, IL-1β and IL-18 have an apparently crucial role in the immune response to Ad infections but also appear to contribute to the pathogenesis of Ad infection as well.

In addition to PRRs recognizing Ad during cell entry, Trim21 mediates innate immune recognition of Ad infection. Trim21 is a cytosolic E3 ubiquitin ligase that binds incoming, antibody-opsonized adenovirus through its spry domain. Trim21 mediates degradation of incoming viral particles and also activates the innate immune response (McEwan, Hauler et al. 2012; McEwan, Tam et al. 2013). *Watkinson et al*. demonstrate that Trim21 plays a role in the production of IL-6 and TNF-α following Ad infection (Watkinson, Tam et al. 2013). As mentioned previously, these proinflammatory cytokines are an important component to the innate immune response to infection. They help to recruit additional immune cells to the site of infection. Additionally, IL-6, in particular, helps to support the adaptive immune response (described in detail in a later section).

ROS as an innate immune signal

As mentioned previously, oxidative stress, including reactive oxygen species (ROS), is a key mediator of the innate immune response to several pathogens. In general, ROS are produced in the context of general cellular processes and signaling and also during cellular stress. Since ROS are produced in response to cellular stress, increasing evidence suggest ROS serve as a danger signal that activates the innate immune response (Nathan and Cunningham-Bussel 2013). ROS include many different reactive oxygen

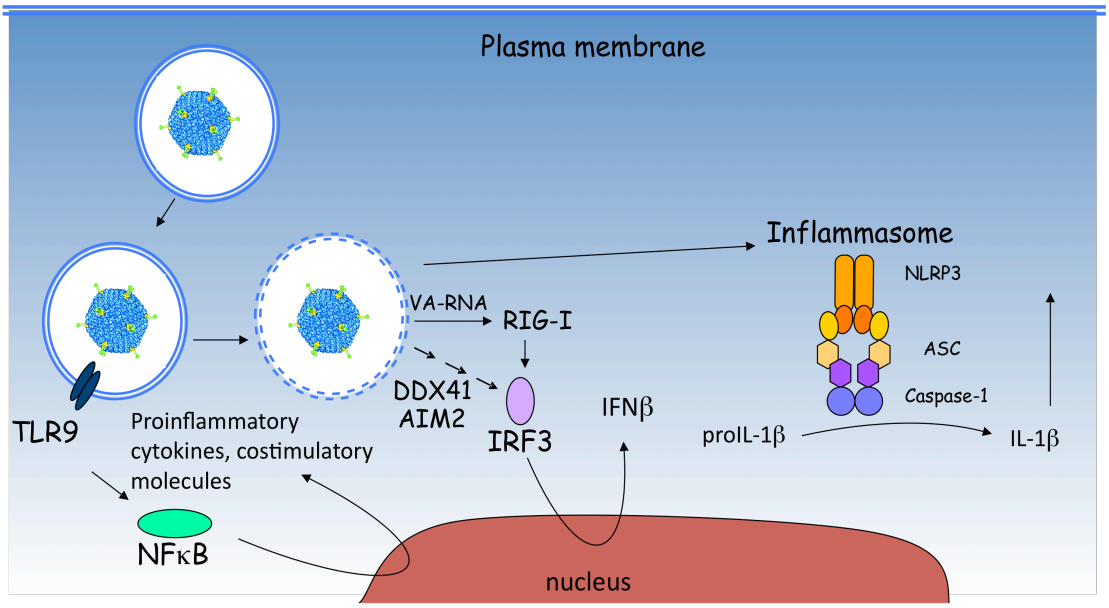

Figure 2. Innate Immune Activation following Adenovirus Infection. During Ad cell entry several pattern recognition receptors contribute to the innate immune response to the virus. TLR9, DDX41, AIM2, RIG-I and NLRP3 all contribute to the innate immune response to Ad.

intermediates such as singlet oxygen, hydrogen peroxide, and superoxide. They are generated by several cellular components such as NADPH (nicotinamide adenine dinucleotide phosphate) oxidase, endoplasmic reticulum (ER)-resident enzymes, and mitochondria (Figure 3). These potential sources of ROS and their role in innate immune activation are discussed below.

NADPH oxidase is a primary source of ROS in the cell. It is a multi-subunit component bound to both the plasma membrane and the phagosomal membrane. It is responsible for the de novo production of ROS through a series of reactions starting with the transfer of electrons from NADPH to oxygen generating superoxide anion (Rada and Leto 2008; Katsuyama 2010). NADPH oxidase is activated by a variety of signals including exogenous signals like ATP or the mitogen, phorbol 12-myristate 13-acetate (PMA). NADPH oxidase can also become activated in phagocytic lymphocytes during the uptake of bacteria or bacterial components (DeLeo, Allen et al. 1999). NADPH oxidase-dependent ROS play a role in the innate immune response by generating a respiratory burst that kills bacteria in the phagosome (Zsengeller, Otake et al. 2000). These responses are thought to be less successful against viruses, which survive by gaining access to intracellular compartments to replicate. Therefore, ROS in general have been traditionally studied and characterized as an innate immune effector known to eliminate extracellular bacteria. However, more recently, ROS have been shown to possess antiviral properties. In particular, ROS have been implicated as a signal activating innate immune pathways critical to the antiviral response. *Fink et al.* found that during infection with respiratory syncytial virus or Sendai virus, NADPH oxidase-

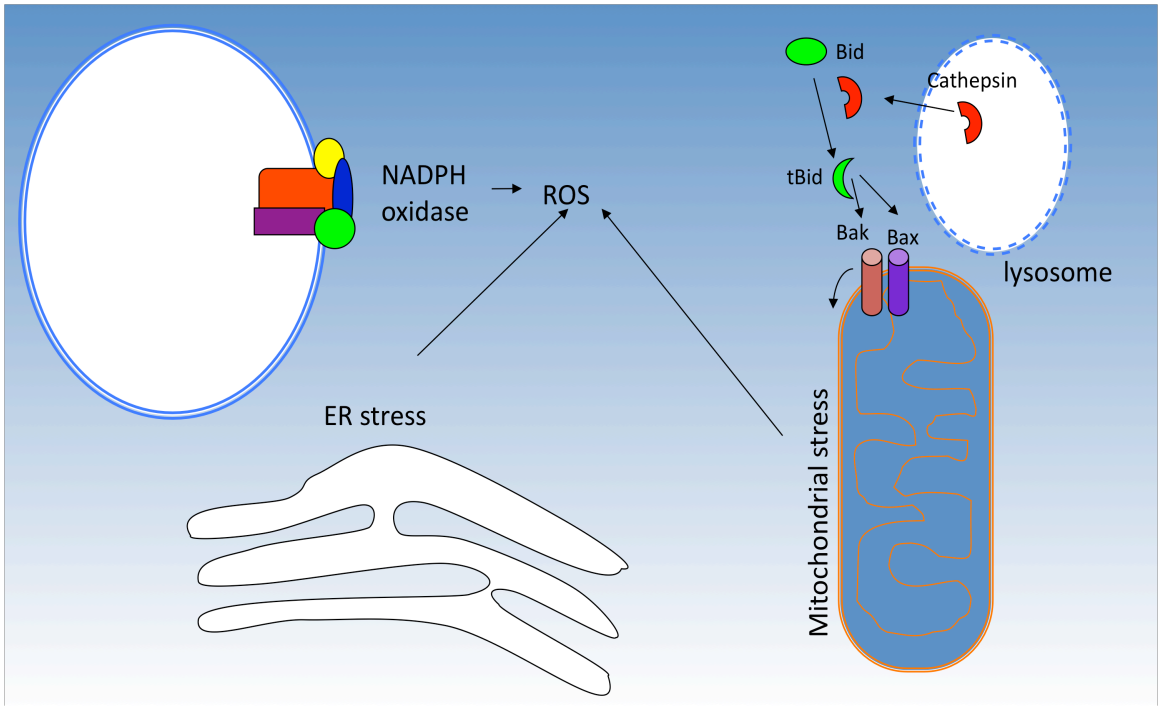

Figure 3. Model of potential sources of ROS upon Ad5 infection. There are 3 potential sources of ROS in the cell: ER stress, NADPH oxidase on the phagosomal membrane and mitochondrial stress.

dependent ROS contribute to activation of NFκB, a key transcription factor activating the production of innate immune cytokines (Fink, Duval et al. 2008). More recently, *Soucy-Faulkner et al.* identify a role for NADPH oxidase and ROS in activating IRF3, a key transcription factor activating IFNβ and interferon-inducible genes (Soucy-Faulkner, Mukawera et al. 2010). Additionally, this ROS-dependent IRF3 activation is mediated by the pattern recognition receptor RIG-I. A different study by *Chiang et al.* also implicates ROS in activating IRF3 in a manner dependent on another pattern recognition receptor TLR4 (Park, Jung et al. 2004; Chiang, Dang et al. 2006). Together, these studies demonstrate that NADPH oxidase-dependent ROS are a key activator of the innate immune response to infection.

The endoplasmic reticulum (ER) is another source for cellular ROS. The ER is responsible for the proper processing and folding of nascent proteins. This protein processing involves redox reactions and the ER contains the requisite enzymes to perform these processes. While some ROS are a natural byproduct of protein processing in the ER, ER stress contributes to the accumulation of ROS (Santos, Tanaka et al. 2009). ER stress is a common consequence of viral infection. Several studies with hepatitis C virus (HCV) implicate a role for ER stress in generating ROS during viral infection (Chan and Egan 2005; Ivanov, Smirnova et al. 2011; Merquiol, Uzi et al. 2011; Ivanov, Bartosch et al. 2013). During viral replication, HCV proteins overload the ER and lead to ER stress and the production of ROS. Two separate studies demonstrate that ER stress and ROS can contribute to the innate immune response during HCV infection. These studies show that the expression of two nonstructural proteins induce ER stress during HCV infection.

Additionally, this ER stress generates ROS. Together, ER stress and ROS lead to NFκB activation (Waris, Tardif et al. 2002; Li, Ye et al. 2009). Taken together, these studies demonstrate that ER stress can serve as a source for ROS and, further, that ER-derived ROS can contribute to innate immune activation during viral infection.

Mitochondrial stress is a third source for cellular ROS. The mitochondria generate chemical energy in the form of ATP via oxidative phosphorylation. Oxidative phosphorylation is generated through the electron transport chain on the inner mitochondrial membrane. This process generates ROS within the mitochondria. When the mitochondria are damaged or under stress ROS can leak into the cytoplasm of the cell where it activates the innate immune system (Nathan and Cunningham-Bussel 2013). Recent studies implicate mitochondrial ROS as a key signal in innate immune activation. One particular study demonstrates that mitochondrial dysfunction and ROS enhance RIG-I activation during infection with vesicular stomatitis virus (VSV). Further, enhanced RIG-I activation led to enhanced secretion of type I interferons, a key antiviral response (Tal, Sasai et al. 2009). Another recent study demonstrates that mitochondrial ROS can contribute to innate immune activation through the pattern recognition receptor NLRP3, the Nod-like receptor responsible for production of proinflammatory cytokines such as IL-1β and IL-18 (Zhou, Tardivel et al. 2009). These studies demonstrate that mitochondrial ROS are yet another important activator of the innate immune response.

Taken together, these studies identify that ROS are a key signal in activating the innate immune response specifically by contributing to activation of innate signaling pathways that lead to activation of NFκB, IRF3 and NLRP3. It is unknown whether ROS

play a role in the proinflammatory response to adenovirus infection. Since Ad is known to activate NFκB, IRF3 and NLRP3 during infection it is likely that ROS contribute to innate immune activation since many studies demonstrate that ROS are a key activator of these innate immune pathways. The data presented in this study demonstrate a role for mitochondrial ROS in activating the innate immune response to Ad.

Cellular Mediators of the Innate Immune Response to Adenovirus

The innate response is critical in activating an antiviral state in infected cells, recruiting immune cells and initiating the adaptive immune response. As mentioned previously, cells sense viral infection through PRRs to initiate the innate immune response. This sensing triggers the antiviral state in target cells and recruits additional cells that mediate the innate immune response. These recruited immune cells contribute to the rapid proinflammatory response, which is characterized by the rapid release of numerous cytokines, including IL-1, TNF-α, IL-6, and type I interferons (Liu and Muruve 2003). This rapid innate response activates the adaptive immune response, which will be discussed in the next section.

Several populations of innate immune cells are responsible for the production of these proinflammatory cytokines. These innate lymphocytes are the initial responders to Ad infection and alert cells of the adaptive immune system to become activated and respond to viral infection. Following infection with adenovirus, resident macrophages of the lung or liver release the majority of these cytokines and chemokines in response to the virus. In fact, within minutes of intratracheal or intravenous administration, the majority of the virus appears to be associated with these phagocytic cells (Zsengeller,

Otake et al. 2000). Additionally, *Di Paolo et al.* demonstrate that Ad also associates with marginal zone macrophages (MZM) in the spleen following intravenous administration. MZMs are responsible for the rapid production of IL-1α, which is a key inflammatory cytokine. IL-1α helps generate an antiviral environment and upregulates innate immune chemokines for the recruitment of additional lymphocytes to the site of infection. IL-1α production results from Ad engagement of integrins. Through its RGD motif, penton base interacts with integrins on macrophages and promotes the secretion of IL-1α in vivo (Di Paolo, Miao et al. 2009; Nemerow 2009). Another innate immune population, natural killer cells also quickly respond to Ad infection. They can kill virally infected cells and produce both IFNγ and TNF-α (Zhu, Huang et al. 2008). These cytokines are important for macrophage activation, dendritic cell maturation and recruitment of other innate cells. Additionally, IFNγ is a key cytokine regulating the adaptive immune response (discussed in a later section).

As mentioned previously, tissue resident macrophages in the lung are key innate immune cells responding to Ad infection. These cells are responsible for the production of proinflammatory cytokines in response to the virus regardless of the route of administration. Upon intravenous administration of Ad, the virus is carried to the liver where Kuppfer cells, specialized macrophages residing in the liver, respond to the virus. This innate immune response is further enhanced by the complement system. Studies show that complement plays a role in the clearance of Ad5 from the bloodstream by Kupffer cells in the liver (Xu, Tian et al. 2008; He, Katschke et al. 2013). Complement consists of a series of proteins that attack and lyse pathogens or antibody-opsonized

pathogens (Dunkelberger and Song 2010). In addition to direct killing of pathogens, complement also enhances the phagocytosis of pathogens and generates chemokines important in recruiting innate immune cells to the site of infection.

Complement components in the blood recognize antibody-opsonized pathogen via the classical pathway, native pathogen via the alternative pathway, and mannose residues via the lectin-binding pathway (Dunkelberger and Song 2010). The classical pathway plays a role in recognizing antibody-opsonized Ad in the blood. Naturally occurring IgM in the blood recognize repetitive viral epitopes and opsonize the viral capsid. These naturally occurring IgM molecules do not require prior exposure to the virus to recognize Ad. In turn, the antibody-opsonized virus activates the classical complement pathway. Pre-existing Ad-specific antibodies can also activate the classical pathway. Complement activation elicits a cascade of proteolytic cleavages that generate the membrane attack complex (MAC). The MAC generates pores in the target membrane to kill cells or pathogens (Dunkelberger and Song 2010). As mentioned, complement assists in clearing Ad from the blood by enhancing phagocytosis of the virus by Kupffer cells (Xu, Tian et al. 2008; He, Katschke et al. 2013). Interestingly, *Xu et al.* identified how Ad evades the complement system. They demonstrate that Ad binds to factor X, a blood coagulation factor, allowing the virus to evade this antibody- and complement-mediated clearance (Xu, Qiu et al. 2013). Further, factor X binding facilitates the CAR-independent transduction of hepatocytes (as described in a previous section). These findings demonstrate that Ad evolved a mechanism to avoid complement-mediated neutralization

thus highlighting the importance of the complement system in the innate immune response to the virus.

Taken together, innate immune cells, particularly natural killer cells and macrophages, encounter and eliminate Ad. Natural killer cells detect and kill virally infected cells. Macrophages recognize Ad alone or opsonized by antibodies. Additionally, the complement system can facilitate this macrophage-dependent clearance of the virus when Ad is present in the bloodstream. As mentioned previously, macrophages are also important in activating the immune system, which is required to generate immunological memory to the virus. The next section describes how these innate immune cells facilitate the initiation of the adaptive immune response.

Innate immunity to Adenovirus is crucial to activation of the adaptive immune response

The innate immune response is the rapid, initial responder to a pathogen or vaccine. This early recognition of the incoming foreign agent is a crucial alarm to activate the host's adaptive immune response. Thus the extent and nature of the innate immune response dictates how the adaptive response reacts to the pathogen or vaccine, in our case. One way the innate response influences the adaptive response is through antigen presentation by antigen-presenting cells (APCs). *Lore et al.* demonstrate that Ad transduction of APCs, such as myeloid and plasmacytoid dendritic cells (DCs), results in DC maturation and upregulation of costimulatory molecules (Lore, Adams et al. 2007). These events promote antigen presentation by these DCs to T cells (described in the next section). In addition to supporting antigen presentation, the innate immune response

triggers the secretion of proinflammatory cytokines that help recruit immune cells, including those of the adaptive immune response, to the site of infection. These proinflammatory cytokines support efficient antigen presentation to, and activation of, adaptive immune cells. The nature of the innate response, particularly the specific PRRs stimulated, determines the nature of the adaptive response. For example, studies show that the concurrent delivery of adjuvants that stimulate multiple PRRs along with antigen delivery to dendritic cells leads to optimal T cell immunity (Coffman, Sher et al. 2010). These studies suggest that robust innate immune stimulation during antigen presentation leads to superb activation of the adaptive immune response.

Adaptive immune response

Following activation of the innate immune response at the site of vaccination or infection, cytokines produced by the innate immune response stimulate the recruitment of adaptive cells to the site of infection (Pulendran and Ahmed 2006). Concurrently, antigen is carried to the local lymphatics by antigen-presenting cells such as dendritic cells. Once in the secondary lymph node, antigen further stimulates the adaptive immune system to generate a specialized response to the target. Unlike the innate immune response, the adaptive response generates memory cells that persist once the antigen is cleared and provide a source of protection upon antigen challenge.

At the site of infection and in secondary lymphoid tissue, adaptive immune cells respond to infection. One adaptive immune cell, the antigen-specific $CD8^+$ T cell, recognizes antigen in the context of major histocompatibility complex (MHC) class I molecules on the surface of infected cells. All nucleated cells express MHCI and virally

infected cells present viral antigens on MHCI. Antigen-specific cytotoxic T cells encounter antigen in the context of MHCI on the cell surface. Alternatively, antigen-specific $CD8^+$ T cell occasionally encounter antigen via cross presentation by dendritic cells. Cross presentation occurs when professional antigen-presenting cells, particularly plasmacytoid dendritic cells (pDCs), take up exogenous antigen and present it on MHCI molecules. This exogenous antigen is taken up from the extracellular milieu or during the clearance of dying cells and cell debris (Pulendran and Ahmed 2011).

After cytotoxic T cells encounter cognate antigen in the context of an MHCI molecule, they must receive a second costimulatory signal to become activated. This second signal can be the engagement of CD28 on $CD8^+$ T cells with the costimulatory molecules CD80/86 on the target cell. Alternatively, this signal can also come from the cytokines produced by activated $CD4^+$ T cells. Upon activation, cytotoxic T cells directly kill infected cells (Szabo, Sullivan et al. 2003). These cells kill their targets by releasing cytolytic granules containing perforin and granzymes. Perforin creates a pore in the plasma membrane of infected cells, allowing granzymes to enter the cell. Granzymes then cleave caspase 9 to its active form. Then active caspase 9 triggers a cascade that leads to apoptosis. Alternatively, cytotoxic T cells can also kill target cells through the Fas ligand-dependent pathway (Bachmann, Wolint et al. 2005). $CD8^+$ T cells upregulate Fas ligand (FasL) on their surface, which then engages Fas on the target cell, facilitating Fas-dependent apoptosis. However, this pathway is thought to be more important in the clearance of T cells during development and contraction following an immune response. In addition to killing target cells, cytotoxic T cells also produce cytokines to support the

immune response. IFNγ is an important cytokine produced by cytotoxic T cells. This cytokine supports the $CD4^+$ T helper cell response (described next) and, in turn, the antibodies produced.

In addition to their well-characterized cytotoxic functions, $CD8^+$ T cells can provide helper functions. These helper functions include supporting the antigen-specific B cell response. $CD8^+$ T cells, through the expression of CD40L can activate antigen-presenting cells, such as B cells. *Frentsch et al.* found that these $CD40L^+$ $CD8^+$ T cells comprise a substantial pool of the memory $CD8^+$ T cells following viral infection. Further, these $CD8^+$ T cells were functionally similar to $CD4^+$ T cells as they are able to produce cytokines, such as IL-4, that are normally produced by helper T cells. These cytokines support B cell activation (Frentsch, Stark et al. 2013). A study from *Serre et al.* underscores the role for $CD8^+$ T cells in providing support similar to $CD4^+$ T cells ($CD4^+$ T cell help described in detail the next section). They demonstrate that IFNγ production by $CD8^+$ T cells induces the upregulation of the chemokine receptor CXCR3 on the surface of antigen-specific B cells (Serre, Cunningham et al. 2012). CXCR3 expression facilitates the migration of B cells to the site of inflammation and, in turn, antibody production and isotype switching. Together, these studies demonstrate that, like $CD4^+$ T cells, $CD8^+$ T cells can provide support to a traditionally T_H2-biased response.

While $CD8^+$ T cells encounter antigen at the site of infection and in secondary lymphoid tissue, $CD4^+$ T cells predominantly become activated in the T cell zone of the lymph node. In the lymph node, dendritic cells present antigen to naïve $CD4^+$ T cells. As described previously, innate immune stimulation in dendritic cells leads to the

upregulation of costimulatory molecules, such as CD80 and CD86, and MHC class II (MHCII) molecules on the surface of the DC (Sallusto, Geginat et al. 2004). These surface molecules facilitate efficient antigen presentation to T cells within the T cell zone of the lymph node. A $CD4^+$ T cell encounters its cognate antigen in the context of MHCII on an APC. In addition to the T cell receptor (TcR) interacting with antigen on MHCII, CD4 acts as a co-receptor. Other surface molecules, such as LFA-1, contribute to strengthening this antigen-TcR interaction. To become fully activated antigen-specific T cells require a secondary interaction between CD28 on its surface and CD80/86 on the APC (Ansel, Djuretic et al. 2006). In the absence of costimulatory molecules, T cells will become anergic. Activated T cells then secrete cytokines such as IL-2 and upregulate surface molecules to support their survival, proliferation and differentiation to different effector populations.

Following activation, $CD4^+$ T cells can differentiate into several $CD4^+$ effector T cell populations. Particular transcription factors, called master regulators, are required for the differentiation of these effector populations. Thus, effector T cell populations are characterized master regulator expression, cytokine production and function. T_H1 and T_H2 are the two major, characterized T helper effector populations activated upon infection. T_H1 cells produce cytokines supporting cell-mediated immunity, such as IFNγ, which supports $CD8^+$ T cell activation and activates antibody class switching to generate opsonizing IgG classes. T_H2 cells secrete cytokines such as IL-4 that support B cell proliferation (Szabo, Sullivan et al. 2003; Ansel, Djuretic et al. 2006). Regulatory T cells (T_{reg}), another important effector population, are also induced upon viral infection.

Through both direct contact and the production of immunosuppressive cytokines T_{reg} cells temper the immune response to prevent excessive immune activation (Sakaguchi, Yamaguchi et al. 2008).

Other effector T helper cell subsets, such as T_H17 cells and T follicular helper (T_{FH}) cells, are likely involved in the immune response to adenovirus but have yet to be fully characterized. T_H17 cells are inflammatory in nature and, as their name implies, produce IL-17. IL-1β is an important factor promoting T_H17 differentiation and since Ad elicits IL-1β it is likely that it also induces T_H17 cells (Korn, Bettelli et al. 2009). Another important effector T cell population, T_{FH} cells, home to the B cell follicle within lymph nodes where they provide help to B cells and support B cell class switching. T_{FH} cells are characterized in part by their ability to produce IL-21, a cytokine that promotes proliferation (Fazilleau, Mark et al. 2009; Crotty 2011; Hale, Youngblood et al. 2013). Importantly, these effector functions of T cells are not fixed, as there is plasticity between different effector subsets. *Wang et al.* found that Ad transduction of monocyte-derived dendritic cells led to the production of IL-17 from T_{reg} cells (Wang, Crome et al. 2011). Further, *Hegazy et al.* found that type I interferons reprogram T_H2 cells to possess a combined T_H1/T_H2 phenotype (Hegazy, Peine et al. 2010). Many studies demonstrate that T_{FH} cells, an important subset in providing B cell help, are the most plastic effector T cell. Many other effector T cell types can home to the follicle and acquire a T_{FH} phenotype (Eto, Lao et al. 2011).

After differentiating to specific effector $CD4^+$ T cells, these activated $CD4^+$ T cells are then primed to provide the requisite help to B cells. They provide help indirectly

through cytokine production and directly by engaging costimulatory molecules on cognate B cells (described in the next section). Once the pathogen is cleared, the antigen-specific T cell population contracts via apoptosis and then a small subset of surviving T cells become long-lasting memory cells (Pepper and Jenkins 2011). There are two types of memory T cells with slightly different functions. Central memory T cells home to lymphoid organs and rapidly proliferate upon secondary exposure to antigen. Effector memory T cells also respond quickly to antigen. While their effector functions are more efficient than those of central effector cells, effector memory T cells do not proliferate extensively upon secondary exposure (Sallusto, Geginat et al. 2004).

When antigen-specific T cells are exposed to antigen in the local lymph node, antigen-specific B cells concurrently encounter antigen within the B cell zone of the lymph node. B cells encounter antigen in the lymph node in several ways. Small antigens enter the lymph node through conduits while larger antigens are transported into the B cell follicle by macrophages or other APCs. Subcapsular sinus (SCS) macrophages are key APCs that carry antigen into the lymph node follicle and present antigen to B cells. B cells themselves can also transport antigen on their Fc receptors to the follicle, where Ag is deposited on follicular dendritic cells (Batista and Harwood 2009). This Ag transport to the follicle occurs independent of the B cell's BcR specificity. Regardless of their mode of transport into the lymph node, antigens are deposited on follicular dendritic cells (FDCs). Unlike conventional DCs of lymphoid and myeloid origin, FDCs arise from mesenchymal stem cells and are non-migratory in nature. FDCs express complement and Fc receptors that mediate the capture of antibody- and complement-opsonized antigen

and thus serve as key mediators in B cell recognition of antigen in the follicle. B cells in the follicle, called Follicular B cells, sample antigen on FDCs (Tarlinton and Good-Jacobson 2013).

B cells encounter antigen on follicular dendritic cells or those carried in by APCs. Similar to T cell interactions with antigen, B cells recognize antigen through its B cell receptor (BcR). This antigen-BcR interaction is strengthened by coreceptors such as the integrin LFA-1 with ICAM-1. When an antigen is membrane-bound the immunological synapse with the BcR is enhanced by adhesion molecules such that membrane-bound antigen can provide a strong activation signal to the BcR that requires less antigen than its soluble counterpart (Carrasco, Fleire et al. 2004; Carrasco and Batista 2006; Depoil, Weber et al. 2009)**.**

Once the B cell receptor encounters its cognate antigen, the BcR crosslinks allowing the B cell to internalize antigen. The B cell upregulates costimulatory molecules on its surface for secondary interactions with T cells and cytokine receptors required for migration to the B-T cell interface within the lymph node (Depoil, Weber et al. 2009). In addition to encountering its cognate antigen, the B cell must receive a second activation signal, provided by $CD4^+$ T cells, to become fully activated. The B cell must engage CD40 on its surface with CD40 ligand (CD40L) on the surface of a T helper cell. Once a B cell encounters a cognate $CD4^+$ T cell, the B cell presents antigen on MHCII molecules to $CD4^+$ T cells. Another interaction between CD80/86 (B7) on the B cell and CD28 on the T cell induces the upregulation of CD40L on the T cell (Depoil, Weber et al. 2009). Then CD40L on the T cell interacts with CD40 on the B cells. Recent studies implicate

T_{FH} cells within the B cell follicle, rather than those in the T cell zone of the lymph node, as the T cells providing the initial T cell help to naïve B cells (Fazilleau, Mark et al. 2009; Crotty 2011). It is thought that naïve $CD4^+$ T cells in the T cell zone are primed by DCs (as described above) and then upregulate the chemokine receptor, CXCR5, to enter the B cell zone of the lymph node follicle. Once in the follicle, T_{FH} cells provide the second signal to B cells. In the context of virus-like particles, B cells themselves can also prime T_{FH} cells to then support B cell proliferation (Hou, Saudan et al. 2011).

Once the B cell has encountered antigen and received the requisite T cell help described above it becomes fully activated. The activated B cell then either leaves the primary B cell follicle or enters the germinal center where it undergoes further proliferation, affinity maturation and class switching to generate high affinity, antigen-specific B cells (McHeyzer-Williams, Okitsu et al. 2012; Tarlinton and Good-Jacobson 2013). Alternatively, T-independent antigens do not require this T cell licensing step. For these antigens, BcR engagement with the antigen provides the two requisite signals for B cell activation. T-independent antigens generally elicit a predominant IgM response and do not generate a strong memory response.

Following activation, antigen-specific B cells differentiate into plasma cells or memory B cells. B cells can differentiate to both short-lived (SLPC) and long-lived plasma cells (LLPC), both of which are generated in the germinal center. Long-lived plasma cells home back to the bone marrow as early as 7 days post-infection and are thought to be the main source of long-lasting serum antibody. Some studies suggest that LLPCs are generated from plasmablasts with the highest affinity for antigen (Tarlinton

and Good-Jacobson 2013). There are no reports extensively characterizing the long-lived plasma cell response upon adenovirus infection or vaccination, however, one study by *de Cassan et al.* does identify that Ad-based vectors generate plasma cells in the bone marrow. Additionally, several studies show that serum antibody levels for many viral antigens persist following vaccination (de Cassan, Forbes et al. 2011). These studies suggest Ad vaccine vectors generate long-lived plasma cells. LLPCs may therefore contribute to the persistence of serum antibody following vaccination with Ad-based vaccine vectors.

Adenovirus as a vaccine vector

Adenovirus is a candidate vaccine vector due to its potent T cell adjuvanticity. It elicits a strong innate inflammatory response that contributes to the activation of both $CD4^+$ and $CD8^+$ T cells upon vaccination (Li, Locke et al. 2007). In general, adenovirus is a practical vaccine vector because replication-defective viruses can be grown to high titer *in vitro*, its genome can be genetically manipulated, it can transduce a variety of cell types and it can induce high levels of transgene expression upon transduction (Li, Locke et al. 2007; Sakurai, Kawabata et al. 2007). Further, many studies have characterized the molecular properties of adenovirus making it easier to design adenovirus-based vectors for different applications.

Traditionally, research groups have explored adenovirus as a gene therapy vector. However, due to the resultant innate proinflammatory response and the adaptive immune response directed against the transgene, transgene expression declines over time (Muruve 2004). In the context of vaccine vectors, these properties of adenovirus are attractive

since the innate and adaptive immune response to the virus will enhance the adaptive immune response to the target vaccine antigen (Rollier, Reyes-Sandoval et al.). In this way adenovirus not only serves as the delivery vehicle for the antigen of interest but also as an adjuvant to this antigen.

Several early vaccine studies utilized replication-defective adenovirus vectors carrying a transgene for the vaccine antigen. In vaccine vectors, a portion of the adenovirus genome can be deleted, after which it can then accommodate up to 7.5 kilobases (kb) of foreign DNA. Replication-defective Ad genomes lack the E1 gene, the key transcriptional activator, such that the viral genome is not transcribed. The E3 region is also deleted in many current Ad vaccine vectors to accommodate the 7.5 kb of foreign DNA. E1-deleted Ad vectors are propagated in E1-complementing cell lines. Recombinant Ad vectors normally contain a strong, constitutive promoter such as the immediate early cytomegalovirus (CMV) promoter. This strong promoter allows for robust transgene expression. *Geiben-Lynn et al.* found that Ad vectors elicit the strongest transgene expression via the intramuscular vaccination route. Further, for all vaccine routes, transgene expression is detectable out to 100 days post-vaccination (Geiben-Lynn, Greenland et al. 2008). These Ad-vectored transgenes elicit both antigen-specific T cells and antibodies. In one of the earliest studies involving replication-defective Ad vaccine vectors, *Xiang et al.* generated an E1/E3-deleted Ad vector expressing the rabies virus glycoprotein (G protein) and found that one immunization with this vector elicited a high G protein-specific antibody response and $CD8^+$ T cell response that protected against rabies virus challenge. In contrast, a vaccinia-based vaccine conferred only partial

protection against rabies challenge (Xiang, Yang et al. 1996). To date, many research groups have generated transgene-expressing Ad vaccine vectors against a variety of pathogens. Compared to other gene-based vectors, Ad-based vectors carrying an exogenous, or foreign, transgene appear to be the most immunogenic (Casimiro, Chen et al. 2003).

In addition to Ad vectors expressing candidate antigens from a transgene, studies demonstrate how adenovirus displaying vaccine epitopes on its capsid also elicits a protective immune response (Wu, Han et al. 2005). Many groups demonstrate how various capsid proteins can accommodate exogenous epitopes. Further, many of these capsid modifications elicit an enhanced humoral immune response compared to an Ad vector expressing the vaccine antigen from a transgene (McConnell, Danthinne et al. 2006; Shiratsuchi, Rai et al. 2010) The Ad Hexon protein is an attractive protein to include foreign antigens due to 9 hypervariable regions (HVRs) that form nonstructural loops (Russell 2009). Replacement of many of these HVRs with foreign sequences does not interfere with viral assembly and structure, but rather presents an antigen of interest on the surface of the viral capsid. *Shiratsuchi et al.* demonstrate that vaccination with an Ad vector containing a B cell epitope of the *Plasmodium yoelii* protein CS in place of HVR1 resulted in a significantly higher level of protection in mice upon *P. yoelii* challenge as compared to an Ad vector expressing the CS protein from a transgene (Shiratsuchi, Rai et al. 2010). Additionally, this observed enhancement in protection to malaria infection correlated with an elevated antibody response to the capsid-displayed malaria epitope. Another study by *Worgall et al.* demonstrate that display of an epitope

from the outer membrane protein (OprF) of *Pseudomonas aeruginosa*, within HVR5 of Hexon, elicits an OprF-specific antibody response. This OprF vaccine also efficiently activates the cellular immune response (Worgall, Krause et al. 2005). The immune response generated by this vaccine protected against lethal *P. aeruginosa* challenge.

One issue with Ad vectors as candidates for vaccine vehicles is pre-existing immunity (PEI). Most individuals have been exposed to adenovirus and thus have neutralizing antibodies to Ad. Studies estimate that PEI to Ad5 is as high as 50% in developed countries and 80-90% in developing countries such as sub-Saharan Africa (Nwanegbo, Vardas et al. 2004). This PEI can greatly decrease the efficacy of an Ad-vectored vaccine. However, depending on the extent of PEI, the effectiveness of Ad-vectored vaccines can vary. *Choi et al.* found that the extent of antibody opsonization influenced the transgene-specific response to Ad-vectored vaccines. They found that antibody-Ad complexes did not greatly attenuate the transgene-specific immune response in naïve animals. In contrast, PEI attenuated this response. However, the lowest concentration of antibody opsonization enhanced the transgene-specific cellular and humoral immune response in animals with PEI (Choi, Dekker et al. 2012). Nonetheless, PEI is still a concern in Ad-vectored vaccine design and current Ad-based vectors often employ ways to evade PEI.

In addition to generating a robust humoral response, Ad-vectored vaccines with Hexon-displayed antigens can circumvent pre-existing immunity. Neutralizing antibodies generated in response to adenovirus predominantly recognize the HVR loops of Hexon. Replacement of several, or all, of these HVRs with those of a less common serotype of

Ad allows common serotypes, like Ad2 or Ad5, to evade pre-existing immunity. In the same study mentioned above from Worgall and colleagues, they found that repeated immunization with an Ad5 vector displaying the *P. aeruginosa* OprF epitope within HVR5 of the Ad capsid enhanced the antibody and cell-mediated immune response to the OprF epitope. In contrast, repeated immunization with a transgene-expressing control virus did not generate a boost in the immune response to the transgene. In another study, *Shiratsuchi et al.* indicate that, in addition to enhancing the antigen-specific antibody response, replacement of HVR1 with the *Plasmodium yoelii* B cell epitope evades Ad5 PEI (Shiratsuchi, Rai et al. 2010). Therefore, replacing Hexon HVRs may help the Ad vector evade neutralizing antibodies while maintaining Ad-specific $CD4^+$ T cell help to antibody generation against the Hexon-displayed vaccine antigen.

Malaria Pathogenesis and Current Vaccine Approaches

Malaria is a disease caused by the protozoan parasite Plasmodium. There are several species of Plasmodium, with *Plasmodium falciparum* being the most pathogenic and most common. The CDC estimates that there are nearly 300 million new cases of malaria and 1 million fatalities annually (Hafalla, Silvie et al. ; Carvalho, Daniel-Ribeiro et al. 2002). There is no available vaccine against malaria, and current drug therapy is not always available or efficacious in endemic areas, such as sub-Saharan Africa, where drug resistant strains are prevalent (Casares and Richie 2009). Further, the mosquito vector, in which the *Plasmodium* species are transmitted, has become insecticide-resistant in many endemic areas. Researchers have explored several vaccine strategies. These include vaccines that protect against disease, as well as those that block transmission to the

mosquito vector from an infected individual after a blood meal (Crompton, Pierce et al. 2010). *Alonso et al.* discussed the research agenda that could lead to the eradication of malaria. They outline current efforts by The Malaria Eradication Research Agenda and emphasize that effective malaria vaccine campaigns must include strategies to prevent transmission to mosquitoes, as vector control is a key component to eliminating malaria infections in endemic areas (Alonso, Brown et al. 2011).

The plasmodium life cycle is complex with one half of the cycle in the mosquito vector and the other in humans (Figure 4). The life cycle begins when a female *Anopheles* mosquito carrying the parasite takes a blood meal, where she transmits infectious sporozoites into her prey's bloodstream. Once plasmodium enters the human bloodstream, it travels to the liver where it grows in hepatocytes. Here, the sporozoite divides into a merozoite, or haploid cell, over the course of a week or two. Merozoites exit the liver to re-enter the bloodstream where they infect red blood cells and multiply exponentially over the next 1 to 3 days. This blood stage is the symptomatic stage in humans, resulting in fever and flu-like symptoms. A fraction of these merozoites develop into male and female gametocytes within the red blood cell. When a mosquito takes a blood meal, it ingests the red blood cells containing these gametocytes. The red blood cells then rupture, releasing the gametocytes within the mosquito midgut. The gametocytes mature to gametes and then undergo a sexual life cycle before they can be transmitted back to a human (Phillips 2001; Zhang, Joyce et al. 2013). The sexual life cycle in the mosquito begins when male and female gametes join to form a diploid zygote. The zygote then becomes a motile ookinete that embeds in the mosquito midgut

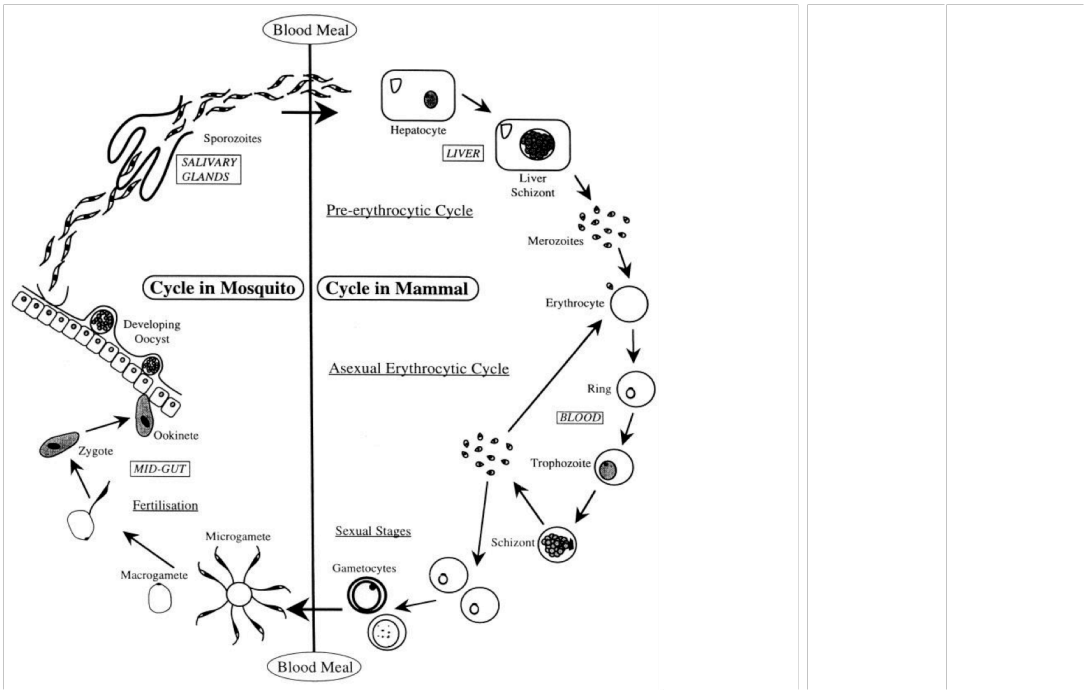

Figure 4. Malaria Pathogenesis. The asexual stage begins when a female *Anopheles* mosquito carrying the parasite takes a blood meal, and transmits infectious sporozoites bloodstream. Plasmodium travels through the bloodstream to the liver where it grows in hepatocytes. The sporozoite divides into a merozoite, or haploid cell, over the course of a week or two. Merozoites exit the liver to re-enter the bloodstream where they infect red blood cells and multiply exponentially over the next 1 to 3 days. This blood stage is the symptomatic stage in humans, resulting in fever and flu-like symptoms. A fraction of these merozoites develop into male and female gametocytes within the red blood cell. When a mosquito takes a blood meal, it ingests the red blood cells containing these gametocytes. The red blood cells then rupture, releasing the gametocytes within the mosquito midgut. The gametocytes mature to gametes and then undergo a sexual life cycle before they can be transmitted back to a human. Adapted from (Phillips 2001).

wall to become an oocyst. The oocyst proliferates over the course of roughly 2 weeks and generates asexual sporozoites. The oocyst bursts, releasing sporozoites that travel to the salivary gland of the mosquito where they are transmitted to a human when the mosquito takes a blood meal and the cycle continues (Phillips 2001).

To infect humans, *Plasmodia* must undergo this sexual life cycle within the mosquito vector. Due to the complexity of this life cycle, no single vaccine against any one stage of the *Plasmodium* life cycle protects against *Plasmodium* infection. There are currently no licensed vaccines against malaria infection (Crompton, Pierce et al. 2010). A novel approach to the development of malaria vaccines is to combine vaccines targeting different life stages simultaneously. Specifically, many research groups are designing vaccines that target both the blood and liver stages of plasmodia to generate protective vaccines. Other current studies focus on generating vaccines that target the Plasmodia life stages within the mosquito to block transmission (Smith, Chitnis et al.). By attacking different stages of the Plasmodia life cycle, protective vaccines will be more effective and, in combination with transmission-blocking vaccines, there is the potential to eradicate malaria from endemic areas.

Plasmodium vaccine candidates are proteins that are normally poorly immunogenic. This poor immunogenicity is a major challenge since for a vaccine to be effective it must generate a robust, long-lived immune response to the target antigen. Therefore, subunit, or protein-based, vaccines have not proven to be very efficacious and thus require several boosts to generate an immune response. As a result, many

researchers are exploring alternative approaches such as virally vectored vaccines for both protective and transmission-blocking vaccines.

Several recent protective vaccines against *P. falciparum* target the circumsporozoite protein (CSP) on the surface of the sporozoite, such that this vaccine blocks infection when the parasite first enters the bloodstream. A recombinant vaccine consisting of CSP fused to the hepatitis B surface antigen (RTS, S) has shown promise when additional adjuvants were incorporated. However, this vaccine only conferred 50% protection in humans when administered alone (Agnandji, Lell et al. 2011; Wilby, Lau et al. 2012). *Rodriguez et al.* were the first to show that a single vaccination with an Ad vector expressing CS partially protected mice against *Plasmodium yoelii* infection, the mouse model for malaria, thus underscoring the effectiveness of Ad-vectored vaccines (Rodriguez, Mintardjo et al. 2009). *Bruna-Romero et al.* demonstrate that a single vaccination with an Ad-expressing CS elicits both antigen-specific $CD4^+$ T cells and antibody production 2 weeks post-vaccination (Bruna-Romero, Gonzalez-Aseguinolaza et al. 2001). Combining this vaccination with boost vaccination with a different (heterologous) Vaccinia-based vaccine conferred 100% protection against malaria infection in mice. *Shiratsuchi et al.* generated a novel Ad-based vector that expresses CS and displays a CS B cell epitope within the HVR1 of the Ad Hexon. Vaccination with this vector generated a higher CS-specific antibody titer than vectors solely expressing CS and conferred partial protection as well. Whether alone or in combination with other vaccine vectors, Ad-based vectors enhance the humoral and cell-mediated immune response to protective malaria antigens (Shiratsuchi, Rai et al. 2010). More recently,

other protective epitopes have been identified and incorporated into current vaccine development to protect against malaria infection.

In addition to protective malaria vaccines, transmission-blocking vaccines (TBVs) could contribute to limiting malaria infection by preventing parasite transmission to the mosquito vector. By expressing or displaying antigens expressed exclusively in the mosquito vector, TBVs would elicit antibodies against these plasmodium proteins in vaccinated individuals. When a mosquito takes a blood meal from an infected individual vaccinated with a TBV the insect takes up the parasite and also the transmission-blocking antibodies. Once in the mosquito host, these antibodies will bind to the parasite and prevent it from developing in the mosquito (Smith, Chitnis et al. ; Hill 2011). TBVs could be very effective because, unlike parasite proteins expressed in the human host, the proteins expressed in the mosquito vector are not highly varied. Thus TBVs could also prevent the spread of mutant parasites that escape protective vaccines.

The *Plasmodium falciparum* protein Pfs25 is a desirable target antigen for TBVs (Kaslow, Quakyi et al. 1988). Pfs25 (*Plasmodium falciparum* surface protein 25) is a membrane-bound protein expressed on the surface of the parasite once it enters the mosquito midgut. It contains 4 extracellular EGF-like domains, DI-DIV (Figure 5). EGF-like domains contain 6 cysteine residues that form 3 disulfide bonds. These domains are normally found in the extracellular portion of many membrane-bound proteins. While the function of Pfs25 is not completely elucidated it is thought to facilitate binding and invasion of the midgut epithelia, one of the early and crucial steps in the parasite life cycle in the mosquito (Kaslow, Quakyi et al. 1988). Antibodies against Pfs25 block

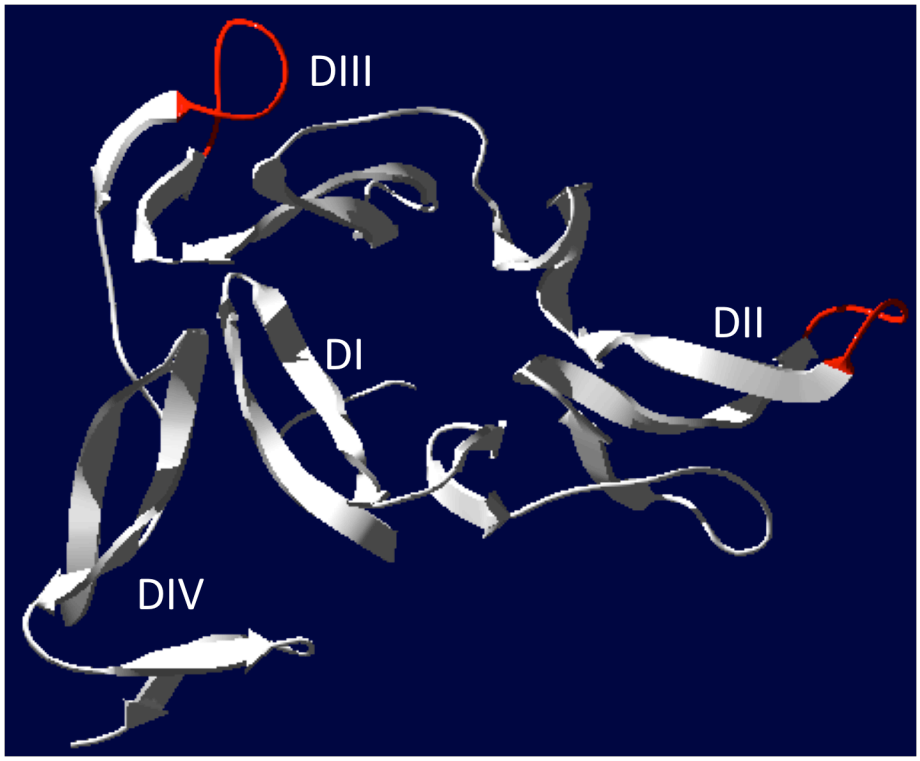

Figure 5. Modeled structure of Pfs25 based on the *P. vivax* homolog, Pvs25. (generated by C. Wiethoff). The DII and DIII loops are in red.

plasmodium development and midgut binding (Tomas, Margos et al. 2001; Sharma 2008). When vaccinated with the Pfs25 protein in the presence of the adjuvants alum or cholera toxin, mice develop transmission-blocking antibodies to Pfs25 (Barr, Green et al. 1991; Kaslow, Bathurst et al. 1994; Gozar, Muratova et al. 2001).

While Pfs25 is an attractive candidate antigen for TBV development, the protein itself as a subunit, or protein, vaccine does not strongly elicit Pfs25-specific antibodies suggesting Pfs25 is not very immunogenic on its own. Sequence analysis suggests that Pfs25 possesses very weak $CD4^+$ T cell epitopes. *Gozar et al.* demonstrate that Pfs25 alone does not elicit T cell activation. Further, fusion of Pfs25 to another plasmodium transmission-blocking antigen, Pfs28, enhanced this T cell response and antibody production. This enhancement is likely due to conserved T cell epitopes within Pfs28 to activate T cells (Gozar, Price et al. 1998). In order to enhance the Pfs25-specific antibody response, an adjuvant must elicit T cell help. To improve T cell help, TBVs must employ adjuvants in Pfs25 vaccine design.

Several preliminary research studies assess the effects of different adjuvants on Pfs25-specific antibody development. However, so far there has only been one clinical trial involving Pfs25 vaccination. This phase 1 human clinical trial used the adjuvant montanide ISA in conjunction with Pfs25 but was prematurely halted as subjects experienced adverse side effects (Wu, Ellis et al. 2008). A more immunogenic Pfs25 subunit vaccine has been recently developed and is currently being tested in phase 1 clinical trials. This vaccine consists of Pfs25 fused to the *Pseudomonas aeruginosa*

exoprotein (EPA) and has been shown to generate about 100-fold higher Pfs25-specific antibody titers in mice (Shimp, Rowe et al. 2013).

Due to their inherent adjuvant properties, several groups have generated TBVs using viral vector platforms in order to overcome the poor immunogenicity of transmission-blocking malaria proteins. For example, *Miyata et al.* demonstrate that adenovirus expressing the Pfs25 homolog of *P. vivax*, pvs25, more potently elicits Pvs25-specific antibodies than alum-adjuvanted Pvs25 (Miyata, Harakuni et al. 2011). More recently, *Goodman et al.* generated Ad-based vaccine vectors expressing Pfs25 as a transgene (Goodman, Blagborough et al. 2011). These vectors express a truncated form of Pfs25 lacking its transmembrane domain and thus are secreted from Ad-transduced cells. They did not assess the efficacy of these Ad-based vectors alone but rather combined primary Ad-based immunizations with boost vaccination with a Vaccinia virus-based vaccine expressing Pfs25. They found that this prime-boost vaccination could block transmission of malaria to mosquitoes both in vitro and in vivo. In this study, mosquitoes fed directly from vaccinated mice infected with a genetically modified strain of mouse plasmodia, which expressed Pfs25 in place of its own Pfs25 homolog, to assess transmission blockade mediated by Pfs25-specific antibodies. When mosquitoes fed on infected mice, parasite load was reduced by 67% in mosquitoes. While these results are promising there is still a need for an effective and safe transmission-blocking vaccine that potently elicits transmission-blocking antibodies against Pfs25. This dissertation describes the generation of novel Ad5-based transmission-blocking vaccines targeting *P.*

falciparum Pfs25. Further this work contributes to our understanding of how Ad-based vectors serve as a natural adjuvant during vaccination.

CHAPTER II

MATERIALS AND METHODS

Cell lines and culture

THP-1 cells, HeLa cells and 293Trex cells were obtained from ATCC. 293β5 cells were a gift from Glen Nemerow. Tissue culture reagents were obtained from Mediatech and HyClone. HeLa cells, 293β5 cells and 293Trex cells were maintained in Dulbecco modified eagle medium (DMEM) supplemented with 1mg/ml streptomycin, 100IU/ml penicillin, 0.25 mg/ml amphotericin B, non-essential amino acids, 2mM glutamine, 10mM Hepes buffer, 1mM sodium pyruvate. 293Trex cells were maintained with 5μg/ml blasticidin purchased from Sigma Aldrich. THP-1 cells and primary mouse cells were maintained in RPMI 1640 made up with the same supplements as DMEM. For all experiments, THP-1 cells were differentiated to macrophage-like cells by overnight stimulation with 100nM phorbol-12-myristate-13-acetate (PMA). PMA and N-acetylcysteine were purchased from Sigma Aldrich. RPMI 1640 was purchased from Mediatech.

Stable cell lines

Lentiviral vectors were employed to generate TLR9 knockdown THP-1 cells (THP-1-TLR9KD), gp91phox knockdown THP-1 cells (THP-1-gp91phoxKD) THP-1 cells expressing control shRNA (THP-1-control). Lentiviral vectors with an LKO.1 backbone

expressing shRNA for TLR9 (Openbiosystems cat#RHS3979- 9624075, cloneID TRCN0000056891), shRNA for NLRP3 (OpenBiosystems cat # RHS3979-19 9629911, clone ID TRCN0000062727), shRNA for gp91phox (OpenBiosystems cat # RHS3979-97052978; clone ID TRCN0000064591) or control shRNA recommended by the RNAi consortium (AddGene #10879) were generated by co-transfection of 293T cells with the packaging plasmids pHEF-VSVG (cat# 4693, NIH AIDS research and reagent program), pRSV-REV (Addgene #12253) and pMDLg/pRRE (Addgene #12251). Supernatants containing these recombinant lentiviral vectors were collected 48 hours post-transfection and used to transduce THP-1 cells by spinoculation. Positive transductants were selected with puromycin. THP-1 cells stably overexpressing bcl-2 were generated similiarly by generating transducing particles carrying either a pBMN-I-GFP vector (Addgene # 1736) or pBMN-I-GFP containing the bcl-2 gene.

Virus Generation

Ad5gfp generation has been previously described (Wodrich, Henaff et al. 2010). The Ad5-pfs25 virus was generated using a modified AdEasy system described by *He et al.* and more recently *Luo et al.* (He, Zhou et al. 1998; Luo, Deng et al. 2007). Briefly, we used lambda red recombineering to insert a shuttle vector containing the pfs25 gene into the E1/E3-deleted Ad5 genome. We used Genescript to synthesize the pfs25 gene after first codon-optimizing it for expression in humans. After cloning the pfs25 gene into the shuttle vector we inserted the tet operator (tetO, TO) sequence from pcDNA4/TO/myc-HisA upstream of the CMV promoter. This allows us to suppress pfs25 expression while

propagating the virus since 293Trex cells constitutively express the Tet repressor (TetR). TetR will bind to TetO and block transcription of the pfs25 transgene. We found that expression of pfs25 during viral propagation decreased viral yields by at least 10-fold making it very difficult to recover virus. We confirmed pfs25 expression is suppressed in 293Trex cells by detecting Pfs25 in infected cells by IFA with Pfs25 antisera. We further confirmed that pfs25 expression is not suppressed in 293 cells by IFA.

For the Ad5-HVR-pfs25 vectors, we used galk recombineering using positive and negative selection steps as described by *Warming et al.* (Warming, Costantino et al. 2005). Briefly, we generated primers with homology to regions within HVR1 or HVR5 flanking the galk sequence (Table 1). Using these primers we amplified galk from a plasmid containing galk (pgalk) obtained from the NCI BRB Preclinical Repository (NCI-FCRDC). GalK flanked by HVR1 or HVR5 was used in recombination into the specified HVRs in the Ad5 genome by bacterial recombineering. Following positive selection for galK insertion and confirmation by restriction enzyme digestion we replaced galk with an epitope from the DII or DIII domains of Pfs25. To do so, we generated primers with the pfs25 epitope flanked by sequences homologous to either HVR1 or HVR5 (Table 1). These primers overlap such that we generated primer dimers in order to generate a PCR product containing the pfs25 epitope flanked by sequences homologous to the Hexon HVR of interest. Following a second round of recombineering we replace galk with the pfs25 epitope within the HVR1 or HVR5 of Hexon and selected positive clones by negatively selecting galk-containing clones. We confirmed positive

recombinants by restriction enzyme digest, PCR amplification of the pfs25 epitopes and sequencing of the Hexon region of the genome. We removed the same number of amino acids that we inserted to conserve the size of the HVR domains. Serum from mice immunized with Pfs25-alum recognizes the Hexon from these recombinant viruses by Western blot (Figure 20B).

Table 1. Primer sequences for recombineering. Ad5 Hexon sequences are highlighted in red.

	Primer name	Sequence
Primers for galk insertion	HVR1 galk forward	gtgccccaaatccttgcgaatgggatgaagctgctactgctcttgaaatac ctgttgacaattaatcatcggca
	HVR1 galk reverse	agaataaggcgcctgcccaaatacgtgagtttttgctgctcagcttgcttc agcactgtcctgctcctt
	HVR5 galk forward	agcaacaaaatggaaagctagaaagtcaagtggaaatgcaatttttctca cctgttgacaattaatcatcggca
	HVR5 galk reverse	gtgtctggggtttctatatctacatcttcactgtacaataccactttaggtcag cactgtcctgctcctt
Primers for pfs25 epitope insertion	HVR1 DIII forward	gtgccccaaatccttgcgaatgggatgaagctgctactgctcttgaaataa tctggatacatctaatcccgtgaagactggagtctgcagt
	HVR1 DIII reverse	agaataaggcgcctgcccaaatacgtgagtttttgctgctcagcttgctca caactgcagactccagtcttcacgggattagatgtatc
	HVR5 pfsDII forward	agcaacaaaatggaaagctagaaagtcaagtggaaatgcaatttttctca attgatgggaacccagtgtcctacgcctgcaagtgtaat
	HVR5 pfsDII reverse	gtgtctggggtttctatatctacatcttcactgtacaataccactttaggatta cacttgcaggcgtaggacactgggttcccatcaat
	HVR5 pfsDIII forward	agcaacaaaatggaaagctagaaagtcaagtggaaatgcaatttttctca ctggatacatctaatcccgtgaagactggagtctgcagt
	HVR5 pfsDIII reverse	gtgtctggggtttctatatctacatcttcactgtacaataccactttaggaca actgcagactccagtcttcacgggattagatgtatc

Virus Preparation

With the exception of Ad5-pfs25, which was propagated in 293Trex cells, all viruses were propagated in HEK293 cells and subjected to cesium chloride gradient

centrifugation twice to purify the virus and subjected to dialysis in 40 mM Tris, 150 mM NaCl, 10% glycerol, and 1 mM MgCl2 (pH 8.2) (Wiethoff, Wodrich et al. 2005). The *ts1* virus was propagated at the non-permissive temperature, 39.5°C, to generate mutant virus unable to penetrate the endosomal membrane (Weber 1976; Wiethoff, Wodrich et al. 2005). Bradford assay determined the viral concentration as 1 mg of protein corresponded to $4x10^9$ viral particles (Bio-Rad Laboratories, Inc.) and aliquots were flash frozen in liquid nitrogen and stored at -80°C. To determine viral titer, the viruses were serially diluted on HeLa cells and flow cytometry quantified GFP expression. For viruses without the GFP reporter, viruses were serially diluted on 293β5 cells and titer determined by IFA detecting adenovirus Hexon with a dylight labeled 9C12 antibody. Of the viruses used in this study, specific infectivity ranged from 100-200 viral particles per GFP-transducing unit (GTU).

Pfs25 expression from Ad5-pfs25

To confirm that Pfs25 is expressed from Ad5-pfs25 we transfected HeLa cells in a 24-well plate (100,000 cells per well) with the Ad5 shuttle vector containing the pfs25 transgene and 24 hours later fixed (3.7% PFA in 0.159 M PIPES buffer for 10 minutes) and permeabilized (10% FBS and 0.5% saponin) and then probed with ID2 (from K. Williamson), a monoclonal antibody recognizing a conformational epitope in the DIII domain of Pfs25, at a 1:250 dilution for 1 hour. Following 5 washes with 1xPBS, cells were incubated with an Alexa fluor 488-conjugated anti-mouse IgG antibody (Invitrogen, cat # A11029).

Reagents and antibodies

2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, cat # D399) was purchased from Invitrogen. CA-074me was purchased from EMD Biosciences. PAM3CSK4 was purchased from InvivoGen. IL-1β, TNF-α, IFNγ ELISA Ready-SET-Go! Kits were obtained from eBioscience. IFNγ ELISPOT kits were also from eBioscience. ConA (concanavalin A) was purchased from Sigma Aldrich (cat # c2272). For the Pfs25-specific ELISAs, 5x assay diluent (cat # 00-4202-56) and 1xTMB substrate were purchased from eBioscience (cat # 00-4201-56). The secondary HRP-conjugated anti-mouse IgG (Fc) was purchased from Abcam (ab97265). HRP-conjugated anti-mouse isotype specific antibodies were purchased from Southern Biotech (cat # 5300-05B). Sodium thiocyanate was purchased from Sigma and resuspended in 1xPBS. All other HRP-conjugated secondary antibodies were obtained from Biomeda Ltd. All other reagents were from Fisher Scientific.

Quantification of IL-1b and TNFα secretion by ELISA

THP-1 cells stably expressing empty vector and THP-1-TLR9 knockdown cells were plated at 50,000 cells per well in a 96-well plate with 100nM PMA to induce macrophage differentiation. The following morning cells were serum starved for 2 hours and left untreated or treated with adenovirus at 30,000 ppc for 6 hours. Supernatants from each sample were collected and an ELISA was performed using the Ready-SET-Go! IL-1β kit from eBioscience (cat # 88-7010-88). Similarly, THP-1 cells stably expressing empty vector and THP-1-bcl-2 cells were plated at 50,000 cells per well in a 96-well

plate with 100nM PMA to induce macrophage differentiation. The following morning cells were serum starved for 3 hours, primed with 30ng/ml of the TLR2 ligand, PAM3CSK4, washed and then left untreated, treated with 3mM ATP (positive control), or treated with adenovirus at 50,000 ppc for 2 hours. Supernatants were used in the IL-1β ELISA assay. For TNFα secretion, THP1 cells were plated as done for the IL-1β ELISA and were serum-starved for 3 hours. They were then left untreated, or were pre-treated for 10 minutes with 30mM N-acetyl-cysteine or 10μM rotenone then they were infected with Ad5 at 30,000 ppc in the continued presence of drug. After 4 hours supernatants were subjected to analysis by ELISA for TNFα (eBioscience, cat # 88-7346-77).

ROS Assay

All THP-1 cells were plated at 200,000 cells per well in a black 96-well plate (Co-star) in RPMI with 10% FBS containing 100nM PMA to stimulate differentiation to macrophage-like cells. Media was replaced the following morning and cells were rested for two days. Following three-hour serum starvation, cells were incubated with the ROS sensitive fluorophore H_2DCFDA (DCF, Invitrogen) at 10 mM for thirty minutes followed by two washes with 1xPBS. If pre-treated with drug, cells were incubated with the drug for one hour following DCF incubation. Virus was added to wells when necessary and fluorescence intensity was measured over time at an excitation wavelength of 485 nm and emission wavelength of 520 nm on a fluorescent plate reader.

Western Blot Analysis

Western blotting confirmed TLR9 knockdown and bcl-2 overexpression in THP-1-TLR9KD and THP-1-bcl-2 cells, respectively. THP-1-TLR9kd and THP-1-bcl-2 cells and their respective control cells were plated in a 6-well plate at 2 million cells per well in RPMI containing 100nM PMA to stimulate macrophage differentiation. The next morning cells were washed and lysed in 25 mM Tris pH 8, 25 mM NaCl, 0.1 mM EDTA, 1% triton X-100, 0.5% Deoxycholate and 15 mM beta-mercaptoethanol (solution B) containing 1mM of the serine protease inhibitor PMSF. Lysates were subjected to SDS-PAGE on 15% polyacrylamide gels, transferred to nitrocellulose and immunoblotted for TLR9 (Santa Cruz, cat # sc-25468), NLRP3 (Enzo, cat#ALX- 22804-881), or Bcl2 (Santa Cruz, cat # sc-7382). As a loading control, both membranes were immunoblotted for Actin (Sigma cat# A5441).

To determine if Ad5 Hexon displayed either Pfs25 epitopes, 2.5 µg of purified virus was subjected to SDS-page alongside .25µg purified Pfs25 protein (obtained from the NIH, yeast-purified protein) as a positive control. Western blotting with serum from Pfs25-alum-immunized mice (at 1:250) followed by an HRP-conjugated anti-ms IgG (H+L) (1:1000) determined whether Ad5 Hexon contained the Pfs25 epitope.

qPCR for gp91phox levels

RNA was isolated from THP-1-gp91phoxKD and THP-1-control cells using the Qiagen RNeasy Plus Mini Kit (cat #74134) and the Qiagen QIAshredder (cat # 79654). 500 ng RNA was subjected to DNase I treatment (Fermentas # EN0521) then reverse transcribed to single strand cDNA (Fermentas RevertAid™First Strand cDNA Synthesis

Kit # K1621). cDNA was then subjected to qPCR on the BioRad Opticon 2 using BioRad IQ SYBR Green Supermix (cat #170-8880). Primers for actin: forward ATGGGTCAGAAGGATTCCTATGTG, reverse CTTCATGAGGTAGTCAGTCAGGTC (Leon, Gomez et al. 2009). Primers for gp91phox: forward CACAGGCCTGAAACAAAAGA, reverse GCTTCAGGTCCACAGAGGAA (QPPD: Quantitative PCR Primers Database, NCI, primer set 10932). Relative gp91phox expression was generated using the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen where the fold change in gp91phox expression was normalized to the housekeeping gene β-actin in each cell type and gp91phox expression in the THP-1-control cells is set to 1 (Livak and Schmittgen 2001).

Measurement of Cytosolic Cathepsin B and Cytochrome C

THP-1 cells were plated in a 6-well plate at 2 million cells per well in RPMI containing 100nM PMA to stimulate macrophage differentiation. The next morning cells were serum starved for 3 hours and left untreated (control), treated with adenovirus at 100,000 ppc or treated with the *ts1* mutant adenovirus at 100,000 ppc for 30 minutes. Cells were put on ice where they were washed and permeabilized with 50 mg/ml Digitonin plus 1 mM PMSF in PBS (pH 7.4) for 15 minutes at 4 ºC to effectively lyse the plasma membrane while leaving the intracellular membranes intact. These cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted for Cathepsin B (Santa Cruz, cat # sc-13985) and Cytochrome C (BioLegend, cat # 612302).

To assess the catalytic activity of cathepsin B in the cytoplasm, PMA-differentiated THP-1 cells ($2x10^5$/well) plated on glass coverslips in 24 well plates were treated with media alone or 10^5 particles of Ad5gfp, or *ts1* for 15 minutes followed be the addition of the fluorogenic cathepsin B substrate, Magic Red™ (Immunochemistry, Inc.) for 30 minutes. Cells were washed in PBS, counterstained with Hoechst 33242 for 15 minutes and then visualized by epifluorescence microscopy.

Assessment of mitochondrial membrane potential

THP-1 cells were plated in a 96-well plate at 100,000 cells per well in RPMI plus 100nM PMA to generate macrophage-like cells. The following day media was replaced and cells were rested for one day in RPMI plus 10% FBS. Cells were serum starved for 3 hours. If necessary, cells were pre-treated with Ca-074me (EMD Biosciences, cat # 205531) during the last 20 minutes of serum starvation. Cells were then left untreated, incubated with 50μM of the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP), infected with adenovirus at 50,000 ppc or infected with the *ts1* mutant adenovirus at 50,000 ppc for 30 minutes. Cells were then washed and incubated with the MitoPT™ JC-1 dye reagent per manufacturer's recommendations (ImmunoChemistry Technologies, LLC, cat # 937). JC-1 oligomerized within the mitochondria where it appears as red puncta throughout the cytoplasm. When mitochondrial membrane potential was lost, JC-1 appeared as diffuse green fluorescence throughout the cytoplasm where it existed as a monomer. Briefly, cells were incubated with JC-1 for 15 minutes, washed twice carefully with wash buffer. Cells were analyzed

by fluorescence microscopy. Then we quantified the percentage of cells containing red fluorescent puncta. The data was expressed as the % cells with intact $Y_{m.}$

Mice and immunizations

All mice used in these studies were C57/BL6 wildtype mice (Jackson laboratories cat #000663) between the ages of 6-12 weeks old. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of Loyola University Chicago (Maywood, IL). Animals were vaccinated intramuscularly in the left quadriceps. The primary immunization was administered between 6-8 weeks of age and the boost immunization or serum collection was given 20-22 days later. Animals were sacrificed and serum collected 21-22 days following the last immunization. For all immunizations, mice were injected with 50µl. For protein immunizations, purified Pfs25 (obtained from the NIH, yeast-produced protein) was adsorbed to aluminum hydroxide (referred to as alum). Aluminum hydroxide was purchased as a gel suspension called Alhydrogel from Invivogen (cat # 21645-51-2). For all protein-alum preparations, 25µl alhydrogel was added to the 50µl solution for a 1:1 ratio of protein to alum in solution. For Ad5-pfs25 primary vaccinations, Ad5-pfs25 was administered at 10^{10} pfu per animal and when used in combination with a heterologous prime-boost, Ad5-pfs25 prime vaccination was administered at 10^{9} pfu. All Ad5-HVR-pfs25 vaccinations were administered at 10^{10} pfu per animal. For the pre-existing immunity experiments, mice were given 10^{10} pfu of the E1/E3-deleted Ad5 containing a luciferase reporter gene (no GFP) or a UV-inactivated Ad12 virus. 21 days later the animals were vaccinated with the specified vectors.

Determination of Pfs25-specific serum antibody titer and IgG subclass production

Blood was taken from sacrificed mice via cardiac puncture. For serum collection, blood samples were kept on ice from 1-4 hours and then red blood cells spun down at 8000 rpm for 5 minutes and cleared supernatants were aliquoted and stored at -80° C until use. High-binding ELISA plates (Costar cat # 07-200-35) were coated with Pfs25 protein at 1µg/ml in coating buffer (eBioscience) shaking overnight at 4° C. Plates were washed 4 times with 1XPBST (.05% Tween) before blocking with 1xAD (eBioscience 00-4202-56) at 200µl/well for 1 hour. After 4 more washes, serum was serially diluted in 1xAD and added to the plate for 2 hours at 100µl/well. A secondary anti-mouse IgG (Fc) was added to the wells following 4 more washes for 1 hour at a 1:5000 dilution in 1xAD. Again we washed the plate and then developed with 1xTMB substrate (at 50µl per well and after a color change stopped the reaction with 1M sulfuric acid at 25µl per well. The plate was read with a KC Junior plate reader at an optical density of 450 nm. For the IgG subclass ELISAs all steps were performed as described above except serum samples were added at one constant dilution and the secondary antibodies added were specific to IgG1, IgG2a or IgG3 (Southern Biotech). Antibody endpoint titers (depicted as ELISA units) were determined by dilution curve where the absorbance value is 3 standard deviations above the O.D. from PBS-immunized control mice.

Determining the relative affinity of Pfs25-specific serum antibodies

The affinity assay was set up similar to the Pfs25-specific ELISAs where plates were coated with Pfs25 and serum was added to the wells. In this case, a fixed dilution of

serum was added to the wells at a dilution where the antibody binding is presumably saturated (determined previously by assessing the O.D. for various dilutions from the antibody titer ELISA). Following serum incubation, the plate was incubated with increasing concentrations of NaSCN at 100µl/well. NaSCN was made up and diluted in 1xPBS. NaSCN was added to the plate and incubated at room temperature for 15 minutes shaking. The plate was then washed 15 times with PBST before adding the secondary antibody (anti-mouse IgG (Fc)) and detection as previously described.

B cell elispots

ELISPOT plates (Millipore, cat # MAHAS4510) were coated with 10µg/ml antigen in coating buffer overnight at 4° C. Plates were blocked with RPMI (complete) for 1-2 hours, washed 3 times with 1xPBS and then cells were added in serial three-fold dilutions starting with the lowest dilution of $1x10^5$ cells per well. Bone marrow cells were harvested from femurs and tibias from both hind legs. Bones were flushed with RPMI, spun down at 300xg for 5 minutes and resuspended in RPMI. Splenocytes were harvested from spleens homogenized on a mesh filter into RPMI. Cells were spun down and resuspended in 1 ml red blood cell lysis buffer and incubated for 1 minute at room temperature. Cells were resuspended in 14 ml PBS, spun down and resuspended in RPMI. Both bone marrow cells and splenocytes were added to the ELISPOT plate for 4-6 hours. Following 4 washes with PBS and 3 washes with PBST (0.1% tween), the plate was incubated with secondary anti-mouse IgG antibody overnight at 4° C (final concentration of 2µg/ml). Wash the plate 4 times with PBST (0.1%) then add

streptavidin-HRP (diluted 1:1000 in PBST with 1%FBS) for 1 hour. After 4 washes with PBS, we add 100µl of AEC substrate (Sigma, prepared as directed) to each well and develop for 15 minutes or until spots appear.

Ex Vivo splenocyte stimulation and cytokine production

We harvested splenocytes as described above for the B cell ELISPOT 10 days post-vaccination. We plated cells in a 96-well tissue culture plate (Costar) with specific antigens. Whole splenocytes were restimulated with Ad5 (10,000 particles per cell) or Pfs25 (10µg/ml). Cells were left without antigen as the unstimulated control and plated with 2.5µg/ml ConA as a positive control. After 24 hours, we collected cell-free supernatants and subjected them to an ELISA to detect IFNγ secretion. We followed the eBioScience protocol for the IFNγ ready-set-go ELISA as described in a previous section.

Parasite binding assays

Purified NF54 strain *P. falciparum* gametes were obtained from Kim Williamson's lab at Loyola University of Chicago's Lakeshore Campus. Intraerythrocytic, asexual gametocytes are maintained in culture in the Williamson lab and are induced to undergo gametogenesis to form gametes, the asexual parasites that have escaped the red blood cell and are capable of infecting the mosquito. Gametogenesis and gamete purification have been previously described by *Eksi et al.*(Eksi, Czesny et al. 2007). In this case, gametocytes in culture were induced for 4 hours to undergo gametogenesis by incubating them in emergence media containing xanthurenic acid.

Following gametogenesis, gametes were purified on a density gradient using Nycodenz (Progen). Gametes were resuspended and counted and plated in a 96-well, V-bottom plate at 100,000 cells per well. The 96-well tissue culture plate was first siliconized with Sigmacote from Sigma Aldrich (cat # SL2) to prevent parasites from sticking to the wells. 200µl Sigmacote was used to rinse each well and then plate was left to air dry before adding gametes. Gametes were plated and then stained with 50µl per well of serial dilutions of sera from immunized mice for 1 hour on ice in 1xSA media (*P. falciparum* media containing glucose). For the first wash, wells were brought up to 250µl with 200µl SA media and spun at 3000rpm for 3 minutes. Supernatant was carefully removed from each well with a micropipette very carefully as not to disturb the pellet. We then washed the wells a second time by resuspending the pellet in 200µl SA media, spun out media and removed the sup carefully. The pellet was resuspended in Alexa fluor 488-conjugated anti-mouse IgG (H+L) antibody diluted 1:1000 in SA media and incubated in the dark on ice for 1 hour. Wells were washed one time as previously described and resuspended in 100µl SA media before fluorescence was analyzed by FACS using a BD Accuri C6 flow cytometer.

Standard membrane-feeding assays (SMFAs)

In collaboration with Kazutoyo Miura and Carol Long at the National Institutes of Health within the Malaria Vaccine Development Branch of the National Institute of Allergy and Infectious Diseases, we performed a standard membrane-feeding assay (MFA) to assess transmission blockade as previously described (Miura, Deng et al. 2013). Briefly, we

diluted each serum sample 1 to 10 in human blood infected with *P. falciparum* (NF54 strain) and normal human serum replenished with complement. Three- to six- day-old Female *Anopheles stephensi* mosquitoes fed on this serum and gametocyte culture through a membrane. Eight days later, we harvested and dissected the mosquitoes and counted *P. falciparum* oocyst number per mosquito to assess the parasite load. Serum from Ad5-immunized mice served as a negative control while purified 4B7, a monoclonal antibody recognizing Pfs25, served as a positive control for transmission blockade.

Statistics

Statistical significance ($p < 0.05$) was assessed using the Student's T Test when two groups were compared. Data are presented as mean ± SEM.

CHAPTER III

RESULTS

Investigating the role of reactive oxygen species in the proinflammatory response to adenovirus infection

Adenovirus (Ad) elicits a rapid proinflammatory response. This innate immune response is initiated early during infection when Ad interacts with antigen-presenting cells (APCs) such as macrophages or dendritic cells. These APCs, such as alveolar macrophages in the lung or Kupffer cells in the liver, are sentinels of the innate immune system. They mediate this rapid response to adenovirus by producing proinflammatory cytokines and chemokines (Lore, Adams et al. 2007). In turn, these molecules generate inflammation in the surrounding tissue and activate the recruitment of additional immune cells to the site of infection (Pulendran and Ahmed 2006; Appledorn, Patial et al. 2008; Xu, Tian et al. 2008).

This process of activating the innate immune response is mediated by conserved pattern recognition receptors (PRRs). These PRRs are responsible for sensing the presence of Ad in target cells. Since Ad rapidly activates the innate immune response, PRRs are likely activated during viral cell entry. Studies demonstrate that Ad activates the PRR, Toll-like receptor 9 (TLR9). TLR9 senses the viral genome when the virus partially uncoats following endosome acidification (Yamaguchi, Kawabata et al. 2007).

TLR9 in turn activates NFκB, a key transcription factor involved in the upregulation of several proinflammatory cytokines (Cerullo, Seiler et al. 2007; Kawai and Akira 2008). In addition to TLR activation, the cytosolic PRRs, Ddx41, AIM2 and RIG-I, activate IRF-3, a key transcription factor activating the production of type I interferons. Another cytosolic PRR, the Nod-like Receptor, NLRP3, is also involved in the innate immune response to Ad infection (Muruve, Petrilli et al. 2008). Ad infection of peritoneal macrophages leads to the secretion of NLRP3-dependent cytokines and, further, requires components of the NLRP3 inflammasome (Muruve, Petrilli et al. 2008). The NLRP3-dependent cytokines IL-1β and IL-18 are important cytokines determining the nature of Ad infection. IL-1R knockout mice or mice given neutralizing IL-1β antibodies prior to infection exhibit a less severe inflammatory response (Shayakhmetov, Li et al. 2005). Taken together, Ad infection activates the PRRs TLR9, AIM2, DDX41, RIG-I and the NLRP3 inflammasome. These PRRs are crucial to alerting the innate immune system to viral infection. Since not much is known about the signals activating NLRP3 during Ad infection, we were particularly interested in characterizing the stimuli required for NLRP3 activation during Ad infection.

NLRP3 responds to many different stimuli. These stimuli are thought to be endogenous danger signals such as extracellular ATP or particulates such as uric acid and silica particles (Petrilli, Dostert et al. 2007). As mentioned previously, the stimuli that activate NLRP3 during Ad infection have not yet been fully elucidated. Upon activation, NLRP3 forms a multicomponent complex containing an adaptor protein, apoptosis-

associated speck-like protein (ASC), and active Caspase-1 (Schroder, Zhou et al. 2010). This complex, called the inflammasome, through Caspase-1 activity, processes the proinflammatory cytokines IL-1β and IL-18 into their mature form to be secreted (Dostert, Petrilli et al. 2008). As mentioned above, NLRP3 is required for the secretion of IL-1β from peritoneal macrophages following Ad infection (Muruve, Petrilli et al. 2008). These data suggest that NLRP3 is activated following Ad infection.

We first asked whether Ad indeed activated the NLRP3 inflammasome in the human monocyte THP-1 cell line. THP-1 cells served as a useful model to assess the innate immune response to adenovirus infection since alveolar macrophages and infiltrating monocytes respond to an Ad infection in the lung. We differentiated THP-1 cells with PMA to generate macrophage-like cells. We infected these macrophage-like THP1 cells with replication-defective Ad5 vector. This Ad vector is commonly explored for gene therapy and vaccination so understanding how it activates the immune system will contribute to more rationally designed Ad-based vectors. Following Ad5 infection we assessed IL-1β production by ELISA as a read out for NLRP3 activation. We found that Ad5 infection resulted in a significant increase in IL-1β production as compared to uninfected control cells (Figure 6A). These data suggested that Ad5 activates NLRP3 since this inflammasome is a key contributor to IL-1β production.

Since IL-1β was produced in response to Ad5 infection of THP-1 cells, we next asked directly whether components of the NLRP3 inflammasome were required for the IL-1β produced in response to Ad5. Several other cytosolic PRRs form inflammasomes

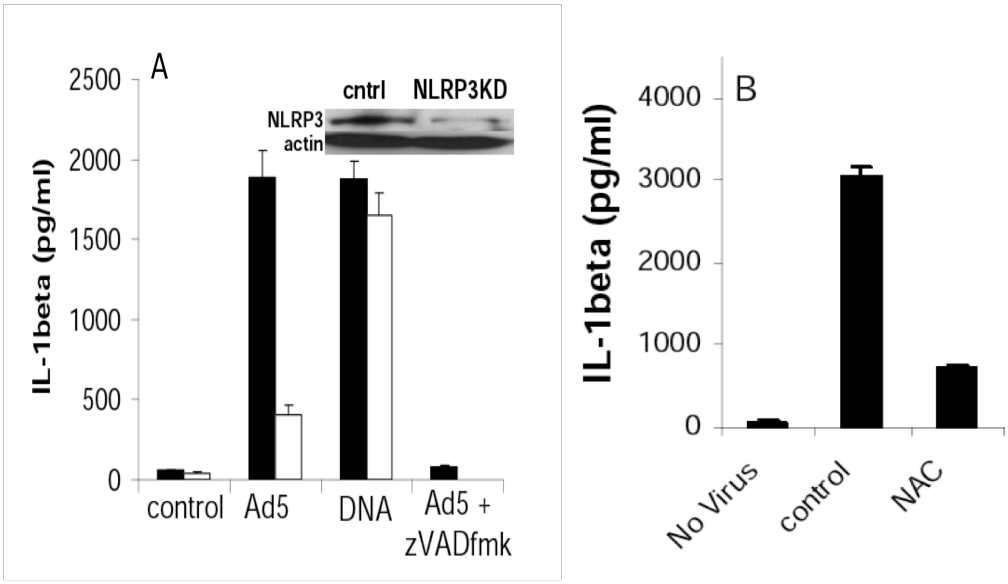

Figure 6. Role of NLRP3, Caspase-1 and ROS in Ad5-induced IL-1β release from PMA-differentiated THP-1 cells. A. Cells stably expressing either control (black bars) or NLRP3-specific shRNA (white bars) were rested in serum free media for 2 hours and then treated with media alone (control), Ad5gfp, or PAM3CSK4 +Lipofectamine/DNA for 6 hrs. Control cells were also pretreated with the pan-caspase inhibitor, zVADfmk, for 30 minutes before infection with Ad5gfp for 6 hrs. Release of IL-1β was quantified by ELISA. Inset: Western blot for NLRP3 and actin loading control in control and NLRP3 knockdown cells. B. THP1 control cells were pretreated with the radical scavenger N-acetylcysteine (NAC) for 30 minutes and then Ad5gfp for 1 hour in the continued presence of drug. Release of IL-1β was quantified by ELISA. Data represent the mean and standard error of 3 replicates. Experiment performed by A.U. Barlan.

and process IL-1β, although, there is no evidence that these other PRRs are activated during Ad infection (Latz 2010). Previous data implicate NLRP3 as the mediator of IL-1β production in response to Ad infection (Muruve, Petrilli et al. 2008). To address whether NLRP3 was required for Ad5-induced IL-1β production we stably knocked down NLRP3 in THP-1 cells and assessed IL-1β by ELISA following Ad5 infection. In cells where NLRP3 was knocked down (NLRP3 KD cells), IL-1β secretion was reduced by 80% as compared to THP-1 control cells following Ad5 infection (Figure 6A). These results correlated with a 70% reduction in NLRP3 protein in NLRP3 KD cells as determined by Western blot (Figure 6A, inset). In contrast, NLRP3 knockdown had no effect on the IL-1β secretion in response to cytosolic DNA, a known activator of the absent in melanoma 2 (AIM2) inflammasome. AIM2 also requires the inflammasome components ASC and caspase-1 (Figure 6A) (Fernandes-Alnemri, Yu et al. 2009; Hornung, Ablasser et al. 2009). These data demonstrated that NLRP3 was required for the IL-1β produced in response to Ad5 infection. We also asked whether Caspase-1, another component of the NLRP3 inflammasome, was required for IL-1β secretion following Ad5 infection. We pretreated PMA-differentiated THP-1 cells with Caspase-1 inhibitor, zVADfmk, before infecting them with Ad5 and then assessed IL-1β secretion by ELISA. We found that IL-1β secretion was abrogated in the presence of zVADfmk suggesting that Caspase-1 was required for Ad5-induced IL-1β production (Figure 6A). Together, these data demonstrated that IL-1β secretion in response to Ad5 required the NLRP3 inflammasome components NLRP3 and Caspase-1. Further, another cytosolic

PRR, AIM2, which forms an inflammasome with Caspase-1 did not play a role in IL-1β release following Ad infection.

So far, we found that Ad5 activates the NLRP3 inflammasome, which is required for IL-1β secretion following Ad5 infection. We next asked what signals or stimuli generated during Ad infection contribute to inflammasome activation. A key mediator of this innate immune response to several pathogens is oxidative stress, or reactive oxygen species (ROS). ROS have been traditionally studied and characterized as a mediator of the innate immune response to extracellular bacteria. ROS contribute to the respiratory burst that kills extracellular bacteria in the phagosome when they are taken up by cells (Zsengeller, Otake et al. 2000). These responses would not eliminate viruses, which escape to various cellular organelles to replicate. However, more recently, studies demonstrate a role for ROS in the antiviral response in activating important innate immune pathways (Nathan and Cunningham-Bussel 2013). *Fink et al.* demonstrate that ROS contribute to NFκB activation upon viral infection (Fink, Duval et al. 2008). Several other studies demonstrate a role for ROS in activating the type I interferon response, which is critical to generating an antiviral state in cells during infection. Specifically, ROS enhance the activation of the PRR, RIG-I, and the transcription factor, IRF3 during viral infection, which activates type I interferon production (Tal, Sasai et al. 2009; Soucy-Faulkner, Mukawera et al. 2010). Yet another study implicates ROS in activating another innate immune PRR, NLRP3, the Nod-like receptor responsible for production of proinflammatory cytokines such as IL-1β and IL-18 (Zhou, Tardivel et al. 2009). Taken

together, these studies demonstrate that ROS are a key signal in activating the innate immune response specifically by contributing to activation of innate signaling pathways that lead to activation of NFκB, IRF3 and NLRP3. It is unknown whether ROS play a role in the proinflammatory response to adenovirus infection. Ad is known to activate NFκB, IRF3 and NLRP3 during infection. Thus it is likely that ROS contribute to innate immune activation since many studies demonstrate that ROS are a key activator of these innate immune pathways (Nociari, Ocheretina et al. 2007; Zhu, Huang et al. 2007; Nociari, Ocheretina et al. 2009; Yamaguchi, Kawabata et al. 2010).

We next asked whether reactive oxygen species (ROS) were required for NLRP3 activation in response to Ad5. Recent studies demonstrate that NLRP3 activation requires ROS (Tschopp and Schroder 2010). Since Ad is a potent activator of the NLRP3 inflammasome, it most likely elicits ROS production. It was not yet known whether ROS are produced in response to Ad5 infection or whether they contributed to the activation of the innate immune response. To determine whether ROS contribute to NLRP3 inflammasome activation during Ad infection, we assessed IL-1β production following Ad5 infection in the presence of the ROS scavenger N-acetyl-cysteine (NAC). We incubated PMA-differentiated THP1 cells with NAC and assessed IL-1β production by ELISA following Ad5 infection. We found that NAC significantly attenuated IL-1β secretion following Ad5 infection. These data suggest that ROS contribute to NLRP3-dependent IL-1β secretion upon infection with Ad (Figure 6B).

Since our data suggest that ROS contribute to IL-1β secretion following Ad5 infection, we next asked whether ROS are involved in other pathways of the innate immune response. As mentioned previously, ROS is known to contribute to other innate immune pathways. To address whether ROS contribute to other innate immune pathways, we determined whether ROS contribute to the production of the proinflammatory cytokine TNFα. This cytokine is produced independent of NLRP3 activation. We asked whether inhibiting ROS with N-acetyl-cysteine (NAC) attenuated TNFα production in response to Ad5 infection. We pretreated PMA-differentiated THP1 cells with NAC and then infected these cells with Ad5 before assessing TNFα secretion by ELISA. We found that pretreatment with NAC significantly reduced TNFα secretion following Ad5 infection (Figure 7A). These data suggest that, in addition to contributing to NLRP3-dependent IL-1β secretion, ROS also contributed to the proinflammatory signals leading to TNFα secretion during Ad infection. Another way to address whether ROS contribute to the TNFα response following Ad5 infection is to determine whether enhancing ROS can augment TNFα production during Ad5 infection. To ask whether ROS augments TNFα production during Ad5 infection, we artificially enhanced the levels of ROS by pretreating PMA-differentiated THP1 cells with rotenone and then infected these cells with Ad5 before assessing TNFα secretion by ELISA. Rotenone inhibits complex I of the electron transport chain and would thus enhance mitochondrial ROS. We found that the enhancement of mitochondrial ROS by treatment with rotenone significantly augmented Ad5-induced TNFα production in PMA-differentiated THP1 cells (Figure 7A). NAC and

rotenone pretreatment did not influence Ad5 transduction such that the observed decreases in cytokine production were not the result of decreased viral load (Barlan, Griffin et al. 2010). These data suggest that ROS also contribute more broadly to the innate immune response to Ad5 by activating proinflammatory cytokine production from NLRP3-dependent and NLRP3-independent innate immune signaling pathways.

Since ROS are required for the proinflammatory response to Ad5, we next directly asked whether Ad5 induces ROS. We incubated the macrophage-like, PMA-differentiated THP-1 cells with the ROS-sensitive fluorophore 2',7'-dichlorofluorescein diacetate (DCFDA). DCFDA is a cell permeable dye that becomes deacetylated to its nonfluorescent form when it enters the cell. In the presence of ROS, this compound is oxidized to its highly fluorescent form, 2',7'-dichlorofluorescein (DCF), such that increases in DCF fluorescence intensity serve as a measure of ROS production.
We infected DCFDA-loaded THP-1 cells with Ad5 and monitored ROS production over time post-infection by measuring increases in the DCF fluorescence intensity using a fluorescence plate reader. We found that increasing doses of Ad5 induced a dose-dependent increase in ROS-dependent fluorescence (Figure 7B). Further, ROS were produced rapidly post-infection. These data demonstrate that Ad5 induces ROS. We next investigated the mechanism of ROS production during Ad5 infection.

Our data demonstrate that ROS are produced rapidly upon Ad5 infection suggesting that ROS are produced during Ad5 cell entry, the earliest event in the viral life cycle. To better understand the stimuli leading to ROS production during Ad infection,

we asked whether key events during Ad cell entry contributed to ROS production upon infection. During cell entry, Ad undergoes receptor-mediated endocytosis and then ruptures the endosome to gain access to the cytoplasm. We have previously demonstrated that this Ad-dependent rupture event is a danger signal contributing to NLRP3 inflammasome activation (Barlan, Griffin et al. 2010). We asked whether this endosomal rupture event was required for Ad5-induced ROS. To address this question, we infected PMA-differentiated THP1 cells with the temperature sensitive mutant virus, *Ad2ts1* (*ts1*) and assessed ROS production with DCF using the same assay as described above. When produced at the non-permissive temperature, *ts1* are hyperstable virions that can bind receptors and become endocytosed but fail to rupture endosomal membranes because the capsid does not properly uncoat in endosomes (Weber 1976). While infection with increasing doses of Ad5 induced a dose-dependent increase in ROS-dependent fluorescence, maximal doses of *ts1* did not elicit ROS above background levels (Figure 7B). Virus multiplicities of infection (MOIs) necessary to observe ROS using this fluorescence assay were much higher than those necessary to elicit the secretion of proinflammatory cytokines. However, at these MOIs, the virus more rapidly associates with the cell, allowing us to detect ROS production at times corresponding to early events in Ad5 cell entry. These data demonstrate that *ts1* cannot elicit ROS. As mentioned, *ts1* has a hyperstable capsid and does not partially uncoat within the endosome to release the membrane lytic protein. By comparison with *ts1*, we concluded that virus uncoating and/or rupture of endosomes is required to elicit ROS.

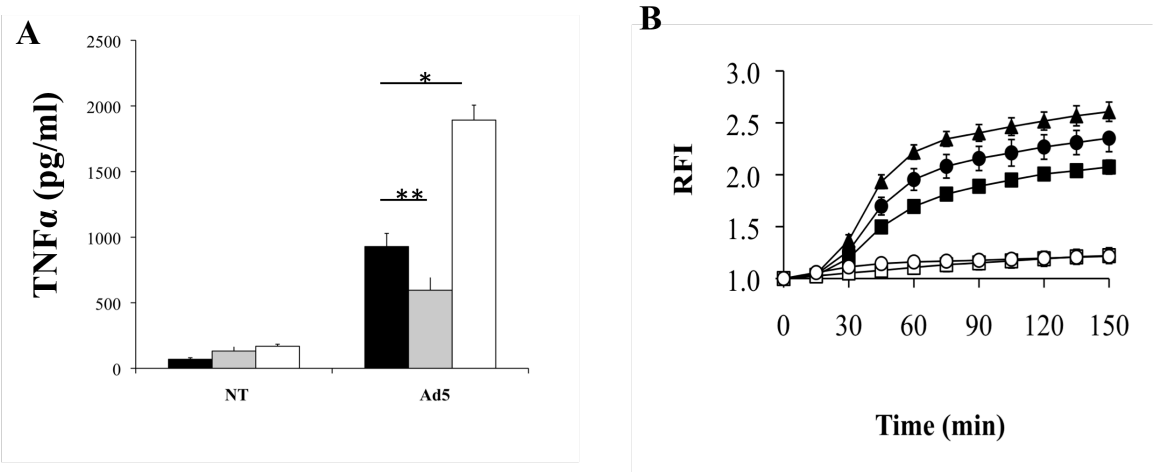

Figure 7. Ad5-induced ROS production and the role of ROS in Ad5-induced TNFα secretion. A. PMA-differentiated THP-1 cells were serum-starved for 3 hours and either left untreated (black bars) or pre-treated for 10 minutes with 30mM N-acetyl-cysteine (grey bars) or with 10mM rotenone (white bars) and then cells were left uninfected (NT) or infected with Ad5 at 30,000 ppc in the continued presence of drug for 4 hours. Secreted TNFα was measured by ELISA. B. PMA-differentiated THP-1 cells were serum starved for 3 hours then incubated with 10μM DCFDA for 30 minutes, washed, and left untreated (open squares), infected with *ts1* mutant adenovirus (*Ad2ts1*) at 500,000 ppc (open circles), or infected with Ad5 at 50,000 ppc (closed squares), 100,000 ppc (closed circles), or 500,000 ppc (closed triangles). DCF fluorescence was measured on a fluorescent plate reader at an excitation wavelength of 485 nm and emission wavelength of 520 nm. Graph depicts DCF relative fluorescence intensity (normalized to time 0) over time.

Since we found that ROS were produced rapidly during infection and that viral endosome rupture was required for their generation, we next asked whether TLR9 activity was required for ROS generation. Since ROS are generated during Ad cell entry it is plausible that TLR9 activation, a viral cell entry event, contributes to its generation. Several studies have reported that signaling through TLRs can lead to ROS production (Park, Jung et al. 2004; Matsuzawa, Saegusa et al. 2005; Yang, Shin et al. 2008). We have shown that the DNA genome of Ad5 is recognized by TLR9 in PMA-differentiated THP-1 cells when the virus uncoats within endosomes (Barlan, Griffin et al. 2010). TLR9 is required for the expression of proinflammatory cytokines, including proIL-1β, during Ad5 infection so TLR9 signaling may play a role in ROS production (Barlan, Griffin et al. 2010). Further, since we found that *ts1* does not elicit ROS we wanted to determine whether its inability to activate TLR9, in addition to its inability to rupture the endosome, was contributing to Ad-induced ROS. As mentioned, *ts1* does not partially uncoat and thus does not expose its genome to activate TLR9 (Barlan, Griffin et al. 2010). To ask whether TLR9 activity is required for Ad-induced ROS, we assessed ROS production (as previously described) following Ad infection in PMA-differentiated THP-1 cells stably expressing control or TLR9-specific shRNA. While TLR9 knockdown leads to a decrease in IL-1β production, as expected (Figure 8A), we found that TLR9 knockdown did not affect Ad5-induced ROS production (Figure 8B). Specifically, we observed a similar increase in DCF relative fluorescence intensity (RFI) between control and TLR9

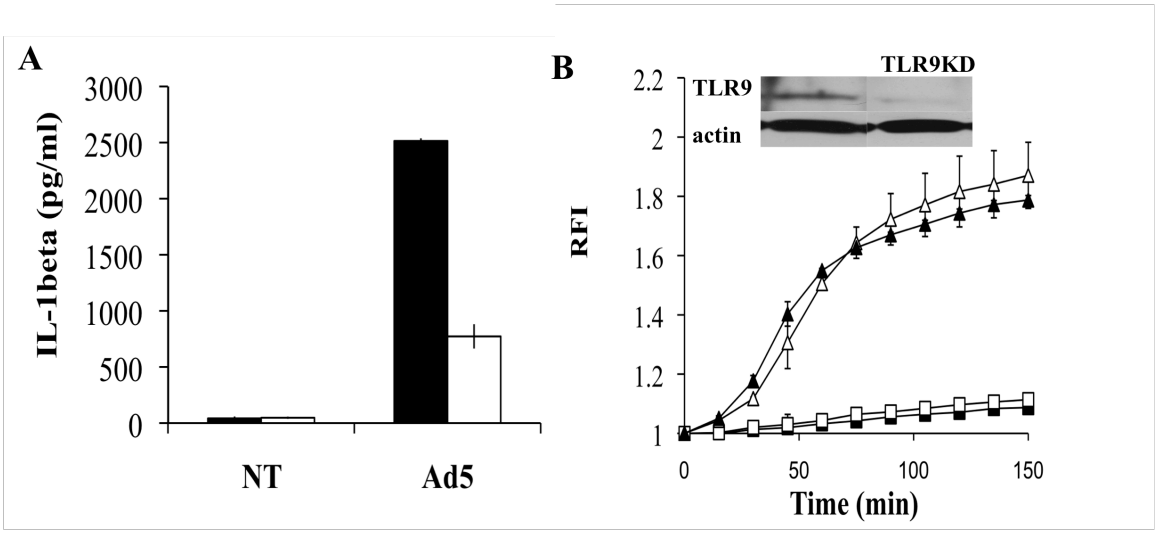

Figure 8. ROS production in response to Ad5 infection in the presence or absence of TLR9 knockdown. A. PMA-differentiated THP-1 control cells (black bars) and THP-1-TLR9 KD cells (white bars) were serum-starved for 3 hours and either left untreated (NT) or infected with Ad5 at 30,000 ppc for 6 hours. Secreted IL-1b was measured by ELISA. B. PMA-differentiated THP-1 and TLR9 knockdown cells were serum starved for 3 hours then incubated with 10μM DCFDA for 30 minutes, washed, then left untreated (squares: open, THP-1 cells; closed TLR9 kd cells) or infected with Ad5 at 500,000 ppc (triangles: open, THP-1 cells; closed, TLR9 kd cells). Graph depicts relative DCF fluorescence intensity (RFI) over time. Inset: Western blot for TLR9 demonstrating efficient knockdown. Significance determined by Student's T test (unpaired) where * $p<0.005$, ** $p<0.05$.

knockdown cells (Figure 8B). Further, these data suggest that endosomal rupture and viral uncoating are two events required for the innate immune response to Ad.

We have found that Ad leads to rapid ROS production requiring virally mediated endosomal rupture. To further characterize the mechanism of Ad-induced ROS we sought to identify the source of ROS following Ad5 infection. There are 3 potential sources of ROS in the cell (Figure 3). ER stress can contribute to ROS; although studies traditionally show ER-derived stress generates ROS upon infection with enveloped viruses. ER stress is generated in response to enveloped viruses when the ER gets overloaded with viral envelope glycoproteins late in infection (Tardif, Waris et al. 2005). Since the nonenveloped Ad5 elicits ROS rapidly we did not assess whether ER-derived ROS play a role upon Ad5 infection but rather focused on more likely sources. However, we have not ruled out whether ER stress contributes to Ad-induced ROS. NADPH oxidase and mitochondrial stress are two other potential sources of ROS (Rada and Leto 2008; Kohchi, Inagawa et al. 2009; Nathan and Cunningham-Bussel 2013). We addressed whether either one of these components contribute to ROS upon Ad infection.

To identify the source of Ad-induced ROS we first asked whether NADPH oxidase was required for Ad-induced ROS. Several studies have highlighted the role for phagosomal NADPH oxidase in ROS production following innate immune activation (Fink, Duval et al. 2008; Soucy-Faulkner, Mukawera et al. 2010). Further, there are numerous examples implicating a role for phagosomal NADPH oxidase in the production of ROS upon infection, particularly during infection with intracellular pathogens such as

bacteria (Sadikot, Zeng et al. 2004; Rada, Hably et al. 2008). Considering these findings and the fact that Ad may encounter NADPH oxidase during cell entry, NAPDH oxidase may contribute to Ad-induced ROS. To determine whether Ad5-induced ROS required NADPH oxidase we stably knocked down expression of an enzymatic subunit of phagosomal NADPH oxidase, gp91phox, in THP-1 cells by RNAi. Gp91phox is the heme-binding subunit of NADPH oxidase and is critical for the production of ROS by this enzyme (Sadikot, Zeng et al. 2004; Katsuyama 2010). We quantified knockdown of gp91phox by RT-qPCR. We detected over a ten-fold reduction in gp91phox expression in THP-1 cells stably expressing gp91phox shRNA (Gp91phox KD cells) as compared to control THP-1 cells (Figure 9A). We treated PMA-differentiated control or Gp91phox KD THP1 cells with Ad5 or ATP, a known activator of phagosomal NADPH oxidase, and measured DCF fluorescence over time as an indication of ROS production (Figure 9B) (Lister, Sharkey et al. 2007; Noguchi, Ishii et al. 2008). Our data indicated that, while knockdown of gp91phox attenuated ATP-induced ROS, as expected, it did not attenuate Ad5-induced ROS. Rather gp91phox knockdown enhanced Ad5-induced ROS. Although this increase in Ad5-induced ROS was statistically significant, it is unclear what role Gp91phox might play in Ad5-induced ROS. These findings suggest that ROS produced in response to Ad5 infection do not require phagosomal NADPH oxidase.

Based on the studies above, we have excluded a role for TLR9 and phagosomal NADPH oxidase in Ad5-induced ROS production. In contrast, our experiments with *ts1* suggest endosomal membrane penetration by Ad5 is required for

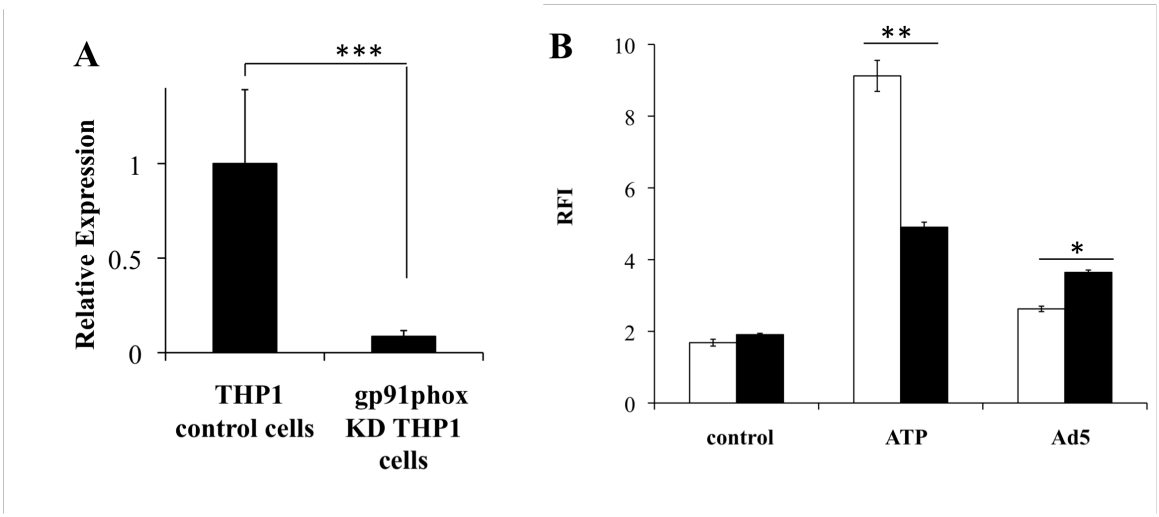

Figure 9. ROS production in response to Ad5 in the presence or absence of gp91phox (Nox2) knockdown. A. RNA was isolated from THP-1 control cells and THP-1 gp91phox knockdown cells, and subjected to qRT-PCR using primer sets for gp91phox and β-actin. Relative gp91phox expression was generated using the $2^{-\Delta\Delta C_T}$ method where the fold change in gp91phox expression was normalized to the housekeeping gene β-actin and gp91phox expression in the THP-1 control cells is set to 1. B. PMA-differentiated THP-1 control cells (white bars) and THP-1 gp91phox knockdown cells (black bars) were serum starved for 3 hours then incubated with 10μM DCFDA for 30 minutes, washed, and left untreated, treated with Ad5 at 100,000 ppc, or treated with 5mM ATP. DCF fluorescence was measured on a fluorescent plate reader. Graph depicts relative fluorescence intensity at 6 hours post-infection normalized to time 0. Significance determined by Student's T test (unpaired) where * $p<0.001$, ** $p<0.005$, *** $p<0.01$.

ROS production. Endosomal membrane rupture may contribute to Ad-induced ROS by generating mitochondrial stress. Mitochondrial stress results from a loss of outer mitochondrial membrane integrity and loss of inner mitochondrial membrane potential (Ψ_m) (Kohchi, Inagawa et al. 2009; Santos, Tanaka et al. 2009). Mitochondrial stress during Ad infection may occur following endosome escape. When Ad ruptures the endosome, resident cysteine proteases, Cathepsins, gain access to the cytoplasm where they can contribute to mitochondrial outer membrane stress (Droga-Mazovec, Bojic et al. 2008). Cytosolic Cathepsin B has previously been implicated in the destabilization of the mitochondrial membrane. Once in the cytosol, Cathepsin can cleave the pro-apoptotic factor, Bid to its active form, tBid, which then promotes the oligomerization of the pro-apoptotic proteins Bak and Bax to form pores in the outer mitochondrial membrane. This event can lead to the leakage of ROS from the mitochondria (Figure 11). Recent evidence demonstrates that mitochondrial stress contributes to ROS production in the context of infection (Deng, Adachi et al. 2008). Further, this mitochondrial stress is thought to contribute to the innate immune response, particularly NLRP3 inflammasome activation (Zhou, Yazdi et al. 2010). Upon discovering that Ad5-induced ROS required membrane disruption and did not involve TLR9 or phagosomal NADPH oxidase, we next investigated whether mitochondrial membrane permeability contributes to Ad5-induced ROS. To ask generally whether mitochondrial membrane permeability contributed to Ad5-induced ROS we first over-expressed bcl-2 to attenuate mitochondrial membrane disruption. When over-expressed, the anti-apoptotic protein Bcl-2 associates with pro-

apoptotic Bcl-2 family members on the mitochondrial membrane and prevents pore formation that leads to cytochrome C release and eventual loss of Ψ_m (Brunelle and Letai 2009; Tait and Green 2010). We stably over-expressed bcl-2 in THP-1 cells (THP-1-bcl-2) by retroviral transduction and assessed ROS production following Ad5 infection using DCF as previously described. This experiment assessed whether Ad-induced ROS production is attenuated when mitochondrial membrane disruption is inhibited. We found that, upon Ad5 infection, THP-1-bcl-2 cells exhibited a ~50% decrease in ROS production as compared to THP-1-control cells (Figure 10A). These data implicate mitochondrial membrane disruption as the key source for Ad-induced ROS. Further, this reduction in ROS production in THP-1-bcl-2 cells correlated with decreased NLRP3 inflammasome activation in THP-1-bcl-2 cells. Specifically, IL-1β production was attenuated in THP-1-bcl-2 cells as compared to THP-1-control cells upon Ad5 infection (Figure 10B). These observed decreases in ROS and IL-1β production in THP-1-bcl-2 cells were not due to decreased Ad infectivity upon bcl-2 overexpression since we saw no significant difference in relative luciferase activity between THP-1-control cells and THP-1-bcl-2 cells infected with an Ad5 luciferase reporter virus (data not shown). These results suggest that mitochondrial stress contributes to Ad5-induced ROS production. So far we have implicated Ad5 penetration of endosomal membranes and mitochondrial stress in the production of ROS upon Ad5 infection. As mentioned, the resident endolysosomal Cathepsin is one way in which both endosomal membrane rupture and mitochondrial stress could be involved in Ad5-induced ROS. Lysosomal Cathepsins such

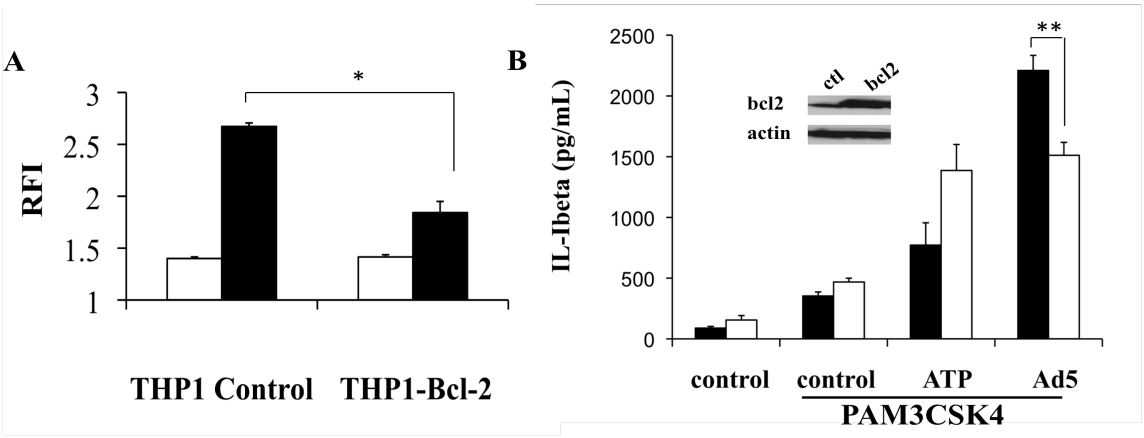

Figure 10. Ad5-induced ROS production and IL-1b Release in the presence or absence of bcl-2 overexpression. A. PMA-differentiated THP-1 control cells and THP-1-bcl-2 cells were serum starved for 3 hours then incubated with 10μM DCFDA for 30 minutes, washed, and left untreated (white bars) or infected with Ad5 at 500,000 ppc (black bars). DCF fluorescence was measured on a fluorescent plate reader. Graph depicts relative fluorescence intensity at 6 hours post-infection normalized to time 0. B. PMA-differentiated THP-1 control cells (black bars) and THP-1-bcl-2 cells (white bars) were serum-starved for 3 hours, pre-treated with the TLR2 ligand PAM3CSK4, and infected with Ad5 at 50,000 ppc for 2 hours. Secreted IL-1b was measured by ELISA. Inset: Western blot demonstrating Bcl-2 protein levels. Significance determined by Student's T test (unpaired) where * $p<0.0005$, ** $p<0.05$.

as Cathepsins B and D, are cysteine proteases found in endosomes and lysosomes (Reiser, Adair et al. 2010). During Ad5 cell entry and rupture of the endolysosome, these Cathepsins can gain access to the cytoplasm where they can contribute to mitochondrial outer membrane stress (Droga-Mazovec, Bojic et al. 2008). Cytosolic Cathepsin B has previously been implicated in the destabilization of the mitochondrial outer membrane and loss of outer mitochondrial membrane potential, Ψ_m (Droga-Mazovec, Bojic et al. 2008; Zhang, Zhong et al. 2009). As mentioned, Cathepsin can cleave the pro-apoptotic factor, Bid to its active form, tBid, to promote pore formation in the outer mitochondrial membrane. This event can lead to the leakage of ROS from the mitochondria (Figure 11). In the next several experiments, we explored whether this mechanism explained how Ad5 infection elicited ROS.

To determine if endosomal rupture and subsequent Cathepsin B release were contributing to mitochondrial membrane disruption and ROS production during Ad infection, we first asked whether Ad5 cell entry and endosomal rupture lead to Cathepsin B release into the cytoplasm. We treated PMA-differentiated THP-1 cells with Ad5 or *ts1* for one hour and then detected catalytically active Cathepsin using the Magic Red™ Cathepsin B Assay Kit. This kit contains a cresyl violet (CV) fluorophore linked to the Cathepsin B substrate (CV-(Arg-Arg)2). CV-(Arg-Arg)2 does not fluoresce in the absence of active Cathepsin B. Active Cathepsin B hydrolyzes the Arg-Arg substrate in CV-(Arg-Arg)2, which ultimately leads to its cleavage from CV. This cleavage releases free CV that can fluoresce such that CV fluorescence serves as a measure of Cathepsin B activity. We

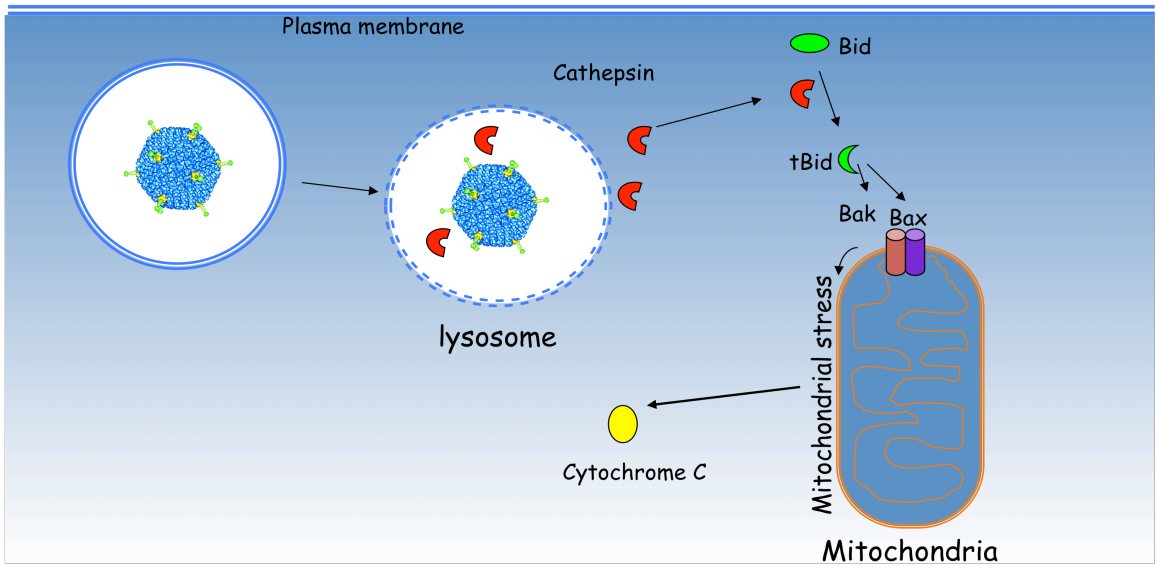

Figure 11. Proposed Mechanism of Ad5-Induced ROS. Ad5 enters the cell through receptor-mediated endocytosis and upon endosomal acidification ruptures and escapes from the endosome. During endosomal rupture resident endo-lysosomal cathepsins leak out into the cytoplasm of the infected cell where they cleave Bid to its active form. Bid cleavage promotes mitochondrial stress and mitochondrial outer membrane permeability by promoting the oligomerization of pro-apoptotic factors on the mitochondrial membrane. This mitochondrial membrane disruption leads to release of ROS into the cytoplasm of infected cells.

incubated PMA-differentiated THP-1 cells with Magic Red™ and detected Cathepsin B activity as CV fluorescence by microscopy. We found that uninfected cells had punctate Magic Red™ staining suggesting Cathepsin B was localized to the lysosomal compartment. In contrast, Magic Red™ staining became diffuse upon Ad5 treatment suggesting that Ad5 elicited the release of active Cathepsin from the lysosomal compartment into the cytosol. In contrast, cells treated with *ts1* exhibited punctate Magic Red™ staining, similar to the staining pattern in uninfected cells (Figure 12A-C). These data suggest that Ad-induced rupture of endosomes is required for the cytoplasmic release of Cathepsin B.

In addition to assessing the subcellular localization of Cathepsin B using Magic Red™ staining, we also assessed the subcellular localization of Cathepsin B by Western blot. Since we hypothesized that Cathepsin release leads to mitochondrial membrane disruption we also asked whether Ad5-mediated Cathepsin release correlated with the release of Cytochrome C, a resident mitochondrial protein, into the cytoplasm. As mentioned previously, Cytosolic Cathepsin B has previously been implicated in the destabilization of the mitochondrial outer membrane and loss of outer mitochondrial membrane potential, Ψ_m (Droga-Mazovec, Bojic et al. 2008; Zhang, Zhong et al. 2009). Once in the cytosol, Cathepsin can promote pore formation in the outer mitochondrial membrane. This event can lead to the leakage of ROS and the release of the proapoptotic mitochondrial protein, Cytochrome C, from the mitochondria (Figure 11). We expected that Ad5 infection would lead to the cytoplasmic release of both Cathepsin B and

Cytochrome C. In contrast, *ts1* infection, due to its inability to rupture the endosomal membrane, would not lead to the release of Cathepsin B and Cytochrome C into the cytoplasm. Cytosolic Cathepsin B serves as a measure of Ad5 rupture of endosome while Cytochrome C serves as a measure of mitochondrial membrane disruption (Brunelle and Letai 2009). We assessed whether Ad5 infection leads to the cytoplasmic release of Cathepsin B and Cytochrome C by Western blot. Thirty minutes after viral infection, PMA differentiated THP-1 cells were lysed with digitonin, which selectively permeabilizes the plasma membrane, to extract the cytoplasmic fraction for Western blot analysis. Following Ad5 infection, we detected Cathepsin B and Cytochrome C in the cytoplasm while we did not detect these proteins in the cytoplasm in untreated control cells (Figure 12D). In contrast to Ad5 infection, *ts1* infection did not induce release of Cathepsin B or Cytochrome C into the cytoplasm indicating that membrane disruption was required for the release of Cathepsin B and Cytochrome C into the cytoplasm (Figure 12D). Taken together, these data suggest that Ad5, through endosomal membrane rupture, induces the cytoplasmic release of Cathepsin B from the endolysomal compartment. Further, Cathepsin B release correlated with the release of Cytochrome C from the mitochondria. These data implicated Cathepsin as the mediator of mitochondrial membrane disruption during Ad5 infection. Since endosomal membrane rupture was required for NLRP3 activation and led to the release of Cathepsin B into the cytoplasm, we hypothesized that endosomal rupture mediates NLRP3 activation through Cathepsin. Specifically, we hypothesized that when Cathepsin B gains access to the cytoplasm from

the endolysosome upon Ad-mediated endosome rupture, it induces mitochondrial membrane disruption and ROS, which are key signals for NLRP3 activation. As mentioned previously, cytosolic Cathepsin B has previously been implicated in the destabilization of the mitochondrial outer membrane and loss of outer mitochondrial membrane potential, Ψ_m (Droga-Mazovec, Bojic et al. 2008; Zhang, Zhong et al. 2009). If Ad-dependent membrane rupture mediates NLRP3 activation through Cathepsin B then Cathepsin activity will be required for NLRP3 activation. To ask whether Cathepsin B activity is required for NLRP3 activation, we pretreated PMA-differentiated THP-1 cells with increasing concentrations of the Cathepsin inhibitor, Ca074me, infected cells with Ad5 and then assessed IL-1β release by ELISA. We found that inhibiting Cathepsin activity attenuated IL-1β secretion in a dose-dependent manner following Ad5 infection. These data suggest that Cathepsin activity is required to activate the NLRP3 inflammasome (Figure 12E). Thus, Cathepsin B release, upon Ad5 membrane rupture, is essential for NLRP3 activation and may be mediating its effects by generating ROS through the induction of mitochondrial membrane disruption.

We have found that Ad endosomal membrane rupture led to Cathepsin release and, further, that Cathepsin activity was required for NLRP3-dependent IL-1β production. Additionally, we have demonstrated that Cathepsin B release correlated with Cytochrome C release upon Ad5 infection. We hypothesized that Cathepsin contributed to activation of NLRP3 by inducing ROS as a result of damage to the mitochondrial membrane. To directly address whether Ad5 infection resulted in mitochondrial membrane disruption

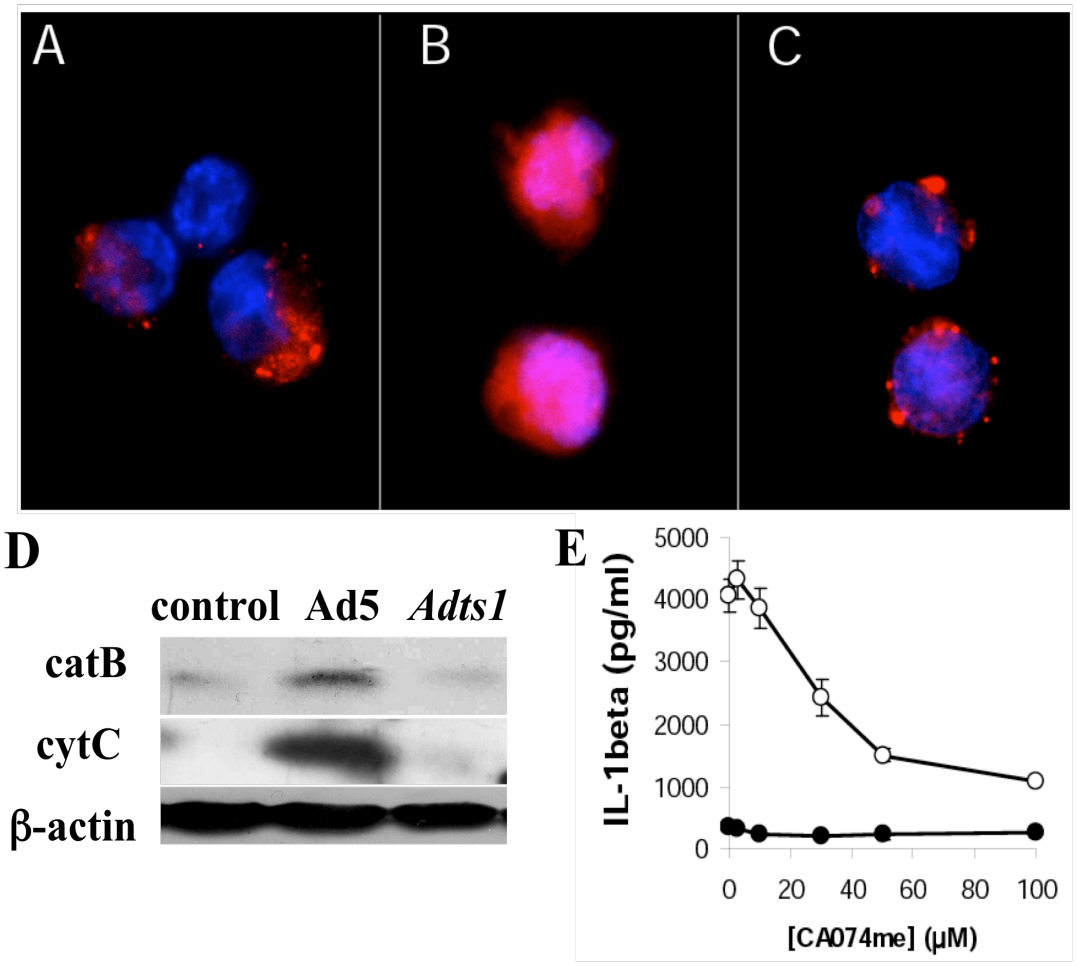

Figure 12. A-C: Cellular Localization of Cathepsin following Ad5 treatment. PMA-differentiated THP-1 cells were treated with A) media alone B) Ad5gfp or C) equivalent ts1 for 1 hour before staining with Magic Red™ Cathepsin B and counterstaining with Hoechst dye. Cells were visualized by fluorescence microscopy. **D.** 2 million PMA-differentiated THP-1 cells were left untreated, infected with Ad5 at 100,000 ppc, or infected with *ts1* mutant adenovirus at 100,000 ppc for 30 minutes. Cell membranes were selectively lysed with cytosolic extraction buffer containing 50μg/ml digitonin. Cell lysates were run on SDS-PAGE and transferred to a nitrocellulose membrane. Western blotting detected cytoplasmic Cathepsin B and Cytochrome C. **E.** IL-1β Release following Ad infection in the presence of the cathepsin inhibitor Ca074me. Cells were rested in serum-free media for 2 hours, primed with PAM3CSK4 for 2 hours and pretreated with increasing concentrations of the cathepsin B inhibitor, Ca074me or vehicle control for 30 minutes before infecting with Ad5gfp for 1 hour. IL-1β release was quantified by ELISA. Data represent the mean and standard error of 3 replicates. Experiment performed in collaboration with A.U. Barlan.

and loss of mitochondrial membrane potential, Ψ_m, we utilized the fluorophore, JC-1. JC-1 accumulates and oligomerizes in mitochondria and emits red fluorescence when Ψ_m is intact (Arnoult 2008). Upon loss of Ψ_m, JC-1 remains diffuse in the cytoplasm and fluoresces green. After treating PMA-differentiated THP-1 cells with Ad5 or the *ts1* mutant, we incubated the cells with the JC-1 reagent and assessed JC-1 fluorescence by microscopy 30 minutes post-infection to monitor the status of the mitochondrial membrane potential. If Ad5 infection led to mitochondrial membrane disruption we expected to see an increase in the percent of cells with diffuse JC-1 staining and thus a loss in Ψ_m. We found that Ad5 treatment reduced the percentage of cells with intact Ψ_m by nearly ten-fold as compared to the negative control (Figure 13). These findings were similar to our positive control, the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which would abrogate Ψ_m. In contrast, infection with the *ts1* mutant did not result in a significant decrease in the percentage of cells with intact Ψ_m (Figure 13). These results confirmed that Ad5 penetration of endosomal membranes is required to destabilize the mitochondrial membrane, an event that likely leads to the ROS produced upon infection.

So far we have shown that Ad5 mediates mitochondrial membrane disruption through its ability to rupture the endosome. When Ad5 ruptures the endosome it releases Cathepsin B into the cytoplasm of the cell. We have shown that this cytoplasmic release of Cathepsin B correlates with Cytochrome C release and that Cathepsin activity is required for the activation of the NLRP3 inflammasome. As mentioned previously, we

hypothesized that Cathepsin B mediates its activation of the innate immune response by inducing mitochondrial membrane damage. We next asked whether Cathepsin B activity was required for the observed loss of mitochondrial membrane potential upon Ad5 infection. Cathepsins have been shown to contribute to mitochondrial membrane stress (Droga-Mazovec, Bojic et al. 2008; Zhang, Zhong et al. 2009). Thus, Ad5-induced Cathepsin release may facilitate the loss in Ψ_m that we observed upon Ad5 infection. We asked directly whether Cathepsin B activity was required for Ad5-induced mitochondrial inner membrane depolarization. We pretreated cells with the Cathepsin B inhibitor, Ca074me, and infected them with Ad5 in the continued presence of drug then assessed loss of Y_m using the JC-1 assay as described above. As expected, we found that inhibiting Cathepsin B activity significantly attenuated the loss of Ψ_m during Ad5 infection (Figure 13). These data suggest that Cathepsin B activity contributes to the mitochondrial membrane damage elicited upon Ad5 infection. One alternative explanation of these results could be that Ca074me inhibits the adenovirus 23K protease, which, like Cathepsin B, is a cysteine protease. The 23K protease resides inside the incoming virion and is required to release the membrane lytic protein, pVI, from the viral capsid where pVI can then rupture the endosomal membrane thus allowing the virus to escape the endosome and infect the host cell. We found that Ca074me does not inhibit Ad5 transduction of THP-1 cells (Barlan, Griffin et al. 2010). Since the 23K protease is required for Ad5 transduction, we conclude that Ca074me does not inhibit the adenovirus 23K protease (Greber, Webster et al. 1996; Moyer and Nemerow 2012). Ca074me

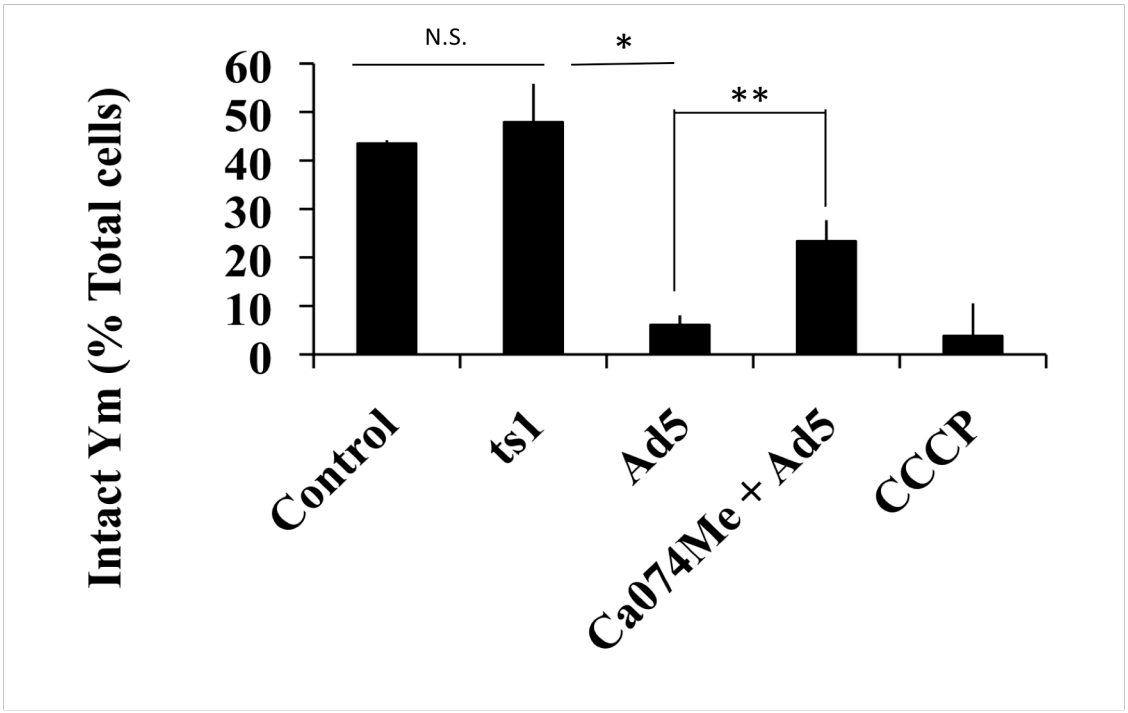

Figure 13. Mitochondrial membrane destabilization upon Ad5 infection in the presence or absence of the cathepsin B inhibitor Ca074me. PMA-differentiated THP-1 cells were serum-starved for 3 hours and pre-treated with 100μM Ca074me for 20 minutes or left untreated. Cells were then incubated with CCCP, infected with Ad5, or infected with the *ts1* mutant adenovirus for 30 minutes, washed, and incubated with JC-1 for 15 minutes. Following two washes, cells were visualized by fluorescence microscopy and counted. Significance determined by Student's T test (unpaired) where * $p<0.0005$, ** $p<0.05$.

treatment therefore exerts its effects by inhibiting Cathepsin activity during Ad infection. These results support our hypothesis that Ad5-induced membrane disruption facilitates Cathepsin-dependent mitochondrial membrane permeabilization.

We next directly investigated whether Cathepsin activity was required for ROS produced in response to Ad infection. We have found that Ad5 membrane penetration leads to the disruption of local lysosomal membranes and, in turn, the release of Cathepsins from the lysosome. Further Cathepsin B facilitates mitochondrial membrane disruption. We hypothesized that this Cathepsin-dependent mitochondrial membrane disruption leads to the release of ROS from the damaged mitochondria. These ROS then serve as a danger signal that activates the innate immune response upon Ad5 infection. To address whether Ad5-induced ROS required Cathepsin activity, DCF-loaded, PMA-differentiated THP-1 cells were pretreated with Ca074me, infected with Ad5 in the continued presence of drug, and then DCF fluorescence was monitored 6 hours post-infection as a measure of ROS production. We found that Ca074me treatment attenuated ROS production upon Ad5 infection (Figure 14). These results were consistent with our data indicating Cathepsin activity was required for mitochondrial membrane disruption. Further, these findings showed that Cathepsin activity contributes to Ad5-induced ROS. Overall, we demonstrated that Cathepsin B activity contributes to Ad5-induced ROS by facilitating mitochondrial membrane disruption. This mitochondrial membrane disruption leads to the release of ROS into the cytoplasm of the infected cell (as proposed in the model described in Figure 11). These data describe the mechanism of how Ad5 induces

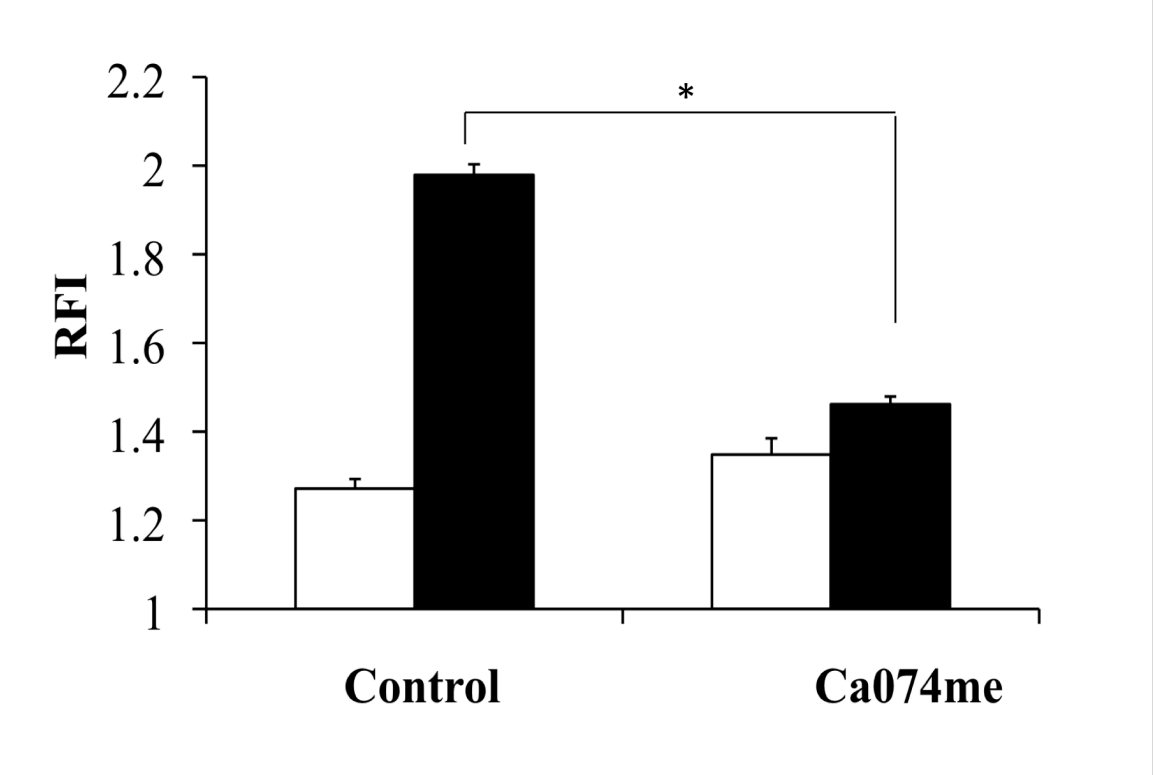

Figure 14. Ad5-induced ROS in the presence of the cathepsin B inhibitor Ca074me. PMA-differentiated THP-1 cells were serum starved for 3 hours then incubated with 10μM DCFDA for 30 minutes, washed, pre-treated with the cathepsin inhibitor, Ca074me (100μM), or DMF control for 1 hour, then left untreated (white bars) or incubated with Ad5 at 250,000 ppc (black bars). DCF fluorescence was measured on a fluorescent plate reader. Graph depicts relative fluorescence intensity at 6 hours post-infection normalized to time 0. Significance determined by Student's T test (unpaired) where * $p<0.0001$.

ROS and, further, identify a role for the mitochondria in generating an endogenous danger signal (ROS) that activates the innate immune response upon viral infection. Additionally, our study contributes to the overall understanding of how Ad rapidly activates the innate immune response and serves as another example of how ROS are a key signal in innate immune activation.

Characterizing the antibody response to Pfs25 following vaccination with Ad5 expressing Pfs25

The innate immune response, including the pathway I elucidated above, is likely a key factor contributing to the success of adenovirus-based vaccine vectors. While it is not yet clear exactly how my findings can be exploited to further improve adenovirus vaccine vectors, I next began to examine the immunogenicity of adenovirus vectors for use in Malaria vaccines. Recombinant adenovirus vectors are an attractive option for vaccine development due to their ability to activate the immune system effectively. While many groups use Ad vectors in vaccine design many do not fully investigate the mechanisms behind the immune response to a target antigen in the context of the Ad vaccine vector. Therefore, we sought to generate improved transmission-blocking vaccines (TBV) for malaria and to further explore the mechanisms by which Ad-vectored vaccines can improve the antibody response to a given antigen. We hypothesize that, due to its natural adjuvant properties to activate both the innate and adaptive immune responses, Ad vectors will provide the necessary T cell help to support the B cell response to a target malaria antigen. This malaria antigen is a surface protein called

Pfs25. Pfs25 is a 25 kilodalton protein containing 4 EGF-like domains (Figure 5). While the function of Pfs25 is not completely elucidated it is thought to facilitate binding and invasion of the midgut epithelia, one of the early and crucial steps in the parasite life cycle in the mosquito (Kaslow, Quakyi et al. 1988). Antibodies against Pfs25 block plasmodium development and midgut binding (Tomas, Margos et al. 2001; Sharma 2008).

Upon vaccination with the Pfs25 protein in the presence of the adjuvants alum or cholera toxin mice develop transmission-blocking antibodies to Pfs25 (Barr, Green et al. 1991; Kaslow, Bathurst et al. 1994; Gozar, Muratova et al. 2001). However, Pfs25 as a subunit, or protein, vaccine does not strongly elicit Pfs25-specific antibodies suggesting that Pfs25 is not very immunogenic on its own (Kubler-Kielb, Majadly et al. ; Kaslow, Bathurst et al. 1994; Gozar, Price et al. 1998; Gozar, Muratova et al. 2001). The exact reasons for the poor immunogenicity of Pfs25 are not completely understood. One hypothesis is that the poorly immunogenic Pfs25 protein does not contain any significant MHCII epitopes and, hence, does not activate T cells upon vaccination. *Gozar et al.* demonstrate that Pfs25 alone does not elicit T cell activation. Further, fusion of Pfs25 to another plasmodium transmission-blocking antigen, Pfs28, enhanced this T cell response and antibody production. This enhancement is likely due to conserved T cell epitopes within Pfs28 to activate T cells (Gozar, Price et al. 1998). To enhance the Pfs25-specific antibody response, an adjuvant must elicit T cell help. To improve T cell help, TBVs must employ adjuvants in Pfs25 vaccine design.

The goal of adjuvants is to enhance the immune response during vaccination. In the case of Pfs25 vaccination, an adjuvant must enhance the quality and level of Pfs25-specific antibodies since these antibodies mediate the malaria transmission blockade. As mentioned, providing the requisite T cell help to this Pfs25-specific antibody response will improve this antibody response. Current adjuvants fused to the Pfs25 protein enhance the immunogenicity of Pfs25 subunit vaccinations but still require multiple boosts in mice (Gozar, Muratova et al. 2001; Coban, Ishii et al. 2004; Coban, Philipp et al. 2004; Kubler-Kielb, Majadly et al. 2007; Shimp, Rowe et al. 2013). Further, limited attempts at vaccinating humans with Pfs25 subunit vaccines have not proven effective. Thus, there is still a need for a vaccine that elicits a heightened and prolonged response to Pfs25. Our solution was to utilize novel Ad5-based Pfs25 transmission-blocking vaccines (TBV) to enhance the Pfs25-specific antibody response.

A current study by Miyata and colleagues demonstrates the potent adjuvanticity of Ad5 vectors as a TBV for malaria caused by *Plasmodium vivax*. An Ad5 expressing the pfs25 ortholog of *P. vixax*, pvs25, induced a slight, but significantly, higher Pvs25-specific antibody titer in mice (Miyata, Harakuni et al. 2011). Additionally, *Goodman et al.* generated an Ad-based vaccine expressing Pfs25 and assessed the capability of this vaccine to elicit transmission-blocking antibodies upon boost vaccination with a Modified Vaccinia Ankara (MVA) vaccine expressing Pfs25 (Goodman, Blagborough et al. 2011). These vectors express a truncated form of Pfs25 lacking its transmembrane domain and thus are secreted from virally transduced cells. They did not assess the

efficacy of these Ad-based vectors alone. They found that this prime-boost vaccination could block transmission of malaria to mosquitoes both *in vitro* and in vivo when mosquitoes fed directly from vaccinated mice infected with a genetically modified strain of mouse plasmodia expressing Pfs25 in place of its own Pfs25 homolog. Parasite load was reduced by 67% in mosquitoes when they fed on infected mice. As these studies demonstrate, we believe that, due to its inherent adjuvant properties, adenovirus could enhance the antibody response to Pfs25, an otherwise poorly immunogenic protein. We propose to improve upon these studies using Ad-based pfs25 vectors by generating novel Ad-based pfs25 vectors. We have generated an Ad5 vector expressing the full-length Pfs25. This protein retains its transmembrane domain and thus will be expressed on the surface of virally transduced cells. There is evidence that membrane-bound proteins can more strongly stimulate B cell activation since the B cell encounters these antigens in the context of cell surface adhesion molecules that can strengthen the interaction between the BcR and its cognate antigen. Therefore, the Ad5-based pfs25 vaccine we have generated may even further improve the Pfs25-specific antibody titer following vaccination. We hypothesize that, due to its ability to provide T cell help, Ad5 expressing full-length Pfs25 will more potently induce anti-Pfs25 antibodies than a Pfs25-alum subunit vaccine.

We asked whether Ad5 expressing Pfs25 (Ad5-pfs25) elicits a more potent transmission blocking, Pfs25-specific antibody response than an alum-adjuvanted Pfs25 subunit vaccine. Prior to vaccination, we confirmed Pfs25 is properly expressed in the context of the Ad5 genome by performing IFA on Ad5-pfs25 transfected cells and

detecting the protein with ID2, a monoclonal antibody recognizing a conformational epitope within the D3 domain of the Pfs25 (Figure 15). We found that Pfs25 was expressed in cells and recognized by ID2. While these studies reflect Pfs25 expression in permeabilized cells it appeared that most the Pfs25 localized to the surface of the cell and extended in fingerlike projections from the surface (Figure 15). Furthermore, Pfs25 on the surface of transduced cells will likely be accessible to Pfs25-specific B cells.

We immunized mice intramuscularly with 10^{10} viral particles of Ad5-pfs25 or 25μg of Pfs25-alum and collected serum and assessed Pfs25-specific antibody titer by ELISA 21 days later to determine whether Ad5-pfs25 elicits transmission-blocking antibodies against Pfs25. Briefly, we coated ELISA plates with Pfs25 protein obtained from the NIH. After incubating serial dilutions of our sera on these plates we detected Pfs25-specific antibodies using an anti-mouse IgG-specific antibody. We found that Ad5-pfs25 vaccination elicited an 8-fold higher titer of Pfs25-specific IgG antibodies in sera as compared to those immunized with alum-adjuvanted Pfs25 (Figure 16A). Further, in this first experiment, mice were immunized with 25μg protein to ensure that we could detect a Pfs25-specific antibody response. However, this dose is 10 times higher than the clinically relevant dose of 2.5μg, which does not elicit detectable antigen-specific IgG after a single immunization. In the prime-boost experiments (described later) all protein immunizations were carried out with 2.5μg per animal.

In addition to generating a higher titer of Pfs25-specific antibodies, we expected that Ad5-pfs25 also generated a higher affinity Pfs25-specific antibody response than that

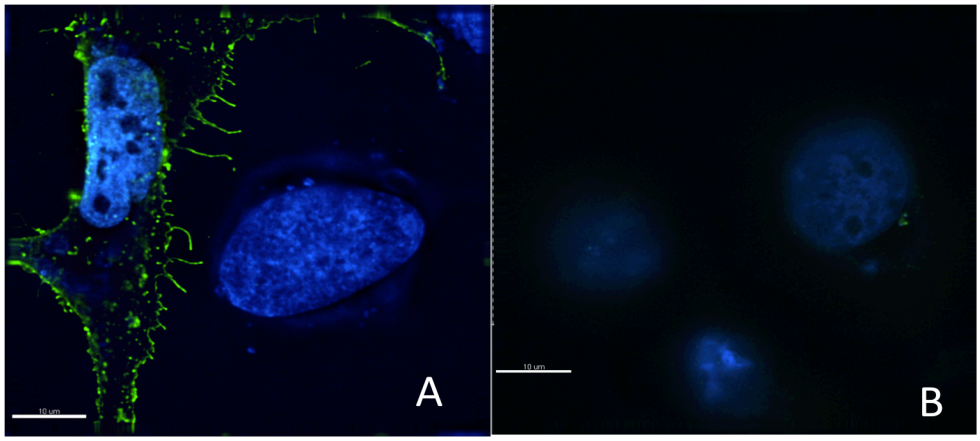

Figure 15. Pfs25 expression from a transgene within the Ad5 genome. Hela cells were transfected with an Ad5 shuttle vector containing a codon-optimized pfs25 under the control of the CMV immediate early promotor. 24 hours post transfection, cells were fixed, permeabilized, and probed with ID2 (A), a conformation-dependent Pfs25-specific monoclonal antibody, or left alone (B), then proved with an Alexa Fluor488-conjugated anti-mouse secondary antibody and visualized by IFA.

generated by Pfs25-alum. We hypothesized that Ad5-pfs25 elicits a more robust Pfs25-specific antibody response than its Pfs25-alum counterpart due to its ability to potently activate the innate and adaptive immune response. In particular, we hypothesized that Ad5-pfs25 recruits the requisite T cell help to support the Pfs25-specific B cell response. T cell help contributes to B cell activation and proliferation (McHeyzer-Williams, Okitsu et al. 2012). It also supports B cell affinity maturation such that Ad5-pfs25 would generate a higher affinity Pfs25-specific antibody response. To further assess the quality of the Pfs25-specific antibodies generated in the presence of Ad5-pfs25 or Pfs25-alum prime, we compared the relative affinities of the Pfs25-specific antibody response. The antibody titers determined by ELISA reflect both the concentration of the antibodies in the serum as well as their affinity for Pfs25. To better assess the affinity of these antibodies for Pfs25 independently of concentration, we examined the sensitivity of antibody binding (under saturated binding conditions) to the chaotropic salt, sodium thiocyanate (NaSCN). This assay works by exposing the Pfs25-specific serum antibodies bound to Pfs25 to increasing amounts of NaSCN. The thiocyanate ions disrupt noncovalent bonds and thus disrupt the noncovalent antibody-antigen interactions. As a result, antibodies with lower relative affinity are more sensitive to NaSCN than antibodies with a higher relative affinity for Pfs25. As the relative affinity of the Pfs25-specific antibody increases so does its resistance to elution by NaSCN. We exposed sera bound to Pfs25 to increasing concentrations of NaSCN and measured the amount of antibody remaining bound to Pfs25 at each concentration. We normalized the amount of

antibody remaining bound to Pfs25 to the initial amount bound in the absence of NaSCN to generate a curve that represented the antibody bound to Pfs25. We then compared the relative affinities of each serum sample by comparing the affinity index, or the NaSCN concentration where 50% of the antibody is still bound to Pfs25. In this way we can compare the relative affinities between two samples. While this assay does not directly measure affinity constants, it is a useful way to compare relative affinities with limited amount of serum sample. To assess the exact affinity, we would need a large serum sample to first purify the Pfs25-specific IgG from the sample. At the time, we did not have enough serum or the ability to purify Pfs25-specific IgG to determine the actual affinities. In the future, we could perform surface plasmon resonance to determine the actual affinities. For the meantime, this method was a useful way to compare the relative affinities between our different vaccine regimens. Further, while this assay can only provide estimates of relative affinities, *MacDonald et al.* demonstrate that comparing the relative affinities obtained by this method is useful to rank order affinities of multiple samples. They found that comparing the order of relative affinities by this method was consistent with the order obtained by comparing the affinity constants, which reflect affinity (Macdonald, Hosking et al. 1988)**.**

As mentioned, the antibody titers determined by ELISA reflect both the concentration of the antibodies in the serum as well as their affinity for Pfs25. By determining the relative affinities of the Pfs25-specific antibody response we can independently assess this aspect of the antibody response. We performed a standard

affinity assay as described above to compare the relative affinities of the Pfs25-specific antibodies from mice vaccinated with Pfs25-alum or Ad5-pfs25. Consistent with the higher antibody titers observed with Ad5-pfs25 prime, we also saw that prime with Ad5-pfs25 had a roughly 4-fold higher affinity index than prime with Pfs25-alum (Figure 16B). These data suggest that Ad5-pfs25 prime generated Pfs25-specific antibodies of higher relative affinity. Further, the Pfs25-alum vaccination assessed in these titer and affinity assays was 10 times higher than the clinically relevant dose. We initially used this higher dose since it is known that a single vaccination at the clinically relevant dose of 2.5μg does not elicit a detectable Pfs25-specific antibody response such that we would not be able to assess Pfs25-specific antibodies raised against protein vaccination.

In addition to affinity maturation, antibody class switching is also an important aspect of B cell affinity maturation and establishment of a memory B cell pool (Durandy 2003). With the exception of IgM, B cell class switching requires T cell help and thus investigating the variability in IgG subclasses can also suggest whether T cell help is involved in the Pfs25-specific B cell response upon Ad5-pfs25 vaccination. Isotype switching occurs following B cell activation and requires interaction with $CD4^+$ T cells and is dictated by the T_H cytokines produced as well. We believe that Ad5-pfs25 vaccination enhances the Pfs25-specific antibody response by providing the requisite T cell help. In addition to generating a high titer Pfs25-specific antibody response, we hypothesized that Ad5-pfs25 vaccination also generates Pfs25-specific antibodies of different IgG subclasses. We assessed the Pfs25-specific IgG subclasses produced upon

immunization with Pfs25-alum or Ad5-pfs25 by ELISA. We performed the ELISA as described above but this time used isotype-specific secondary antibodies to detect different IgG subclasses recognizing Pfs25. As expected, immunization with Pfs25-alum generated a significant Pfs25-specific response in IgG1 titers but not in the other IgG subclasses assessed (Figure 16C). Alum adjuvant in mice generates a Th_2-biased response and IgG1 is a result of this skewing. In contrast, immunization with Ad5-pfs25 generated a diverse array of IgG subclasses as indicated by a significant increase in IgG1, IgG2b and IgG3 Pfs25-specific antibodies generated. These data suggest Ad5-pfs25 elicits a robust Pfs25-specific antibody response as demonstrated both by a high antibody titer and a greater diversity of IgG subclasses.

In addition to the high titer, high relative affinity and broad diversity of IgG subclasses, the antibody longevity is another important characteristic of a high quality antibody response. The transient nature of the antibody response is a major problem with protein-based vaccines. For a TBV to be most effective, it must generate a long-lasting antibody response. A long-lived response would limit the number of required boost vaccinations. One hallmark of long-lasting antibody responses are the presence of long-lived plasma cells (LLPCs) in the bone marrow, as these cells are thought to be the main source of serum antibody. They home back to the bone marrow as early as 7-14 days following vaccination where they secrete high levels of antibody (Tarlinton and Good-Jacobson 2013). In mice, long-lived plasma cells are identified from the bone marrow using FACS to identify cells with the appropriate surface molecules. Long-lived plasma

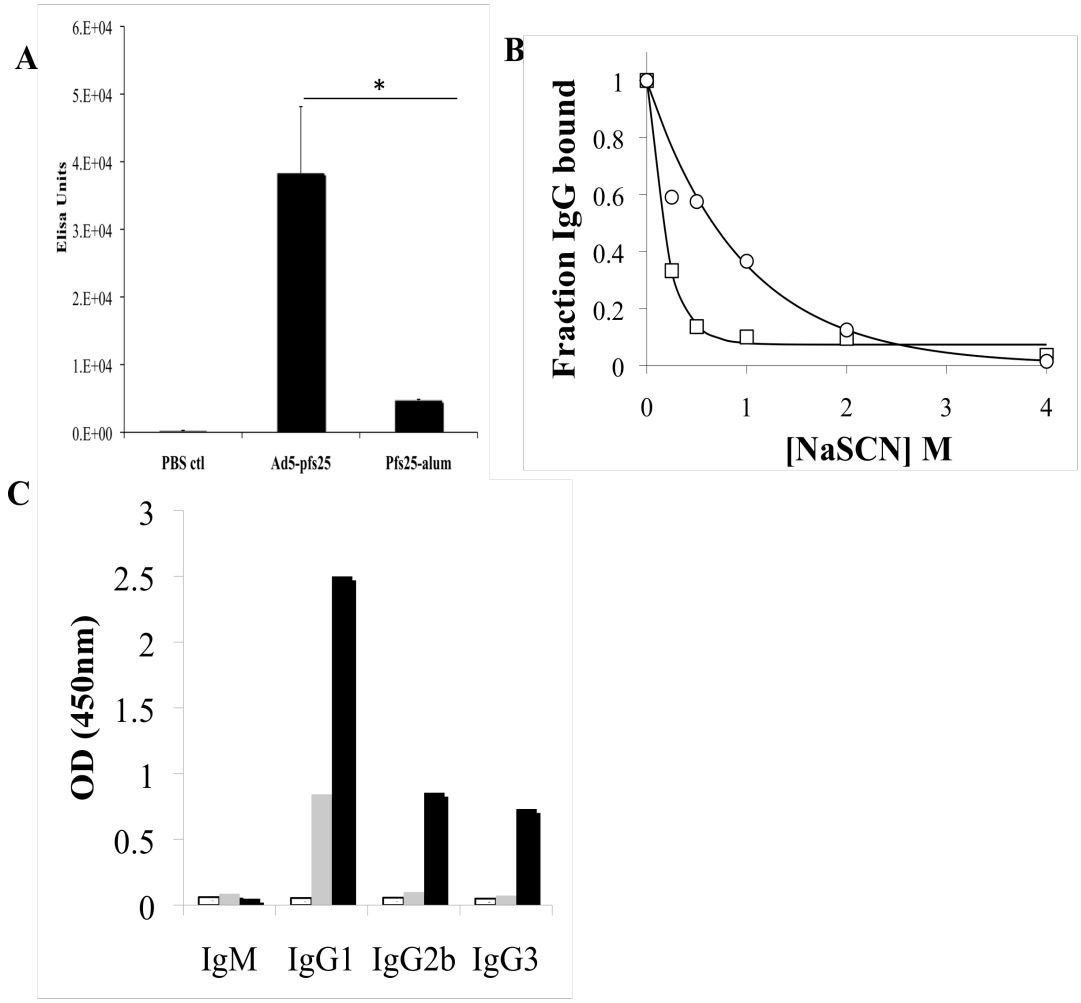

Figure 16: Pfs25-specific serum antibody response upon primary immunization with an Ad vector expressing Pfs25. A. Pfs25-specific ELISA was performed as described with serum collected from mice 21 days after immunization with 10^{10} particles Ad5 expressing pfs25 or Pfs25-alum (25µg/mouse) followed by an HRP-conjugated anti-mouse IgG (Fc) antibody. Data depict 3 animals per group. Elisa units are determined by endpoint dilution. **B. Affinity of Pfs25-specific antibody response.** Sera were added at a single dilution and then incubated with increasing concentrations of NaSCN and pfs25-specific antibodies were detected with an HRP-conjugated anti-mouse IgG (Fc). Data points depict OD normalized to the OD without NaSCN. Lines are exponential decay fitted curves. Squares: Pfs25-alum; circles: Ad5-pfs25 **C. Isotype response to Pfs25.** Pfs25-specific ELISA was performed and the pfs25-specific isotypes were detected with HRP-conjugated isotypic antibodies. Data depict the OD for each isotype assessed. Data depict 3 animals per group. White bars: PBS; Grey: Pfs25-alum; Black: Ad5-pfs25. Significance determined by Student's T test (unpaired) where * is $p<0.01$.

cells express CD138 (syndecan-1), a reliable plasma cell marker, and do not express B220 that is commonly expressed on B cells (O'Connell, Pinkus et al. 2004). Another way to assess the LLPCs upon vaccination is to enumerate the antibody-secreting cells in the bone marrow by ELISPOT. The ELISPOT method also allows us to assess antigen-specific LLPCs since the ELISPOT plates can be coated with specific antigens. By assessing the Pfs25-specific long-lived plasma cell compartment we can determine relatively shortly after vaccination if the serum antibody levels will be maintained over time.

To assess Pfs25-specific and Ad5-specific antibody-secreting cells we performed an ELISPOT on cells isolated from the bone marrow and the spleen 10 days following vaccination with Pfs25-alum or Ad5-pfs25 (10^{10} viral particles). Briefly, we coated wells with purified adenovirus or Pfs25 and then added serial dilutions of bone marrow cells or splenocytes followed by an anti-mouse IgG (H+L) antibody. We found that Ad5-Pfs25 vaccination elicited a significant increase in the antibody-secreting cells specific to Pfs25 and Ad5 in the bone marrow compared to control mice. In contrast, mice immunized with Pfs25-alum did not generate antibody-secreting cells to Pfs25 (Figure 17A). These results indicated that Ad5-pfs25 vaccination generates a pool of long-lived plasma cells residing in the bone marrow while Pfs25-alum vaccination does not generate LLPCs. It is possible that the antibody-secreting cells we detected were not LLPCs. To further confirm that these cells are indeed LLPCs, FACS analysis will be performed on future experiments to confirm phenotypically that these cells are LLPCs. Both Pfs25-alum and

Ad5-pfs25 vaccination generated Pfs25-specific antibody secreting cells in the spleen as would be expected since both vaccinations did generate Pfs25-specific antibodies (Figure 17B). Interestingly, the Pfs25-specific responses in the spleen were comparable between Pfs25-alum vaccination and Ad5-pfs25 vaccination suggesting, consistent with current literature, that the LLPC response is the main contributor to the serum antibody response. However, Pfs25-alum vaccination elicited only a transient Pfs25-specific response in the spleen, since, due to variability, there was no significant difference between the Pfs25-specific, antibody-secreting cells between PBS control mice and Pfs25-alum-immunized mice (Figure 17B). As mentioned previously, we observed a negligible Pfs25-specific serum antibody response upon Pfs25-alum vaccination and a significantly higher response upon Ad5-pfs25 vaccination at day 10 post-vaccination when the LLPC populations were assessed. These observed differences in serum antibody levels correlated with the observed differences in the LLPC response. In addition to generating a high titer of Pfs25-specific antibodies with high relative affinity, Ad5-pfs25 vaccination generates a significant population of Pfs25-specific LLPCs, which are thought to be the main source of serum antibody over time.

We next asked whether the observed increase in the Pfs25-specific antibody affinity and class switching following Ad5-pfs25 correlated with Ad-specific T cell activation. We hypothesized that the Ad vector provides the necessary T cell help to support the Pfs25-specific B cells by activating T cells and cytokine production. As mentioned previously, Pfs25 is not very immunogenic on its own. It is not clear whether

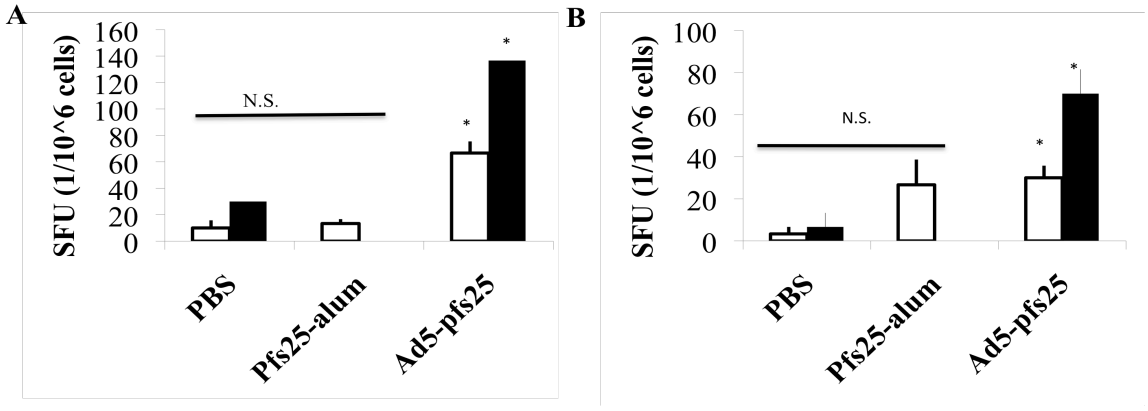

Figure 17. Antibody-secreting cells from the spleen and bone marrow following primary immunization with Ad5-pfs25 or Pfs25-alum. Cells were isolated from the bone marrow (A) or spleen (B) from mice immunized with PBS control, 2.5μg Pfs25-alum or Ad5-pfs25 (10^{10} pfu) and added in serial dilutions to ELISPOT plates coated with Pfs25 (white bars) or Ad5 (black bars) at 10ug/ml. After 4 hours cells were decanted and antibody-secreting cells detected with an HRP-conjugated anti-mouse secondary antibody. Data depict the mean and standard error from 3 replicates of pooled samples from 3 mice. Significance determined by Student's T test (unpaired) where * $p<0.05$ and reflects significance as compared to PBS control and Pfs25-immunized mice for (A) or compared to PBS control mice for (B). Experiment performed in collaboration with the Witte Lab (Loyola University Chicago, Stritch School of Medicine. Maywood, IL).

Pfs25 contains MHC class II epitopes. Without these epitopes, antigen-presenting cells may not efficiently present antigen to $CD4^+$ cells. Thus $CD4^+$ T cell activation in response to Pfs25 is thought to be suboptimal. Adenovirus is a potent T cell activator and thus will activate $CD4^+$ T cells that can, in turn, provide the necessary help to support B cell activation and antibody production. Since Ad has well-defined and conserved T cell epitopes we expected that Ad-specific T cells get activated upon Ad5-pfs25 vaccination and produce cytokines to support the Pfs25-specific antibody response. We examined the antigen-specific T cell activation ex vivo 10 days after vaccination with Ad5-pfs25 or Pfs25-alum. After restimulating splenocytes in vitro with purified Pfs25 or adenovirus we assessed antigen-specific T cell activation by measuring the IFNγ production by ELISA. We found a significant increase in IFNγ secretion upon Ad5 restimulation but not following Pfs25 stimulation as compared to unstimulated control cells suggesting that the Ad5-pfs25 immunized mice generate Ad-specific T cells while the Pfs25-alum immunized mice do not generate a detectable T cell response (Figure 18A). We did not detect IL-4 production following ex vivo stimulation (data not shown). IL-4 is an important cytokine produced by activated T_H2 cells. Taken together, these results indicated that T_H1 cytokines were produced following Ad5-pfs25 vaccination. This cytokine profile correlated with the observed increase in the IgG2A specific to Pfs25. However, Ad5-pfs25 still elicited the generation of IgG1 antibodies to Pfs25. T_H2 cytokines support the generation of IgG1 isotypes. *Pine et al.* demonstrate that Ad5-based HIV vaccines generate a mixed Th1/Th2 cytokine profile following ex vivo stimulation

(Pine, Kublin et al. 2011). Consistent with our data, Pine and colleagues detected IFNγ production but not the T_H2-dependent cytokine IL-4 following ex vivo stimulation. However they did detect IL-13, another cytokine produced during a T_H2-biased response. In future experiments, we will look at whether IL-13 is produced during Ad5-pfs25 vaccination as this could explain how Ad5-pfs25 supports the T_H2-dependent IgG isotypes such as IgG1. These data demonstrate that Ad5-pfs25 generates Ad5-specific IFNγ production and, presumably, Ad5-specific T cell activation following Ad5-pfs25 immunization. This Ad5-specific T cell response correlated with the observed increase in the Pfs25-specific antibody response following Ad5-pfs25 vaccination. Our studies demonstrate that vaccination with either Pfs25-alum or Ad5-pfs25 did not generate Pfs25-specific T cells. These results are consistent with the thought that Pfs25 does not efficiently activate T cells. Further, at this early time point, we saw a significant and detectable Pfs25-specific antibody response upon Ad5-pfs25 and no detectable response in those mice immunized with Pfs25-alum, thus underscoring the importance of T cell activation to the humoral immune response to Pfs25 (Figure 18B).

Characterizing the antibody response to Pfs25 following heterologous prime-boost vaccination with Ad5-pfs25 followed by Pfs25-alum or homologous prime-boost with Pfs25-alum

Adenoviral vectors remain one of the best T cell-inducing adjuvants available. We have demonstrated that adenovirus is capable of inducing adenovirus-specific T cells that, through cytokine production, presumably support the activation and proliferation of the

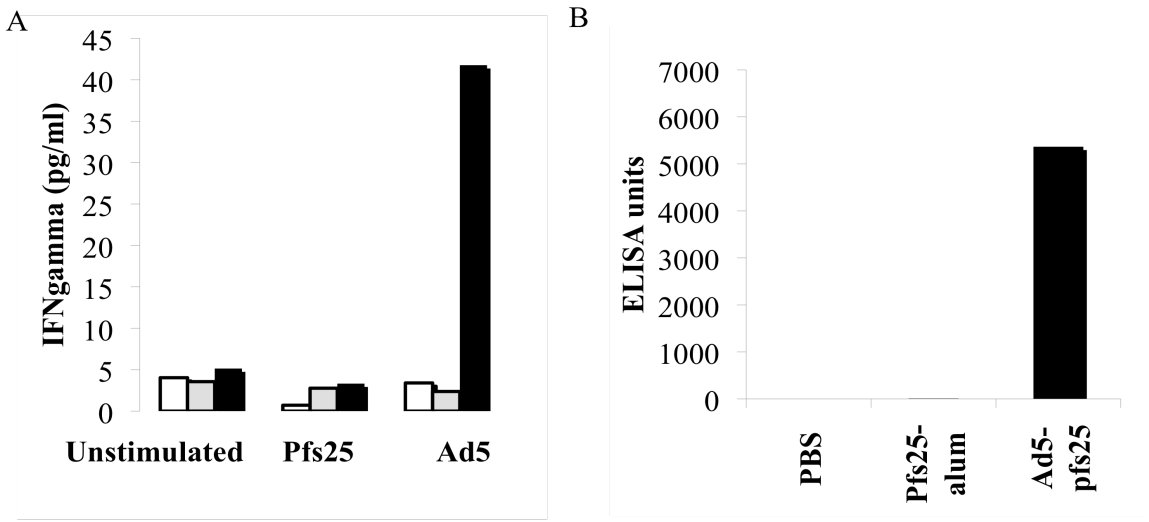

Figure 18. T cell activation following Ad5-pfs25 primary vaccination. A. IFNγ secretion following ex vivo antigen stimulation. At day 10 post-vaccination with 10^{10} particles Ad5 expressing pfs25 or Pfs25-alum (2.5μg/mouse) splenocytes were harvested and incubated with Pfs25 at 10μg/ml or Ad5 at 10,000 particles per cell for 24 hours. IFNγ secretion was detected by ELISA. **B. Pfs25-specific serum antibody response upon primary immunization day 10 post vaccination.** Pfs25-specific ELISA was performed as described probing with serum collected from mice 10 days after immunization followed by an HRP-conjugated anti-mouse IgG (Fc) antibody. Data depict 3 animals per group. Elisa units are determined by endpoint dilution.

Pfs25-specific B cell response following primary vaccination. Like other vaccine regimens, Ad5-pfs25 vaccination will likely require a boost vaccination in order to achieve the high levels of antibodies required to block malaria transmission. Since Ad5-pfs25 elicits a stronger Pfs25-specific antibody response than Pfs25-alum upon primary vaccination, we hypothesized that this response will be further enhanced upon boost immunization. This response will be much more potent than a boost immunization following Pfs25-alum prime. We first asked whether Ad5-pfs25 or Pfs25-alum priming improves the Pfs25-specific immune response upon boost immunization with Pfs25-alum. We immunized mice with Ad5-pfs25 or Pfs25-alum as previously described and then boosted the mice with Pfs25-alum (2.5 μg per mouse). Consistent with the improved Pfs25-specific antibody response upon Ad5-pfs25 primary vaccination, we saw that heterologous prime-boost vaccination with Ad5-pfs25 followed by Pfs25-alum elicited about a 60-fold higher Pfs25-specific antibody titer than Pfs25-alum homologous prime-boost vaccination (Figure 19A). These data reflect the importance of generating a robust primary response. The enhanced Pfs25-specific antibody response we observe during primary vaccination with Ad5-pfs25 as compared to Pfs25-alum likely generates a larger pool of Pfs25-specific B cells and, in turn, improves the antibody response upon boost vaccination.

In addition to generating a higher titer of Pfs25-specific antibodies, we expected that Ad5-pfs25 prime and Pfs25-alum boost also generates a higher affinity Pfs25-specific antibody response than that generated by homologous prime-boost with Pfs25-

alum. As mentioned previously, T cell help is required for B cell affinity maturation and class switching. We observed that primary vaccination with Ad5-pfs25 generated Pfs25-specific antibodies of high affinity relative to Pfs25-alum vaccination. This antibody response correlated with the increase in Ad-specific T cell activation following Ad5-pfs25 vaccination. We hypothesized that boosting the Ad5-pfs25 vaccination with Pfs25-alum will not only increase the titer of Pfs25-specific antibodies produced, but will also generate high affinity antibodies as well. To further assess the quality of the Pfs25-specific antibodies generated in the presence of Ad5-pfs25 or Pfs25-alum prime followed by Pfs25-alum boost we assessed the relative affinities of the Pfs25-specific antibody response. The antibody titers determined by ELISA reflect both the concentration and affinity of the antibodies in the serum. Using the affinity assay described previously, we determined the relative affinities of the Pfs25-specific antibody response from mice primed with Pfs25-alum or Ad5-pfs25 and boosted with Pfs25-alum. Consistent with the higher antibody titers observed with Ad5-pfs25 prime, we also saw that prime with Ad5-pfs25 had a nearly three-fold higher affinity index upon Pfs25-alum boost than prime with Pfs25-alum, suggesting that Ad5-pfs25 prime generated Pfs25-specific antibodies of higher relative affinity (Figure 19B).

As mentioned, another measure of B cell maturation is class switching. By measuring the IgG subclasses produced following immunization, we can better understand how Ad5-pfs25 vaccination generates a more robust Pfs25-specific antibody response. We expect that heterologous prime-boost with Ad5-pfs25 followed by Pfs25-

alum would generate a greater diversity of IgG subclasses than homologous prime-boost with Pfs25-alum suggesting that Ad5-pfs25 more potently activates T cells and cytokine production required to support B cell activation, proliferation and class switching. Both B cell affinity maturation and class switching require the activation-induced cytidine deaminase (AID). The upregulation of AID is mediated by cytokines, such as IL-4 produced by T helper cells, and CD40L-CD40 interactions between T_H and B cells, respectively (Oppezzo, Dumas et al. 2005; Bhattacharya, Cheah et al. 2007). We assessed the Pfs25-specific immunoglobulin isotypes produced upon prime immunization with Pfs25-alum or Ad5-pfs25 followed by boost immunization with Pfs25-alum. Consistent with the isotype response for Ad5-pfs25 single vaccination, primary immunization with Ad5-pfs25 followed by Pfs25-alum boost generated a more diverse array of Pfs25-specific IgG subclasses as compared to Pfs25-alum homologous prime-boost vaccinations. Further, there was a greater ratio of IgG2b and IgG3 to IgG1 following heterologous prime-boost indicating that there was a broader distribution of IgG subclasses than when mice are primed with Pfs25-alum and boosted with this same protein vaccine (Figure 19C). In contrast, homologous prime-boost with Pfs25-alum generated a smaller ratio of IgG2b and IgG3 to IgG1 indicating that there was an IgG1-biased response over the other IgG isotypes. Similar to the primary immunizations alone, these data suggest that Ad5-pfs25 prime immunization enhances the antibody class switching upon boost with Pfs25-alum while homologous Pfs25-alum prime-boost solely generates a significant IgG1 response.

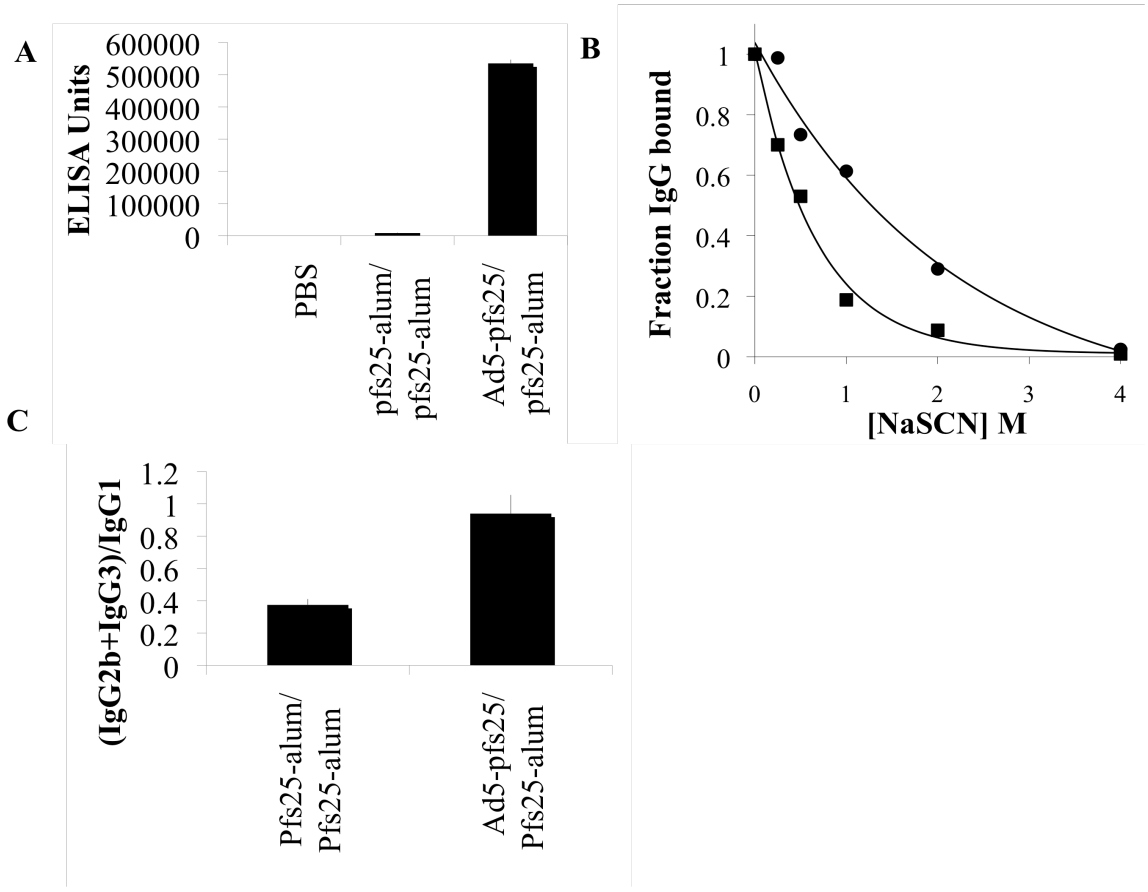

Figure 19. Pfs25-specific serum antibody response upon heterologous prime-boost with Ad5-pfs25 followed by Pfs25-alum or homologous prime-boost with Pfs25-alum. Pfs25-specific ELISA with serum collected from Pfs25-alum or Ad5 vector-primed mice 21 days after boost immunization with Pfs25-alum (2.5μg/mouse) followed by an HRP-conjugated anti-mouse IgG (Fc) antibody. Elisa units are determined by endpoint dilution. Data depict 3 animals per group for the Ad5-pfs25-primed animals and 6 pooled animals for the Pfs25-alum homologous prime-boosts. **B. Affinity of Pfs25-specific antibody response.** Sera were added to a Pfs25-coated ELISA plate at a single dilution and then incubated with increasing concentrations of NaSCN and Pfs25-specific antibodies were detected with an HRP-conjugated anti-mouse IgG (Fc). Data points depict O.D. normalized to the O.D. without NaSCN. Lines are exponential decay fitted curves. Squares: Pfs25-alum/Pfs25-alum; circles: Ad5-pfs25/Pfs25-alum. **C. Isotype response to Pfs25.** Pfs25-specific ELISA was performed and the Pfs25-specific isotypes were detected with HRP-conjugated isotypic antibodies. Data depict the ratio of the O.D. for IgG1 to the O.D.s of IgG2b and IgG3.

Since we saw a higher antibody titer and class switching upon boost vaccination with Pfs25-alum following Ad5-pfs25 prime, as compared to Pfs25-alum prime, we next asked if we could exploit these Ad5-specific memory T cells upon boost vaccination. Ad5-specific $CD4^+$ memory T cells may enhance T cell help and thus Pfs25-specific antibody production upon boost vaccination with Ad5 displaying Pfs25 B cell epitopes. We hypothesized that priming with Ad5-pfs25 followed by boosting with Ad5 displaying a B cell epitope of Pfs25 will elicit a heightened anti-Pfs25 antibody response.

In addition to the poor immunogenicity of protein-based vaccines, vaccines with whole proteins generate antibodies to many epitopes, some of which are not neutralizing or, in our case, transmission-blocking epitopes. Therefore, we attempted to overcome the poor immunogenicity of Pfs25 by first immunizing with Ad5-pfs25 and then boosted with an Ad-vectored vaccine containing the relevant, transmission-blocking Pfs25 epitopes on the capsid to enrich for antibodies recognizing neutralizing epitopes rather than irrelevant epitopes.

Characterizing the antibody response to Pfs25 following boost vaccination with Ad5 containing capsid-displayed Pfs25 B cell epitopes

Several studies demonstrate how various Ad capsid proteins can accommodate exogenous epitopes. Further, many of these capsid modifications elicit an enhanced humoral immune response compared to an Ad vector expressing the vaccine antigen from a transgene. The Ad Hexon protein is an attractive protein to include foreign antigens because it contains 9 hypervariable region loops (HVRs). These HVRs, particularly since

many of the Ad-specific antibodies recognize them, are known to be immunogenic. *Wu et al.* first demonstrated that histidine tags can be inserted into the HVRs and that these HVR-modified virions maintain proper viral assembly, structure and infectivity (Wu, Han et al. 2005). More recently, *Shiratsuchi et al.* demonstrate that vaccination with an Ad vector containing a B cell epitope of the *Plasmodium yoelii* protein CS in place of HVR1 resulted in a significantly higher level of protection in mice upon *P. yoelii* challenge as compared to an Ad vector solely expressing the CS protein from a transgene (Shiratsuchi, Rai et al. 2010). Additionally, this observed enhancement in protection to infection correlated with an elevated antibody response to the capsid-displayed malaria epitope.

We have generated Ad5 vectors containing Pfs25 B cell epitopes taken from domains 2 or 3 of Pfs25 (we refer to them as DII or DIII) within its capsid by lambda red recombineering. Specifically, we have introduced these pfs25 epitopes in the hypervariable regions 1 or 5 (HVR) of the Ad5 Hexon protein (Figure 20). For simplicity, we will refer to these Ad5 vectors with Hexon-displayed Pfs25 epitopes as Ad5-HVR-pfs25. When we refer to a specific D loop of Pfs25 in a specific HVR this will be specified, i.e. Ad5-HVR1-DIII (DIII of pfs25 displayed within HVR1 of Hexon). We have inserted epitopes found within the EGF-like domains II or III of Pfs25 within the HVR1 or HVR5 of the Ad Hexon. Studies demonstrate that domain III of Pfs25 is immunogenic since monoclonal antibodies to this domain have been isolated from Pfs25-immunized mice. We found that the Pfs25 epitopes are recognized within the purified Ad5-HVR-pfs25 viruses. Briefly, we performed Western blotting with serum from Pfs25-

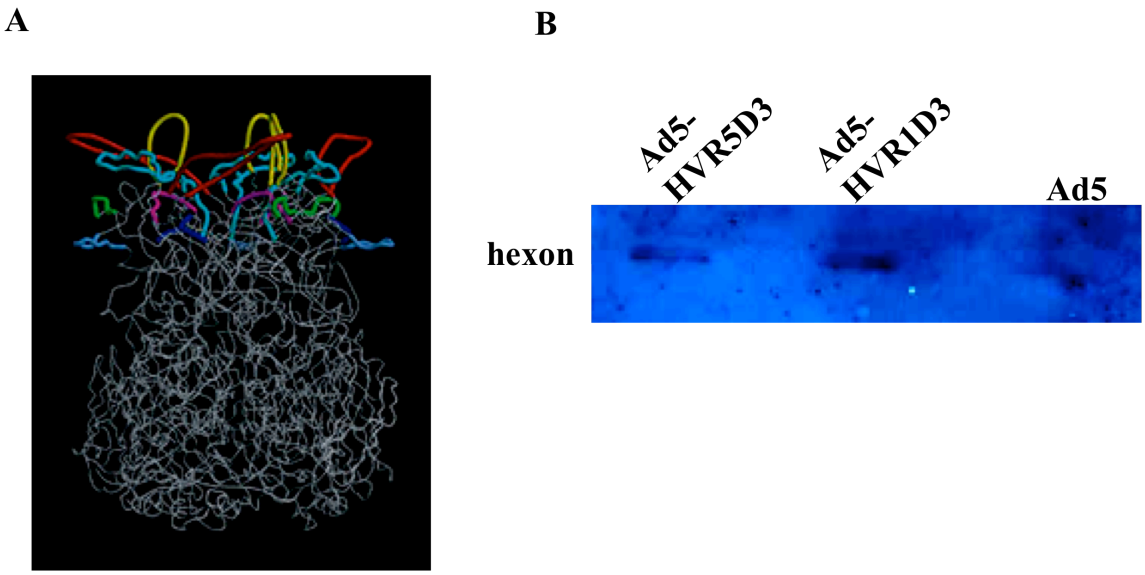

Figure 20. A. Pfs25 epitope expression within Ad5 Hexon. A. Ad5 Hexon trimer structure (Protein Data Bank 1P30) with modeled and highlighted HVRs (HVR1, red; HVR2, green; HVR3, pink; HVR4, light blue; HVR5, yellow; HVR6, blue; HVR7, cyan). B. Detection of Pfs25 epitopes with Pfs25 antisera within Ad5 Hexon.

alum-immunized mice on purified viruses and found that Pfs25-specific sera recognized the Pfs25 epitope within Hexon (Figure 20). We determined the specific infectivities of these Ad5-HVR-pfs25 viruses by measuring GFP reporter gene expression by FACS following infection of Hela cells in vitro. The Ad5-HVR5-pfs25 viruses that display a Pfs25 epitope within HVR5 exhibited specific infectivities comparable to wildtype viruses with at most a roughly 2.5-fold difference in viral particles per GFP transducing unit (GTU). The specific infectivities (particles/GTU) are: Ad5-HVR5D2 is 41.78, Ad5-HVR5D3 is 53.85, and Ad5-HVR1D3 is 275.15. When we inserted the DIII Pfs25 into HVR1 of Ad5 we saw a moderate defect in specific infectivity as the Ad5-HVR1D3 virus exhibited about a 10-fold defect in specific infectivity as compared to wildtype virus and about a 5-fold difference in infectivity as compared to Ad5-HVR5-pfs25 viruses.

It has yet to be determined which Pfs25 epitopes provide the best transmission blockade in the context of Ad5 capsid display. Additionally, we did not know which HVR of Hexon is the optimal platform for displaying these epitopes. We have immunized mice with Pfs25-alum and then boosted with Ad5-HVR1-DIII, Ad5-HVR5-DII or Ad5-HVR5-DIII to determine whether DII or DIII epitopes are most immunogenic and in which HVR is display of these epitopes most effective at generating Pfs25-specific antibodies. We found that primary immunization with Ad5-HVR1-DIII, AD5-HVR5-DII or AD5-HVR5-DIII elicits a low but detectable Pfs25-specific antibody response. To generate a stronger response where we can better compare the contribution of each Pfs25 epitope and each HVR to the Pfs25-specific antibody response we first immunized mice

with Pfs25-alum. We did not use our Ad5-pfs25 prime vaccination because pre-existing immunity could influence the response to Pfs25 and we addressed the role of pre-existing immunity in another section. Previous studies with epitopes for other pathogens demonstrate that display in HVR1 is the most immunogenic. Additionally, one study directly compared the transmission-blocking functions of each Pfs25 domain and found that antibodies to DII correlate with the strongest transmission blockade (Stowers, Keister et al. 2000). Keeping these studies in mind, we expect to see a higher Pfs25-specific antibody titer in mice boosted with an Ad5 vector displaying the DII loop. We performed Pfs25-specific ELISAs as previously described and assessed the Pfs25-specific antibody titers following heterologous prime-boost with Pfs25-alum followed by Ad5-HVR-pfs25 boost. We found that boost with Ad5-HVR-pfs25 elicited a comparable Pfs25-specific antibody response as compared with Pfs25-alum boost (Figure 21A). We also looked at the isotype specific antibody response with the isotype-specific ELISA as previously described and, somewhat surprisingly, found that all these prime-boost regimens reflected the same antibody repertoire as the Pfs25-alum homologous prime-boost with a skewed response towards IgG1 (Figure 21B). This skewing to IgG1 persisted when mice were boosted with Ad5-HVR-pfs25, despite the fact that Ad-vectored immunogens are capable of generating significant levels of IgG1, IgG2b and IgG3 Pfs25-specific antibodies (data not shown and Figure 22C). These data suggest that the B cells produced during the primary vaccination tend to dictate the nature of the antibody response produced. Therefore boost vaccination with any of the Ad5-HVR-pfs25 vectors may be activating

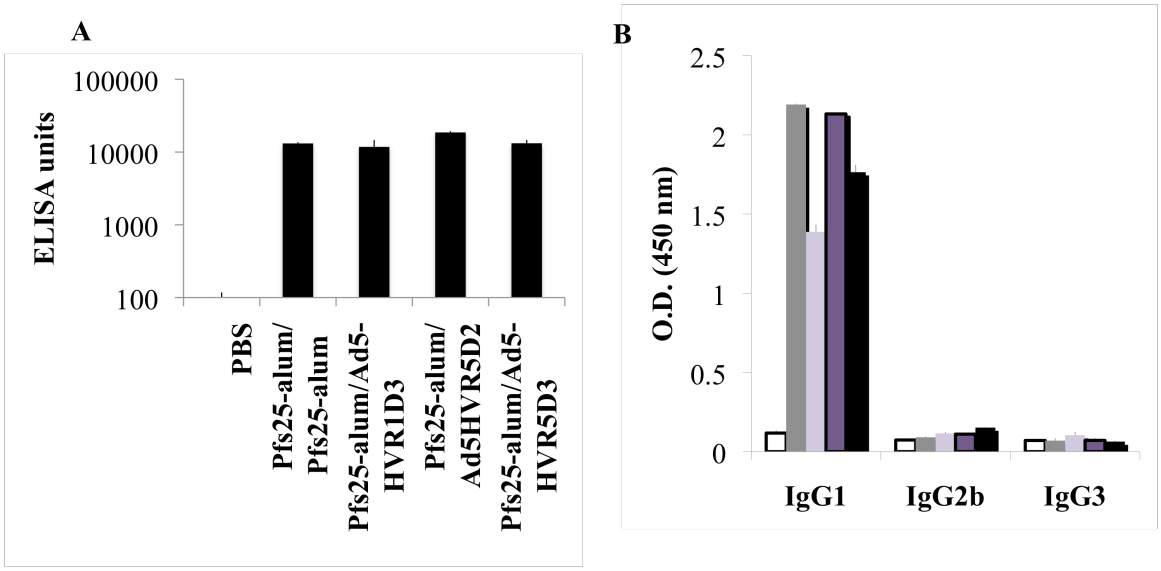

Figure 21. Pfs25-specific serum antibody response upon primary immunization with Pfs25-alum and boost with Ad containing capsid-displayed Pfs25 B cell epitopes. A. Pfs25-specific ELISA as previously described with ELISA units determined by endpoint dilution. **B.** Isotype specific Pfs25 ELISA with O.D. for each isotype displayed. White bars: PBS control; Grey bars: Pfs25-alum/Pfs25-alum; Light Purple: Pfs25-alum/Ad5-HVR1D3; Dark Purple: Pfs25-alum/Ad5-HVR5D2, Black bars: Pfs25-alum/Ad5-HVR5D3. Data depict 3 animals per group.

the existing pool of Pfs25-specific B cells, as we found with our previous studies comparing Pfs25-alum and Ad5-pfs25 prime vaccinations upon boost vaccination with Pfs25-alum.

Since we know that Ad5-pfs25 prime generates a stronger Pfs25-specific antibody response than Pfs25-alum prime upon Pfs25-alum boost, we next addressed whether we could improve the antibody response to Pfs25 with Ad5-pfs25 prime and Ad5-HVR-pfs25 boost. We immunized mice with Ad5-pfs25 as previously described and boosted the mice with Ad5-HVR1-DIII, Ad5-HVR5-DII, or Ad5-HVR5-DIII. We found that, by ELISA, boost with Pfs25-alum following Ad5-pfs25 prime generated a higher Pfs25-specific antibody titer than boost with any Ad5-HVR-pfs25 vector (Figure 22A). Nonetheless, we saw a significant increase in the Pfs25-specific antibody response upon boost with Ad5-HVR5-DII and Ad5-HVR5-DIII vectors, but not with Ad5-HVR1-DIII, as compared to a single primary vaccination with Ad5-pfs25 (Figure 22A). It is possible that the decreased specific infectivity of Ad5-HVR1-DIII was contributing to the inability of Ad5-HVR1-DIII boost vaccination to significantly increase the Pfs25-specific antibody titer. Additionally, Ad5-HVR5-DII boost generated a slight but significantly higher Pfs25-specific antibody titer as compared to Ad5-HVR5-DIII boost vaccination (Figure 22A). These data suggest that the DII Pfs25 epitope elicits a significantly higher Pfs25-specific antibody response than the DIII loop. Therefore we have identified another transmission-blocking Pfs25 epitope that appears to be more immunogenic than the previously identified DIII Pfs25 epitope. We can assume that the antibodies generated in

the presence of our Ad5-HVR-pfs25 viruses recognize the capsid-displayed Pfs25 epitope that is known to block transmission. So this observed increase in Pfs25-specific antibodies following Ad5-HVR-pfs25 boost should reflect an increase, and enrichment of, the transmission-blocking Pfs25 antibodies recognizing the DII or DIII epitopes. In contrast, the observed increase in the Pfs25-specific antibody levels following Pfs25-alum boost in the Ad5-pfs25-primed animals reflects an increase in the antibodies recognizing all available epitopes within Pfs25. Many of these epitopes do not block transmission.

In addition to assessing the Pfs25-specific antibody titer following heterologous prime-boost with Ad5-pfs25 then Ad5-HVR-pfs25 we also assessed the relative affinities of these prime-boost regimens. We compared the relative affinities of these prime-boosts to those generated by homologous prime-boost with Pfs25-alum. We hypothesized that Ad5-pfs25 prime followed by Ad5-HVR-pfs25 boost would generate a Pfs25-specific antibody response with higher relative affinity than Pfs25-alum homologous prime-boost because both the primary and secondary vaccination would activate Ad-specific T cells required to support B cell activation and affinity maturation. As mentioned previously, the antibody titers determined by ELISA reflect both the concentration and affinity of the antibodies in the serum. Thus we approximated the relative affinity of the Pfs25-specific antibody response between Pfs25-alum and Ad5-HVR-pfs25 boosts to better characterize the Pfs25-specific antibody response. We performed a standard affinity assay with NaSCN, as previously described, to determine the relative affinities of the Pfs25-specific

antibodies from mice primed with Ad5-pfs25 and boosted with Pfs25-alum or Ad5-HVR-pfs25. Consistent with the higher antibody titers observed with Ad5-pfs25 prime, we also saw that prime with Ad5-pfs25 generated a three-fold higher affinity index upon boost with any Ad5-HVR-pfs25 as compared to homologous prime-boost with Pfs25-alum. These results suggest that Ad5-pfs25 prime followed by Ad5-HVR-pfs25 boost generates Pfs25-specific antibodies of higher relative affinity (Figure 22B). Interestingly, while the Ad5-pfs25 prime and Pfs25-alum boost generated the highest titer of Pfs25-specific antibodies, the relative affinity from this protein boost was comparable to those of the Ad5-HVR-pfs25 boosts. These data suggest that the affinity of antibodies generated during boost vaccination with Ad-HVR-pfs25 may be strongly influenced by the antibodies generated during the primary vaccination (Figure 22B).

In addition to assessing the titer and relative affinity of the Pfs25-specific antibody response following Ad5-pfs25 or Pfs25-alum prime and Ad5-HVR-pfs25 boost vaccinations, we also examined the variability of IgG subclasses recognizing Pfs25. As mentioned previously, class switching is an important component to B cell maturation since it occurs following B cell activation. If a vaccination generates more IgG subclasses then that vaccine regimen may more strongly activate B cells, allowing them to enter germinal centers where they proliferate, undergo affinity maturation and immunoglobulin class switching. We assessed the Pfs25-specific IgG subclasses produced upon primary immunization with Ad5-pfs25 followed by boost with Ad5-HVR-pfs25 vectors. Consistent with the Pfs25-alum prime and Ad5-HVR-pfs25 boosts, we found that the IgG

subclasses generated in response to these prime-boost vaccinations were dictated by the nature of the primary immunogen. As described previously, homologous prime-boost with Pfs25-alum generated an IgG1-dominated response, as would be expected with an alum-adjuvanted vaccine. In contrast, primary immunization with Ad5-pfs25 generated a broad IgG repertoire as indicated by a significant increase in IgG1, IgG2b and IgG3 Pfs25-specific antibodies generated. This IgG response was maintained upon boost immunization with Pfs25-alum or Ad5-HVR-pfs25. The IgG2b and IgG3 to IgG1 ratio was fairly low upon heterologous prime-boost with Ad5-pfs25 followed by Ad5-HVR1-DIII. However, this low ratio could be the result of the overall lower antibody titer for this vaccine regimen, likely due to the infectivity defect of Ad5-HVR1-DIII. Heterologous prime-boost with Ad5-pfs25 followed by Pfs25-alum or AD5-HVR-pfs25 elicited a greater ratio of IgG2b and IgG3 to IgG1 as compared to homologous prime-boost with Pfs25-alum (Figure 22C). These data suggest Ad5-pfs25 prime immunization dictates the isotypes generated upon boost vaccination. These results are consistent with the fact that antibody affinity is similar between the sera from all Ad5-pfs25-primed mice regardless of the boosting vaccine.

Determining the parasite-binding and transmission-blocking functions of Pfs25-specific antibodies generated by heterologous prime-boost vaccination with Ad5-pfs25 followed by boost with Ad5 containing capsid-displayed Pfs25 epitopes

To further characterize the antibodies generated by these heterologous prime-boost regimens we next asked whether these Pfs25-specific antibodies bind to the surface

of purified *P. falciparum* gametes. While the transmission-blocking function of Pfs25-specific antibodies has been shown to be a function of antibody titer, determining parasite binding by these antibodies is another, more relevant way to assess the specificity of these antibodies (Miura, Keister et al. 2007). To ask this question, we obtained purified *P. falciparum* gametes from Dr. Kim Williamson's laboratory at Loyola University Chicago and performed parasite-binding assays. Briefly, we incubated the purified *Plasmodium falciparum* mosquito-stage parasites with serial dilutions of serum from our vaccinated mice. To detect antibody binding to the parasites we next incubated the gametes with an Alexafluor488-conjugated anti-mouse IgG. We determined sera binding to the parasites by FACS. Consistent with our ELISA data, we found that serum from mice immunized with a heterologous prime-boost of Ad5-pfs25 followed by Pfs25-alum or Ad5-HVR-pfs25 readily bound purified *P. falciparum* and, further, parasite binding by these sera was significantly higher than that of mice immunized with a homologous prime-boost of Pfs25-alum (Figure 22D). In fact, the homologous prime-boost with Pfs25-alum did not bind *P. falciparum* gametes any better than serum from PBS-immunized control mice (Figure 22D). This could be both the result of a low titer of Pfs25-specific antibodies and the generation of antibodies that recognize Pfs25 by ELISA but do not block transmission. Taken together, these data suggest that the heterologous prime-boosts with Ad5-pfs25 vaccination elicit a higher Pfs25-specific antibody response that recognize and bind to the parasite surface and likely more effectively block

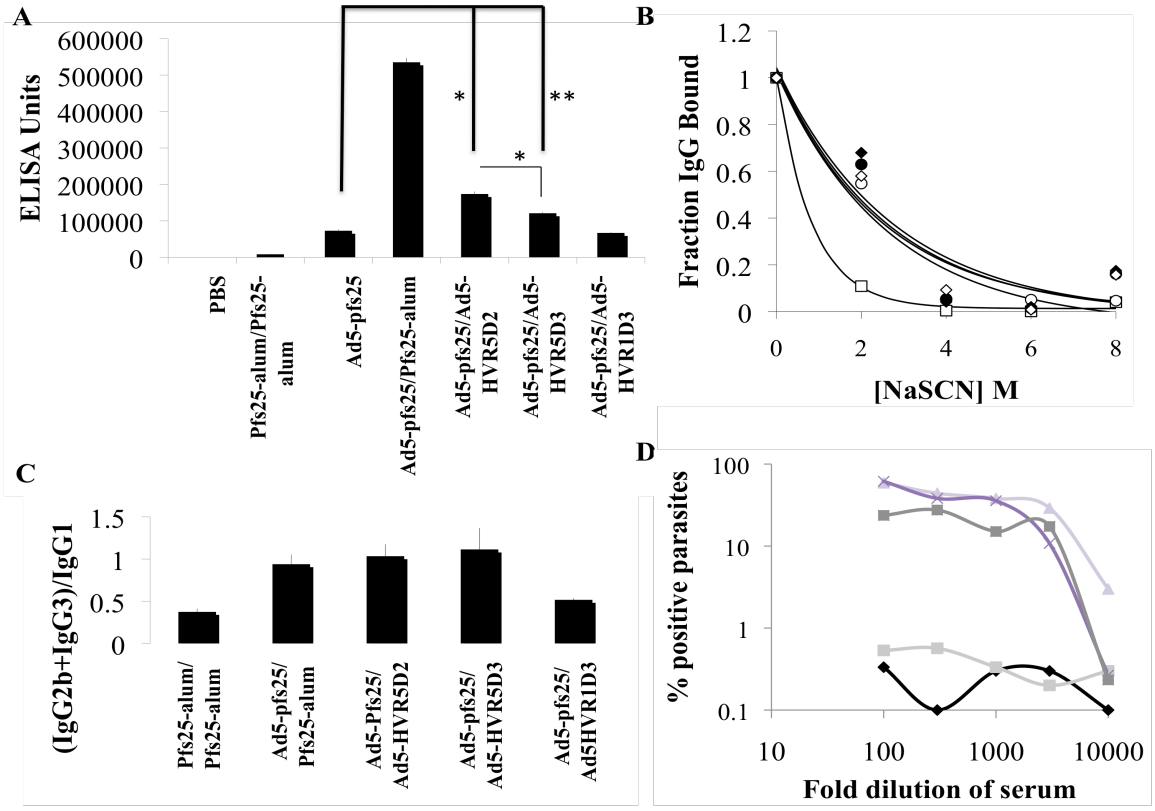

Figure 22. Pfs25-specific serum antibody response upon heterologous prime-boost with Ad5-pfs25 followed by Ad5-HVR-pfs25 boost. Pfs25-specific serum antibody from Pfs25-alum or Ad5 vector-primed mice 21 days after boost immunization with Pfs25-alum (2.5µg/mouse) or Ad5-HVR-pfs25 (10^{10} pfu). Data depict 3 animals per group for the Ad5-pfs25-primed animals and 6 pooled animals for the Pfs25-alum homologous prime-boosts. **B. Affinity of Pfs25-specific antibody response.** Assay as previously described. Data reflect O.D. normalized to a no NaSCN control. Open squares: Pfs25-alum/Pfs25-alum prime boost; open circles: Ad5-pfs25/Pfs25-alum; closed circles: Ad5-pfs25/Ad5-HVR5D2; open diamonds: Ad5-pfs25/Ad5-HVR1D3; closed diamonds: Ad5-pfs25/Ad5-HVR5D3. **C. Isotype response to Pfs25.** Assay as previous described. Data depict the ratio of the O.D. for IgG1 to the O.D.s of IgG2b and IgG3. **D. Serum antibody binding to *P. falciparum* gametes.** Sera from immunized mice were incubated with purified gametes followed by an AlexaFluor488-conjugated anti-mouse secondary antibody and percent of parasites bound to antibody determined by FACS. Black line: PBS; Light grey: Pfs25-alum/Pfs25-alum, dark grey: Ad5-pfs25/Ad5-HVR5D3; dark purple: Ad5-pfs25/Ad5-HVR5D2; light purple: Ad5-pfs25/Pfs25-alum. Significance determined by Student's T test (unpaired) where * $p<0.005$, ** $p<0.01$.

transmission of *P. falciparum* than those antibodies generated by homologous prime-boost with Pfs25-alum.

Since we found that heterologous prime-boosts with Ad5-pfs25 recognized and bound to the parasite surface we next asked whether the sera from these mice more effectively blocked transmission of *P. falciparum* than those antibodies generated by homologous prime-boost with Pfs25-alum. In collaboration with Kazutoyo Miura and Carol Long at the National Institutes of Health within the Malaria Vaccine Development Branch of the National Institute of Allergy and Infectious Diseases, we performed a standard membrane-feeding assay (MFA) to assess transmission blockade as previously described (Miura, Deng et al. 2013). Briefly, we diluted each serum sample 1 to 10 in human blood infected with *P. falciparum* and normal human serum replenished with complement. Three- to six- day-old Female *Anopheles stephensi* mosquitoes fed on this serum and gametocyte culture through a membrane. Eight days later, we harvested and dissected the mosquitoes and counted *P. falciparum* oocyst number per mosquito to assess the parasite load. Serum from Ad5-immunized mice served as a negative control while purified 4B7, a monoclonal antibody recognizing Pfs25, served as a positive control for transmission blockade. Consistent with our parasite binding data, we found that all the heterologous prime-boost vaccines with Ad5-pfs25 significantly reduced parasite load compared to our negative control, Ad5-immunized serum (Figure 23). These data demonstrated that Ad5-pfs25 prime followed by Pfs25-alum, Ad5-HVR5-DII, or Ad5-HVR5-DIII boost all significantly reduced transmission to the *Anopheles*

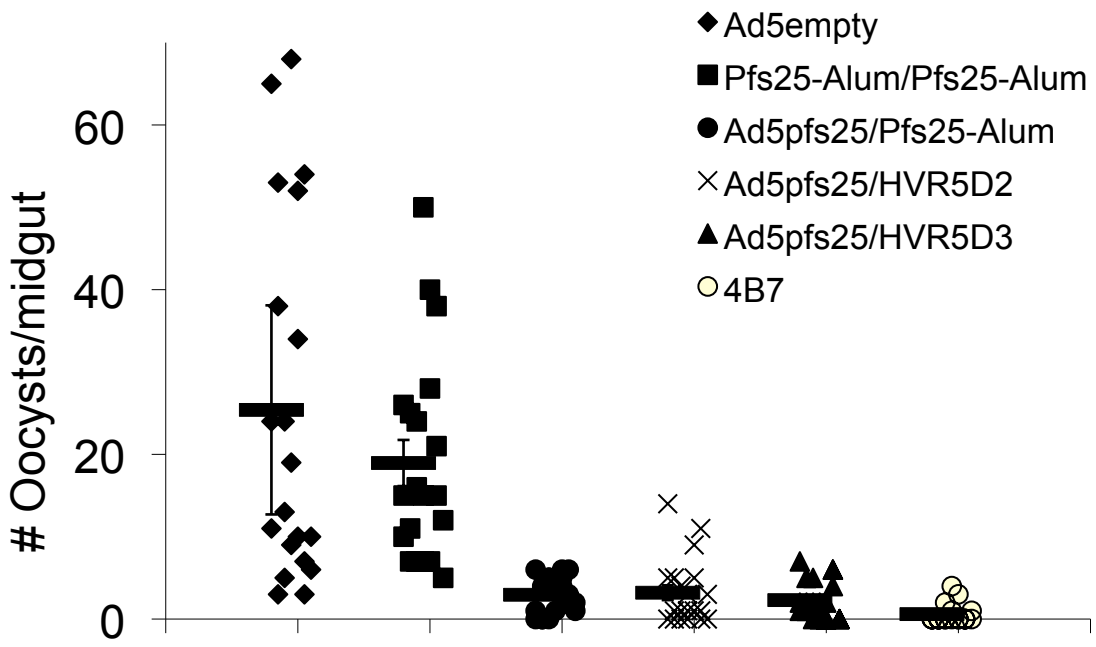

Figure 23. *P. falciparum* load in *Anopheles* mosquitoes in the presence of vaccine serum. The ability of serum antibody to block transmission of sexual stage *P. falciparum* to mosquitoes was determined by measuring parasite load in *Anopheles* mosquitoes following a standard membrane-feeding assay (SMFA). The SMFA is performed by feeding mosquitoes a *P. falciparum* gametocyte culture and human red blood cells through a membrane. Mosquitoes were fed in the presence of a 1/10 dilution of vaccine sera. Vaccine sera is pooled serum from each mouse (n=5 except for Ad5empty or Ad5pfs25/Pfs25-alum where n=3). Each data point represents the parasite load (number of oocysts) in a single mosquito. Horizontal lines represent the average oocyst number for each vaccination and vertical lines represent the standard error for each vaccine group.

mosquito. In contrast, homologous prime-boost with Pfs25-alum did not significantly block transmission as compared to our negative control. Interestingly, while we saw the highest Pfs25-specific antibody titer by ELISA with the Ad5-pfs25 prime and Pfs25-alum boost, we observed no difference in the transmission blockade by this vaccination regimen and the Ad5-pfs25 prime followed by boost with either Ad5-HVR5-DII or Ad5-HVR5-DIII. These data suggest that, despite having differences in titer by ELISA, all these heterologous prime-boost regimens containing the Ad5-pfs25 prime vaccination significantly blocked transmission. These findings further support the notion that Pfs25-alum vaccination elicits some Pfs25-specific antibodies that recognize Pfs25 but may not block transmission. Together, these data suggest that our Ad5-vectored Pfs25 vaccine regimens are capable of generating Pfs25-specific antibodies that block transmission to the mosquito vector.

Determining the effect of pre-existing immunity on the antibody response to Pfs25 following vaccination with Ad5 containing capsid-displayed Pfs25 B cell epitopes

We have shown thus far that heterologous prime-boost with Ad5-pfs25 followed by Ad5-HVR-pfs25 generated a higher titer of Pfs25-specific antibodies with higher relative affinities than those generated by homologous prime-boost with Pfs25-alum. Further, these heterologous prime-boost vaccinations blocked transmission of plasmodia to the mosquito vector. In addition to generating a high quality Pfs25-specific antibody response, another advantage to using this heterologous prime-boost with Ad5-HVR-pfs25 boost is that Ad5-HVR-pfs25 can evade Ad5-specific preexisting immunity. Several

studies have shown that replacement of HVR1 prevents Ad5 pre-existing immunity (PEI) from neutralizing Ad5 vaccine vectors. We find that Ad5-HVR1-DIII evades neutralization by Ad5-specific sera and pooled human sera in vitro. It is important to determine the impact of Ad5 PEI on vaccination because most individuals have pre-existing immunity to Ad5. Further, if this pre-existing immunity enhances the Pfs25-specific antibody response upon vaccination with Ad5-HVR-pfs25 then Ad5 PEI could be exploited in this way to enhance the antibody response of Ad-vectored vaccines. Additionally, if this approach is effective then it could bypass the need for a boost vaccination. A vaccine regimen with a single dose is much more practical and economical than those with multiple boosts. Further, pre-existing immunity, particularly Ad-specific T cells, could support the Pfs25-specific B cell response upon vaccination with Ad5-HVR-pfs25. We hypothesized that, similar to the $CD4^+$ memory T cells generated by prime vaccination with Ad5-pfs25, pre-existing Ad5-specific $CD4^+$ T cell immunity would also enhance the antibody response to Pfs25 upon single immunization with Ad5 containing capsid-displayed Pfs25. Additionally, this Ad5-HVR-pfs25 should avoid neutralization by the pre-existing Ad5-neutralizing antibodies because the HVR loops 1 or 5 were replaced with the Pfs25 epitopes.

To determine if pre-existing immunity to Ad5 affected the Pfs25-specific antibody response upon vaccination with Ad5-HVR-pfs25 we immunized mice with Ad5 or Ad12 to generate PEI. We used Ad12 to specifically address the role for pre-existing T cell immunity because Ad12 does not elicit Ad5-neutralizing antibodies directed towards

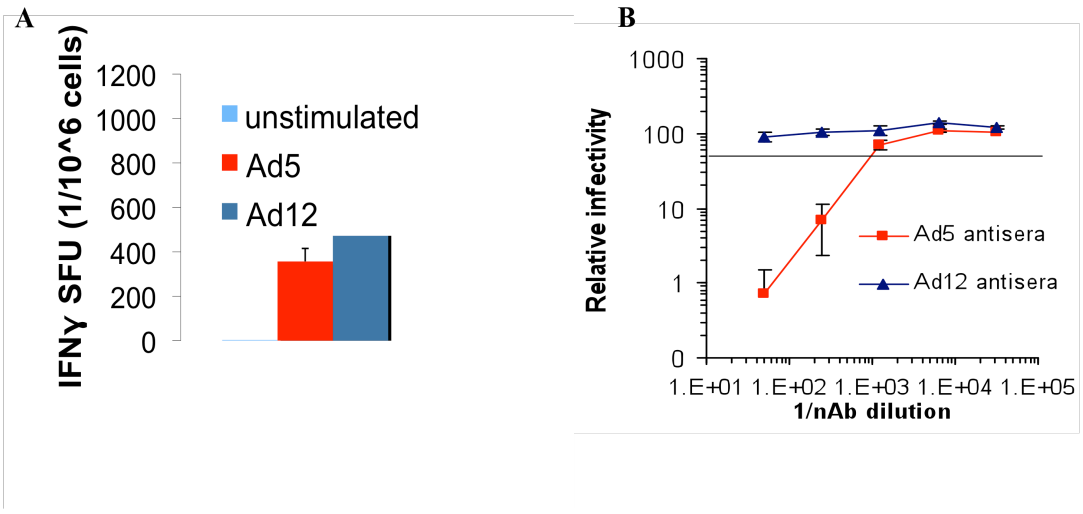

Figure 24. Ad12 cross-reactivity with Ad5 pre-existing immunity. A. Ad5 luciferase reporter virus infectivity of Hela cells in the presence of Ad5 or Ad12-specific serum. Relative infectivity is determined by luciferase activity normalized to luc activity of an uninfected control. B. IFNγ-producing T cells (splenocytes) from Ad5-infected mice stimulated ex vivo with replication-defective Ad5 or UV-inactivated Ad12 then IFNγ-producing cells determined by ELISPOT.

the adenovirus type 5 Hexon since the HVRs vary between serotypes (Figure 24A). However, Ad12 will elicit $CD4^+$ T cell immunity that reacts with Ad5 since Ad12 and Ad5 share highly conserved $CD4^+$ T cell epitopes in their capsids (Figure 24B). After generating PEI to Ad5 or Ad12, we immunized with Ad5-HVR-pfs25 21 days later and assessed the Pfs25-specific antibody response by ELISA. We found that pre-existing immunity slightly increased the Pfs25-specific antibody titer as compared with a single Ad5-HVR5-DIII vaccination. These results suggest that pre-existing T cell immunity is not detrimental to the antibody response to capsid-displayed antigens as it is to virally expressed antigens (Figure 25). In fact, these data suggest that preexisting T-cell immunity may aid the antibody response to capsid-displayed epitopes. Since Ad5-HVR1-DIII evades pre-existing neutralizing antibodies (nAbs) it would be interesting to determine how pre-existing immunity influences the Pfs25-specific antibody response with this vaccine. We would expect that evading nAbs could enhance the antibody response to Pfs25. Although, several studies suggest that the extent of neutralizing antibodies prior to vaccination and the amount of opsonization of Ad by nAbs can differentially affect the immune response. Unfortunately, these studies characterized the immune response to a transgene-encoded antigen rather than a capsid-displayed antigen.

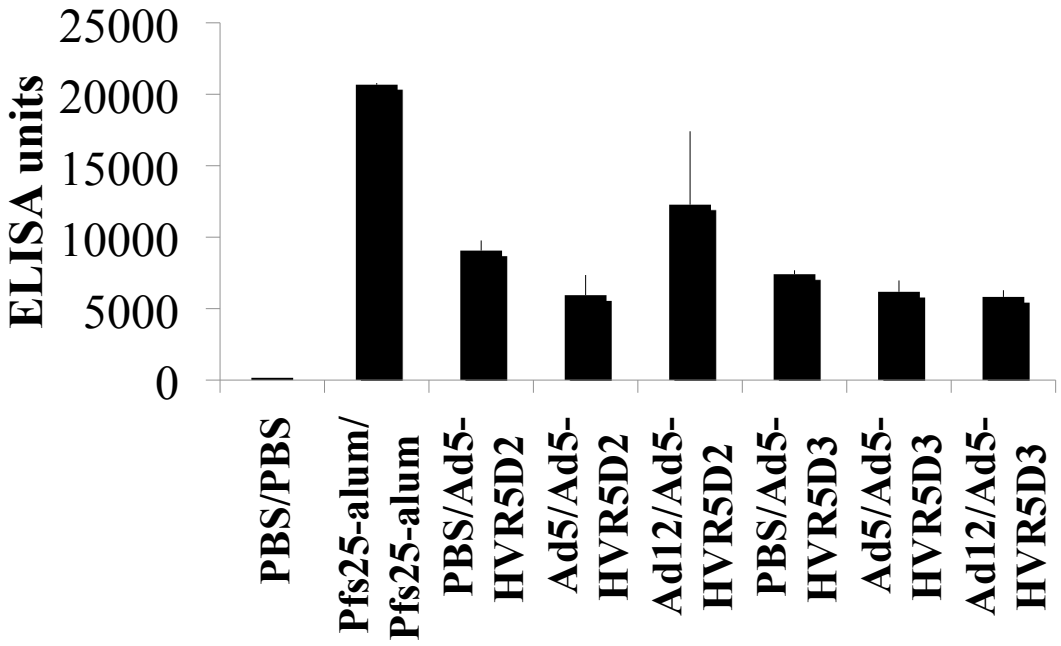

Figure 25. Pfs25-specific serum antibody response upon primary immunization in the presence of pre-existing immunity. Pfs25-specific ELISA with serum from mice primed with PBS control or 10^{10} particles Ad5 or Ad12 collected 21 days after boost immunization with 10^{10} Ad5-HVR-pfs25 or homologous prime-boost with Pfs25-alum (2.5μg/mouse) and detected by HRP-conjugated anti-mouse IgG (Fc) antibody.ELISA units are determined by endpoint dilution. Each group represents 3 animals per group.

Chapter IV

Discussion

ROS play a key role in the innate immune response to adenovirus type 5 infection

Adenovirus (Ad) is an attractive vaccine vector due in part to its ability to efficiently activate the immune system (Hartman, Appledorn et al. 2008). The immune system initially responds to infection, or, in this case, vaccination, with the innate response. The innate response senses Ad through pattern recognition receptors (PRRs) that sense molecular patterns, called pathogen-associated molecular patterns (PAMPs), associated with infection. During cell entry, Ad generates PAMPs that are recognized by several PRRs to rapidly activate the innate proinflammatory response. One such PAMP is the viral genome, which is sensed by the PRR Toll-like receptor 9 (TLR9) (Yamaguchi, Kawabata et al. 2007). TLR9 in turn activates NFκB, a key transcription factor involved in the upregulation of several proinflammatory cytokines (Cerullo, Seiler et al. 2007; Kawai and Akira 2008). In addition to TLR activation of NFκB, the cytosolic PRRs, Ddx41 and AIM2, also recognize viral nucleic acids to activate IRF-3, a key transcription factor activating the production of type I interferons (Stein and Falck-Pedersen 2012). The cytosolic PRR RIG-I recognizes virally-associated RNAs to also activate IRF-3 (Yamaguchi, Kawabata et al. 2010; Minamitani, Iwakiri et al. 2011). Another cytosolic PRR, the Nod-like Receptor, NLRP3, is also involved in the innate immune response to Ad infection, however the exact PAMPs NLRP3 recognizes are unclear

(Muruve, Barnes et al. 1999). One group recently found that NLRP3 was required for the proinflammatory response to Ad in peritoneal macrophages (Muruve, Petrilli et al. 2008). Additionally, this study also demonstrates that NLRP3 plays a role in the proinflammatory response to Ad by contributing to IL-1β production. We found that NLRP3 and its inflammasome component, Caspase-1, are required for IL-1β production following Ad infection in the human macrophage-like, differentiated THP-1 cells (Barlan, Griffin et al. 2010). Further, IL-1β was produced rapidly following Ad infection. We sought to further understand the signals, or PAMPs, that activate NLRP3 during Ad infection.

Several PAMPs are known to activate the NLRP3 inflammasome. These PAMPs are usually danger signals, called danger-associated molecular patterns (DAMPs), associated with cellular stresses such as viral infection. DAMPS such as oxidative stress (reactive oxygen species), exogenous ATP and particulates such as uric acid crystals are known to activate NLRP3 (Martinon, Burns et al. 2002; Martinon 2008; Latz 2010). We found that one particular DAMP, oxidative stress (reactive oxygen species), activates NLRP3 in response to Ad infection (McGuire, Barlan et al. 2011). These findings are consistent with one study from Jornot and colleagues. They found that co-administration of reducing agents such as N-acetylcysteine with Ad5 vectors in the lungs reduces the inflammatory response in mice and augments the level and duration of transgene expression (Jornot, Morris et al. 2002). Taken together, ROS may play a broad role in the

innate immune response to Ad, thus contributing to other innate immune signals in addition to its role in activating NLRP3.

In response to several pathogens, ROS contribute to the activation of many innate immune pathways. Traditionally, ROS contribute to the innate response to extracellular bacteria by killing these microbes in the phagosome when phagocytic cells take them up. More recently, ROS have been shown to play a role in activating innate signaling pathways during bacterial infection. Following bacterial infection, ROS are involved in the activation of transcription factors IRF-3 and NFκB (Chiang, Dang et al. 2006; Fink, Duval et al. 2008). While the role for ROS in the inflammatory response to extracellular bacteria is better characterized, recent studies have implicated ROS as a mediator of inflammatory signals in response to viruses. For example, ROS are known to enhance RIG-I-dependent IRF-3 activation in response to RSV infection (Soucy-Faulkner, Mukawera et al. 2010). While we have shown that ROS contribute to NLRP3 activation, it is unclear how ROS affect other innate immune pathways in response to Ad5 infection. We have demonstrated that Ad5-induced ROS contributes to the production of TNFα. Further, artificially enhancing ROS augments this process (McGuire, Barlan et al. 2011). These findings highlight the importance of ROS as a key mediator in innate immune activation of both NLRP3- and NFκB-dependent pathways upon Ad infection. While we haven't shown that ROS directly contributes to NFκB activation our studies thus far implicate ROS as a signal activating several innate immune pathways.

ROS have not yet been shown to contribute to NFkB- and IRF-3-dependent transcription. However, previous observations show that ROS activates NFkB and IRF-3 through the p38 kinase and JNK kinase, respectively (Matsuzawa, Saegusa et al. 2005; Muruve, Petrilli et al. 2008; Tattoli, Carneiro et al. 2008). Since Nociari and colleagues demonstrate that p38 and JNK MAP Kinases are involved in the innate immune response to Ad5 infection, ROS may contribute to these proinflammatory signaling pathways to exert its broad effects on the innate response to the virus (Nociari, Ocheretina et al. 2009). The role of ROS in these other signaling pathways must still be elucidated. Our current studies provide additional insight into ROS-dependent innate immune responses and have identified molecules whose contributions to innate immune signaling during Ad5 cell entry can now be further explored.

Since ROS play a role in innate immune activation we next hypothesized that ROS are produced during Ad infection. We investigated the generation of ROS overtime following Ad infection and found that Ad rapidly induces ROS. Within thirty minutes post-infection, we detected maximal levels of ROS. These data suggest that Ad cell entry triggers rapid ROS production. These findings are consistent with the fact that inflammatory responses to adenovirus have been tightly linked to cell entry. As mentioned, following receptor-mediated endocytosis, TLR9 activation in response to the Ad dsDNA genome leads to NFκB activation while endosomal escape activates IRF-3 through the cytosolic DNA sensors AIM2 and DDX41 (Stein and Falck-Pedersen 2012). Further, both Ad cell entry and endosomal escape contribute to NLRP3 activation (Di

Paolo, Miao et al. 2009; Barlan, Griffin et al. 2010; Barlan, Danthi et al. 2011) As mentioned, ROS-dependent signaling reportedly enhances much of these signaling pathways, which are also activated during the early phases of Ad infection (Zsengeller, Otake et al. 2000; Nociari, Ocheretina et al. 2007). To better understand how ROS contribute to the proinflammatory response to Ad5 we investigated how ROS are generated during adenovirus infection.

Ad5 induces ROS from the mitochondria

We found that ROS produced in response to adenoviral infection were derived from mitochondrial sources, independently of TLR signaling or phagosomal NADPH oxidase. This finding is striking because both TLR signaling and NADPH oxidase are known contributors to ROS production in response to many infections (Park, Jung et al. 2004; Fink, Duval et al. 2008). Since Ad enters the cell through endocytosis it is conceivable that NADPH oxidase on the endosomal membrane could have been a likely contributor to ROS generation during viral entry. However, our findings are consistent with recent observations highlighting an important role for mitochondria in antiviral innate immune signaling. Particularly, studies demonstrate that, following activation of RIG-I-like receptors or inflammasomes, many signaling intermediates localize to mitochondria upon viral infection (Moore, Bergstralh et al. 2008; Tattoli, Carneiro et al. 2008; Castanier, Garcin et al. ; Soucy-Faulkner, Mukawera et al. 2010). Taken together, mitochondria are capable of responding to a diverse array of virally-associated molecular patterns and danger signals to elicit an innate immune response.

We have shown a novel manner by which a nonenveloped virus triggers an innate immune response by activating a well-established danger signal in cells: the permeabilization of endo-lysosomal membranes. This membrane permeabilization leads to the release of cathepsin B into the cytosol of the infected cell. Cathepsin then triggers a pro-apoptotic mitochondrial stress. These findings are consistent with other studies demonstrating that the antiviral innate response is linked to an apoptotic response. Mitochondrial stress induced by incoming virions or via RIG-I recognition of viral RNA has also been linked to a proapoptotic response, which is thought to limit viral replication (Danthi, Kobayashi et al. 2008) (Chattopadhyay, Marques et al. 2010). Therefore, our findings show that a traditional proapoptotic signal, or danger signal, plays an important role in activating the innate response, particularly NLRP3 activation, following Ad infection.

Several recent studies implicate mitochondrial damage in activating the NLRP3 inflammasome. *Zhou et al.* demonstrate that mitochondrial dysfunction can activate the NLRP3 inflammasome (Zhou, Yazdi et al. 2011). Further, one study suggests that mitochondrial ROS activates the cation channel Trpm2 leading to a Ca^{2++} influx and NLRP3 activation (Zhong, Zhai et al. 2013). More recently, *Iyer et al.* suggest that inflammasome activation by damaged mitochondria is triggered by the release of the mitochondrial inner membrane component cardiolipin (Iyer, He et al. 2013). Furthermore, cardiolipin can interact with NLRP3. While we see that cathepsin activity and ROS are required for NLRP3 inflammasome activation upon Ad infection we have

not ruled out that cardiolipin may also contribute to inflammasome activation independent of ROS production. It is possible that these pathways occur simultaneously and thus have an additive effect on NLRP3 activation. Alternatively, ROS and cardiolipin could synergistically activate NLRP3 upon Ad infection. *Zhou et al*. found that upon mitochondrial damage, NLRP3 relocalizes from ER structures to the perinuclear area containing both ER and mitochondria. Therefore, cathepsin-dependent mitochondrial damage results in ROS that activates the inflammasome and, presumably, the release of cardiolipin. This event could trigger the relocalization of NLRP3 to the perinuclear space and the sustained and efficient activation then may involve an interaction with NLRP3 and cardiolipin. These hypotheses remain to be tested.

Ad5-mediated membrane rupture is required for the generation of ROS

During Ad5 cell entry, endo-lysosomal rupture is a danger signal that triggers ROS production. In this way, endo-lysosomal rupture allows cathepsin, a resident endo-lysosomal enzyme, to gain access to the cytosol of the cell. In turn, cathepsin activates a cascade that leads to mitochondrial stress, as previously described (Droga-Mazovec, Bojic et al. 2008). The mode of membrane rupture could influence this response. For example, we have previously shown that reovirus, which escapes the endosome by forming a pore in the membrane, does not activate the NLRP3 inflammasome to the extent of Ad5 despite enhanced colocalization with cathepsin-enriched lysosomes (Danthi, Guglielmi et al. 2010; Barlan, Danthi et al. 2011). As the endosomes fuse with later endosomes and lysosomes along the endocytic pathway, cathepsins become further

enriched such that late endosomes and lysosomes have a higher content of cathepsins than early endosomes (Reiser, Adair et al. 2010). Our lab found previously that the extent to which Ad vectors colocalize with cathepsin-enriched lysosomes influenced the inflammatory response to vectors (Barlan, Danthi et al. 2011). Specifically, when Ad traffics to late endosomes prior to endosome escape, as compared to early endosomes, they elicit a stronger inflammatory response. These observations have many implications for the use of adenovirus vectors in gene therapy and vaccine development. Since release of lysosomal cathepsins leads to ROS production, altering Ad intracellular trafficking by modifying the receptors used for cell entry could alter this ROS-dependent inflammatory response. It is not yet clear whether altering Ad membrane lytic activity could attenuate or enhance the inflammatory response. However, neutralizing ROS during Ad5 transduction could increase transgene expression by dampening the inflammatory response and, in turn, could increase the antigen-specific adaptive immune response due to prolonged antigen expression. Alternatively, amplifying the inflammatory response could promote dendritic cell maturation and lymphocyte migration. Increasing inflammation could also modify the helper T cell response as IL-1β can enhance T_H17 differentiation and IL-6 supports T_{FH} differentiation (Pepper and Jenkins 2011). Modifying the inflammatory response is an important way to tailor the adaptive response for a particular antigen in the context of Ad-based vectors. Our findings provide important insight into how to design Ad-based vectors rationally to generate the appropriate immune response depending on the application.

Cathepsin activity facilitates mitochondrial membrane destabilization

We found that Cathepsin activity contributes to Ad-induced ROS. Further, we demonstrated that Cathepsin activity is required for mitochondrial membrane damage. Specifically, inhibiting Cathepsin activity significantly attenuated loss in mitochondrial membrane potential during Ad infection. As mentioned, Cathepsins have been shown to cleave the pro-apoptotic factor Bid to facilitate mitochondrial membrane permeabilization and caspase-dependent apoptosis (Droga-Mazovec, Bojic et al. 2008; Zhang, Zhong et al. 2009). Our results demonstrate that stabilization of mitochondrial membrane potential by overexpressing bcl-2 reduces Ad5-induced ROS production and IL-1β secretion. Bcl-2 is an antiapoptotic protein that associates with proapoptotic factors, such as Bak or Bax, on the mitochondrial membrane to prevent pore formation and mitochondrial membrane damage and thus prevents apoptosis (Tait and Green 2010). The adenovirus E1B-19K gene is a bcl-2 family member, which is one of the first viral proteins to be expressed during infection (Berk 2005). Although E1B-19K is important for preventing apoptosis in response to viral infection, it is interesting to consider that this protein, detectable several hours post infection, may function in some capacity to dampen the inflammatory response elicited by incoming virions or during viral replication. The E1B-19K protein has been previously shown to inhibit NFκB transcriptional activity (Schmitz, Indorf et al. 1996), which fits with our model of Ad-induced ROS-dependent augmentation of NFκB pathway. Further study is required to explore this possibility.

As a whole, our data indicate that Ad5 membrane rupture and subsequent Cathepsin B activity in the cytoplasm contribute to mitochondrial stress and the ROS produced during infection. Specifically, Cathepsin B activity is required for both mitochondrial membrane damage and Ad-induced ROS. Ad-induced ROS is significantly attenuated when mitochondrial membrane damage is inhibited by bcl-2 overexpression. This mechanism of ROS production could play a role in the innate response to particulate adjuvants, such as silica and asbestos as well since phagocytic cells can take up particulates and activate the NLRP3 in an ROS-dependent manner (Dostert, Petrilli et al. 2008). Our findings elucidate how ROS are produced and also identify their requirement in NLRP3 activation upon Ad infection. Additionally, recent studies demonstrate that the common vaccine adjuvant, alum, exerts its immunostimulatory effects by activating the NLRP3 inflammasome (Eisenbarth, Colegio et al. 2008; Franchi and Nunez 2008; Li, Willingham et al. 2008). Further, phagocytosis of alum is required to activate the NLRP3 inflammasome (Eisenbarth, Colegio et al. 2008). Therefore, particulates may activate the NLRP3 inflammasome in a manner similar to Ad. The mechanism of particulate-induced NLRP3 activation still needs to be further investigated.

This mechanism of Ad5-induced ROS is likely a common response to pathogens that rupture membranes such as bacteria that replicate in the cytoplasm. ROS is a key component in the antiviral and proinflammatory response to both bacterial and viral infection. These results will help with future studies of innate immune responses to Ad5. We do not yet know how the extent of ROS influences the innate response. It is possible

that the extent of ROS influences the extent of cell death upon Ad infection. If this is the case, then altering the amount of ROS induced by Ad could alter the amount of cell death. The amount of cell death, in turn, could influence both the innate and adaptive response by generating DAMPs that activate both innate and adaptive immune cells. These hypotheses still need to be explored.

The natural adjuvant properties of Ad5 vaccine vectors can be exploited to generate improved transmission-blocking vaccines against malaria

Our studies elucidating the mechanism of ROS production in response to Ad5 contribute to our understanding of the innate immune response to Ad5 vaccine vectors. The innate immune response is the rapid, initial responder to a pathogen or vaccine. This early recognition of Ad5 serves as the crucial alarm to activate the adaptive immune response such that the extent and nature of the innate immune response dictates how the adaptive immune response reacts to the vaccine. The robust and rapid inflammatory response to Ad5 vaccine vectors will likely contribute to a more robust adaptive response to Ad5 and encoded vaccine antigens. However, it is unclear whether ROS production directly influences the adaptive immune response or how ROS influences the adaptive response. The role for ROS in the adaptive response to Ad still needs to be explored. Nonetheless, adenovirus vectors are attractive vaccine platforms due to their ability to potently activate the immune system (Hartman, Appledorn et al. 2008). In addition to their immunostimulatory properties, adenoviral vaccine vectors have well-characterized physical properties. Ad has a genetically tractable genome that can accommodate

exogenous transgenes. Since we have a fairly good understanding of both the physical and immunostimulatory properties of adenoviruses and how these vectors interact with antigen presenting cells, we would like to exploit this knowledge to rationally design better vaccines. We assessed how we could improve the antibody response to Pfs25, a candidate antigen for transmission-blocking vaccines (TBV) against malaria by generating an Ad5-based TBV expressing Pfs25 from a transgene. A single immunization with Ad5-pfs25 generated a high titer of Pfs25-specific antibodies and this titer was significantly higher than that generated by single immunization with alum-adjuvanted Pfs25 or a homologous prime-boost vaccination with Pfs25-alum. It is important to point out that another study recently compared the Pfs25-specific antibody response with vaccine regimens involving an Ad5 expressing pfs25 (Goodman, Blagborough et al. 2011). However, this study employs an Ad5 expressing a soluble form of Pfs25. Our Ad5-pfs25 vaccine is novel since it expresses the full-length, membrane-bound Pfs25. Several studies demonstrate that membrane bound antigens more effectively activate B cells since adhesion molecules expressed on the cell surface can strengthen the interaction between a B cell and cells expressing it's cognate antigen on the cell surface. These additional interactions thus lower the threshold for the B cell to become activated (Carrasco, Fleire et al. 2004). Further, it presumably helps having the innate response and cytokines in very close proximity to the target antigen. It is therefore possible that soluble antigen is not as effective as membrane-bound antigen since soluble antigen may not be adjacent to both adhesion molecules and costimulatory molecules since it is not bound to

the membrane. *Zhu et al.* demonstrate that type I interferon signaling on B and T cells is required for the optimal production of antibodies to adenovirus indicating that the innate immune response generates cytokines that directly affect the function of B and T cells (Zhu, Huang et al. 2007). *McConnell et al.* found that immunization with an Ad5-vectored vaccine generated a significant antibody response to the target vaccine antigen while co-injection with the Ad vector and the target antigen as a soluble protein did not elicit a detectable antibody response (McConnell, Danthinne et al. 2006). Considering these data, we would expect that our novel Ad5-pfs25 expressing the membrane-bound Pfs25 would elicit a more robust antibody response than an Ad5 expressing soluble Pfs25. Preliminary data from our lab suggests that this is the case. However, this hypothesis and proposed mechanism for the enhanced antibody response remains to be tested.

In addition to generating a higher Pfs25-specific antibody titer, we also found that Ad5-pfs25 generated a broad array of IgG subclasses recognizing Pfs25. While Ad vectors have been traditionally explored as vectors to elicit a T_H1-skewed, or cellular immune response there is increasing evidence, in our study and others, that Ad-based vectors generate IgG subclasses supported by both T_H1 and T_H2 responses. We found that Ad5-pfs25 vaccination generates a mixed T_H1/ T_H2 response. Specifically, Ad5-pfs25 generated a significant IgG1 and IgG2b response to Pfs25. IgG1 is generated predominantly in response to a T_H2-biased response while IgG2b is produced in a T_H1-

biased response. These findings support the notion that Ad5-based vectors can be used to generate a humoral response in addition to generating a cellular response.

As mentioned, we found that Ad5-pfs25 generates a higher Pfs25-specific antibody response than vaccination with Pfs25-alum. In fact, vaccination with Pfs25-alum did not generate a detectable increase in the Pfs25-specific antibody titer. There are several reasons why the Pfs25-specific antibody response is improved with an Ad-based vector as compared with protein vaccination. For one, transgene expression is persistent and, therefore, the Pfs25 antigen is likely present longer in Ad5-pfs25-vaccinated mice than in Pfs25-alum-immunized mice. *Geiben-Lynn et al.* found that transgene expression following Ad-based vector vaccination is detectable out to 100 days post-vaccination (Geiben-Lynn, Greenland et al. 2008). These Ad-vectored transgenes elicit both antigen-specific T cells and antibodies. Secondly, Ad-based vectors likely activate antigen-specific T cells. We found that Ad5-pfs25 vaccination generated a broader array of IgG subclasses, a process that requires T cell help. These findings support the notion that Ad5-pfs25 activates T cells.

Vaccination with either Ad5 expressing pfs25 or Pfs25-Alum does not elicit Pfs25 specific IFNγ-producing T cells

The observed increase in the Pfs25-specific antibody response with greater switching to IgG2b upon Ad5-pfs25 vaccination as compared to Pfs25-alum vaccination correlates with a significant increase in T cell activation. We found that Ad5-pfs25 immunization generated an Ad5-specific IFNγ response suggesting Ad-specific T cells

are activated upon Ad5-pfs25 immunization. In contrast, there was no significant increase in IFNγ to Pfs25 suggesting that Ad5-pfs25 immunization elicits T-cell help, which predominantly recognized adenovirus antigens. Additionally, Pfs25-alum vaccination did not activate Pfs25-specific IFNγ-producing T cells. These data correlated with the high titer antibody response generated upon Ad5-pfs25 vaccination. These results suggest that Ad5-pfs25 vaccination generate a robust Pfs25-specific antibody response by providing the requisite T cell help to support B cell activation, affinity maturation and class switching. Ad vectors have been traditionally thought to predominantly activate T helper cells that support the cellular immune response. These T_H1 cells support the activation of cytotoxic T cells through the production of IFNγ. However, our antibody data indicate that Ad5-pfs25 also generates IgG subclasses that are generated in response to T_H2-biased cytokines. T_H2 cells support the humoral immune response by producing cytokines, such as IL-4 and IL-13, which support B cell activation, proliferation, and class switching. We did not detect IL-4 production, the key cytokine in supporting the T_H2 response. However, *Pine et al.* also found that Ad-based vectors could generate a mixed T_H1/T_H2 response and did not detect IL-4 but did detect another T_H2 cytokine IL-13 (Pine, Kublin et al. 2011). IL-13 production following Ad5-pfs25 vaccination still remains to be assessed.

Ad5 could support the humoral immune response and Pfs25-specific B cells in a manner independent of IL-4 production by T_H2 cells. Ad is known to induce production of the proinflammatory cytokine IL-6 (Muruve, Barnes et al. 1999). IL-6 signaling on T

cells induces IL-21 production, which is required for the induction of follicular helper T cells (T_{FH}) (Nurieva, Yang et al. 2007; Suto, Kashiwakuma et al. 2008). T_{FH} cells produce IL-21 and home to the B cell follicle within lymph nodes where they provide help to B cells and support B cell class switching. *Avery et al.* demonstrate that IL-21 supports naïve B cell class switch to $IgG3^+$ and IgA^+ cells *in vitro* while IL-4 supported class switching to IgG1 (Avery, Bryant et al. 2008). They found IL-4 and IL-21 together synergistically supported IgG1 class switching but abrogated isotype switching to IgA. We found that Ad5-pfs25 generates both IgG1 and IgG3 subclasses. Another study shows that Ad-based vectors generate a robust IgA response in the lung following sublingual immunization (Shim, Stadler et al. 2012). Since Ad5 appears to generate a broad array of immunoglobulin isotypes, and, in our hands, a broad range of IgG subclasses it is likely that Ad5 vectors generate a mixed helper T cell response that may be influenced by the relative amount, location or timing of cytokine production. It would be interesting to assess T_{FH} cell induction upon Ad5-pfs25 vaccination. As mentioned, since Ad induces the proinflammatory cytokine IL-6, which is a key inducer of T_{FH} cells (Dienz, Eaton et al. 2009), Ad5-pfs25 likely generates a significant T_{FH} response.. This T_{FH} response, could in turn, support B cell activation, proliferation and class switching. Since Pfs25-alum does not generate a robust proinflammatory response it may not generate a significant T_{FH} population. These hypotheses remain to be tested.

Another possibility is that Ad5-pfs25 does predominantly generate a T_H1-biased response and that there is some plasticity allowing both T_H1 cells and $CD8^+$ T cells to

support the diversification of IgG subclasses to Pfs25. $CD8^+$ T cells, through the expression of CD40L can activate antigen-presenting cells. *Frentsch et al.* found that these $CD40L^+$ $CD8^+$ T cells comprise a substantial pool of the memory $CD8^+$ T cells following viral infection (Frentsch, Stark et al. 2013). Further, these $CD8^+$ T cells were functionally similar to $CD4^+$ T cells as they are able to produce cytokines, such as IL-4, that are normally produced by helper T cells to support B cell activation. Another study demonstrates that IFNγ production by $CD8^+$ T cells induces the upregulation of the chemokine receptor CXCR3 on the surface of antigen-specific B cells that facilitates the migration of B cells to the site of inflammation and, in turn, antibody production and isotype switching (Serre, Cunningham et al. 2012). Taking these studies into account, Ad5-pfs25 may elicit $CD8^+$ T cells that, similar to $CD4^+$ T cells, provide support to a traditionally T_H2-biased response. Further, as mentioned previously, Ad5-pfs25 likely generates T_{FH} cells that are an important part of the antibody response. It is thought that most effector T helper subsets can become T_{FH} cells to support antibody production in the germinal center.

As expected, Pfs25-alum primary immunization did not generate antigen-specific T cells upon immunization. There are several proposed reasons why Pfs25 does not activate T cells. It is likely that Pfs25 does not have strong MHC class I or II epitopes. Another possibility is that Pfs25 is tolerizing in some way and is therefore suppressing the immune response. These hypotheses have not been tested. Thus far, our results support the idea that Pfs25 does not effectively activate T cells and this is likely due to its

lack of strong MHC epitopes. If Pfs25 does not have strong MHCII epitopes then helper T cells will not get activated. Further, a lack of MHCII epitopes within Pfs25 would also support the idea that Pfs25-alum does not generate T_{FH} cells. In the absence of these cells, short-lived plasma cells can still be generated but long-lived plasma cells and the serum antibody response will be greatly diminished. Long-lived plasma cells are thought to be the main source of serum antibody and are sustained in the bone marrow over long periods of time where they secrete antibody in the absence of antigen (McHeyzer-Williams, Okitsu et al. 2012; Tarlinton and Good-Jacobson 2013).

Vaccination with Ad5 expressing pfs25 generates antibody-secreting cells in the bone marrow

Our studies demonstrate that Ad5-pfs25 vaccination generates a high titer, Pfs25-specific antibody response. Further, these antibodies have high relative affinity for Pfs25 as compared to Pfs25-alum vaccination. Additionally, Ad5-pfs25 generates a diverse array of IgG subclasses recognizing Pfs25. In addition to these key properties that suggest Ad5-pfs25 elicits a robust Pfs25-specific response, we hypothesized that this antibody response is also long-lived. To ask whether the Pfs25-specific antibody response is long-lived without waiting many months to assess antibody titers we asked whether Ad5-pfs25 vaccination generates antibody-secreting cells (ASCs) in the bone marrow and spleen. ASCs represent the plasma cells present in these lymphoid organs. The ASCs found in the spleen may reflect short-lived plasma cells (SLPCS) that are generated in response to vaccination and then contract over time (McHeyzer-Williams, Okitsu et al. 2012). ASCs

in the bone marrow may reflect a population of long-lived plasma cells (LLPCs). LLPCs are thought to be the main source of serum antibody and are sustained in the bone marrow over long periods of time where they secrete antibody in the absence of antigen (McHeyzer-Williams, Okitsu et al. 2012; Tarlinton and Good-Jacobson 2013). We found that Ad5-pfs25 vaccination generated antibody-secreting cells (ASCs) specific to Pfs25 and adenovirus in the bone marrow and spleen. As mentioned, these ASCs in the bone marrow may reflect a population of LLPCs. In contrast, Pfs25-alum vaccination did not elicit ASCs in the bone marrow. We found no difference in the antibody-secreting cells recognizing Pfs25 in the spleen between Ad5-pfs25- and Pfs25-alum-vaccinated mice. These results demonstrate that both Ad5-pfs25 and Pfs25-alum vaccinations generate ASCs in the spleen while only Ad5-pfs25 vaccination is capable of generating ASCs in the bone marrow. It is important to point out that the ELISPOT assessing the antigen-secreting cells in both the spleen and bone marrow reflects the cells secreting any immunoglobulin isotype. Therefore, it is possible that the plasma cells in the spleen of Pfs25-alum immunized mice may reflect low affinity, IgM-secreting plasmablasts that are known to peak at day 10 post-vaccination, the time at which we are assessing this response. Additionally, these data imply that Ad5-pfs25 and Pfs25-alum vaccinations elicit short-lived plasma cells in the spleen while Ad5-pfs25 vaccination generates LLPCs in the bone marrow. However, another limitation to this assay is that it enumerates the antibody-secreting cells, which includes any plasma cell. To be sure that these cells from the bone marrow are indeed LLPCs we should also perform FACS

analysis. Nonetheless, our findings are one of the first assessing the antigen-specific LLPCs in the bone marrow generated by Ad5-based vectors. Due to their ability to generate a significant pool of antigen-specific LLPCs, Ad-based vectors are an attractive option for vaccines requiring an antibody response.

A recent study from Rasheed and colleagues identifies the importance of IL-21 in supporting LLPC generation in response to viral infection (Rasheed, Latner et al. 2013). Follicular helper T (T_{FH}) cells predominantly produce IL-21. Therefore, Ad5-pfs25 may generate LLPCs by inducing a population of T_{FH} cells to support LLPC formation. It would be interesting to assess T_{FH} cell populations and their activation following Ad5-pfs25. *Rasheed et al.* found that while IL-21 was not required for germinal center formation or the initial IgG response to infection, it was required to maintain germinal centers 15 days post-infection, generate LLPCs and support antibody levels as far as 300 days post-infection. This study also underscores the importance of LLPCs in the long-lived antibody response. We would hypothesize that Ad5 vectors are potent inducers of IL-21 production by T_{FH} cells as this response is dependent on the proinflammatory cytokine IL-6 (Dienz, Eaton et al. 2009). Ad is known to induce IL-6 production and this, in turn, could activate IL-21 production and thus support LLPC production (Muruve, Barnes et al. 1999).

Since Pfs25-alum does not generate a robust proinflammatory response it may not generate a significant T_{FH} population. We found no significant difference in the antibody-secreting cells specific to Pfs25 in the spleen from mice immunized with Pfs25-alum or

Ad5-pfs25 despite there being no detectable serum antibody response to Pfs25 upon Pfs25-alum immunization. In contrast, at day 10 post-vaccination when the antibody-secreting cells were assessed, Ad5-pfs25 generates an antibody titer that is 2 orders of magnitude higher than that following Pfs25-alum vaccination. These data are consistent with those from *Rasheed et al.* where the germinal center response was initiated appropriately but the LLPC and serum antibody response was disrupted when IL-21, the key T_{FH} cytokine, was absent (Rasheed, Latner et al. 2013).

Heterologous prime-boost vaccination with Ad5-pfs25 followed by Pfs25-alum induces a more robust anti-Pfs25 antibody response than homologous prime-boost with Pfs25-alum.

We have demonstrated that adenovirus is capable of inducing adenovirus-specific T cells that, through cytokine production, presumably support the activation and proliferation of the Pfs25-specific B cell response upon primary vaccination. Further, like other vaccine regimens, Ad5-pfs25 vaccination will likely require a boost vaccination to achieve the high levels of antibodies required to block malaria transmission. Since Ad5-pfs25 elicits a stronger Pfs25-specific antibody response upon primary vaccination we hypothesized that this response will be further enhanced upon boost immunization and that this response will be much more potent than a boost immunization following Pfs25-alum prime. Consistent with the improved Pfs25-specific antibody response upon Ad5-pfs25 primary vaccination, we saw that heterologous prime-boost vaccination with Ad5-

pfs25 followed by Pfs25-alum elicited about a 60-fold higher Pfs25-specific antibody titer than Pfs25-alum homologous prime-boost vaccination.

Consistent with our findings from Ad5-pfs25 primary immunization, heterologous prime-boost with Ad5-pfs25 followed by Pfs25-alum generated antibodies with high relative affinity as compared to homologous prime-boost with Pfs25-alum. Additionally, Ad5-pfs25 prime and Pfs25-alum boost generated a broader array of IgG subclasses recognizing Pfs25. In contrast, homologous prime-boost with Pfs25-alum generated an IgG1-dominated response. These data demonstrate the importance in generating a robust primary immune response. Both these vaccine regimens have the same boost vaccination yet there is a substantial difference in the antibody response. These data suggest that the memory B cell pool generated upon primary immunization with Ad5-pfs25 plays an important role in the secondary response. Therefore, it is important to choose a primary vaccine that generates the most robust response.

Boost vaccination with novel adenovirus-vectored vaccines displaying Pfs25 B cell epitopes enhances the Pfs25-specific antibody response

In addition to the poor immunogenicity of protein-based vaccines, vaccines with whole proteins generate antibodies to many epitopes, some of which are not neutralizing or, in our case, transmission-blocking epitopes. In fact, these antibodies could directly compete with transmission blocking antibodies for binding to the parasite surface. Therefore, we attempted to overcome the poor immunogenicity of Pfs25 by first immunizing with Ad5-pfs25 and then boosting with an Ad-vectored vaccine containing

the relevant, transmission-blocking Pfs25 epitopes on the capsid to enrich for antibodies recognizing neutralizing epitopes rather than irrelevant epitopes. We have generated Ad5 vectors containing Pfs25 B cell epitopes taken from domains 2 or 3 of Pfs25 (referred to as DII or DIII) within its capsid by lambda red recombineering. Specifically, we have introduced these Pfs25 epitopes in the hypervariable regions 1 and 5 (HVR) of the Ad5 Hexon protein. The Ad5-HVR-pfs25 viruses generated a low but detectable Pfs25-specific antibody response. To generate a stronger response to better compare the contribution of each Pfs25 epitope and each HVR to the Pfs25-specific antibody response we first immunized mice with Pfs25-alum. We did not use our Ad5-pfs25 prime vaccination because pre-existing immunity could influence the response to Pfs25 and we addressed the role of pre-existing immunity separately. We have found that the DII Pfs25 epitope elicits a comparable Pfs25-specific antibody response than the DIII loop following Pfs25-alum prime vaccination. While the DIII loop was selected based on its known recognition by several transmission blocking monoclonal antibodies, we chose the DII loop based on its location within the predicted structure of Pfs25. Our results suggest that we have identified a novel B-cell epitope in Pfs25. This result is exciting and promising in light of a study from *Stowers et al.* that demonstrated that antibodies recognizing the EGF-like domain 2 have the most effective transmission-blocking activity (Stowers, Keister et al. 2000). If Ad5-HVR5-DII directs the antibody response to the most effective transmission-blocking epitope then immunization with this vector may reduce the number of boost vaccinations required to generate an effective transmission-

blocking antibody titer in humans. Interestingly, Pfs25-alum prime followed by Ad5-HVR-pfs25 boost generated an IgG1-biased antibody response regardless of the boost vaccination. These results again emphasize the importance of generating an appropriate primary vaccination as the memory B and T cells appear to dictate the nature of the secondary response.

Heterologous prime-boost vaccination with Ad5-pfs25 followed by Ad5-HVR-pfs25 boost generates a robust Pfs25-specific antibody response

During primary immunization we observed the greatest Pfs25-specific response when immunizing with Ad5-pfs25 presumably because it potently activates T cells that support the B cell response. Additionally, boost immunization with Pfs5-alum following Ad5-pfs25 prime vaccination further increased the Pfs25-specific antibody titer. In contrast, homologous prime-boost with Pfs25-alum did not greatly enhance the Pfs25-specific antibody titer despite vaccinating twice. Taken together, these data suggest that Ad5-pfs25 primary vaccination generates a pool of memory B and T cells that, upon boost vaccination, rapidly respond to further increase the Pfs25-specific antibody titers. Therefore, we can exploit this pool of memory B and T cells upon boost vaccination and, further, enrich for those memory B cells that recognize the relevant transmission-blocking epitopes by boost immunizing with Ad5-HVR-pfs25. Since Ad5-HVR-pfs25 displays the Pfs25 epitope in the context of adenovirus, pre-existing Ad5-specific T cells could provide help to support the Pfs25-specific antibody response.

We found that heterologous prime-boost with Ad5-pfs25 prime followed by Ad5-HVR-pfs25 vaccination further increased the Pfs25-specific antibody titer as compared to Ad5-pfs25 prime alone. Additionally, boosting with Ad5-HVR5-DII generated a significantly higher Pfs25-specific antibody response as compared to Ad5-HVR1-DIII or Ad5-HVR5-DIII boost vaccination. These data suggest that the DII is more immunogenic than the DIII epitope, a known transmission blocking epitope in this context. A slight defect in Ad5-HVR1-DIII specific infectivity could contribute to the differences in the Pfs25-specific antibody response seen with this vaccination. However, Ad5-HVR5-DII and Ad5-HVR5-DIII have comparable specific infectivities suggesting that the differences in Pfs25-specific antibody titer may be due to the higher immunogenicity of DII as compared to DIII. These observed increases in Pfs25-specific antibodies upon boost with Ad5-HVR-pfs25 should reflect an increase in those antibodies recognizing the relevant transmission blocking, DII or DIII, epitopes. This remains to be determined.

In addition to generating a higher titer of Pfs25-specific antibodies, we found that these prime-boost vaccinations generated antibodies with a higher relative affinity than homologous prime-boost with Pfs25-alum. Interestingly, while the Ad5-pfs25 prime and Pfs25-alum boost generated the highest titer of Pfs25-specific antibodies, the relative affinity from this protein boost is comparable to the Ad5-HVR-pfs25 boosts. These findings support the notion that the immune response generated during primary vaccination dictates the nature of the secondary response. For these Pfs25-specific antibodies, both titer and affinity will contribute to their transmission-blocking efficacy.

In addition to generating antibodies of higher relative affinities, heterologous prime boost with Ad5-pfs25 followed by any boost vaccine generated a broader array of IgG subclasses similar to those generated upon Ad5-pfs25 prime vaccination alone. These data suggest that the specificity, relative affinity, and IgG subclass of antibodies generated during boost vaccination with Ad-HVR-pfs25 may be strongly influenced by the antibodies generated during the primary vaccination.

We believe that Ad5-specific memory T cells support the Pfs25-specific antibody response in the context of heterologous prime-boost with Ad5-pfs25 prime followed by Ad5-HVR-pfs25 boost. Following recognition of DII or DIII on the viral capsid and internalization of Ad5-HVR-pfs25, Pfs25-specific memory B cells can present Ad5 antigens to Ad5-specific T cells. Studies suggest that B cell selection in germinal centers is regulated by B cell competition for interactions with T_{FH} cells (Tarlinton and Good-Jacobson 2013). Further, B cells that were able to efficiently present antigen to T_{FH} cells led to their activation and subsequent proliferation and differentiation. This selection process can occur in the absence of BCR engagement (Victora, Schwickert et al. 2010). Displaying Pfs25 epitopes on the surface of the Ad5 vector should enhance the antigen presentation to T cells by Pfs25-specific B cells since Ad vectors contain well-conserved T cell epitopes. Ultimately, this could lead to selection of more Pfs25-specific B cells. These hypotheses still need to be tested.

Ad5-vectored prime-boost vaccinations generate Pfs25-specific antibodies that bind mosquito stage *Plasmodium falciparum* gametes and block transmission

Heterologous prime-boost with Ad5-pfs25 prime followed by Ad5-HVR-pfs25 boost generated antibodies that bind *Plasmodium falciparum* gametes *in vitro*. In contrast, we did not detect parasite binding by serum from homologous prime-boost with Pfs25-alum and this is likely the result of the low titer of Pfs25-specific antibodies following these vaccinations as homologous prime-boost with Pfs25-alum generated a low titer of Pfs25-specific antibodies. In addition to binding to the parasite surface, we found that sera from mice immunized with these heterologous prime-boosts with Ad5-pfs25 significantly blocked transmission of *P. falciparum* to the *Anopheles* mosquito as determined by a standard membrane-feeding assay (SMFA). Ad5-pfs25 prime followed by Pfs25-alum, Ad5-HVR5-DII, or Ad5-HVR5-DIII boost all significantly reduced transmission to the *Anopheles* mosquito. In contrast, serum from mice vaccinated with a homologous prime-boost of Pfs25-alum did not block transmission. Interestingly, while we saw the highest Pfs25-specific antibody titer by ELISA with the Ad5-pfs25 prime and Pfs25-alum boost, we observed no difference between the transmission blockade by this vaccination regimen and the Ad5-pfs25 prime followed by boost with either Ad5-HVR5-DII or Ad5-HVR5-DIII. These findings further support the notion that Pfs25-alum vaccination elicits some Pfs25-specific antibodies that recognize Pfs25 but may not block transmission. If this is the case, then our Ad5-HVR-pfs25 vaccines will better direct the Pfs25-specific antibody response to generate functional, transmission-blocking antibodies. This is particularly important for TBV design since TBVs must generate and maintain high titers of Pfs25-specific serum antibodies to block transmission. In SMFAs,

the positive control, 4B7, a monoclonal antibody against domain 3 of Pfs25, blocks transmission at a concentration of 100μg/ml (Saxena, Wu et al. 2007; Miura, Deng et al. 2013). Since we see that our Ad5-vectored vaccines have similar transmission-blocking efficiency as 4B7, our vaccines may achieve these antibody concentrations. However, antibody concentration alone does not necessarily dictate transmission blockade. Differences in antibody affinities and specificities could contribute to differences in transmission. For example, *Stowers et al.* found that depleting sera of antibodies recognizing domain 2 of Pfs25 significantly reduced transmission-blocking activity despite the fact that antibody titers to this domain were relatively low (Stowers, Keister et al. 2000). In agreement with these findings, we found that all Ad5-pfs25 prime-boosts significantly blocked transmission despite Pfs25-alum boost eliciting the highest titer of Pfs25-specific antibodies. Nonetheless, TBVs must achieve a high titer of Pfs25-specific antibodies such that the mosquito takes up circulating transmission-blocking antibodies during a blood meal. To better characterize the antibody response produced by our vaccine regimens we determine the affinities and concentrations of the Pfs25-specific antibodies to determine how these properties affect transmission blockade in our vaccine regimens.

Pre-existing immunity does not affect the immune response to capsid-displayed antigens upon primary immunization with Ad5-HVR-pfs25

We hypothesized that, similar to the $CD4^+$ memory T cells generated by prime vaccination with Ad5-pfs25, pre-existing Ad5-specific $CD4^+$ T cell immunity would also

enhance the antibody response to pfs25 upon single immunization with Ad5 vector with capsid-displayed pfs25. Additionally, Ad5-HVR-pfs25 should avoid neutralization by the pre-existing Ad5-neutralizing antibodies (nAbs) because the HVR loops 1 or 5 will be replaced with the Pfs25 epitopes. We would expect that evading nAbs could enhance the antibody response to Pfs25. It is important to determine the impact of Ad5 pre-existing immunity (PEI) on vaccination because most individuals have pre-existing immunity to Ad5. Further, if this PEI enhances the Pfs25-specific antibody response upon vaccination with Ad5-HVR-pfs25 then Ad5 pre-existing immunity could be exploited in this way to enhance the antibody response of Ad-vectored vaccines. Several studies suggest that the extent of neutralizing antibodies prior to vaccination and the amount of opsonization of Ad by nAbs can differentially affect the immune response. Unfortunately, these studies characterized the immune response to a transgene-encoded antigen rather than a capsid-displayed antigen (Mathews 1995; Ahi, Bangari et al. 2011; Choi, Dekker et al. 2012). We found that pre-existing Ad immunity slightly increased the Pfs25-specific antibody titer as compared with a single Ad5-HVR5-DIII vaccination suggesting that pre-existing T cell immunity is not detrimental to the antibody response to capsid-displayed antigens as it is to virally expressed antigens. Since we found that Ad5-HVR1-DIII evades pre-existing neutralizing antibodies it would be interesting to determine how pre-existing immunity influences the Pfs25-specific antibody response with this vaccine. We would expect that evading nAbs could enhance the antibody response to Pfs25. While several studies demonstrate that nAbs can reduce transgene expression following vaccination

with Ad-based vectors, less studies have addressed the effect neutralizing antibodies could have on capsid-displayed antigens. Our studies demonstrate that pre-existing immunity does not negatively impact the Pfs25-specific antibody response but rather has no effect on this response. We still hypothesize that the pre-existing Ad-specific T cells may enhance the Pfs25-specific antibody response but additional studies must be done to determine if this is the case.

In some cases nAbs could support the antibody response upon immunization with viruses displaying capsid-encoded antigens. Phagocytic antigen-presenting cells could take up antibody-opsonized virus via their Fc receptors. In this case APCs could present capsid epitopes to T cells. Further, antibody-opsonized virus could be taken up and recognized by Trim21. While this pathway ultimately leads to the degradation of the virus, it also induces the production of IL-6, an important cytokine that supports the helper T cell response and, in turn, the antibody response.

Initially, researchers sought to determine how neutralizing antibodies affected transgene expression to determine how nAbs would affect Ad-based vectors for gene therapy. Gene therapy vectors require persistent expression of the transgene to be effective. Vaccine vectors require robust transgene expression but PEI has variable effects on the transgene-specific immune response. Transgene expression is reduced in the presence of PEI and overall studies show that, as a result, cytotoxic T cell responses are attenuated. It is less clear how PEI influences the antibody response to a vaccine antigen encoded as a transgene (Nayak and Herzog ; Ahi, Bangari et al. 2011). Ad

vaccine vectors have been traditionally explored to elicit a CTL-based immune response, however studies demonstrate that Ad5 generates a mixed T_H1/ T_H2 response and should therefore be considered for vaccines to support the humoral immune response. Our studies show that Ad-based vectors generate a robust antibody response to Pfs25 and that this response is a great improvement over the antibody response elicited following alum-adjuvanted protein immunization. We still need to assess how PEI affects the Pfs25-specific antibody response following Ad5-pfs25 immunization. If nAbs are extremely detrimental in the context of Ad5-pfs25 vaccination then these studies will serve as a model to understand the effectiveness of Ad-based vectors expressing a membrane-bound Pfs25. Using what we have learned from these studies about these novel vaccines, we can generate Pfs25-based vaccines in other adenovirus serotypes for which there is less seroprevalence and thus very little pre-existing immunity.

For transmission blocking vaccines to be effective they must elicit a high titer of Pfs25-specific antibodies. In humans if PEI is going to decrease pfs25 transgene expression our TBVs could be improved to elicit optimal pfs25 expression. To do so, we could generate hybrid Ad5-pfs25 vaccines with the capsid modifications from our Ad-HVR-pfs25 vaccines. We found that Ad-HVR1-DIII evaded nAbs to Ad5. In this way, we could still recruit Ad5-specific T cell help but avoid neutralizing antibodies to Ad5. Another way to further improve our Ad-based TBVs for use in humans would be to prepare these vaccines for intranasal (I.N.) administration. Studies would have to be performed to assess whether I.N. vaccination is as effective as the intramuscular route.

An I.N. administered would be easier and safer to administer since it would not require a needle or necessarily a health professional to vaccinate an individual.

Conclusions

Our study explores how Ad5-based vectors can be employed to enhance the humoral immune response to a target vaccine antigen. Ad is known to potently activate both the innate and adaptive immune responses. We have characterized some of the ways in which replication-defective Ad vectors activate the innate and the adaptive immune system. These findings contribute to our overall understanding of how Ad activates these two arms of the immune system. We have found that Ad5 elicits ROS by inducing mitochondrial membrane damage, a process that is dependent on endosomal membrane rupture and cathepsin release. Mitochondrial dysfunction is now recognized as a common signal involved in activating the pattern recognition receptor, the NLRP3 inflammasome. Additionally, we found that Ad-induced ROS is a key signal in the proinflammatory response to Ad. This ROS-dependent inflammatory response likely contributes to the adaptive immune response by supporting DC maturation and activation, lymphocyte recruitment to the site of vaccination, and cytokine production that influence B and T cell proliferation and differentiation. A role for ROS in activating the adaptive response still must be explored. We generated several novel Ad5-based vaccines to prevent transmission of malaria by *Plasmodium falciparum*. We found that Ad5-pfs25, a vector expressing the transmission blocking antigen, Pfs25, generated a robust Pfs25-specific antibody response characterized by a higher titer, relative affinity and broader IgG

subclass switching as compared to alum-adjuvanted Pfs25 protein vaccination. We found that Ad5-specific T cell activation correlated with the observed increase in Pfs25-specific antibody titer following As5-pfs25 vaccination. We also designed Ad-based vectors that display relevant, transmission blocking Pfs25 epitopes on its surface within the major capsid protein, Hexon. Boost vaccination with these vectors increases the Pfs25-specific antibody titer and further increased the relative affinities of Pfs25-specific antibodies as compared to homologous prime-boost with Pfs25-alum. Taken together, our data demonstrate that Ad-based vectors can enhance the humoral immune response to target antigens and likely does so by enhancing T cell activation. These novel transmission-blocking vaccines can be further investigated as they achieve a more robust Pfs25-specific antibody response with fewer vaccinations than the antibody response elicited by an alum-adjuvanted Pfs25 protein vaccination. Importantly, these novel Ad5-based TBVs block transmission of *P. falciparum* to the mosquito vector while the Pfs25-alum vaccine does not. Therefore, we have generated and characterized novel Ad5-based TBVs for malaria that may be more effective than the currently explored protein-based vaccines.

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VITA

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During graduate school, Kathleen found time to be with her family, participate in a variety of city and park district sport teams and to cheer on her beloved Blackhawks. She also shares parenting a rambunctious two-year-old Boston terrier named Lord Stanley after the Stanley Cup's namesake. After experiencing two Stanley cup victories in Chicago, Katie is moving to her dog Stanley's hometown of Boston, Massachusetts where she will be working as a Postdoctoral Fellow in Dr. Shannon Turley's lab at Harvard University Medical School. While she may live in Boston, she will never forsake her Blackhawks in exchange for the Bruins.