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Immunobiology of Adenovirus-Vector Vaccines for MRSA

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IMMUNOBIOLOGY OF ADENOVIRUS-VECTOR VACCINES FOR MRSA

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Staphylococcus aureus is a gram-positive, extracellular bacterium that has emerged as an important human pathogen. This bacterium is a leading cause of skin and soft tissue infections (SSTIs) in humans, often leading to invasive and life-threatening infections. Treatment of *S. aureus* infections is becoming more complicated due to the rise of methicillin-resistant *S. aureus* (MRSA) strains, which are becoming increasingly resistant to a number of antibiotics. In the United States, invasive MRSA infections result in more deaths annually than any other infectious agent.

Despite a dire need, there is currently no vaccine against *S. aureus* infections. The failure of past vaccine candidates may be due to a lack of understanding of immune correlates of protection and how to obtain them through vaccination. Additionally, attempts to develop vaccines have, up to this point, focused solely on eliciting high titer antibody responses to antigens. Accumulating evidence from human patients and *in vivo* studies suggest that $CD4^+$ T cells are important mediators of protection from *S. aureus* infections.

T_H17 cells, and their namesake cytokine IL-17, are involved in protection against cutaneous infections. T_H17 cells and IL-17 are important for neutrophil recruitment to sites of infection, which is required for clearance of *S. aureus*, as well as antimicrobial peptide (AMP) production from epithelial cells. Therefore, a protective vaccine will likely require a strong T_H17 response to *S. aureus* antigen. Data also implicates T_H1 cells and the associated cytokine IFNγ as important mediators of protection against *S. aureus*, especially during invasive infection. Based

on these data, the most effective vaccine will likely be one that can elicit strong T_H17 and T_H1 responses against vaccine antigens.

My goal was to develop an adenovirus (Ad) vector based vaccine that could provide protection from cutaneous MRSA infections. Ad is an attractive vaccine vector due to its potent T cell adjuvant capabilities and high level and duration of transgene expression. As T cells have been shown to be important for immunity to *S. aureus*, I hypothesized that Ad vectors expressing domains from MRSA surface proteins as transgenes would elicit more potent T cell responses to these antigens than protein immunization and would provide protection from cutaneous infection. I generated Ad vectors expressing MRSA antigens and assessed the immune response induced by vaccination. I also assessed the ability of these Ad vectors to provide protection against MRSA infection using a mouse model of cutaneous infection.

CHAPTER ONE

INTRODUCTION

Staphylococcus aureus

Staphylococcus aureus is a gram positive, cocci shaped extracellular bacterium. It is transmitted mainly through direct skin to skin contact, however transmission from fomites can also occur (Desai et al. 2011). While it is typically a commensal bacterium present on the skin and mucous membranes of healthy individuals, *S. aureus* has the potential to cause superficial and invasive infections that can be life threatening. It is estimated that *S. aureus* colonizes the nares of more than 30% of the human population (Krismer et al. 2017). Certain populations, including health care workers, hospitalized patients, and needle users have increased colonization rates. Importantly, nasal carriers of *S. aureus* have a higher risk of bloodstream infection, and these infections are typically caused by the colonizing strain.

While *S. aureus* does not normally cause infection in healthy skin it can cause serious infections following skin damage, or if allowed to enter the bloodstream. In this way, commensal bacteria can become pathogenic following tissue damage. *S. aureus* is the leading cause of skin and soft tissue infections (SSTIs) which include impetigo, cellulitis, subcutaneous abscesses, and infected ulcers and wounds. These SSTIs are the greatest contributors to *S. aureus* morbidity and are often chronic and frequently recurrent (Brown et al. 2014). Furthermore, SSTIs may lead to invasive, life-threatening infections.

In addition to SSTIs, *S. aureus* can cause invasive infections including bacteremia, abscesses, pneumonia, meningitis, endocarditis and sepsis. These invasive infections are associated with significant morbidity and mortality, contributing to more deaths annually in the United States than HIV/AIDS, viral hepatitis, and influenza combined (Boucher and Corey 2008). Treatment of these infections has become complicated due to the emergence of methicillin-resistant *S. aureus* (MRSA) strains, which are becoming resistant to a growing number of antibiotics.

The defining characteristic of MRSA is the presence of the staphylococcal cassette chromosome (SCC) *mec*, a mobile genetic element (MGE) that confers broad spectrum beta-lactam resistance (Ito et al. 2014). MRSA strains can be divided into either hospital associated (HA) or community associated (CA) strains. These two groups tend to present different clinical features and have differing susceptibility to antibiotics. Hospital associated (HA) MRSA strains cause infections in hospitalized patients and are a leading cause of hospital acquired infections worldwide. Community associated (CA) MRSA stains cause infection of otherwise healthy individuals. The USA300 strain is the most prevalent CA-MRSA strain in the United States and is commonly associated with skin and soft tissue infections (DeLeo et al. 2010).

In addition to antibiotic resistance, *S. aureus* pathogenesis depends on a number of enzymes, toxins and surface proteins, many of which are encoded for on MGEs. Secreted enzymes such as coagulase, staphylokinase, and proteases help the bacteria evade host defenses and invade new tissues. During infection *S. aureus* also secretes hemolysins and leukotoxins. These toxins lyse red and white blood cells, respectively, by forming pores in the cell membrane leading to necrotic cell death (Kong, Neoh, and Nathan 2016). *S. aureus* enterotoxins, including toxic shock syndrome toxin 1 (TSST-1), are superantigens that non-specifically bind to MHCII

and stimulate T cell activation and proliferation. As a result, these toxins interfere with intestinal functioning, causing vomiting and diarrhea (Otto 2014). Some *S. aureus* strains also produce serine proteases, known as exfoliative toxins, that cause detachment of the epidermis leading to blistering of the skin and secondary infections (Kong, Neoh, and Nathan 2016).

S. aureus surface adhesion proteins are important virulence factors that facilitate adhesion, tissue invasion and evasion of host defenses. Adherence to the extracellular matrix (ECM) and host cells is mainly mediated by proteins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMM). The defining feature of these proteins are multiple IgG-like folded domains within the ligand binding region. MSCRAMM proteins are anchored to the cell wall peptidoglycan by a peptidoglycan binding motif (LPXTG) as well as wall and membrane spanning regions on their C-terminal end. The N-terminal portion contains the signal sequence and the ligand binding region. (Foster and Hook 1998)

The MSCRAMM family consists of clumping factor (Clf) and serine-aspartate repeat (Sdr) proteins, fibronectin-binding proteins (FnBPs), and collagen adhesin (Cna) proteins. ClfA, the archetypal MSCRAMM protein, binds fibrinogen which then mediates attachment to host cells through its interaction with αv□3 integrins (McDonnell et al. 2016). Alternatively, ClfA can use self-secreted von Willebrand factor binding protein to adhere to host cells (Claes et al. 2017). These interactions allow for *S. aureus* adhesion to endothelial cells, an important step in the development of *S. aureus* associated endocarditis (Josse, Laurent, and Diot 2017). During sepsis fibrinogen binding induces platelet aggregation, abscess formation and the development of thromboembolic lesions (McAdow et al. 2011).

Other representative MSCRAMM proteins include SdrD and FnBPA. SdrD binds to Desmoglein 1 on keratinocytes and nasal epithelial cells (Josse, Laurent, and Diot 2017). SdrD is

important for nasal colonization and has been shown to support bacterial survival *in vivo* (Askarian et al. 2016). Fibronectin binding proteins (FnBP), including FnBPA, promote adhesion to the ECM and invasion of new tissues by binding fibronectin. Fibronectin acts to form a bridge between bacteria and host cells through its interaction with α5□1 integrins (Fowler et al. 2003). FnBP-Fibronectin- α5□1 integrin interactions can also induce internalization of the bacteria through the FAK-Src pathway (Josse, Laurent, and Diot 2017). Internalization may support infection by promoting bacterial dissemination and by shielding *S. aureus* from detection by the immune system.

The MSCRAMM proteins ClfA and SdrE both contribute to immune evasion by suppressing complement activation. ClfA binds complement regulator factor I (fI) which cleaves the complement protein C3b into its inactive form, leading to decreased phagocytosis of the bacteria (Hair et al. 2010). In a similar manner, SdrE binds to factor H (fH) which acts as a cofactor for fI and also accelerates the decay of the alternative complement pathway C3 convertase, C3bBb (Sharp et al. 2012). SdrE mediated suppression of complement results in reduced bacterial killing by PMNs, thus promoting infection.

Immune Response to *S. aureus* Infection

Innate immune sensing of bacterial components is a critical first step in mounting an immune response to *S. aureus*. Initial innate responses to bacterial components induce the production of proinflammatory cytokines and chemokines including TNF-α, IL-1□, IL-12, IL-8, GM-CSF, CCL2, and CCL3 (Fournier and Philpott 2005). These proinflammatory factors are produced by cells of the innate immune system, as well as epithelial and endothelial cells. The induction of rapid inflammatory responses by epithelial cells is particularly important, as

invasive *S. aureus* infections (including sepsis) often develop from skin and soft tissue infections.

During infection lipopeptides, including lipoteichoic acid (LTA), present on the surface of extracellular bacterial are recognized by heterodimers of TLR2 and TLR1 or TLR6 (Krishna and Miller 2012). These interactions, along with engagement of co-receptors CD14 and CD36, activate signaling through MYD88 which leads to activation of NFκB and the production of proinflammatory cytokines and chemokines (Krishna and Miller 2012). Following internalization of *S. aureus* or release of bacterial components from the phagolysosome the intracellular sensor NOD2 senses muramyl dipeptide, a breakdown product of *S. aureus* peptidoglycan. NOD2 signaling further activates NFκB dependent expression of proinflammatory cytokines and chemokines, including pro-IL-1□. Pore formation, caused by hemolysins or activation of the purinergic receptor P2X7, or phagosome rupture allow for muramyl dipeptide mediated activation of the NLRP3 inflammasome. The NLRP3 inflammasome is then able to cleave pro-IL-1□ into its mature form (Munoz-Planillo et al. 2009).

In addition to TLR and NLR recognition of pathogen associated molecular patterns (PAMPs), it has also been shown that *S. aureus* protein A binds to TNF-α receptor 1 (TNFR1) on innate immune cells and epithelial cells. This interaction induces activation of NFκB and downstream production of proinflammatory cytokines including IL-8, as well as recruitment of PMNs (Gomez et al. 2004). Additionally, keratinocytes contain pre-made stores of IL-1α that are released in response to tissue damage and infection. Both IL-1α and IL-1□ bind to the IL-1R and induce production of neutrophil attracting chemokines such as CXCL1, CXCL2, and CXCL8 (Olaru and Jensen 2010).

Neutrophils are rapidly recruited to sites of *S. aureus* infection and are an essential part of the host defense against this pathogen. This is evidenced by the fact that patients with defects in neutrophil number or function (e.g. congenital neutropenia or myeloperoxidase deficiency) have increased susceptibility to *S. aureus* infections (Andrews and Sullivan 2003; Lakshman and Finn 2001). Recognition of bacterial PAMPs by pattern recognition receptors (PRRs) leads to increased expression of adhesion molecules on neutrophils and endothelial cells, as well as production of neutrophil attracting chemokines, which facilitate neutrophil diapedesis and entry into infected tissue (Ley et al. 2007). Once recruited to the sites of infection, neutrophils help to control infection though the formation of abscesses. Upon encountering *S. aureus* within abscesses neutrophils use a variety of mechanisms to mediate bacterial killing.

Following engagement of Fc or complement receptors neutrophils phagocytose opsonized bacteria. Inside the phagosome oxidative burst generates reactive oxygen species which can kill bacteria. Furthermore, antimicrobial peptides, proteinases, and acid hydrolases present within the phagosome have direct antimicrobial effects. Neutrophils also release neutrophil extracellular traps (NETs), containing antimicrobial peptides and proteases, that trap and kill extracellular bacteria (Pilsczek et al. 2010).

Accumulating evidence suggests that macrophages play an important role in the immune response to *S. aureus* infections, and that M1 macrophages are particularly important for bacterial clearance (Accarias et al. 2015; Pozzi et al. 2015). In mice a predominately M2 response is associated with greater susceptibility to *S. aureus* infection whereas inoculation with M1 cells rescues abscess formation and helps prevent bacterial spread (Pozzi et al. 2015). In contrast to the anti-inflammatory M2 macrophage subset, M1 macrophages secrete

proinflammatory cytokines that recruit neutrophils, activate antimicrobial mechanisms and promote polarization of T_H1 and T_H17 cells.

Bacterial proteins located in the cytoplasm or phagosomal compartments of antigen presenting cells (APCs) can be processed and presented to naïve T cells via MHCII, inducing differentiation into effector and memory T cell populations. Both mouse and human studies have shown that effector and memory T cells are induced by *S. aureus*, particularly T_H1 and T_H17 subsets (Broker, Mrochen, and Peton 2016). Furthermore, the susceptibility of patients with T_H17 or other $CD4^+$ T cell deficiencies to *S. aureus* infections highlights the importance of these cell types in the immune response against this pathogen. In addition to $CD4^+$ T cells, memory $CD8^+$ and γδ T cell subsets are induced by *S. aureus* infection (Broker, Mrochen, and Peton 2016; Murphy et al. 2014).

In models of systemic infection T_H1 produced IFNγ has been shown to promote neutrophil recruitment and provide protection against *S. aureus* infection by enhancing the activation and bactericidal activities of phagocytic cells (Krishna and Miller 2012). Additionally, in mouse models of systemic infection IFNγ production from T_H1 cells was shown to be required for vaccine mediated protection (Lin et al. 2009). While T_H1 cells are thought to be important for protection against sepsis, data suggests that IL-17 and T_H17 cells also play an important role in defending against *S. aureus* infections, particularly during cutaneous infections.

T_H17, as well as γδ T cells, natural killer (NK), and NKT cells produce IL-17 which binds to keratinocytes and innate immune cells to induce expression of granulopoiesis factors and chemokines. These molecules promote maturation and recruitment of neutrophils to the sites of infection. IL-17 also induces neutrophil and keratinocyte production of antimicrobial peptides (AMPs), which have bactericidal activities that are important for clearance of infection. Along

with IL-17, T_H17 and γδ T cells also produce IL-22. IL-22 activates keratinocytes to produce AMPs and proliferate, playing an important role in bacterial clearance and wound healing during infection. (Figure 1)

T cells also support B cell activation and IgG class switching during infection, as evidenced by the fact that immune-competent individuals generate high titers of class switched anti-*S. aureus* antibodies during infection (Broker, Holtfreter, and Bekeredjian-Ding 2014; Kolata et al. 2011). The majority of anti-*S. aureus* antibodies generated during human infections are directed against toxins and cell surface components. During infection, these antibodies can bind to *S. aureus* toxins and prevent their harmful effects. Additionally, antibody binding to the bacterial surface allows for the opsonization of bacteria by phagocytic cells or activation of the classical complement pathway. Unfortunately, *S. aureus* has evolved a number of mechanisms to evade the host antibody response and therefore the generation of *S. aureus* specific antibodies does not reliably provide protection against infection. It should be noted that while an antibody response may not be sufficient to prevent infection, several studies have shown the importance of anti-toxin antibodies in limiting disease severity and tissue damage (Broker, Holtfreter, and Bekeredjian-Ding 2014).

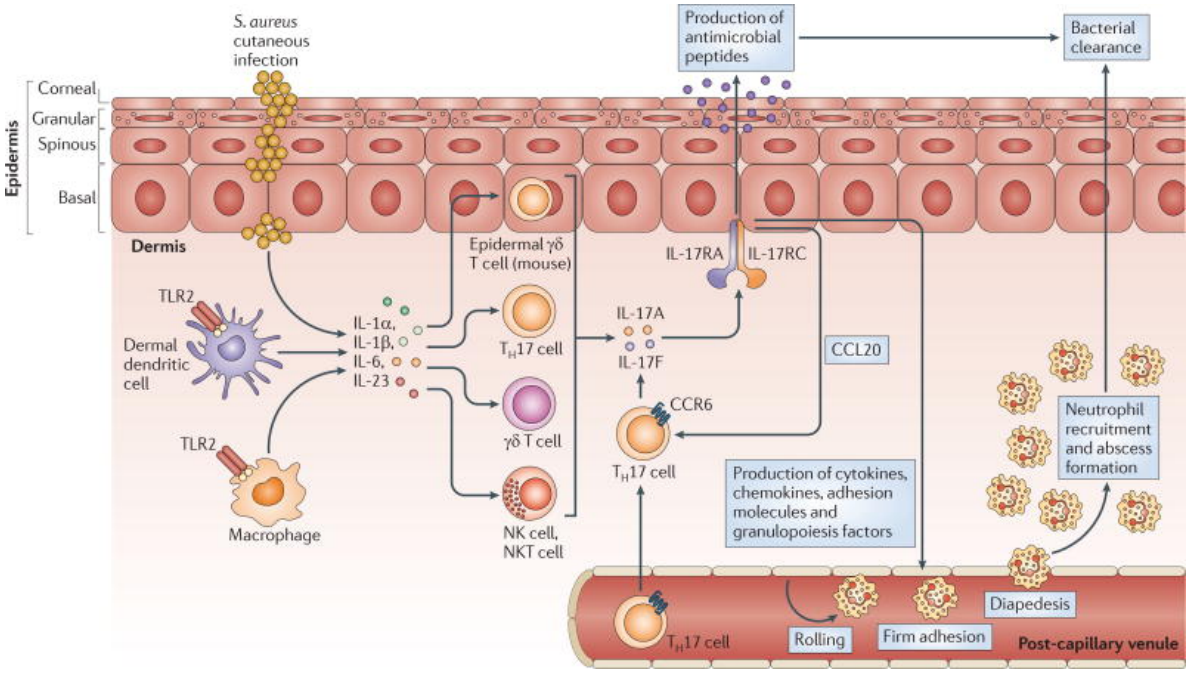


Figure 1. The Cutaneous Immune Response Against *S. aureus*. During cutaneous *S. aureus* infection, bacterial components activate TLR2 and other PRRs on keratinocytes and APCs in the epidermis and dermis. This activation leads to the production of cytokines such as IL-1α, IL-1□, IL-6 and IL-23, which stimulate the production of IL-17 by T_H17 cells, γδ T cells, NK cells, and NKT cells. IL-17 binds to IL-17 receptors (IL-17R) on keratinocytes and innate immune cells to induce expression of CCL20, granulopoesis factors (G-CSF and GM-CSF) and chemokines such as CXCL1, CXCL2, CXCL5, and CXCL8, which promote maturation and recruitment of neutrophils and T_H17 cells. Once recruited to the site of infection, neutrophils phagocytose opsonized bacteria and mediate bacterial killing. Additionally, IL-17 and IL-22 induce the production of AMPs from keratinocytes and neutrophils which mediate bacterial killing. IL-22 also induces keratinocyte proliferation and is therefore important in wound healing during infection. Taken from (Miller and Cho 2011).

Current *S. aureus* Vaccine Approaches

Due to the severe morbidity and mortality associated with *S. aureus* infections, and the rise of antibiotic resistance strains, developing a vaccine against *S. aureus* is of the utmost importance. To date several vaccine candidates have been developed, however none have demonstrated efficacy in late stage clinical trials (Giersing et al. 2016). Ideally, a vaccine would provide sterilizing protection from *S. aureus* however that may be an overly ambitious goal. A

more realistic goal would be to develop a vaccine that can reduce the severity of infection, which would significantly reduce the mortality and cost associated with *S. aureus* infections. While prior infection does not provide protection against recurrent *S. aureus* infection, human carriers do tend to develop less severe infections than noncarriers which suggests that carriers are able to develop some immunity to *S. aureus* (Giersing et al. 2016).

Several challenges have hindered the development of an effective *S. aureus* vaccine to date. Compared to other bacteria for which vaccines have been developed, *S. aureus* is a commensal organism in humans and therefore has evolved numerous defenses and evasion strategies against the host immune response in order to maintain persistent colonization. Antigenic variation between strains, as well as the numerous invasion and colonization mechanisms used by the bacteria have also made it challenging to create an effective vaccine. Finally, a lack of understanding of correlates of protection have impeded the development of vaccines that induce protective immunity.

In the past, *S. aureus* immunization strategies have been aimed at generating high titers of antibodies against a single surface antigen or virulence factor (Proctor 2015). While this strategy has proven effective for vaccines against other bacteria it has not been effective for *S.* aureus, likely due to the many redundant virulence factors and surface proteins encoded by the bacteria. Two subunit vaccines, StaphVAX and V710, underwent phase III testing but neither showed consistent efficacy in humans. StaphVAX, a polysaccharide and protein-conjugated vaccine directed against two *S. aureus* capsular polysaccharides (CP), failed to provide protection from bacteremia in hemodialysis patients (Fattom et al. 2015). A phase III clinical trial for the subunit vaccine candidate V710, which directs an immune response against the cell-wall anchored iron scavenger protein IsdB, was terminated when it was discovered that vaccination induced

significantly greater mortality and other adverse events compared to the placebo (Fowler et al. 2013).

Analysis of the failed efficacy trails led researchers to hypothesize that a multi-antigen approach targeting virulence factors, toxins and adhesion proteins would prove more effective. The most notable candidate is Pfizer's SA4Ag vaccine, composed of CP5, CP8, and recombinant ClfA and manganese transporter MntC. Results from phase I studies have shown that vaccination induces high titers of functional antibodies against vaccine antigens (determined by opsonophagocytic activity assays) (Frenck et al. 2017). A phase II study is currently underway to evaluate the safety and efficacy of the vaccine in patients undergoing spinal fusion surgery. A number of other multi-antigen subunit vaccine candidates are being evaluated in pre-clinical trials (Giersing et al. 2016). Additionally, two monoclonal antibody based (mAb) vaccine candidates are currently undergoing clinical trials, however previous studies using mAb have not shown efficacy in humans (Bagnoli 2017; Ruzin et al. 2018).

All vaccine candidates to date have been designed to generate an antibody response against *S. aureus* antigens, however there is a growing consensus that T-cell immunity is important for vaccine mediated protection. Observations from individuals with deficiencies in humoral immunity, such as agammaglobulinemia, show that they are not at increased risk for *S. aureus* infections (Proctor 2012). In contrast, patients with T_H17 cell or neutrophil deficiencies are more prone to *S. aureus* infections. Individuals with $CD4^+$ T cell deficiencies (such as HIV/AIDS patients) or individuals with defective IFNγ production are also more susceptible to infection, highlighting the importance of cell mediated immunity in protection.

These observations support recent findings from animal studies that show T_H17 and other T cell subsets are important mediators of protection against *S. aureus* infection. T_H17 cells

produce IL-17, which has been shown to be critical for protecting mice and humans from *S. aureus* skin infections (Cho et al. 2010; Puel et al. 2011). Mice with defects in either T_H17 cells or IL-17 production are more susceptible to *S. aureus*, and both have been shown to be required for vaccine mediated protection in mouse models (Lin et al. 2009). The same study showed that T_H1 cells and T_H1 produced IFNγ were also required for vaccine mediated protection. *S. aureus* infections in IL-17 receptor or γδ T cell deficient mice are more severe, with deficient mice exhibiting larger abscess sizes and increased bacterial burden in the wound (Fischer 2008). Given that γδ T cells are a major producer of IL-17 in the skin, these findings further highlight the importance of IL-17 signaling in immunity against *S. aureus*.

Mounting evidence points to T cells and cell mediated immunity as the mediators of protection against *S. aureus* infections, therefore a successful vaccine will likely need to induce potent T cell responses to vaccine antigens. Recently the vaccine candidate NDV-3 has been shown to induce protective T_H17 and T_H1 responses against *S. aureus*. NDV-3 is composed of the recombinant Als3 antigen from *Candida albicans*, which shares structural similarity with *S. aureus* clumping factors, formulated with alum in PBS. NDV-3 was shown to provide protection against sepsis in mice and has completed phase I clinical trials demonstrating safety and tolerability in humans (Yeaman et al. 2014). While this vaccine candidate holds promise, it is formulated with Alum which characteristically induces a predominantly T_H2 response. Given the importance of T_H17 and T_H1 cells in vaccine mediated protection, different adjuvant strategies that more potently induce these T cell subsets may be more effective and should be considered for future vaccine development.

Vaccines and Common Adjuvants

Vaccines represent one of the single greatest public health measures in human history. It is estimated that vaccines prevent almost 6 million deaths annually (Andre et al. 2008). Targeted vaccination strategies have led to the eradication of smallpox and rinderpest, and diseases such as polio and measles are close to eradication (Gardner et al. 2018; Orenstein et al. 2018; Simonsen and Snowden 2018). Despite the past success of vaccines, there is still much work to be done in creating effective vaccines against HIV, malaria, *S. aureus*, and other pathogens.

Vaccines work by providing an initial, controlled, exposure to an infectious agent. This allows the host immune system to develop a protective immune response against the pathogen. The development of a protective immune response ultimately depends on the creation of effective adaptive immune responses, which provide immunological memory after the initial exposure. Traditional vaccine approaches have utilized either live attenuated or killed versions of the pathogen. The advent of molecular biology and recombinant DNA technology has allowed for the development of subunit vaccines, which involve immunization with a component of the disease-causing agent rather than the pathogen itself. Subunit vaccines are considered to be safer than vaccines using live attenuated pathogens because they eliminate the risk of reversion (Moyle and Toth 2013). A major problem with subunit vaccines is that they are not as immunostimulatory as live attenuated or killed versions, and therefore require the use of an adjuvant to elicit a robust and effective immune response (Purcell, McCluskey, and Rossjohn 2007).

An adjuvant is something that is administered as part of a vaccine in order to enhance the immunogenicity of the vaccine. Adjuvant types include insoluble salts, emulsion based adjuvants, and PAMPs. Aluminum salts, or Alum, was the first identified adjuvant and is

currently used in a number of vaccines, including the diphtheria and tetanus toxins and pertussis vaccine and vaccines for hepatitis A and B (McKee and Marrack 2017). The adjuvant effects of Alum are characterized by a T_H2 and T follicular helper (TF_H) cell response and a predominantly IgG1 isotype response (McKee and Marrack 2017).

Alum binds to protein antigen and increases it's stability and retention at injection sites, facilitating a slow release of the antigen (Oleszycka and Lavelle 2014). In addition, alum increases activation of APCs which then present antigen to T_H cells in the draining lymph nodes (dLNs) (Kool et al. 2008). These activated T_H cells are then able to support B cell activation leading to high antibody titers against the vaccine antigen. Interactions between alum and lipids on the membrane of dendritic cells (DCs), as well as alum induced release of uric acid crystals and dsDNA, have been shown to induce signaling via the Syk-PI3 kinase pathway (Kool et al. 2008; Marichal et al. 2011). Activation of the Syk-PI3 kinase pathway promotes a T_H2 response, while inhibiting IL-12 production and a T_H1 response (Mori et al. 2012). Alum has also been shown to activate the NLRP3 inflammasome, however there is conflicting data regarding the role of this activation in the adjuvanticity of alum.

The first emulsion based adjuvant, Freund's Complete Adjuvant (FCA) was developed in the 1930s. FCA consists of a water-in mineral oil emulsion containing *Mycobacterium tuberculosis*. Despite its ability to generate strong antibody and cellular immune responses FCA is not approved for use in humans due to its propensity to cause necrotic abscesses. Incomplete Freund's Adjuvant, which lacks *Mycobacterium tuberculosis*, has been used in vaccine development and has been shown to enhance cellular immunity and generate enhanced IgG1/IgG2a antibody responses (Shibaki and Katz 2002). Squalene based emulsions, such as MF59 and AS03, are currently approved for use in humans and induce both humoral and cellular

immune responses. It is thought that cell injury and damage signals induced by squalene based adjuvants activate innate sensors leading to MyD-88 activation (Vono et al. 2013).

As scientists have gained increased understanding of the immune response to various PAMPs the use of PAMPs as adjuvants has allowed for the development of more efficient and effective vaccines. PAMPs that have been utilized as adjuvants include monophosphoryl lipid A (a derivative of LPS) and the synthetic TLR4 agonist glucopyranosyl lipid A (Pantel et al. 2012; Thompson et al. 2005). Unmethylated CpG motifs and small molecule TLR7/8 agonists are also being investigated as potential vaccine adjuvants due to their ability to activate TL9 or TLR7/8, respectively (Scheiermann and Klinman 2014; Smith et al. 2016).

Another PAMP that is being increasingly used as an adjuvant is flagellin. Flagellin (FliC) is a component of the bacterial protein flagellum. FliC is an ideal adjuvant because it potently activates both the innate and adaptive arms of immunity. Furthermore, the plasticity of flagellin allows for the insertion of peptide sequences to create fusion proteins of FliC and vaccine antigens. The adjuvant effects of FliC are observed both when it is coadministered with vaccine antigens and when it is used to create fusion proteins.

One mechanism through which FliC activates the immune response is through TLR5 signaling. The N and C terminal domains of FliC are recognized by TLR5 leading to activation of NFκB (MyD88 dependent) and IRF3 (MyD88 independent) (Lu and Sun 2012; Mizel et al. 2003; Vijay-Kumar et al. 2008). NFκB activation leads to the production of proinflammatory cytokines, additionally IRF3 activation leads to production of IFN-□. In addition to activating TLR5, the 35 C-terminal amino acids of FliC activate the NLRC4 inflammasome, resulting in the cleave of pro-IL-1□ and pro-IL-18 into their mature form which can then be secreted from the cell (Lightfield et al. 2008; Miao et al. 2006). *In vitro* studies have shown that administration

of flagellin or flagellin-based vaccines increase antigen accumulation in the dLN and activation of DCs, T cells and B cells (Mizel and Bates 2010). Flagellin also promotes class switching in B cells (Bennett et al. 2015).

Viral vectors have also been used as adjuvants in vaccine development. Adenovirus vectors have been used as vaccine vectors due to their ability to potently activate both innate and adaptive immune responses. In addition, these vectors are capable of carrying large transgenes and can also displaying antigen on the viral capsid. Adenoviruses and their use as vaccine vectors will be discussed in the following sections.

Adenovirus

Adenoviruses (family *Adenoviridae*) are non-enveloped viruses whose genome consists of a 34-43kb linear segment of double stranded DNA. Currently, over 60 different types of human adenoviruses (Ad) have been identified and are grouped into species A-G (Jones et al. 2007). Traditionally human Ads have been grouped into species based on hemagglutination and serum neutralization, however more recently identified virus types (52-68) have been grouped based on genomic sequencing (Ghebremedhin 2014; Huang and Xu 2013). Adenovirus type 5 (Ad5), belonging to species C, was the focus of our experiments. In humans Ad causes a variety of respiratory, ocular and gastrointestinal infections. These infections, affecting mainly children, are typically self-limiting however more severe and even fatal infections can occur in immunocompromised patients (Bruno et al. 2003; Hoffman 2006).

The icosahedral viral capsid is ~90nm in diameter and consists primarily of 240 trimers of the hexon protein. At each of the capsids five-fold apices a penton base anchors a trimer of fiber proteins to the capsid. The outer surface of the capsid is also decorated with pIX, which helps to stabilize the viral capsid. Other minor proteins, including pIIIa, pVI and pVIII are also

associated with the capsid. The interior of the capsid contains the virion protease, the dsDNA molecule and five other polypeptides that are associated with the viral DNA (Figure 2).

Initiation of infection begins with binding of the fiber knob domain to a cellular receptor, typically the Coxsackie and Adenovirus receptor (CAR) or CD46 (Bergelson et al. 1997; Gaggar, Shayakhmetov, and Lieber 2003). Following initial binding to the cellular receptor, secondary interactions between the penton base and αv integrins facilitate internalization of the virus via clathrin-mediated endocytosis (Wickham et al. 1993). Following endocytosis partial disassembly of the capsid occurs as capsid proteins including penton base, fiber and pIIIa are released into the acidified endosome. This partial disassembly allows pVI to be released into the endosome where it induces endosomal lysis and allows for escape of the virus into the cytoplasm (Maier et al. 2010; Wiethoff et al. 2005).

Upon release into the cytoplasm, viral particles associate with microtubule motors and traffic along microtubules to the nucleus (Bremner et al. 2009). At the nucleus the partially uncoated virions dock at the nuclear pore complex (NPC) and the viral genome is released into the nucleus where replication occurs (Trotman et al. 2001). Replication of the viral genome is divided into two stages, early phase and late phase. Early genes encode for proteins involved in regulating expression of viral or host genes required for viral DNA replication, as well as genes involved in preventing premature cell death. Late phase genes encode for structural proteins and proteins involved in capsid assembly. Following DNA replication and capsid assembly the newly formed virions are released from the cell via virally induced cell lysis.

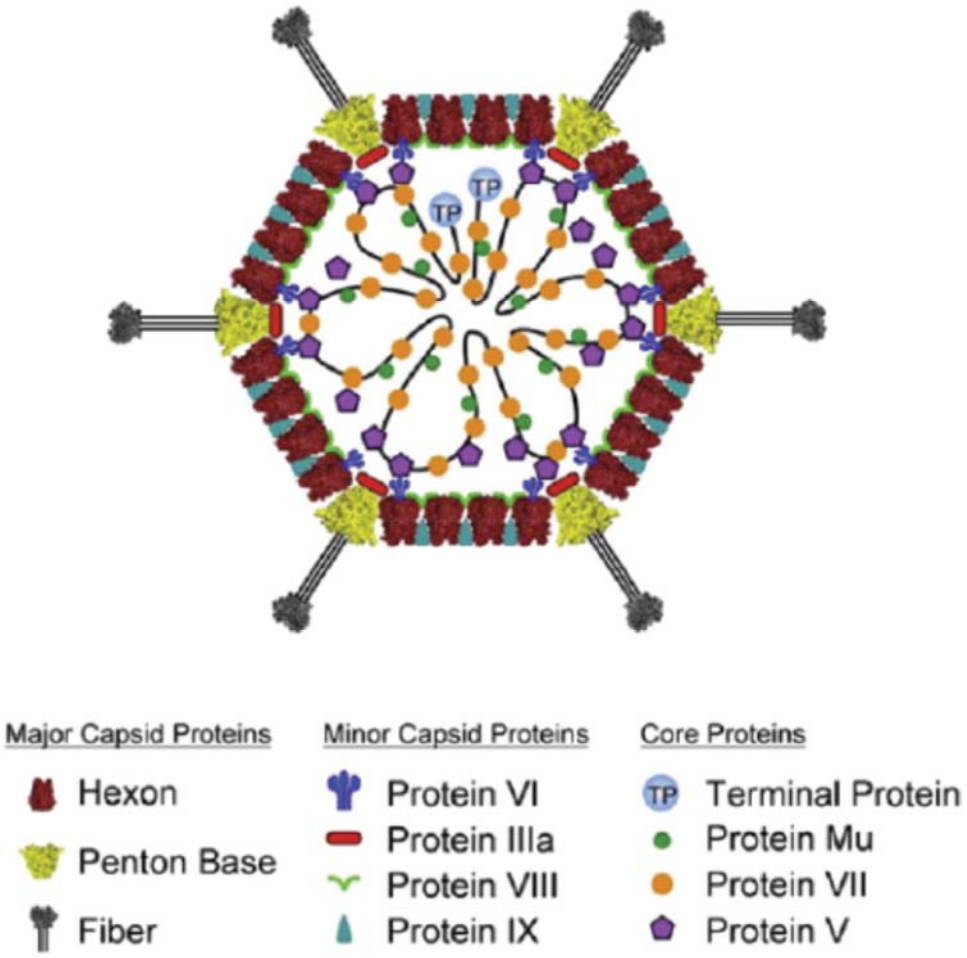


Figure 2. Diagram of the Adenovirus Capsid. The viral capsid is comprised mainly of hexon protein. At each vertex of the capsid a penton base anchors the fiber protein to the capsid. The minor proteins IIIa, VI, VIII, and IX make up the remainder of the capsid structure. The vial protease (not shown) and dsDNA molecule reside within the capsid. The core proteins V, VII, Mu, Terminal protein, and IVa2 (not shown) are associated with the viral DNA. Taken from (Thaci et al. 2011).

Innate Immune Response to Adenovirus

Upon infection Adenovirus is able to rapidly and potently activate the immune system, making Adenovirus an attractive vaccine vector. Innate sensing of Ad infection triggers the release of cytokines such as IL-6, TNF-α, and IL-1□ into the bloodstream, creating a

proinflammatory state (Mistchenko et al. 1994). This proinflammatory state is important for the recruitment of cytotoxic immune cells to the sites of infection. In addition, virally infected cells produce type 1 interferons which induce an antiviral state in neighboring cells (Randall and Goodbourn 2008). The initial innate immune response to Ad is also important for inducing a robust adaptive immune response.

Adenovirus in the bloodstream can interact with a variety of factors including components of the complement system as well as IgG and IgM antibodies. Both the classical and alternative complement pathways seem be involved in Ad induced complement activation (Tian et al. 2009). Naturally occurring IgM, as well as pre-existing IgG antibodies bound to Ad are able to mediate viral opsonization via the classical complement pathway (Cichon et al. 2001). In addition, complement protein C3 has been shown to directly bind to the Ad capsid, activating the alternative complement pathway (Jiang et al. 2004). Complement activation leads to a proteolytic cleavage cascade that results in the formation of the membrane attack complex, creating pores in the membrane and killing the cell or pathogen. In the liver IgM has been shown to mediate Ad clearance by Kupffer cells (Khare et al. 2013), however binding of coagulation factor X (FX) to Ad allows the virus to evade this complement mediated clearance (Xu et al. 2013).

The primary inflammatory response to Ad comes from tissue resident macrophages including alveolar macrophages and Kupffer cells. Cellular pattern recognition receptors (PRRs) detect viral components and rapidly induce expression of various cytokines and chemokines. Within 30 minutes following Ad infection alveolar macrophages express mRNA for proinflammatory cytokines TNF-α, IL-12, and IL-6 (Zsengeller et al. 2000). Similarly it has been shown that marginal zone macrophages begin transcribing IL-1α, IL-I□, TNF-α, and CXCL2 within 30 minutes following Ad infection (Di Paolo et al. 2009). The expression of these genes

was shown to be induced independent of TLR9 or IFN-IR signaling. This same study found that binding of Ad penton base to integrins present on the cell surface leads to expression of IL-1α and subsequent signaling through the IL-1R1 (Di Paolo et al. 2009). This signaling activates expression of polymorphonuclear (PMN) leukocyte chemokines CXCL1 and CXCL2 which are important for recruiting PMNs, such as neutrophils, to the sites of infection (Di Paolo et al. 2014).

Like macrophages, dendritic cells (DCs) produce proinflammatory cytokines, including IL-12, IL-1□ and TNF-α in response to Ad infection (Philpott et al. 2004; Zhang et al. 2001). However, compared to macrophages, DCs are much more efficient antigen presenting cells (APCs) and are therefore extremely important in directing the adaptive immune response to Ad infection. Recognition of Ad by PRRs stimulate DCs to produce proinflammatory cytokines and results in DC maturation and upregulation of costimulatory molecules (Hensley and Amalfitano 2007; Lore et al. 2007; Molinier-Frenkel et al. 2002). The cytokines produced by innate immune cells, in conjunction with antigen presentation via MHCII molecules direct the adaptive immune response to Ad (discussed in a later section).

During Ad infection type I IFNs, produced from infected cells, directly activate NK cells (Zhu, Huang, and Yang 2008). These activated NK cells are then able to perform effector functions including killing of virally infected cells and production of IFN-γ and TNF-α. These cytokines are important for recruitment and activation of immune cells. Furthermore, IFN-γ plays an important role in regulating the adaptive immune response to Ad infections. Neutrophils are also recruited to sites of Ad infection where they internalize opsonized virus and kill virally infected cells (Cotter, Zaiss, and Muruve 2005). As a result, they are responsible for the majority of early tissue injury during Ad infection.

Ad infection triggers TLRs, NLRs, and RLRs that contribute to the innate immune response to Ad. As mentioned above, interactions during Ad attachment result in production of IL-1α leading to IL-1R1 signaling. Additionally, pathogen associated molecular patterns (PAMPs) generated during adenovirus entry and replication are recognized by innate sensors.

Several TLRs are thought to recognize and respond to Ad infection. Both TLR2 and TLR4 on the cell surface have been shown to contribute to the innate immune response to Ad (Appledorn et al. 2008; Doronin et al. 2012). Following partial disassembly of the viral capsid in the late endosome, TLR9 senses viral dsDNA leading to MyD88 dependent activation of NF-κB. Activated NF-κB then induces upregulation of proinflammatory cytokines including IL-6 and IL-12 (Cerullo et al. 2007). TLR9 is the main point of Ad DNA recognition in plasmacytoid DCs and is important for generating a type I IFN antiviral response (Basner-Tschakarjan et al. 2006). Type I IFN responses in primary macrophages and conventional DCs are induced by detection of Ad DNA by TLR independent cytosolic sensors as well as by TLR9 (Nociari et al. 2007; Zhu, Huang, and Yang 2007).

Following endosomal escape, viral DNA in the cytosol can be detected by sensors such as DNA-dependent activator of IFN-regulatory factors (DAI), generating IRF3 and IRF7 mediated type I IFN responses (Fejer et al. 2008; Takaoka et al. 2007). Additionally, Ad can activate the Nod-like receptor NLRP3 (Barlan et al. 2011; Muruve et al. 2008). NLRP3, along with the adaptor protein ASC, recruits caspase-1 into a complex known as the NLRP3 inflammasome. Upon activation of this inflammasome complex caspase-1 processes pro-IL-1 and pro-IL-18 into their mature forms which can then be secreted. Virally associated RNAs (VA-RNAs), expressed early during Ad infection, can also be detected by innate sensors. RIG-I

recognizes these dsRNAs which activates production of type I IFNs (Yamaguchi et al. 2010). (Figure 3A)

Cytokines produced in response to innate sensing of Ad infection activate professional APCs and recruit immune cells to the site of infection. Therefore, not only are they important mediators of the innate immune response to Ad infection but they also help direct the adaptive immune response. IL-6 supports B cell proliferation and, along with type I interferons, promotes optimal B and T cell responses to antigen (Hirano et al. 1986; Tough, Borrow, and Sprent 1996; Vasconcellos et al. 1999). Additionally, IL-6 and IL-1□ support T_H17 differentiation (Veldhoen et al. 2006), whereas IL-12 and IL-18 produced in response to Ad infection help to support differentiation of naïve T cells into the T_H1 subtype (Dinarello 1999; Hsieh et al. 1993). (Figure 3B)

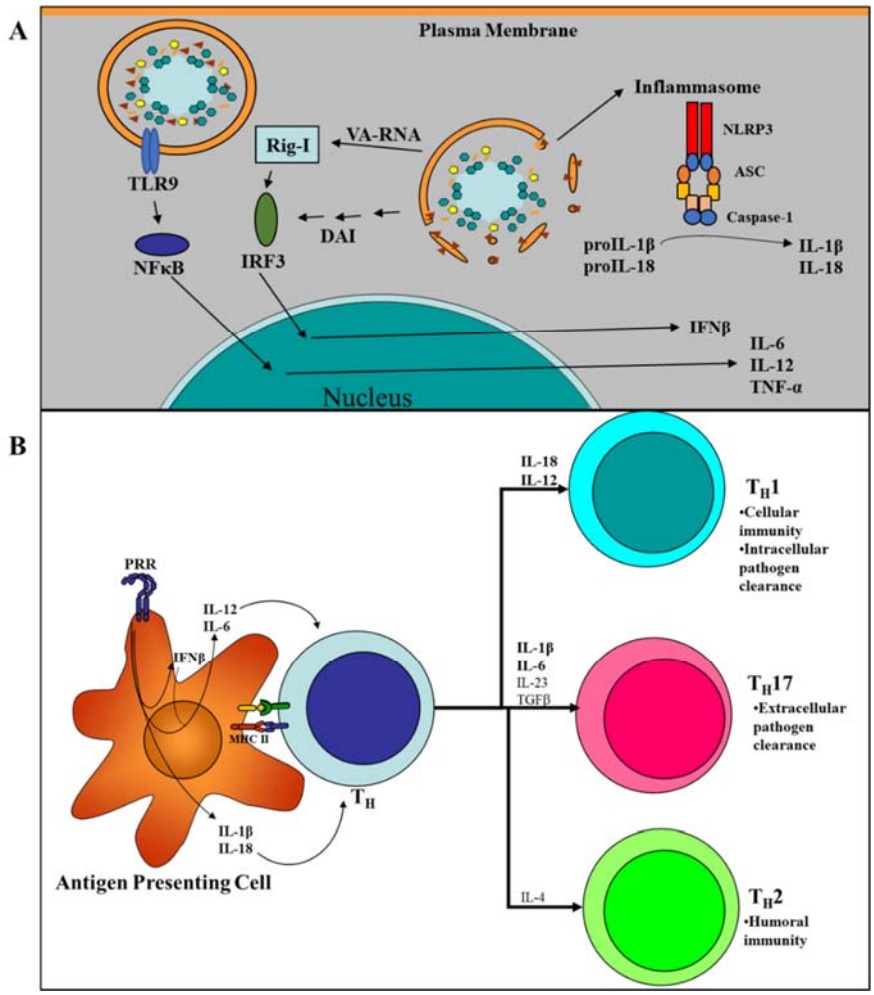


Figure 3. Immune Activation in Response to Adenovirus Infection. (A) Following partial disassembly of the viral capsid within the endosome, TLR9 senses viral dsDNA. TLR9 activates NFκB, leading to the expression of proinflammatory cytokines. Viral DNA in the cytosol is sensed by DNA-dependent activator of IFN-regulatory factors (DAI), which activates IRF3 and IRF7 to induce a type I interferon response. Additionally, virally associated RNAs (VA-RNAs) are detected by RIG-I which activates production of type I interferons. Ad DNA has also been shown to activate the NLRP3 inflammasome. Inflammasome activation leads to the proteolytic cleavage of pro-IL-1□ and pro-IL-18 into their mature forms. (B) In conjunction with antigen presentation via MHCII, cytokines produced in response to innate sensing of Ad infection direct the adaptive immune response to Ad. IL-12 and IL-18 produced in response to Ad support the differentiation of naïve $CD4^+$ T cells into the T_H1 subset. T_H1 cells secrete cytokines that support cell mediated immunity and clearance of intracellular pathogens. Differentiation of $CD4^+$ T cells into the T_H17 subset is supported by cytokines including IL-6 and IL-1□ which are produced in response to Ad infection. T_H17 cells are important for clearance of extracellular pathogens. IL-4 supports differentiation of T_H2 cells, which support humoral immunity.

Adaptive Immune Response to Adenovirus

During Adenovirus infection $CD8^+$ T cells play a primary role in the recognition and destruction of virally infected cells (Yang, Ertl, and Wilson 1994). Virally infected cells present viral antigen on MHCI molecules which can then be recognized by antigen specific cytotoxic T cells (CTLs). In order to become activated CTLs must encounter antigen in the context of MHCI along with a second costimulatory signal. The interaction between CD28 on $CD8^+$ T cells and CD80/86 on the target cell can serve as the second costimulatory signal (Linsley et al. 1991). Alternatively, the costimulatory signal can come from $CD4^+$ T cells including the T_H1 subset (Yang et al. 1995).

Upon activation CTLs use a variety of mechanisms to kill virally infected cells. One mechanism by which CTLs kill target cells is by releasing cytotoxic granules containing perforin and granzymes. Perforin forms a pore in the target cell membrane, thereby allowing granzymes to enter the infected cell. Once inside the cell, granzymes cleave and activate caspase proteins leading to apoptosis of the target cell. Additionally, binding of FasL on the surface of CTLs to its receptor, Fas, on the surface of target cells induces a caspase signaling cascade that leads to apoptosis. CTLs further support immune defenses against pathogens by releasing proinflammatory cytokines such as TNF-α and IFN-γ. IFN-γ production enhances leukocyte migration to sites of infection, increases expression of MHC molecules, and promotes isotype switching (Carneiro et al. 2015; Rubtsova et al. 2016; Thelemann et al. 2014).

In addition to $CD8^+$ T cells Adenovirus infection also induces a $CD4^+$ T cell response. APCs present viral antigen in the context of MHCII to naïve $CD4^+$ T cells and engage antigen specific TCRs on the T cell surface. TCR engagement along with engagement of costimulatory molecules induces activation of antigen specific T cells. During this activation cytokines play a

critical role in the differentiation of naïve $CD4^+$ T cells into distinct T helper (T_H) effector populations (Zhu, Yamane, and Paul 2010). IL-12 and IL-18 support the differentiation of naïve $CD4^+$ T cells into T_H1 cells, whereas IL-4 supports the differentiation of T_H2 cells (Gutcher and Becher 2007). Differentiation into T_H17 cells is supported by cytokines such as IL-1□, IL-6, and TGF□ (Nalbant and Eskier 2016). (Figure 3B)

The different T helper subsets mediate distinct effector functions during Ad infection. T_H1 cells secrete cytokines, including IL-2 and IFN-γ, that support cell mediated immunity and clearance of intracellular pathogens. Specifically, IFN-γ helps to induce CTL differentiation and macrophage activation. As described previously, CTLs and macrophages are important responders to Ad infection. T_H2 cells support humoral immunity and are characterized by secretion of IL-4. Cytokines, such as IL-4, secreted by T_H2 cells support the differentiation of B cells into plasma cells which produce neutralizing antibodies against Ad (Chen and Ross 2007).

The T_H17 response to Ad infection is not well characterized. Given that innate immune responses to Ad infection result in secretion of cytokines important for T_H17 differentiation, such as IL-6 and IL-1□, it is likely a T_H17 response is induced during Ad infection. Indeed, recent studies have shown that a T_H17 response is induced during mouse adenovirus type 1 infection (McCarthy et al. 2014). The role of T_H17 cells in adenovirus infection remains unclear, as this subset is generally involved in clearance of extracellular bacteria, however it has been shown that IL-21 and IL-17 produced by T_H17 cells support germinal center formation and IgG class switching (Mitsdoerffer et al. 2010).

In addition to generating effector T cells, Ad infection also induces formation of memory T cells that can rapidly respond to future infections. Both $CD4^+$ and $CD8^+$ memory T cells are induced during Ad infection (Barnes et al. 2012; Bolinger et al. 2013). There are two main types

of memory T cells. Central memory T cells reside primarily in lymph tissues and rapidly proliferate following restimulation with antigen. In contrast, effector memory T cells are found in the peripheral circulation and tissues. Effector memory T cells rapidly and efficiently respond to antigen; however, they have limited proliferative capabilities compared to central memory T cells.

Naïve B cells in the lymph nodes encounter soluble antigen as well as antigen on follicular DCs (FDCs) or other APCs (Koppel et al. 2005; Qi et al. 2006). During Ad infection binding of viral antigen to the BCR on the surface of naïve B cells provides the primary signal for B cell activation. Interactions with coreceptors including CD19 and integrins strengthens this activation (Harwood and Batista 2010). The receptor bound antigen is then internalized, digested, and complexed with MHCII molecules on the B cell surface. A second activation signal is provided by interactions with antigen specific $CD4^+$ T cells and MHCII complexed antigen on the surface of the B cell. In addition, CD40 on the B cell surface engages with CD40 ligand on the surface of the T cell in order to fully activate the B cell to proliferate and develop into either memory B cells or antibody secreting plasma cells (Good-Jacobson and Tarlinton 2012). Ad-specific antibodies bind to Ad in the bloodstream, preventing cell entry and promoting opsonization of the virus by macrophages (Yu et al. 2013).

Adenovirus Vaccine Vectors

Adenoviruses have been explored extensively as tools for gene replacement therapy, however the strong innate and adaptive immune responses against these vectors have limited their utility. While the potent immune response to Ad vectors poses a challenge for gene therapy, it makes Ad an ideal vaccine vector candidate. In addition to inducing antibody responses against vaccine antigens, Ad vectors are potent T cell adjuvants. They have been shown to induce both

$CD8^+$ and $CD4^+$ T cell responses to vaccine antigens (Appaiahgari and Vrati 2015) (Figure 4). Furthermore, Ads have a broad cellular tropism, can be grown to high titers in tissue culture, induce high levels of transgene expression, and have a well characterized and easy to manipulate genome.

Detailed understanding of Ad molecular biology allowed for the development of first-generation replication-incompetent (FGV) vectors. These vectors lack the E1 gene, a critical transcriptional activator for viral replication, and thus are unable to replicate outside of E1-complementing cell lines. Many vectors also contain deletions in the E3 gene, allowing for increased transgene insertion capacity up to 7.5kb. Typically, robust transgene expression is achieved by having transgene expression under the control of a constitutively active promoter such as the cytomegalovirus (CMV) IE promoter. The Ad vectors used in these studies are E1/E3 deleted replication-incompetent vectors containing the CMV IE promoter.

One of the earliest studies using Ad vectors, performed by *Xiang et al*, used E1 deleted Ad5 vectors to induce protection against the rabies virus. This Ad5 vector expressing the rabies virus glycoprotein induced protective titers of neutralizing antibodies, as well as $CD8^+$ and $CD4^+$ T cell responses against the vaccine antigen (Xiang et al. 1996). Since then Ad5 vectors have been successfully used to generate immune responses against a number of pathogens, including malaria, influenza, and HIV (Chuang et al. 2013; Kanagavelu et al. 2014; Peters et al. 2013). In general, Ad based vectors have been shown to induce superior antigen specific antibody titers and $CD8^+$ T cell responses compared to other subunit vaccines (poxvirus, vaccinia virus, DNA vaccines, etc.) (Tatsis and Ertl 2004).

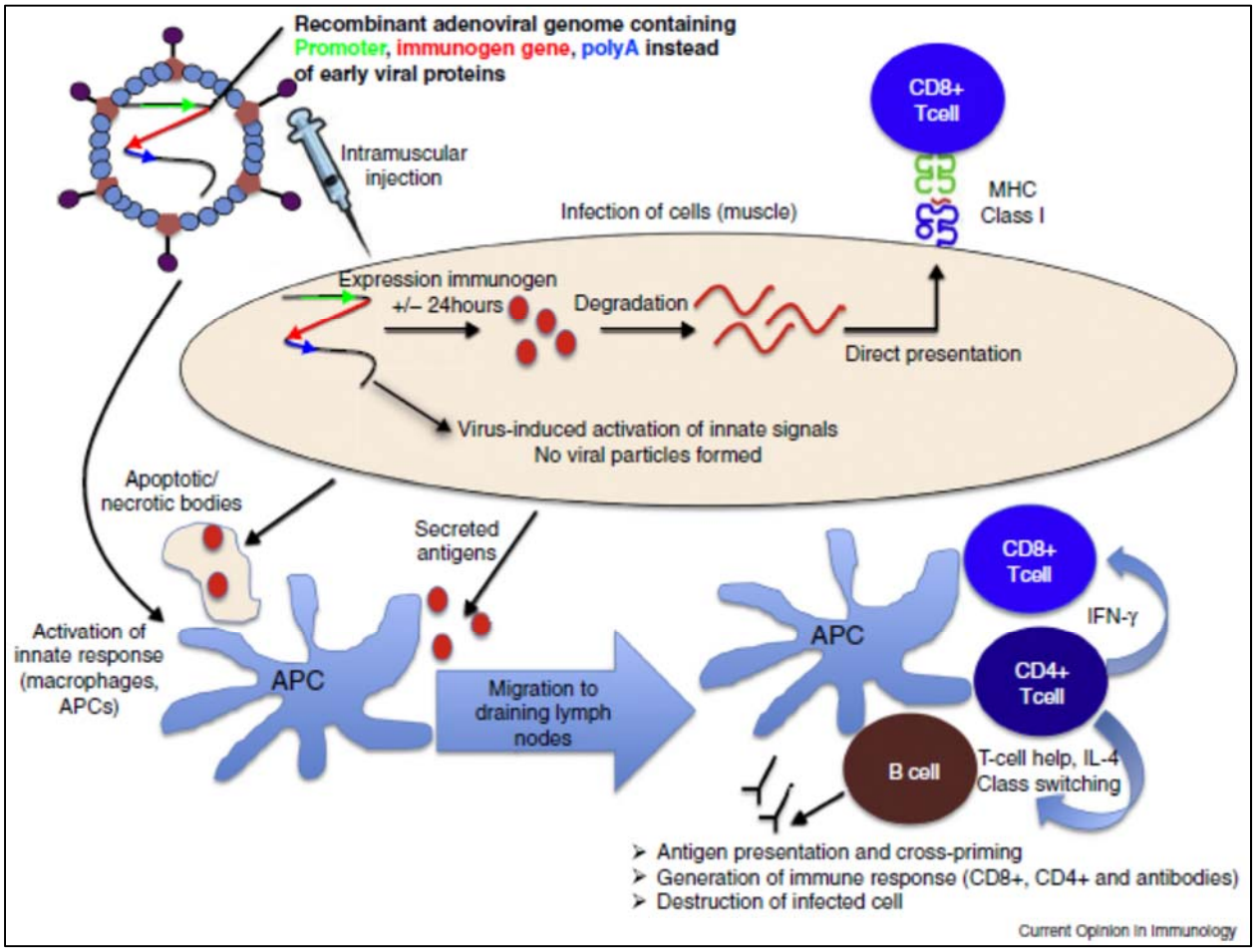


Figure 4. Induction of Transgene-Specific Immune Responses Following Immunization with Adenovirus Vectors. Intramuscular immunization with replication defective Ad vectors results in infection (non-productive) of cells followed by transgene expression. Innate sensing of viral components results in expression of proinflammatory cytokines and type I interferons. Expressed proteins undergo proteasomal degradation and are presented to $CD8^+$ T cells via MHCI. Alternatively, proteins may be secreted and taken up by antigen presenting cells (APCs). Activated APCs then migrate to the draining lymph node where they are able activate $CD8^+$ and $CD4^+$ T cells and B cells. Taken from (Ewer et al. 2016).

One major limitation of Ad vectors is preexisting immunity. As a result of natural Ad infection, the majority of individuals have neutralizing antibodies against Ad. Depending on the region, preexisting immunity to Ad5 ranges from 30-100% (Appaiahgari and Vrati 2015). Neutralizing antibodies against the Ad vector have been shown to reduce Ad uptake and

transgene expression, leading to a greatly diminished transgene specific immune response (Tatsis and Ertl 2004). Several approaches have been investigated in order to try and circumvent the issue of preexisting immunity to Ad5 vectors, including the use of alternative serotype Ad vectors and capsid displayed antigen.

In recent years replication defective chimpanzee Ad vectors (ChAd) and human Ad vectors with lower seroprevalence have been explored as a means to overcome the issue of preexisting immunity. Human Ad4 and Ad6 vaccine vectors have been used in vaccine development for influenza and HCV, respectively. ChAd3 vectors have also been used in HCV vaccine development. In human trials Ad6 and ChAd3 based vaccines generated $CD8^+$ and $CD4^+$ T cell responses, including both central and effector memory populations (Appaiahgari and Vrati 2015). Strong $CD8^+$ and $CD4^+$ T cell responses have also been demonstrated in human subjects following immunization with human Ad35 vectors expressing *Mycobacterium tuberculosis* antigens (Abel et al. 2010). These studies indicate that alternative Ad vectors are able to induce anti-transgene immune responses while also overcoming the obstacle of preexisting immunity.

Another approach that has been used to circumvent preexisting immunity is to use capsid displayed epitopes of the vaccine antigen. Foreign sequences can be inserted into the capsid proteins hexon, penton, fiber and protein IX. Replacement of the hexon hypervariable regions (HVRs) with epitopes from the vaccine antigen allows for presentation of the antigen and prevents recognition of the Ad vector by neutralizing antibodies raised against the HVRs (Bradley et al. 2012).

This document describes the development of novel Ad5-vectors that are capable of inducing potent T and B cell immunity to transgene antigens. Furthermore, this work shows that these vectors are able to provide protection against cutaneous MRSA infection. The results

described in this work contribute to our understanding of the adjuvant properties of Ad5 vectors and the correlates of vaccine mediated protection against MRSA infections. Importantly, this work shows that Ad5-based vectors represent a novel potential *S. aureus* vaccine strategy.

CHAPTER TWO

MATERIALS AND METHODS

Cells Lines and Cell Culture

Our lab obtained 293□5 cells as a gift from Glen Nemerow (Smith et al. 2010). RAW264.7 cells and HeLa cells were purchased from ATCC. All tissue culture reagents were obtained from Mediatech and HyClone. 293□5 and HeLa cells were maintained in Dulbecco Modified Eagle Medium (DMEM) and supplemented with 1mg/mL streptomycin, 100IU/mL penicillin, 0.25mg/mL amphotericin B, non-essential amino acids, 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2mM glutamine, and 10mM HEPES buffer. RAW264.1 cells and murine splenocytes were cultured in RPMI1640 media containing the same supplements as DMEM.

Generation of Recombinant Virus

Ad5gfp was generated as previously described (Wodrich et al. 2010). To generate Ad5ClfA and Ad5SdrD we used the Ad5Easy system as described previously (He et al. 1998; Luo et al. 2007). To summarize, we cloned DNA containing the sequence for the ligand binding domain of ClfA (amino acids 40-559) or SdrD (amino acids 53-853) from *S. aureus* strain USA300 into a shuttle vector (Foster et al. 2014; McDevitt et al. 1995; Wang et al. 2013). We then used recombineering to insert the shuttle vector into an E1/E3 deleted Ad5 genome. We confirmed expression of ClfA or SdrD by western blot of Ad5ClfA or Ad5SdrD infected cell lysates.

Ad5PFgfp was generated using the galK recombineering system described by Warming et al. (2005). Primers encoding the galK sequence flanked by sequences homologous to the intended insert site within the pIX gene were used to amplify galK from a plasmid (pgalK). pgalK was obtained from the NCI BRB Preclinical Repository (NCI-FCRDC). Homologous recombination was then used to insert the galK cassette into the end of the pIX gene, then positive selection by growth on minimal media plates containing galK was used to obtain clones of successful recombinants. To replace galK with the flag-fliC35 sequence, we amplified flag-FliC35 products using primers for flag-FliC35 flanked by sequences homologous to either end of the galK cassette. We then used homologous recombination to replace the galK cassette with flag-fliC35. To identify successful recombinants, we grew bacteria on minimal media plates containing X-gal, which is converted to a toxic metabolite in cells still containing the galK cassette. We were therefore able to use negative selection to screen for successful recombinants. Finally, we used PCR amplification of the pIX gene to confirm successful recombination.

Virus Purification

Viruses were propagated using HEK293 cells. We purified the viruses by subjecting them to two rounds of cesium chloride gradient centrifugation followed by dialysis in 40mM Tris, 150mM NaCl, 1mM MgCl2 and 10% glycerol (Wiethoff et al. 2005). To determine virus concentration, we performed Bradford assays, with 1mg of protein corresponding to $4x10^9$ viral particles (Bio-Rad Laboratories, Inc.). Viruses were aliquoted, frozen in liquid nitrogen, then stored at -80□. We determined viral titers of Ad5gfp and Ad5PFgfp by serially diluting virus on HeLa cells and using flow cytometry to quantitate the number of GFP expressing cells. To determine the titers of Ad5ClfA and Ad5SdrD we serially diluted virus on HeLa cells then used IFA to detect Ad hexon.

Bacterial Strains and Cultures

USA300 and SA113 strains of *S. aureus* were a gift from Dr. Katherine Radek. RN4220 was a gift from Dr. Francis Alonzo III. To construct USA300gfp and RN4220gfp a PCR amplicon of GFP was generated, digested, and subcloned into pJC1111. pJC1111 was then stably integrated into the *S. aureus* SaPl-1 site using phage transduction as previously described (Alonzo et al. 2012; DuMont et al. 2014). pJC1111 was a gift from Dr. Francis Alonzo III. *S. aureus* was gown at 37□ on tryptic soy broth (TSB) solidified with agar or in liquid TSB culture shaking at 180 rpm.

USA300 Whole Cell Lysate Preparation

USA300 whole cell lysate was prepared by incubating bacterial culture with lysostaphin (1:100) and homogenizing using a Dounce homogenizer. Following homogenization lysates were treated with DNase (1:100) and centrifuged at 4□. The concentration of protein in the supernatant of the lysate was then determined by Bradford assay.

Recombinant Protein Construction and Purification

We used PCR to amplify DNA encoding for amino acids 40-559 of ClfA, 53-853 of SdrD, 53-606 of SdrE, and 37-603 of FnBPA from USA300. These amino acids encode for ligand binding regions of ClfA, SdrD, SdrE and FnBPA (Deivanayagam et al. 2002; Foster et al. 2014; Keane et al. 2007; McDevitt et al. 1995; Wang et al. 2013; Wann, Gurusiddappa, and Hook 2000). We then ligated these PCR products into the pGEMT-easy vector. The resulting pGEMT-easyClfA, pGEMT-easySdrD, pGEMT-easySdrE, and pGEMT-easyFnBPA vectors were digested with NdeI and BamHI to release the ClfA, SdrD, SdrE and FnBPA inserts, and those fragments were then ligated into the protein expression vector pET15b. The pET15b vector allows for IPTG inducible, T7 promoter driven expression of recombinant proteins and encodes

for a N-terminal 6X His-tag. We confirmed successful ligation reactions by digesting pET15bClfA, pET15bSdrD, pET15bSdrE and pET15bFnBPA with NdeI and BamHI. We ran the digestion reactions on agarose gels using gel electrophoresis to confirm the identity of bands corresponding to ClfA, SdrD, SdrE or FnBPA sequences.

To express the recombinant proteins, we transformed BL21 *E. coli* with pET15b vectors encoding for ClfA, SdrD, SdrE or FnBPA. To obtain recombinant GFP we transformed BL21 cells with pETHis6GFPTEVLIC (Addgene cat #29663). We grew cultures overnight at 37□ in LB with ampicillin or kanamycin (pETHis6GFPTEVLIC) to maintain expression of the pET vectors. The next day we diluted the overnight cultures 1:20 in 500mLs of LB with ampicillin or kanamycin and continued growing the cultures at 37□ until they reached an OD_{600} of 0.6. At that time, we induced protein expression by addition of 1mM IPTG and continued growing cultures for an additional 4 hours. We harvested cells by centrifugation at 0□. All subsequent steps were performed on ice or at 0□, and 1:100 PMSF was added to all buffers to minimize protein degradation. We lysed cells by incubating the cell pellet in 20mLs of 1% Triton X-100, 0.5mg/mL lysozyme and 1:100 DNAse in 50mM PBS. The solubilized pellets were then centrifuged at 13,000xG for 15min and supernatant, containing the soluble recombinant proteins, was collected.

We added lysozyme and glycerol to the supernatant for a final concentration of 10mM lysozyme and 10% glycerol. We then loaded the supernatant onto 0.5mL TALON cobalt resin columns (Clontech cat #635501). Column flow through was reloaded onto the column one time, followed by 10mL of Wash Buffer 1 (50mM Phosphate, 500mM NaCl, 10mM Imidazole and 1% triton X-100) and 10 1mL washes with Wash Buffer 2 (50mM Phosphate, 500mM NaCl, 10mM Imidazole). We eluted protein using 150mM Imidazole in 50mM PBS and dialyzed protein in

PBS with 1% glycerol for 4hrs. Protein concentration was determined by Bradford assay. Sample purity was confirmed by 15% SDS-PAGE followed by Coomassie stain.

Western Blot Analysis

To demonstrate that immunization with recombinant proteins elicit antibodies that recognize purified proteins as well as USA300 whole cell lysate (WCL) we utilized western blot analysis. We loaded 2µg of purified recombinant ClfA, SdrD, SdrE or FnBPA and 2µg of USA300 WCL onto 15% polyacrylamide gels and performed SDS-PAGE. Proteins were subsequently transferred to nitrocellulose and immunoblotted for ClfA, SdrD, SdrE or FnBPA using 1:250 diluted serum from mice immunized with the respective recombinant protein. Primary antibody was detected using HRP-conjugated anti-ms IgG at a 1:1000 dilution.

We performed western blotting to confirm the expression of ClfA and SdrD in cells following infection with Ad5ClfA or Ad5SdrD, respectively. 293□5 cells were plated at 300,000 cells per well in a 6-well plate. The following morning cells were infected with 300ppc of either Ad5ClfA or Ad5SdrD. 48hrs post infection cells were harvested and cell lysates were subjected to SDS-PAGE (alongside 2ng recombinant ClfA or SdrD run as positive controls) followed by transfer to a nitrocellulose membrane and immunoblotted for ClfA or SdrD using 1:500 diluted serum from ClfA or SdrD immunized mice. Both membranes were also blotted for Actin (Sigma cat #A5441) which served as a loading control. HRP-conjugated anti-ms IgG (1:1000) was used to detect the primary antibody.

Mouse Experiments

Mice used in these studies were either C57BL/6 wildtype mice (Jackson laboratories cat #000663) or BALB/c mice (Jackson laboratories cat #000651) aged 6-12 weeks. All studies were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago

(Maywood, IL). All immunizations were performed by intramuscular vaccination of 50µl into the left quadricep. For protein immunizations purified recombinant ClfA, SdrD, SdrE and/or FnBPA were adsorbed to aluminum hydroxide (alum) using 25µl of alhydrogel for a 1:1 protein to alum solution ratio. Alum was purchased as Alhydrogel gel suspension from Invivogen (cat # 21645-51-2). To obtain polyclonal serum raised against either purified recombinant ClfA, SdrD, SdrE or FnBPA, 6-8 week old C57BL/6 mice were immunized with 10µg of protein followed by a second immunization 21 days later. Mice were sacrificed and serum collected 21 days after the last immunization.

To compare the immune response between ClfA and Ad5ClfA immunized animals, C57BL/6 mice were immunized with either 10^9 viral particles (vp) of Ad5ClfA or 2µg of recombinant ClfA (administered as a protein-Alum solution). For immunization and challenge experiments evaluating the ability of Ad5ClfA and Ad5SdrD to provide protection against USA300 challenge, C57BL/6 mice aged 6-8 weeks were immunized with 10^9 vp of Ad5ClfA and 10^9 vp of Ad5SdrD. To compare the protective effects of Ad immunization to those of protein immunization, additional animals were immunized with 2µg of each ClfA, SdrD, SdrE, and FnBPA. Ad5gfp (10^9vp) immunization was used as a vector control, and PBS (50µl) immunization was used as an unimmunized control. All other immunizations, including those comparing Ad5gfp and Ad5PFgfp vectors, were performed on BALB/c mice using 10^{10} vp of the respective virus. For all single immunization experiments, animals were sacrificed and serum, splenocytes and lymph nodes were collected 10 days post immunization.

For all *S. aureus* immunization and challenge experiments mice were immunized as described above. Mice were then challenged with either USA300, USA300gfp, or RN4220gfp 28 days post immunization. Mice were challenged by intradermal injection at four sites on their

back. At each of the four injection sites, $2x10^6$ CFU of bacteria resuspended in PBS were administered in a 1:1 ratio with Cytodex microcarrier beads in PBS (Sigma cat #C646-5G 1.60-87μm). Total volume per injection site was 100μl. Three to six days following challenge mice were sacrificed and serum, splenocytes, lymph nodes and wounds were collected. For vaccine efficacy experiments immunized mice were challenged with varying doses of USA300 as described above. To confirm that recombinant proteins could generate an IL-17 response, unimmunized mice were infected as described above with USA300, SA113, or mock infected using PBS. Six days post infection mice were sacrificed and splenocytes were collected.

Determination of Wound Bacterial Load and Wound Size

To determine the wound bacterial load following intradermal challenge, we isolated wound biopsy punches and digested them with 10U/mL Collagenase (Sigma cat #C0130) for 1 hour at 37□. The samples were then homogenized in 1mL of sterile PBS by 1.00mm Zirconium silica beads. We then made serial ten-fold dilutions of the homogenized samples in sterile PBS and plated dilutions onto TSA plates. Plates were incubated at 37□ overnight. The following day we determined the number of colony forming units (CFUs) present and multiplied by the appropriate dilution factor to determine CFUs/mL. Lesion area was determined by measuring the diameter of the wound and using the equation $A= \pi r^2$.

IL-17 ELISPOT

ELISPOT assays were performed using the Mouse/Rat IL-17A ELISPOT Ready-SET-Go! kit from eBioscience (cat # 88-7370). Briefly, ELISPOT plates (Millipore cat #MAIPS4510) were coated with an anti-IL-17A capture antibody overnight at 4□. Plates were blocked with complete RPMI for 1 hour at room temperature then splenocytes were added to the wells at $1x10^6$ cells/well. Splenocytes were harvested by homogenizing spleens on a mesh filter in RPMI.

Cells were spun down at 300xg for 5 minutes then resuspended in 2mL of red blood cell lysis buffer for 2 minutes and resuspended in 3mLs of RPMI. Finally, cells were again spun down and the pellet was resuspended in 2mL of RPMI and plated. Antigen (ClfA, SdrE, or USA300 WCL) was added to the appropriate wells for a final concentration of 2μg/mL. Plates were incubated for 42 hours at 37□ then washed with PBST (0.05% tween) and incubated with a biotinylated anti-IL-17A detection antibody at room temp for 2 hours. Following the 2 hour incubation the plates were washed again then incubated with Avidin-HRP at room temperature for 45 minutes. After washing the plates, we developed them by adding AEC substrate (Sigma #AEC101) and incubating them until spots developed.

Ex Vivo Splenocyte Stimulation

We harvested splenocytes as described above for ELISPOT. We then plated cells in a 96-well plate at a density of $1x10^6$ cells/well and stimulated them with specific antigens. Splenocytes were stimulated with Ad5 (10,000 particles per cell) or ClfA, SdrD, or GFP (1μg/mL). Cells were left untreated to serve as an unstimulated control or incubated with 20ng/mL PMA and 1μg/mL Ionomycin to serve as a positive control. Cell free supernatants were harvested 24hrs (for IFNγ ELISAs) or 5 days (for IL-17 ELISAs) after stimulation and subjected to ELISA to detect IFNγ or IL-17 as described below.

ELISA

Mouse serum was subjected to ELISAs to determine endpoint dilution titers and IgG isotype responses to antigen. Blood was obtained from sacrificed mice by the cardiac puncture method. Blood was kept on ice for 1 hour then spun down at 8000 rpm for 5 minutes to separate out coagulated RBCs. Serum was aliquoted and stored at -80□ until use.

High binding ELISA plates (Costar ca #07-200-35) were coated overnight at 4□ with 1µg/mL of antigen in coating buffer (BioLegend cat #421701) while shaking. The next day plates were washed 4 times in PBST (0.05% tween) and blocked with assay diluent (ebioscience cat #00-4202-56) for 1 hour. We washed plates again then added 100µ/well of serially diluted mouse serum and incubated the plates shaking at room temperature for 2 hours. After washing the plates, we added 100µl/well of 1:1000 diluted HRP conjugated anti-mouse IgG (Fc) (Abcam ab97265) and incubated plates for another hour. We washed the plates then developed them using 50µl/well TMB Substrate and stopped the reaction using 1M sulfuric acid. Plates were read on a KC Junior plate reader at 450nm. Dilutions curves were used to determine the antibody endpoint dilution titer (ELISA units), defined as the dilution at which the absorbance value is 3 standard deviations above that of PBS-immunized control mice. ELISAs to determine IgG isotypes were performed as described above except that a single serum dilution was used and the secondary antibodies used were specific to IgG1, IgG2b, IgG2c, or IgG3.

BioLegend Mouse IFNγ (Cat No. 430801) and BioLegend Mouse IL-17A (Cat No. 432501) kits and protocols were used to measure IFNγ and IL-17 levels in cell free supernatants following ex vivo splenocyte stimulation. To summarize, plates were coated overnight at 4□ with the appropriate capture antibody, then washed, blocked and incubated with diluted standards and samples at room temperature for 2 hours while shaking. After washing, plates were incubated with the appropriate biotinylated detection antibody for 1hr, washed and incubated with Avidin-HRP. Plates were developed with TMB substrate and read as described above. The IFNγ or IL-17 response was determined by comparing test OD_{450} values to a standard curve.

Opsonophagocytic Assays (OPAs)

OPAs were used to evaluate the ability of serum from immunized animals to mediate opsonophagocytosis of *S. aureus*. USA300 cultures were grown overnight in TSB at 37□ while shaking. The following day we diluted the cultures 1:100 and incubated them at 37□ for an additional 3 hours. After 3 hours the cultures were spun down at 4000 rpm for 6 minutes, washed with sterile PBS and resuspended in 5mL of sterile PBS. Cultures were normalized to an OD_{600} corresponding to $1x10^8$ CFU/mL. One mL of normalized culture was spin down, resuspended in 1mL of RPMI, 10mM HEPES, and 1% serum mouse serum (collected as previously described) and incubated, shaking, at room temperature for 30 minutes. After 30 minutes cultures were spun down, washed and resuspended in 1mL RPMI with 10mM HEPES.

10µl of culture was added to RAW264.7 cells (in RPMI, 10mM HEPES) plated in 96 well plates at a cell density of $1x10^5$. Bacteria and RAW264.7 cells were synchronized by spinning plates at 1500 rpm for 7 minutes. Plates were either incubated at 37□ (5% CO_2) for 30 minutes (T30) or immediately placed on ice (T0). Following incubation, T30 samples were treated with 50µg/mL gentamicin for 15 min, then a 1% saponin solution was used to permeabilize RAW264.7 cell membranes. T0 samples were treated with 1% saponin immediately following synchronization. Serial 10-fold dilutions of T0 and T30 samples were plated onto TSA plates and the CFU/mL for each sample was determined as described previously. Percent bacterial uptake was calculating by normalizing the CFU/mL of the T30 sample to the CFU/mL of the T0 sample.

RT-qPCR

RNA was isolated from mouse tissues using TRIzol Reagent (Life Technologies cat #15596-026) and its accompanying protocol. 2µg of RNA was DNase I treated (Fermentas cat

#EN0521) and reverse transcribed to single strand cDNA using Fermentas RevertAid First Strand cDNA Synthesis Kit (cat #K1621). The cDNA was then used to perform qPCR on the BioRad CFX96. We used BioRad IQ SYBR Green Supermix (cat #170-8880) and the primer sets shown in Table 1. Relative expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) where the expression of the genes of interest were normalized to GAPDH expression within each sample type and the gene of interest expression level in control samples is set to 1.

Table 1. Primer Sequences for qPCR

Primer name	Sequence	
msIFNγ forward	cggcacagtcattgaaagccta	(Xia et al. 2010)
msIFNγ reverse	gttgctgatggcctgattgtc	
msIL-17 forward	aaggcagcagcgatcatcc	(Krauthausen et al. 2011)
msIL-17 reverse	ggaacggttgaggtagtctgag	
msIL-21 forward	gccagatcgcctcctgatta	(Pesce et al. 2006)
msIL-21 reverse	cggggtaccggagagatgctgatg	
msCXCL1 forward	atccagagcttgaaggtgttg	(Roy et al. 2012)
msCXCL1 reverse	gtctgtcttctttctccgttactt	
msCXCL2 forward	gaacaaaggcaaggctaactga	(Narita et al. 2010)
msCXCL2 reverse	aacataacaacatctgggcaat	
msCXCL5 forward	acagtgccctacggtggaagt	(Roy et al. 2012)
msCXCL5 reverse	cgaggtgcattccgcttagctt	
msGAPDH forward	tgcccccatgtttgtgatg	(Martowicz, Grass, and Bresnick 2006)
msGAPDH reverse	tgtggtcatgagcccttcc	

Immunofluorescence Microscopy

A 3mL culture of USA300 was grown in TSB overnight at 37□. The following day 0.5mL of overnight culture was spun down at 4000 rpm for 6 minutes and used to inoculate 20mL of TSB. The culture was allowed to grown at 37□ for 2 hours, then 0.5mL was spun down, washed 3 times with sterile PBS, applied to glass coverslips and allowed to air dry for 30 minutes. Cells were fixed in 95% ethanol, washed with PBS then incubated with mouse serum (1:100) overnight at 4□. The following day the coverslips were washed in PBS then incubated with an anti-mouse-568 secondary antibody (1:4000) and counterstained with DAPI for 2 hours at room temperature. The coverslips were then mounted on glass slides with Flouro-Gel (Electron Microscopy Sciences) and images were acquired using a confocal microscope.

Reagents and Antibodies

Assay Diluent (cat #00-4202-56) and TMB substrate (cat #00-4201-56) used for ELISA were purchased from eBioscience. Phorbol-12-myristate-13-acetate (PMA), Ionomycin, and Lysostaphin were purchased from Sigma-Aldrich. HRP-conjugated anti-mouse IgG isotype antibodies were purchased from Southern Biotech (cat #5300-05B). HRP-conjugated anti-mouse IgG secondary antibody was purchased from Abcam (cat #ab97265). The Goat anti-mouse IgG secondary antibody, conjugated to Alexa Fluor 568 used for immunofluorescence microscopy was purchased from ThermoFisher (cat #A-11004). SuperSignal West Dura Extended Duration Substrate was used to develop and image western blots (Fisher cat #34075). All other reagents were purchased from Fisher Scientific.

Statistics

We assessed statistical significance ($p < 0.05$) between two groups using the Student's T Test. Data are presented as the mean ± SEM.

CHAPTER THREE

RESULTS

Immunization with Recombinant *S. aureus* Proteins Induces High Titer Antibodies

It has previously been shown that protection from *S. aureus* induced disease in mice correlates with production of antibodies against several *S. aureus* surface adhesion proteins, including FnBPs, ClfA, and Sdr proteins (Kim et al. 2011). However, emerging evidence suggests that protection against *S. aureus* infection likely requires T cell help, including T_H17 and T_H1 responses (Lin et al. 2009). Given that Adenovirus vaccine vectors are potent inducers of T cell responses, we sought to use replication defective Adenovirus vectors to express domains from *S. aureus* surface proteins. I hypothesized that Adenovirus vaccine vectors would induce more potent T cell responses to vaccine antigens than protein in Alum immunization and would therefore provide greater protection against MRSA challenge. To be able to compare the protective effects of Adenovirus vaccines with recombinant protein in Alum immunization we generated recombinant ClfA, SdrD, SdrE, and FnBPA proteins corresponding to the ligand binding domains of each. We also generated E1, E3-deleted Ad5 vectors expressing these same proteins under the control of the CMV promoter.

To confirm that the recombinant proteins were able to induce an antibody response we immunized C57BL/6 mice with the individual recombinant proteins. Following prime and boost immunizations with 10μg of the appropriate recombinant protein, serum was harvested and used to perform western blot analysis. Purified recombinant protein (either ClfA, SdrD, SdrE, or

FnPBA) was run alongside USA300 WCL on a 15% polyacrylamide gel. The gels were then transferred to nitrocellulose membrane and probed with serum from mice immunized with the respective protein. Serum from immunized animals bound to the recombinant protein, as well as protein in USA300 WCL (Figure 5). The differences seen in the size of the recombinant protein vs the protein recognized in WCL may be due to different glycosylation of the recombinant protein when expressed in *E. coli*. Additionally, ELISA data showed that immunized mice had endpoint dilution titers approaching 10^6 ELISA units (data not shown). These data indicate that immunization with recombinant protein is able to produce high titers of antibodies that recognize not only the recombinant protein but also protein from *S. aureus*.

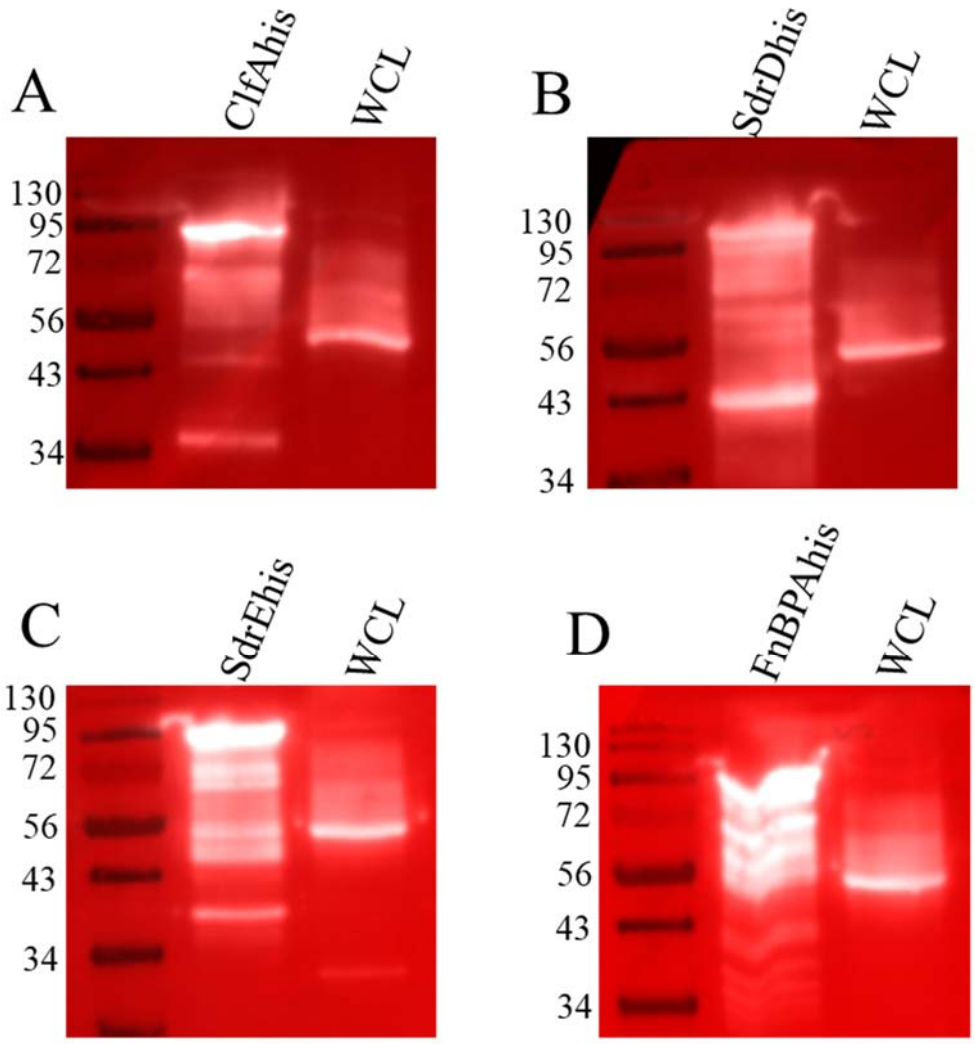


Figure 5. Western Blot Using Serum From Mice Immunized with Recombinant Protein. 2μg of recombinant protein was ran alongside 2μg of USA300 WCL on a 15% polyacrylamide gel. After transferring to nitrocellulose the membrane was immunoblotted for ClfA (A), SdrD (B), SdrE (C) or FnBPA (D) using serum from mice immunized with the respective recombinant protein.

Recombinant Proteins Contain T Cell Epitopes and Induce an IL-17 Response Following Restimulation of Splenocytes

Several studies have shown IL-17 to be critical for protecting mice and humans against *S. aureus* SSTIs, thus a protective vaccine will likely need to generate memory T_H17 cells against *S. aureus* antigens (Cho et al. 2010; Puel et al. 2011). I therefore sought to confirm that the recombinant proteins I used to generate Ad vaccine vectors contain T cell epitopes and are capable of eliciting a T_H17 response. To do this, I obtained splenocytes from mice that had been intradermally infected with the MRSA strains USA300 or SA113. I stimulated splenocytes with either USA300 whole cell extract (WCE) or recombinant proteins for 42 hours, then measured the number of IL-17 producing cells by ELISPOT (Figure 6).

Mice inoculated with MRSA demonstrated IL-17 responses to WCE, as well as to ClfA and SdrE recombinant proteins that was significantly greater than the response seen in naïve mice. Furthermore, recombinant proteins from the USA300 strain were able to induce an IL-17 response in mice that had been previously infected with the SA113 *S. aureus* strain. Our lab has also generated data showing that humans with a history of *S. aureus* infection generate an IFNγ and IL-17 response to domains from recombinant ClfA, SdrD, SdrE and FnBPA (data not shown).

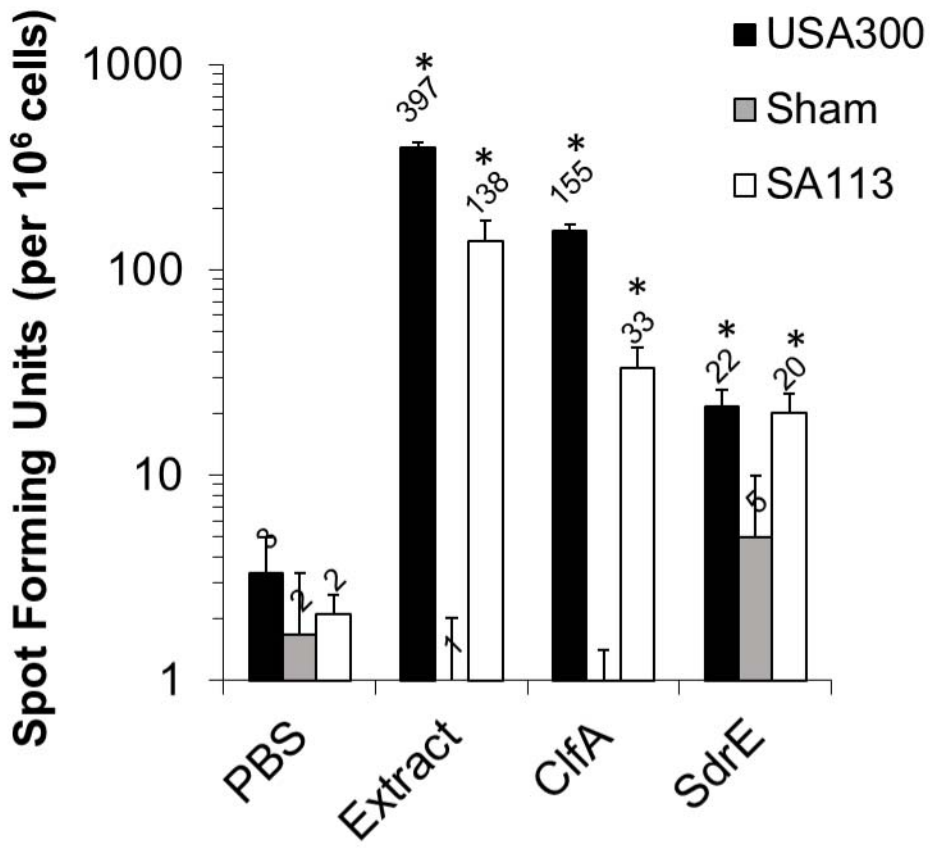


Figure 6. IL-17 Response to *S. aureus* Antigens. C57BL/6 mice were inoculated intradermally with 10^6 CFU of MRSA strains USA300 or SA113. 10 days later splenocytes were harvested and were stimulated with WCE or recombinant purified ClfAhis or SdrEhis. After 42 hours of stimulation the number of IL-17 producing cells was determined by ELISPOT. *$p < 0.05$

Transgenes are Expressed in 293□5 Cells Following Infection with Ad5ClfA or Ad5SdrD

Due to the potent T cell adjuvancy associated with Ad vectors, I hypothesized that a vaccine comprised of Ad vectors expressing *S. aureus* antigens would provide protection against infection. I generated Ad vectors containing domains from the *S. aureus* surface adhesion proteins ClfA or SdrD as transgenes. To confirm that ClfA and SdrD are expressed in cells following infection with Ad5ClfA or Ad5SdrD I performed western blots using Ad5ClfA or Ad5SdrD infected cell lysate. I electrophoresed cell lysates from infected cells alongside cell

lysates from mock-infected cells on a polyacrylamide gel and then transferred proteins onto a nitrocellulose membrane. I then immunoblotted for ClfA or SdrD using serum from ClfA or SdrD immunized mice. Bands corresponding to ClfA and SdrD were detected in lanes containing cell lysates from Ad5ClfA or Ad5SdrD infected cells, confirming that the transgenes are expressed during Ad infection of 293□5 cells (Figure 7). Again, the difference in size of recombinant proteins express in *e. coli* compared to the proteins expressed in mammalian cells is likely due to glycosylation of the proteins expressed in *e coli*.

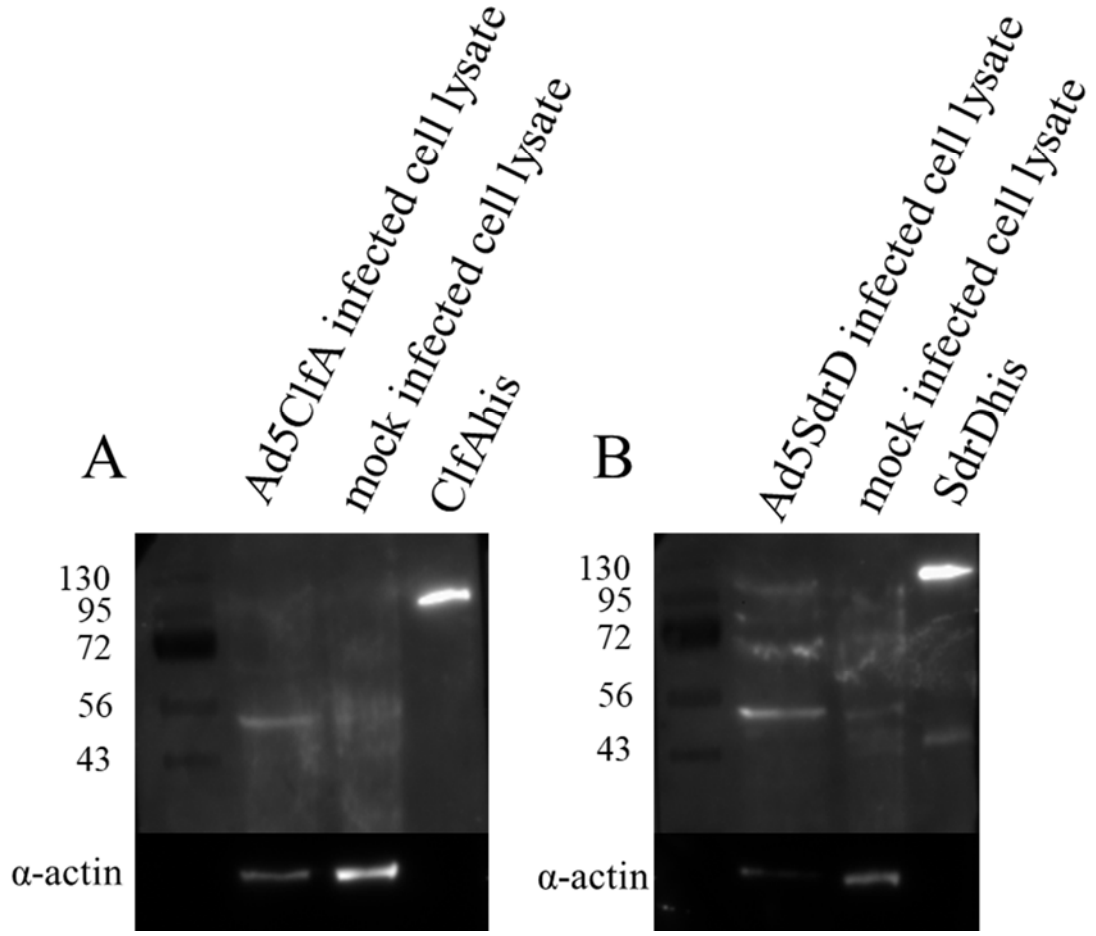


Figure 7. Immunoblot to Detect Transgene Expression in Ad5ClfA and Ad5SdrD Infected Cells. Cell lysates from Ad5ClfA (A) or Ad5SdrD (B) infected cells were subjected to SDS-PAGE, then transferred onto nitrocellulose membranes. Membranes were immunoblotted for either ClfA (A) or SdrD (B) using serum from mice immunized with the respective recombinant protein. Recombinant ClfAhis or SdrDhis were ran as positive controls. Mock infected cell lysates served as a negative control for transgene expression. Membranes were also blotted for Actin which served as a loading control.

Immune Response to Ad5ClfA Immunization

For Ad5ClfA to be an effective vaccine candidate it will need to elicit both T and B cell responses against ClfA. Numerous studies have shown that T_H17 cells and IL-17 are important

mediators of vaccine induced protection. While an IL-17 response has been shown to be protective in a variety of infection models, it is particularly important in protecting against cutaneous infection (Archer, Harro, and Shirtliff 2013; Cho et al. 2010). Additionally, a number of studies have shown that T_H1 cells and the associated production of IFNγ are required for vaccine mediated protection during invasive infections (Brown et al. 2015; Lin et al. 2009). Given that Ad vectors potently induce T cell responses, I hypothesized that Ad vaccine vectors would induce both IL-17 and IFNγ memory responses to vaccine antigens following immunization. I also hypothesized that Ad vaccine vectors would induce greater IL-17 and IFNγ responses compared to protein in Alum immunization, which is associated with a more dominant T_H2 response.

To assess the immune response to ClfA following either protein or Ad5ClfA immunization, I intramuscularly immunized C57BL/6 mice with either 10^9 vp of Ad5ClfA or 2μg of recombinant purified ClfAhis. I harvested the spleens 10 days post immunization, and restimulated splenocytes with recombinant ClfAhis for 24 hours (IFNγ) or 5 days (IL-17). Following restimulation, I determined the levels of IL-17 and IFNγ in the supernatants by ELISA. Both ClfAhis and Ad5ClfA immunization induced an IL-17 response to ClfA, however the IL-17 response induced by Ad5ClfA immunization was significantly greater than the response induced by immunization with ClfAhis in Alum (Figure 8A). Additionally, Ad5ClfA immunization induced an IFNγ response to ClfA that was not seen in protein immunized mice (Figure 8B).

While effective T cell responses to vaccine antigens appear to be the most important biomarker for protection, a B cell response to vaccine antigens may also be important. During infection, antibodies neutralize toxins and bind to the bacterial surface, triggering activation of

the classical complement pathway. Additionally, IgG antibodies bound to the bacterial surface mediate phagocytosis of *S. aureus* by phagocytes through interactions with Fcγ receptors (FcγRs). Given the potential functionality of antibodies in mediating bacterial clearance, I wanted to confirm that immunization with Ad vaccine vectors induced anti-ClfA antibodies. It is known that the different IgG isotypes have differing affinities for antigen and FcγRs, and therefore have differing abilities to mediate effector functions such as opsonophagocytosis and complement dependent cytotoxicity (CDC). Therefore, I also wanted to determine the istoypes of IgG antibodies generated in response to immunization.

To determine anti-ClfA antibody titers I obtained serum from mice immunized with either Ad5ClfA, or ClfAhis in Alum. I used the serum to perform ELISAs to determine endpoint dilution titers to ClfAhis, as well as to determine the IgG isotype response to ClfAhis. Immunization with Ad5ClfA induced a strong antibody response against ClfAhis, although endpoint dilution titers were reduced compared to ClfAhis+Alum immunization (Figure 8C). Serum from ClfAhis+Alum immunized animals contained anti-ClfA antibodies that were predominantly IgG1, whereas immunization with Ad5ClfA induced predominantly IgG2b and IgG2c istoypes (Figure 8D).

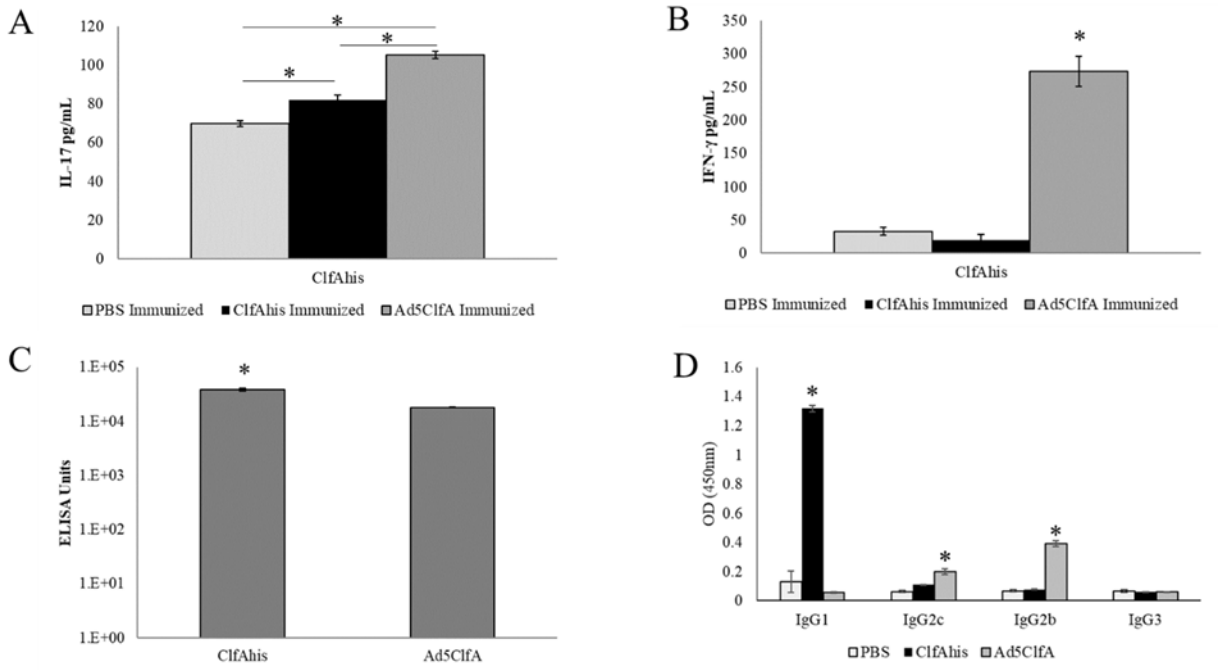


Figure 8. Immune Response to ClfA Following Ad5ClfA or ClfAhis Immunization. (A & B) C57BL/6 mice were intramuscularly immunized with 10^9 vp of Ad5ClfA, 2μ ClfAhis in Alum, or PBS. 10 days post immunization splenocytes were harvested and stimulated with recombinant purified ClfAhis. 24 hours (IFNγ) or 5 days (IL-17) later IFNγ (B) and IL-17 (A) levels in the supernatants were determined by ELISA. (C & D) Serum samples were collected from the mice in the above experiment. Pooled antibody titers to ClfA were determined by endpoint dilution assays (C). Antibody titer was determined by the highest dilution at which the absorbance value differed significantly from those of negative controls. (D) Anti-ClfA IgG isotypes were determined by ELISA and isotype response was determined based on the absorbance at OD450nm. *$p<0.05$

Antibodies Generated Following Ad5ClfA or ClfAhis Immunization Bind to *S. aureus*

Anti-ClfA antibodies are generated in response to both Ad5ClfA and ClfAhis+Alum immunization. My previous data shows that these antibodies bind to recombinant purified ClfAhis, however that does not mean that the antibodies will recognize full length ClfA on the surface of *S. aureus*. For antibodies generated in response to Ad5ClfA immunization to be protective they need to be able to recognize ClfA expressed from *S. aureus* so that they can mediate functions such as opsonophagocytosis and CDC during infection.

To determine if antibodies raised following Ad5ClfA or ClfAhis+Alum immunization can bind to ClfA on *S. aureus* I performed immunofluorescence microscopy. I struck *S. aureus* cultures onto glass coverslips and incubated them with serum from either naïve mice, or mice immunized with Ad5ClfA or ClfAhis+Alum. I used an Alexa Flor 568-conjugated anti-mouse-IgG secondary antibody to detect anti-ClfA antibodies, and counterstained with DAPI. Serum from naïve mice did not show any binding of mouse antibodies to the bacteria; however serum from Ad5ClfA or ClfAhis immunized animals both contain antibodies that bind to *S. aureus* (Figure 9).

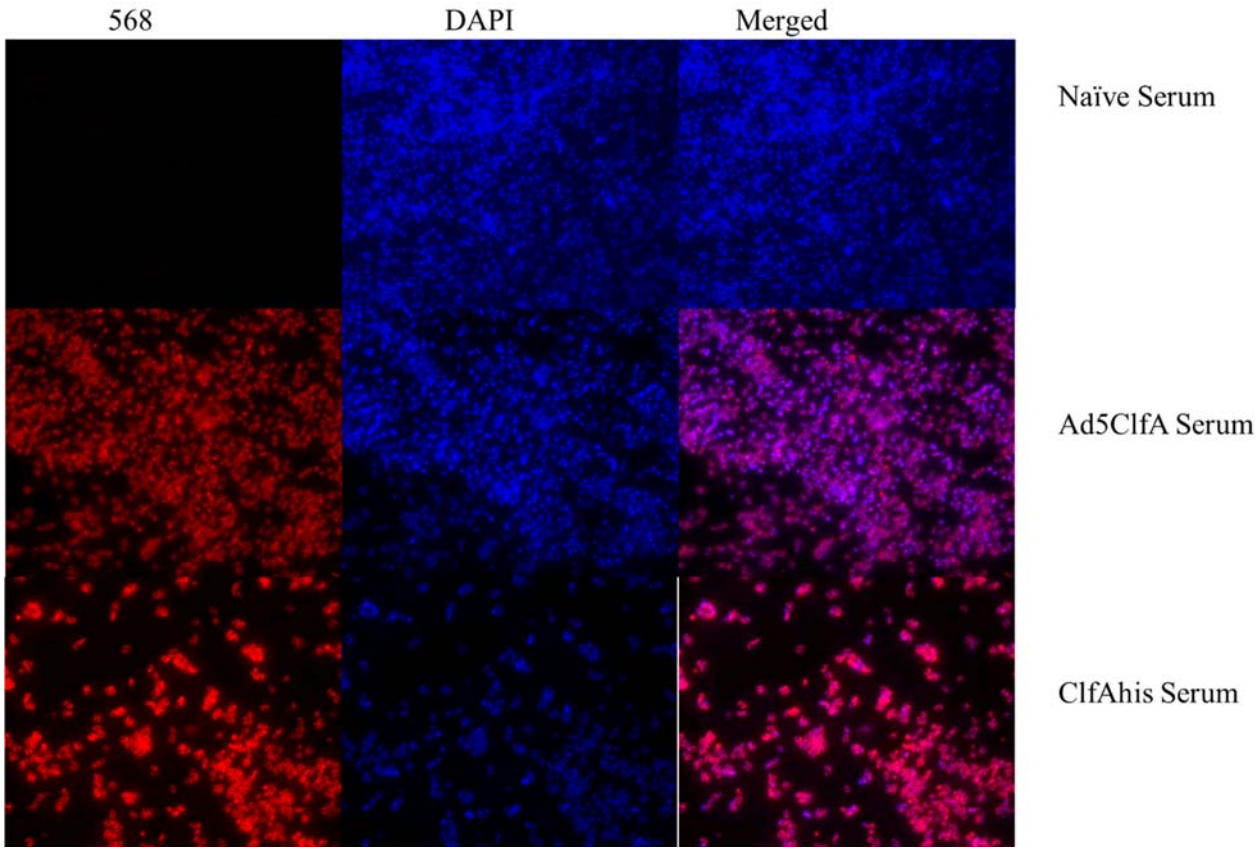


Figure 9. IFA of *S. aureus* Incubated with Serum from Immunized Mice. *S. aureus* cultures were grown overnight, washed with sterile PBS, and fixed onto glass coverslips. Cells were then incubated overnight with mouse serum (1:100 dilution in PBS) from naïve mice, or from mice immunized with Ad5ClfA or Clfahis+Alum. Cells were washed with sterile PBS then incubated with an Alexa Flor 568-conjugated anti-mouse-IgG secondary antibody and counterstained with DAPI. Coverslips were mounted on glass slides and images were acquired using a confocal microscope.

Ad5ClfA/Ad5SdrD Immunization Provides Protection from Cutaneous MRSA Infection

Data implicate IL-17 and T_H17 cells as having an important role in protecting against cutaneous *S. aureus* infection (Archer, Harro, and Shirtliff 2013; Cho et al. 2010; Miller and Cho 2011). Given the ability of Ad vectors to activate T cell subsets, I hypothesized that immunization with Ad vectors expressing the adhesion domains from the *S. aureus* proteins ClfA and SdrD would provide protection from cutaneous MRSA infection by eliciting enhanced

antigen specific T cell responses. Specifically, I hypothesized that Ad vaccine vectors would provide enhanced protection by inducing a more potent T_H17 and IL-17 response to vaccine antigens. I further hypothesized that if these Ad vectors are capable of eliciting stronger T_H17 responses then they would provide better protection from cutaneous infection than protein+Alum immunization, which invokes a strong T_H2 response (Coffman, Sher, and Seder 2010)

To determine whether immunization with Ad vaccine vectors could provide protection from cutaneous MRSA infections, I first generated Ad5 vectors expressing the ligand binding domains from either ClfA or SdrD as transgenes. Mice were intramuscularly immunized with 10^9 particles each of Ad5ClfA and Ad5SdrD. Protein-immunized mice were immunized with 2μg each of ClfAhis, SdrDhis, SdrEhis, and FnBPAhis. Mice injected with PBS were used as an unimmunized control, and mice immunized with $2x10^9$ vp of Ad5gfp served as a vector control.

28 days post immunization mice were challenged by intradermal inoculation at four sites with $2x10^6$ CFU/site of the USA300 strain of MRSA. On day six post challenge mice were sacrificed and bacterial load in the wounds was determined by plating dilutions of wound homogenates onto Tryptic Soy Agar (TSA) plates. Measurements of the wound sizes were also obtained and used to evaluate protection. Mice immunized with Ad5ClfA/Ad5SdrD had significantly lower bacterial titers in the wounds than PBS and Ad5gfp immunized mice, and had reduced titers compared to protein-immunized mice that were approaching significance (Figure 10A). In addition, Ad5CflA/Ad5SdrD immunized mice had significantly reduced wound sizes compared to PBS and Ad5gfp immunized mice (Figure 10B-C). To determine the limit of vaccine efficacy, I challenged mice with various doses of USA300 and assessed bacterial load in the wounds as a measure of protection. The data obtained indicate that Ad5ClfA/Ad5SdrD does

not afford protection against high doses of bacteria, but that at challenge doses in the range of 10^6 CFU/site it is able to provide protection (Figure 10D).

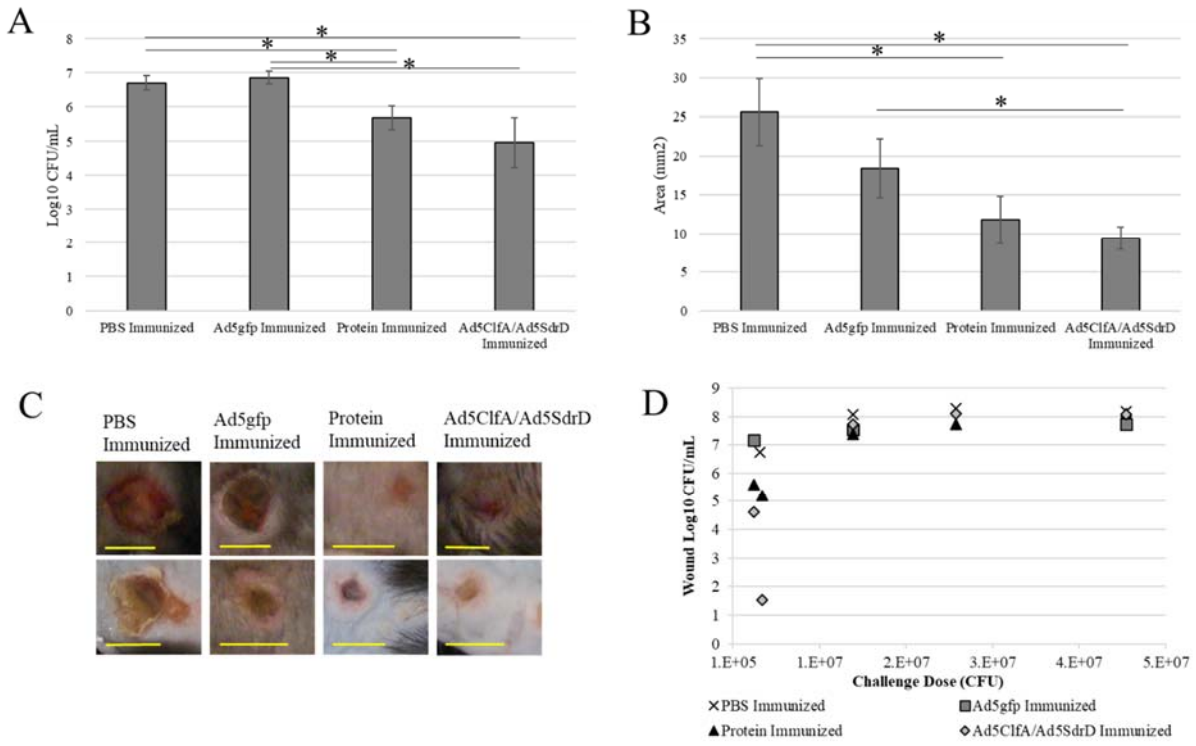


Figure 10. Effects of Ad5ClfA/Ad5SdrD Immunization on Cutaneous MRSA Challenge. C57BL/6 mice were intramuscularly immunized with 10^9 vp/mouse of Ad5ClfA and 10^9 vp/mouse of Ad5SdrD. Alternatively, mice were immunized with 2µg each of ClfAhis, SdrDhis, SdrEhis and FnBPAhis, $2x10^9$ vp of Ad5gfp, or PBS, with 9-12 mice per group. 28 days post immunization mice were intradermally challenged with $2x10^6$ CFU/site on 4 sites on their back. Six days post challenge mice were sacrificed and the bacterial load in the wound (A) and average wound sizes (B) were determined. (C) Representative wound images from the experiments described above. (D) To determine vaccine efficacy, C57BL/6 mice were immunized as described above. 28 days post immunization mice were challenged with the indicated CFU on 4 sites on their back. Six days post challenge mice were sacrificed and the bacterial load in the wounds was determined as a measure of protection. *$p<0.05$

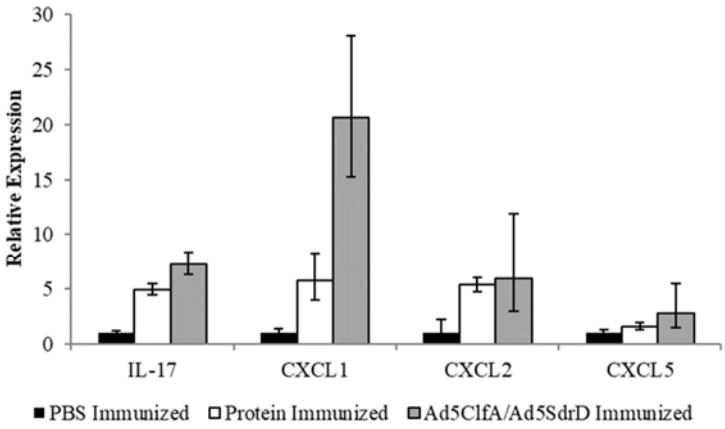
Wounds from Ad5ClfA/Ad5SdrD Immunized Animals Exhibit Enhanced Expression of mRNA Encoding for IL-17 and Neutrophil Chemokines

My data suggest that immunization with Ad vectors expressing *S. aureus* antigens provides protection against cutaneous MRSA challenge. Numerous studies have shown IL-17 to be required for vaccine mediated protection, particularly in response to cutaneous infections (Narita et al. 2010; Yeaman et al. 2014). Data from Ad5ClfA immunization experiments show that Ad5ClfA immunization induces an IL-17 response to ClfA. Therefore, I expected that Ad5ClfA/Ad5SdrD immunized animals would have a greater IL-17 response that would provide protection against challenge.

During cutaneous *S. aureus* infections IL-17 stimulates keratinocytes to produce chemokines and adhesion molecules, including CXCL1, CXCL2 and CXCL5, that recruit neutrophils to the infection site (Herjan et al. 2013; Miller and Cho 2011). I expected that if there was an increased IL-17 response in Ad5ClfA/Ad5SdrD immunized mice then there would also be greater expression of neutrophil recruiting chemokines. Neutrophils are crucial in the clearance of *S. aureus*, therefore I hypothesized that an IL-17 mediated increase in neutrophil recruitment may be one mechanism of vaccine mediated protection.

To determine whether there was increased expression of mRNA encoding for IL-17 and neutrophil recruiting chemokines in the wounds of Ad5ClfA/Ad5SdrD immunized animals I isolated RNA from wound samples and performed RT-qPCR to quantify mRNA expression. Ad5ClfA/Ad5SdrD immunized animals had greater IL-17 mRNA expression than either PBS or protein immunized animals (Figure 11). Compared to both PBS and protein immunized animals Ad5ClfA/Ad5SdrD animals also had increased expression of CXCL1 and CXCL5 mRNA, consistent with the increase in IL-17 mRNA expression (Figure 11). Both Ad and protein-

immunized animals had increased expression of CXCL2 mRNA compared to PBS immunized animals. These data indicate that a possible mechanism of Ad5ClfA/Ad5SdrD mediated protection may be through IL-17 mediated neutrophil recruitment to sites of infection.



Fold increase in expression compared to PBS	IL-17	CXCL1	CXCL2	CXCL5
Protein Immunized	4.5-5.4	4.0-8.2	4.8-6.1	1.3-2.0
Ad5ClfA/Ad5SdrD Immunized	6.3-8.4	15.3-28.0	3.0-11.9	1.5-5.5

Figure 11. mRNA Expression in the Wound Following USA300 Challenge. C57BL/6 mice were intramuscularly immunized with 10^9 vp of Ad5ClfA and 10^9 vp of Ad5SdrD, 2μg each of ClfAhis, SdrDhis, SdrEhis and FnBPAhis, or PBS. 28 days post immunization mice were intradermally challenged with $2x10^6$ CFU on 4 sites on their back. Six days post challenge mice were sacrificed and RNA was isolated from wound samples. IL-17, CXCL1, CXCL2, and CXCL5 mRNA expression from pooled wound RNA samples were determined by quantitative RT-PCR using the $2^{-\Delta\Delta CT}$ method.

Ad5ClfA/Ad5SdrD Immunized Mice Generate High Titer Anti-ClfA Antibodies Following USA300 Challenge That Have a Distinct IgG Profile Compared to Protein Immunization

Although vaccine strategies aimed solely at generating antibody responses to *S. aureus* antigens have largely been unsuccessful, antibodies generated in conjunction with a strong T cell

response may be important in Ad5ClfA/Ad5SdrD vaccine mediated protection. Following production of cytokines from activated T cells, neutrophils are recruited to sites of cutaneous infection. Neutrophils are then able to mediate clearance of bacteria through a variety of mechanisms, including antibody mediated phagocytosis and killing of opsonized bacteria (Segal 2005). In this way antibodies can help facilitate bacterial clearance and may be involved in Ad5ClfA/Ad5SdrD vaccine mediated protection.

To determine the antibody response to vaccine antigens following immunization and challenge, I collected serum from mice in the above described immunization and challenge experiments and determined the endpoint dilution titers by ELISA (Figure 12A). I found that, as expected, protein in Alum immunized animals had robust antibody responses to all four vaccine antigens. Animals immunized with PBS or Ad5gfp did not have detectable antibody responses to ClfA, SdrD, SdrE, or FnBPA. Importantly, immunization with Ad5ClfA/Ad5SdrD yielded antibody titers against ClfA that were comparable to those elicited by immunization with the purified protein.

While protein and Ad immunized animals show comparable levels of anti-ClfA antibodies following USA300 challenge, the isotypes of anti-ClfA antibodies may differ between the two groups of animals. Indeed, data from my immunization studies show that Ad5ClfA immunization induces antibodies that are predominantly IgG2b and IgG2c whereas antibodies generated in response to immunization with recombinant protein in Alum are predominantly IgG1. IgG isotypes have differing capacities to mediate antibody effector functions, therefore differences in the isotypes of antibodies elicited could account for some of the difference in protective capacity seen between protein and Ad immunized animals.

I next asked whether immunization with Ad5ClfA/Ad5SdrD elicited a different profile of anti-ClfA IgG isotypes following USA300 challenge than immunization with protein in Alum. Consistent with the IgG profiles seen in data from immunization studies, protein in Alum immunization induced mainly IgG1 antibodies while Ad5ClfA/Ad5SdrD immunization induced anti-ClfA antibodies that were predominantly IgG2b and IgG2c (Figure 12B). These data indicate that while the total levels of anti-ClfA IgG are comparable between protein and Ad5ClfA/Ad5SdrD immunized animals, the isotype profile of the IgG antibodies are dependent on whether the vaccine antigen is delivered via an alum or Ad adjuvant.

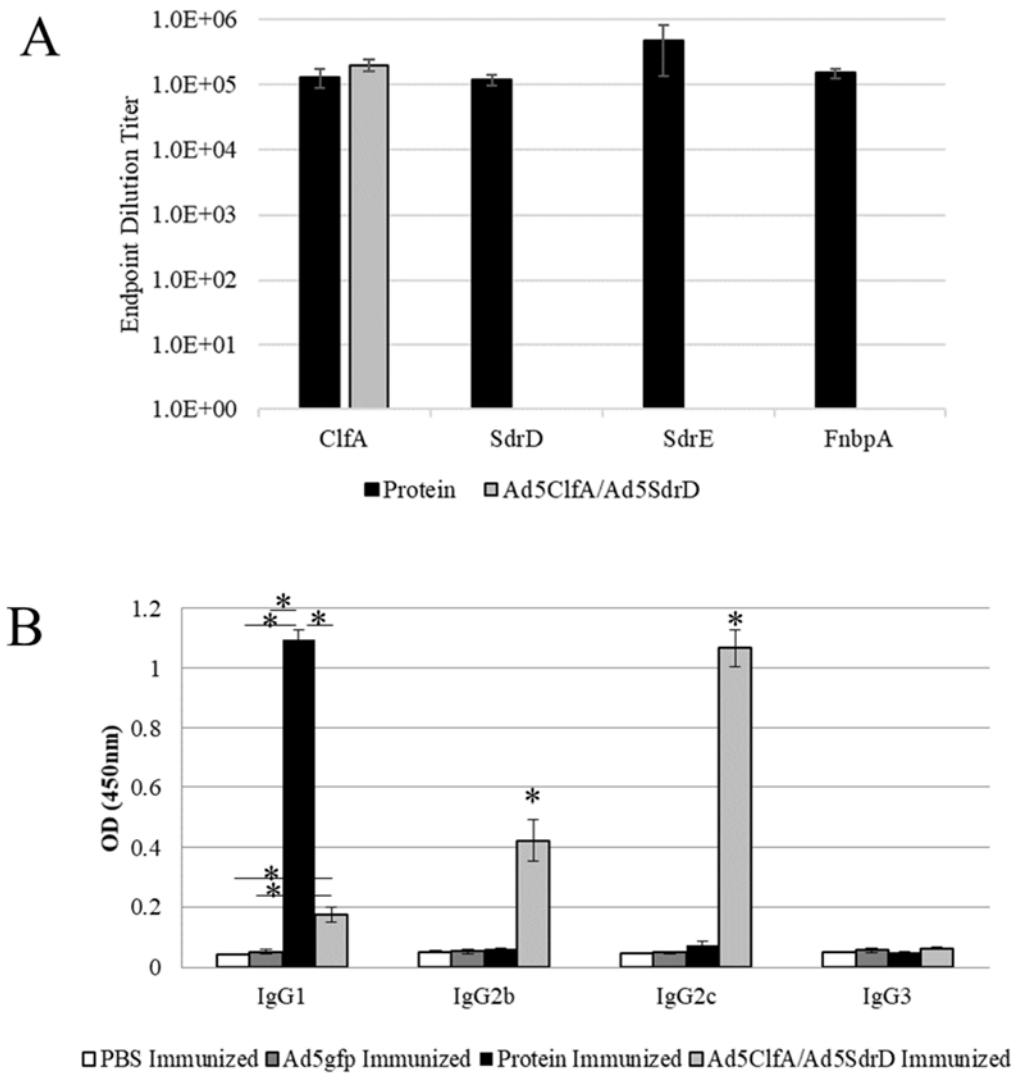


Figure 12. Antibody Response to ClfA Following USA300 Challenge. C57BL/6 mice were intramuscularly immunized with 10^9 vp of Ad5ClfA and 10^9 vp of Ad5SdrD, 2μg each of ClfAhis, SdrDhis, SdrEhis, and FnBPAhis, $2x10^9$ vp of Ad5gfp, or PBS. 28 days post immunization mice were intradermally challenged with $2x10^6$ CFU on 4 sites on their back. Six days post challenge mice were sacrificed and serum was collected. (A) Total IgG antibody titers against ClfA, SdrD, SdrE, and FnBPA were determined by endpoint dilution assays using pooled serum samples. Antibody titer was determined by the highest dilution at which the absorbance value differed significantly from those of negative controls. (B) Anti-ClfA IgG isotypes were determined by ELISA and isotype response was determined based on absorbance at OD450nm. *$p<0.05$

Serum from Ad5ClfA/Ad5SdrD Immunized Mice Mediates Uptake of USA300 by Macrophages

Data from my immunization experiments, as well as immunization/challenge experiments, show that anti-ClfA IgG isotypes in Ad5ClfA/Ad5SdrD immunized animals consist mainly of IgG2 isotypes. This is in contrast to protein immunized mice, which develop anti-ClfA IgGs that are mainly comprised of IgG1. In mice IgG2 isotypes have the highest binding affinity to activating FCγRs, whereas IgG1 isotypes have relatively low binding affinity to FCγRs (Jonsson and Daeron 2012; Stewart et al. 2014). Binding of high levels of antigen bound IgGs activates FCγRs and leads to FCγR mediated effector functions such as ADCC and antibody mediated opsonization by phagocytic cells. Following internalization of bacteria, phagocytic cells mediate bacterial killing through a variety of mechanisms. Therefore, I hypothesized that one mechanism of Ad5ClfA/Ad5SdrD vaccine mediated protection may be enhanced IgG2 mediated opsonization of bacteria.

To determine if serum from Ad5ClfA/Ad5SdrD immunized animals mediated bacterial uptake more efficiently than serum from protein, vector control, or PBS immunized animals I performed OPAs using a macrophage cell line and serum from mice in the previously described immunization and challenge experiments. Serum from Ad5ClfA/Ad5SdrD immunized mice mediated increased uptake of opsonized bacteria by RAW264.7 macrophages compared to serum from PBS, Ad5gfp or protein immunized animals (Figure 13). This suggests that a possible mechanism of vaccine mediated protection may be through enhanced opsonophagocytosis of *S. aureus*.

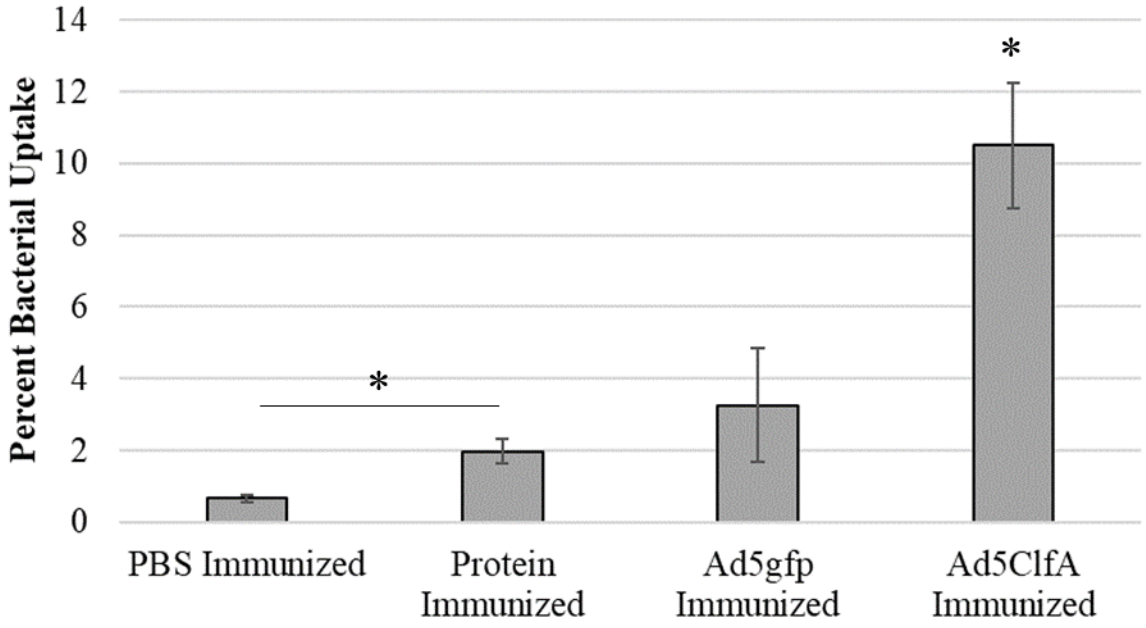


Figure 13. Serum Mediated Opsonophagocytic Activity. Pooled serum was obtained from animals in the previously described immunization and challenge experiments. Pooled serum was diluted 1:100 and incubated with USA300 for 30 minutes. Antibody coated bacteria were incubated for 30 minutes with RAW264.7 cells, then treated with 2μg/mL gentamycin and the total number of opsonized bacteria was determined by plating dilutions of the lysed RAW264.7 cells onto tryptic soy agar (TSA). Total bacteria taken up was normalized to the number of bacteria added to the RAW264.7 culture at time 0 minutes. *$p<0.05$

Ad5 Vectors with Enhanced Inflammasome Activation Elicit Enhanced IL-17 and IFNγ

Data from infection models and vaccine studies suggest that T_H1 and T_H17 cells, as well their associated cytokines IFNγ and IL-17, are important mediators of protection against *S. aureus* infection. My data show that Ad5 vectors expressing *S. aureus* antigens are able to provide protection against cutaneous MRSA infection. I hypothesized that modified Ad vectors, capable of eliciting increased T_H1 and T_H17 responses may provide even greater protection against MRSA challenge.

Cytokines IL-1□ and IL-18, which are secreted following inflammasome activation, drive differentiation of T_H17 and T_H1 cells, respectively. Therefore, a modified Ad5 vector with

increased inflammasome activation may be able to more potently induce T_H1 and T_H17 T cell subsets. Our lab has previously generated Ad vectors with enhanced inflammasome activation due to the appendage of the 35-C terminal amino acids from FliC onto the Ad capsid protein pIX (Ad5PF). Given that Ad5PF shows augmented inflammasome activation compared to Ad5 vectors, I expected that Ad5PF would also induce greater T_H1 and T_H17 responses. To determine whether Ad5PF vectors are able to induce stronger T_H1 and T_H17 responses to vaccine antigens compared to unmodified Ad5 vectors I used Ad5 vectors expressing GFP as a model transgene.

I immunized BALB/C mice with Ad5gfp or the modified vector Ad5PFgfp and harvested splenocytes 10 days post immunization. I re-stimulated splenocytes with GFP, or Ad5 empty capsid to determine the immune response to the vector, and determined IL-17 and IFNγ levels in the supernatant by ELISA. As expected, splenocytes from animals immunized with Ad5PFgfp showed significantly more production of IFNγ and IL-17 in response to GFP than the unmodified vector Ad5gfp (Figure 14). The IFNγ and IL-17 response to the vector was also increased in Ad5PFgfp immunized animals.

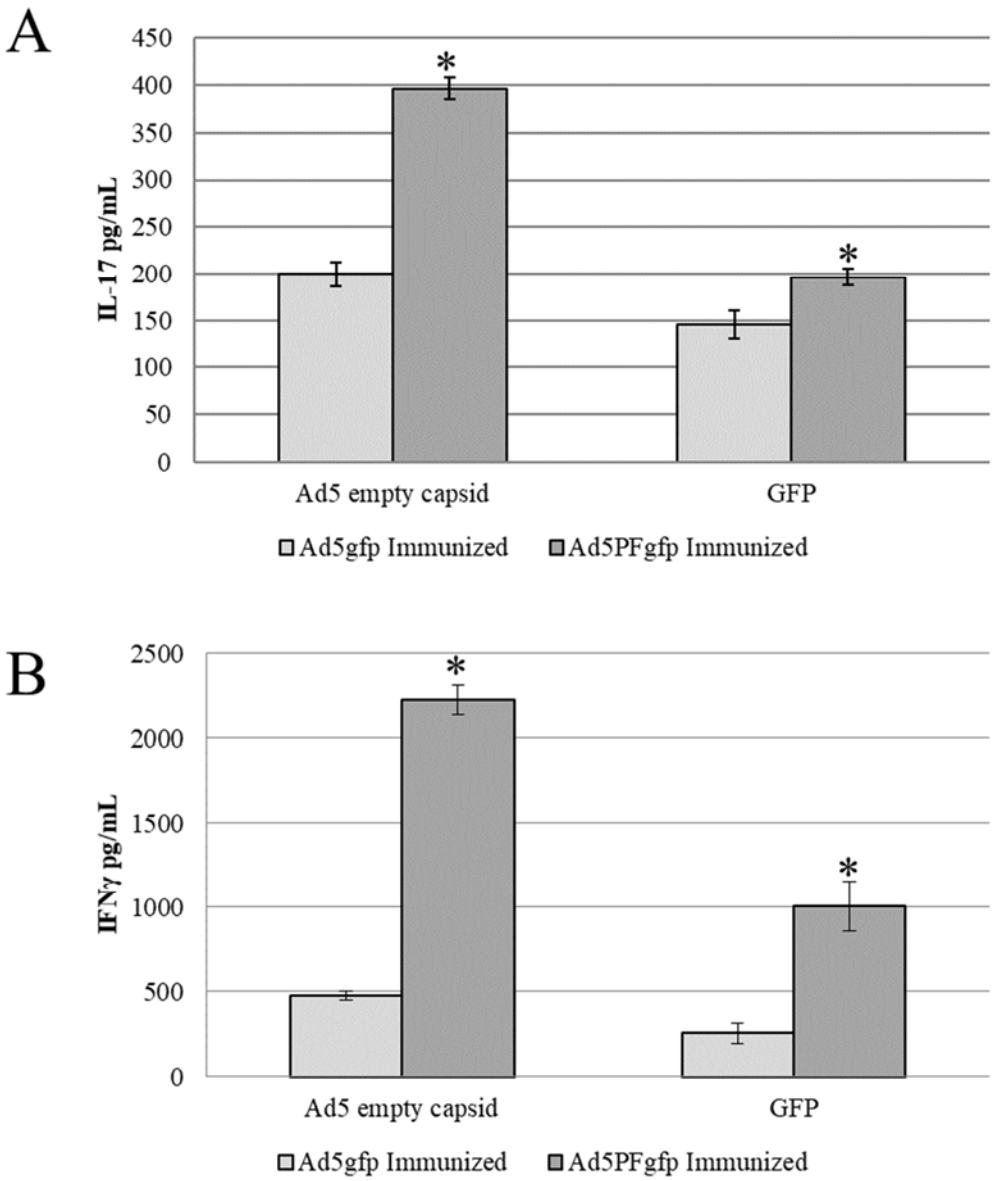
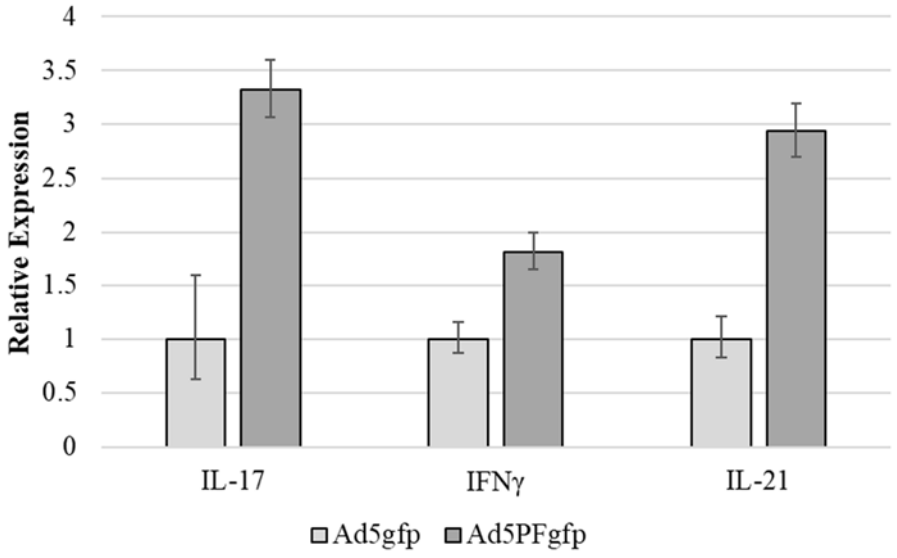


Figure 14. T Cell Response to Ad5PFgfp Immunization. BALB/C mice were intramuscularly immunized with 10^{10} vp of Ad5gfp or Ad5PFgfp. Ten days post immunization mice were sacrificed and splenocytes were harvested. Splenocytes were restimulated with GFP or Ad5 empty capsid. 24 hours (IFNγ) or five days (IL-17) post restimulation the supernatant was collected and the amount of IL-17 (A) and IFNγ (B) present was determined by ELISA. *$p<0.05$

Ad5PFgfp Immunized Mice Show Increased Expression of IL-17, IFNγ, and IL-21 mRNA

Splenocytes from Ad5PFgfp immunized mice show increased production of IL-17 and IFNγ in response to restimulation with GFP. This suggests that Ad5PFgfp immunization induces more potent T cell responses to vaccine antigens than unmodified Ad5 vectors. To further evaluate the effects of Ad5PFgfp immunization on T cell activation I also looked at expression of T cell cytokines in the draining lymph nodes from Ad5PFgfp and Ad5gfp immunized mice. If Ad5PFgfp induces greater T cell activation then I expected to see increased expression of IL-17 and IFNγ in the draining lymph nodes, consistent with the increased responses seen in splenocytes. I would also expect to see increased expression of IL-21, which is predominantly produced by $CD4^+$ T cells including T follicular helper (T_{FH}) and T_H17 cells (Mitsdoerffer et al. 2010). Lymph nodes from animals immunized with Ad5PFgfp had greater expression of IL-17, IFNγ and IL-21 mRNA than Ad5gfp immunized animals (Figure 15), supporting the hypothesis that the pIXFliC vector is able to enhance activation of T cells.



Fold increase in expression compared to Ad5gfp Immunized	IL-17	IFNγ	IL-21
Ad5PFgfp Immunized	3.06-3.59	1.66-2.00	2.70-3.19

Figure 15. mRNA Expression in the Lymph Node of Ad5PFgfp and Ad5gfp Immunized Mice. BALB/C mice were intramuscularly immunized with 10^{10} vp of Ad5gfp or Ad5PFgfp. Ten days post immunization mice were sacrificed and the draining lymph nodes were harvested. IL-17, IFNγ, and IL-21 mRNA expression in the draining lymph nodes were determined by quantitative RT-PCR using the $2^{-\Delta\Delta CT}$ method.

Ad5PFgfp Immunization Provides Protection Against *S. aureus* Challenge

Ad5PFgfp immunization induces increased IL-17 responses to vaccine antigens compared to immunization with Ad5gfp. Given the importance of IL-17 in protecting against cutaneous infections, I hypothesized that immunization with the FliC expressing vector would provide protection against cutaneous infection. Furthermore, since Ad5PF vectors induce a stronger IL-17 response I expected that Ad5PF immunization would provide greater protection against cutaneous infection than immunization with unmodified Ad5 vectors.

As Ad5PFgfp and Ad5gfp viruses had been previously purified I sought to determine whether immunization with these vectors could provide protection against cutaneous *S. aureus* infection. Although these vectors do not express *S. aureus* antigens, I hypothesized that the augmented IL-17 response to the vector could induce a protective immune response. I also used *S. aureus* strains expressing GFP, with the rationale that the anti-GFP response may still afford protection by further augmenting the IL-17 response at the site of infection. In this way the anti-GFP response could mimic the response seen from vectors expressing *S. aureus* antigens.

I first asked whether Ad5PFgfp could provide protection against a less virulent, methicillin sensitive, *S. aureus* strain. Ad5PFgfp and Ad5gfp immunized mice were challenged with the RN4220gfp strain of *S. aureus* and protection was assessed by determining the wound bacterial loads. PBS immunized mice were used as an unimmunized control. I found that while animals immunized with Ad5gfp or PBS had comparable bacterial titers in their wounds, animals immunized with Ad5PFgfp had significantly lower bacterial titers in their wounds compared to PBS immunized animals (Figure 16A). The reduction in bacterial load corresponded to an increased IL-17 response by splenocytes (Figure 16B).

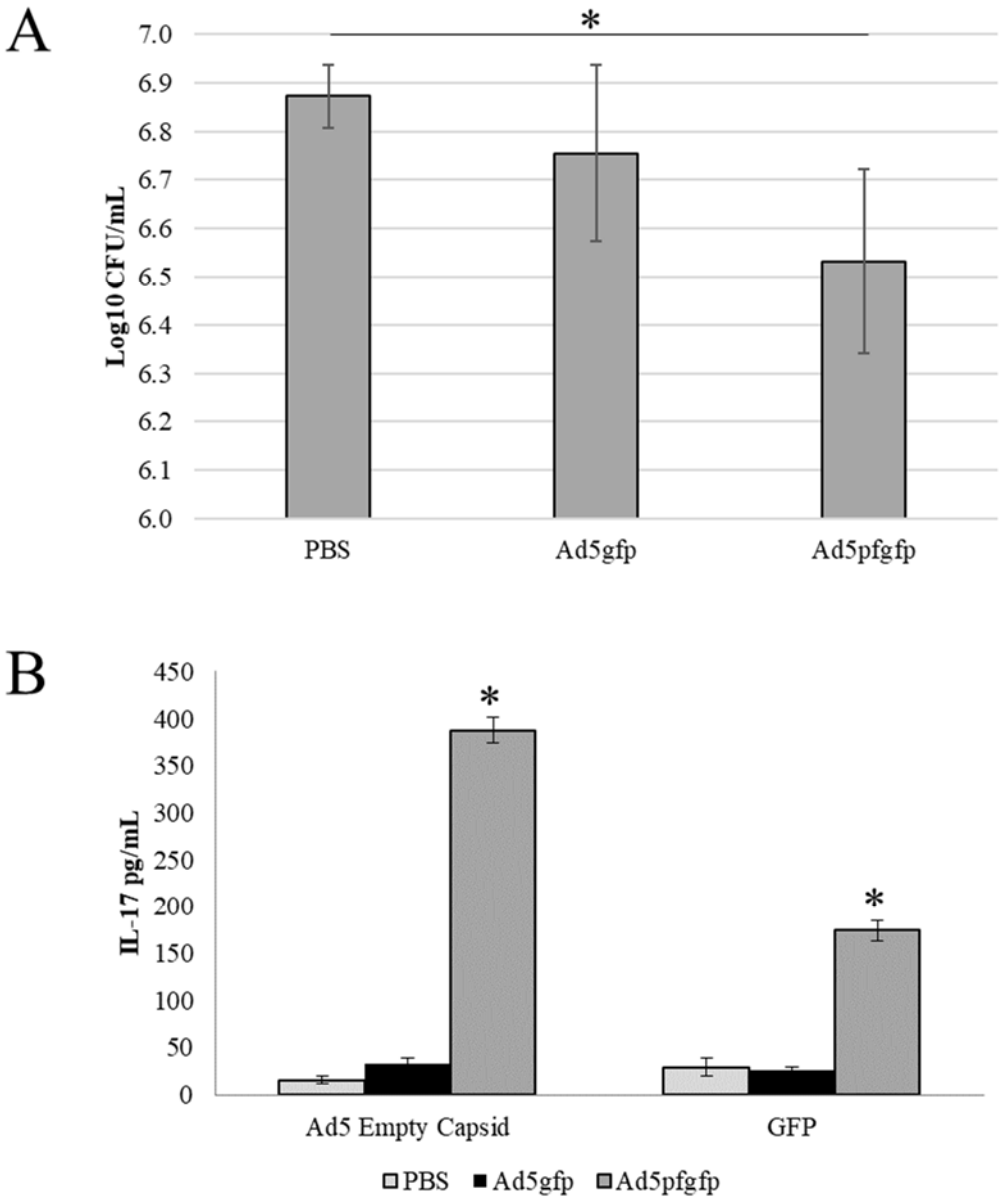


Figure 16. Effects of Ad5PFgfp Immunization on Cutaneous Challenge with RN4220gfp. C57BL/6 mice were intramuscularly immunized with 10^{10} vp of Ad5gfp or Ad5PFgfp. PBS immunized mice were used as an unimmunized control. 28 days post immunization mice were intradermally challenged with $2x10^6$ CFU of RN4220gfp on 4 sites on their back. Three days post challenge mice were sacrificed and bacterial load in the wound was determined (A). (B) Splenocytes were restimulated with Ad5 empty capsid or GFP. Five days post restimulation the supernatants were collected and the amount of IL-17 present was determined by ELISA. *$p<0.05$

Since Ad5PFgfp is able to protect against cutaneous *S. aureus* infections with methicillin sensitive strains I next asked whether this protection extended to MRSA infections. To determine whether Ad5PFgfp immunization could provide greater protection than Ad5gfp against MRSA challenge I used a GFP expressing strain of USA300 to challenge mice that had previously been immunized with Ad5gfp or Ad5PFgfp. Mice immunized with Ad5PFgfp had significantly reduced bacterial load in their wounds compared to mice immunized with Ad5gfp (Figure 17A). Although not statistically significant, protection did correlate with an increased IL-17 response (Figure 17B). These data suggest that the increased IL-17 response induced by the Ad5PF vector is capable of providing protection against MRSA infection, and that this protection is greater than that afforded by unmodified Ad vectors.

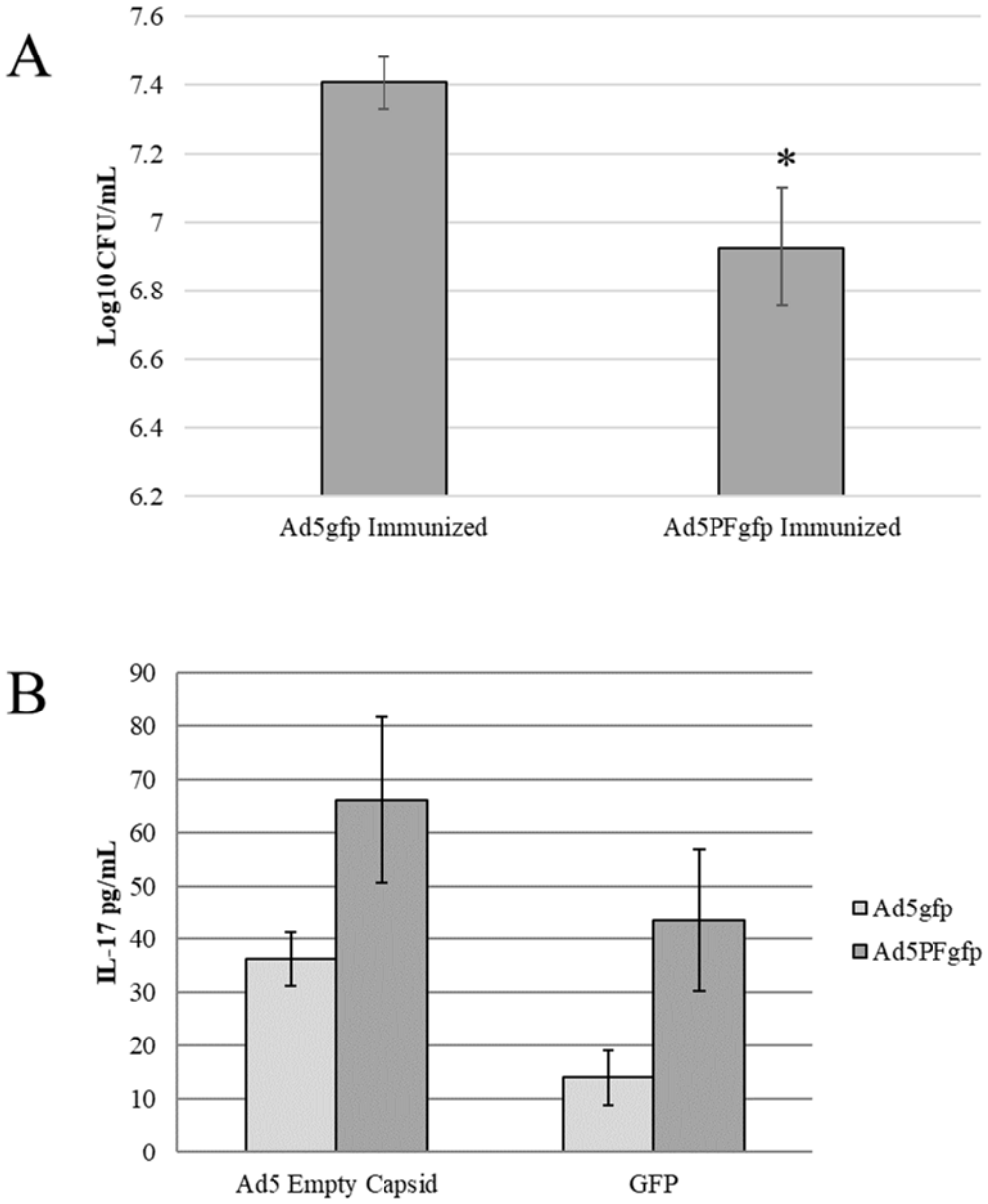


Figure 17. Effects of Ad5PFgfp Immunization on Cutaneous Challenge with MRSA. BALB/C mice were intramuscularly immunized with 10^{10} vp of Ad5gfp or Ad5PFgfp. 35 days post immunization mice were intradermally challenged with $2x10^6$ CFU of USA300gfp on 4 sites on their back. Six days post challenge mice were sacrificed and bacterial load in the wound was determined (A). (B) Splenocytes were restimulated with Ad5 empty capsid or GFP. Five days post restimulation the supernatants were collected and the amount of IL-17 present was determined by ELISA. *$p<0.05$

CHAPTER FOUR

DISCUSSION

S. aureus is a major cause of hospital and community acquired infections worldwide. These infections range from less serious SSTIs to invasive, life-threatening infections such as bacteremia, pneumonia, endocarditis, and sepsis. Treatment of these infections is becoming more challenging due to the rise of methicillin resistant *S. aureus* strains that are resistant to an increasing number of antibiotics. MRSA infections are associated with high rates of morbidity and mortality, and the treatment of these infections represent a significant financial burden. Development of a vaccine capable of providing protection against MRSA infections would significantly advance public health worldwide. Unfortunately, current vaccine strategies have been largely aimed at generating high titer antibody responses to vaccine antigens and have proven unsuccessful in clinical trials.

My goal in this work was to use Adenovirus vectors to develop a vaccine that could provide protection against cutaneous MRSA infections, and to begin to explore the mechanism of vaccine mediated protection. I first wanted to determine whether Ad vectors expressing *S. aureus* antigens could protect against cutaneous challenge in mice. I next asked whether Ad vectors that more potently activate the inflammasome could provide enhanced protection against MRSA challenge due to their ability to induce greater activation of T cells

Immunogenicity of Recombinant *S. aureus* Proteins

The majority of *S. aureus* vaccine candidates have consisted of active immunization approaches using proteins antigens, and have focused on generating high titer functional antibodies against antigen (Giersing et al. 2016). Many of these candidates have shown efficacy in mouse models, however this has not translated to success in humans. Failure of past vaccines has been attributed, in part, to failure to induce T cell mediated responses and functional antibodies. We hypothesized that Ad vectors, which are potent T cell adjuvants, would help to induce a strong anti-*S. aureus* T cell response that would provide greater protection than immunization with protein antigen. We further hypothesized that enhanced T cell responses would induce greater functional responses against *S. aureus*, including increased neutrophil recruitment and killing of opsonized bacteria and higher affinity class-switched antibodies. These features would overcome many of the failings seen in protein-based vaccines and could translate into an efficacious human vaccine.

To evaluate the efficacy of our vaccine vectors compared to protein immunization, which has previously been shown to be protective in mice, we generated recombinant proteins containing the ligand binding domains from the *S. aureus* surface proteins ClfA, SdrD, SdrE, and FnBPA. Data from western blots and ELISAs confirm that the recombinant purified proteins contain B cell epitopes. Antibodies generated against the recombinant proteins bind to protein in *S. aureus* WLC, and IFA data show that serum from ClfA immunized mice contain antibodies that bind to the surface of *S. aureus*. These data suggest that the recombinant proteins are either folded correctly or that the antibodies generated are not conformation dependent.

Emerging data suggest that T cells, and T_H17 cells specifically, are important mediators

of anti-*S. aureus* immunity. It is therefore essential that any potential vaccine antigen contain high affinity T cell epitopes. Due to the importance of IL-17 and IFNγ in mediating clearance of *S. aureus* I wanted to confirm that the domains I used to construct the recombinant proteins and Ad vaccine vectors contained T cell epitopes capable of inducing T_H17 and T_H1 responses. ELISPOT data from mice and humans with prior *S. aureus* infections show that re-stimulation of splenocytes with recombinant proteins induces an IL-17 and IFNγ response. This suggests that these antigens are capable of inducing a memory T_H17 and T_H1 response. Additionally, IL-17 responses to recombinant proteins from USA300 were seen in animals that had previously been infected with the SA113 strain, suggesting these antigens can elicit cross-reactive immunity against numerous different strains of *S. aureus* – a requirement for any potential vaccine antigen.

Anti-ClfA Immunity Induced by Ad5ClfA Immunization

For Ad vector vaccines for MRSA to be effective they will likely need to induce both T and B cell responses to vaccine antigens. $CD4^+$ T cell responses have been shown to be required for a number of vaccine candidates that have shown efficacy in mice, and deficiencies in T cell subsets are associated with increased risk for *S. aureus* infections (Lin et al. 2009; Yeaman et al. 2014). While a B cell response alone does not appear to be sufficient to provide protection against *S. aureus* infections, antibodies will likely be important mediators of bacterial clearance following T cell induced recruitment of phagocytic cells.

Both T_H1 and T_H17 responses, as well as their archetypal cytokines IFNγ and IL-17, have been shown to be critical for vaccine mediated protection in a variety to *S. aureus* infection models (Joshi et al. 2012; Lin et al. 2009; Narita et al. 2010). ELISA data from my immunization experiments show that Ad5CflA immunization is capable of eliciting IL-17 and IFNγ responses to ClfA, suggesting that immunization induces T_H17 and T_H1 responses. I expect that the

majority of activated antigen-specific cells expressing these cytokines in response to ClfA will be $CD4^+$ T_H1 and T_H17, however it is possible that other cell types are producing these cytokines. A number of cell types, including $CD8^+$ and γδ T cells, have also been shown to produce IL-17 and IFNγ. It is known that Ad potently induces $CD8^+$ T cells, so it is possible that the IL-17 and IFNγ responses are coming from this cell type rather than $CD4^+$ T cells. In the future intracellular cytokine staining and FACs could be used to determine the phenotype of cells expressing IL-17 or IFNγ in response to vaccine antigen.

Immunization with *S. aureus* surface proteins has shown limited protection in mouse models, and protection following protein immunization is thought to depend on IL-17 and IFNγ production from $CD4^+$ T cells (Lin et al. 2009; Narita et al. 2010). Given that Ad vectors more potently activate T cell responses compared to protein in Alum immunization I expected that an Ad vector vaccine would provide greater protection against MRSA challenge compared to protein immunization due to increased T cell responses. In support of this theory, ELISA data showed that Ad5ClfA immunization induced significantly more IL-17 and IFNγ compared to ClfAhis immunization. In fact, ClfAhis immunization failed to induce IFNγ responses to ClfA that were above the levels seen in PBS immunized controls.

Although anti-ClfA antibody titers are significantly higher in protein immunized mice, Ad5ClfA immunization does induce a strong antibody response to ClfA. Antibodies induced following Ad5ClfA immunization not only recognize recombinant protein, but also bind to whole *S. aureus* bacterial as shown by our IFA data. Furthermore, Ad5ClfA immunization induces a different anti-ClfA IgG profile than does protein immunization. Anti-ClfA antibodies in protein immunized mice were primarily IgG1, whereas antibodies in Ad5ClfA immunized mice were composed of IgG2b and IgG2c isotypes. This has important implications for vaccine

development, as it is known that mouse IgG2 isotypes have the highest binding affinities to FCγRs and would likely be more efficient at mediating bacterial clearance via opsonophagocytosis than IgG1 antibodies (Stewart et al. 2014). To determine whether Ad5ClfA induced antibodies are actually more functional in mediating opsonophagocytosis than antibodies from protein immunized mice OPAs could be performed.

To date I have purified both Ad5ClfA and Ad5SdrD. In the future it would be of value to perform immunization experiments using Ad5SdrD to determine whether we see a similar immune response to SdrD as we do to ClfA following Ad vector immunization. Studies suggest that immunization with ClfA induces antigen specific IL-17 responses, however other *S. aureus* surface adhesion proteins have not been extensively studied and therefore much less is known about the immunogenicity of SdrD, SdrE and FnBPA as vaccine antigens (Narita et al. 2010).

Ad5ClfA/Ad5SdrD Mediated Protection Against MRSA

Immunization with Ad5ClfA/Ad5SdrD was protective against cutaneous MRSA challenge. Compared to PBS and vector control immunized mice, Ad5ClfA/Ad5SdrD immunized mice had significantly reduced wound sizes and bacterial loads. Additionally, Ad immunized mice had reduced wound size and bacterial load compared to protein immunized mice. The majority of vaccine candidates to date have consisted of active immunization with protein antigen or passive immunization with monoclonal antibodies (Giersing et al. 2016). Unfortunately, none of these vaccine candidates have shown success in clinical trials. Our data suggest that Ad vectors represent a novel *S. aureus* vaccine approach that could be used to generate a vaccine for cutaneous MRSA infections.

Our vaccine efficacy data show that Ad vectors expressing *S. aureus* antigens are protective at challenge doses in the range of 10^6 CFU, but not at CFUs in the range of 10^7. While

a vaccine that is capable of protecting against the highest bacterial load would be ideal, a vaccine capable of protecting against 10^6 CFU would still be a significant public health achievement. Previous studies have shown that immunization with multiple antigens affords greater protection than immunization with individual antigens (Zuo et al. 2013), therefore co-immunization with Ad vectors expressing additional antigens, such as SdrE and FnBPA could enhance the protective efficacy of our vaccine. Additionally, booster immunizations may afford additional protective immunity. If Ad5ClfA/Ad5SdrD is to be further explored as a possible vaccine candidate it will need to be protective against not only USA300, but a wide variety of *S. aureus* strains. It will therefore be important to determine whether Ad5ClfA/Ad5SdrD immunization is able to afford protection against additional MRSA strains.

Cutaneous *S. aureus* infections are a leading cause of skin and soft tissue infections (SSTIs) worldwide, and these infections have the potential to go on to cause invasive and life-threatening infections. Development of a vaccine that can protect against cutaneous infections would be extremely beneficial not only in preventing SSTIs, but also in preventing more invasive infections that result from cutaneous infections. While IL-17 is commonly associated with protection against cutaneous infection it has also been shown, along with T_H1 cells and IFNγ, to provide protection in murine bacteremia models (Lin et al. 2009; Narita et al. 2010). It is therefore possible that the T cell responses induced by Ad5ClfA/Ad5SdrD immunization could also provide protection against other types of *S. aureus* infection. It would be worthwhile to perform additional immunization challenge experiments using models such as bacteremia or endocarditis to determine whether Ad vector vaccines can protect against these types of infections in addition to cutaneous infections.

mRNA expression of IL-17 is increased in the wounds of Ad5ClfA/Ad5SdrD immunized mice and correlates with protection from cutaneous MRSA infection. Ad5ClfA immunization generates an IL-17 response to ClfA, therefore it is likely that the increased IL-17 expression in the wounds of Ad5ClfA/Ad5SdrD immunized mice is due to vaccine mediated enhancement of IL-17 responses to ClfA and SdrD. Further studies would be needed to confirm whether the increased IL-17 expression is due to increased IL-17 responses to vaccine antigens and if this correlates with increased IL-17 production in the wounds. To understand the mechanisms involved in vaccine mediated protection it will be important to determine the cellular source of the increased IL-17 response. As T_H17 cells are a major source of IL-17, I expect that Ad5ClfA/Ad5SdrD immunization will have generated memory T_H17 cells to ClfA and SdrD that are responsible for the increased IL-17 response. It is also possible that $CD8^+$ T cells or γδ T cells, which are abundant in the skin, are responsible for the increased production of IL-17 in Ad5ClfA/Ad5SdrD immunized animals.

Given that IL-17 is known to stimulate production of neutrophil recruiting chemokines, it is not surprising that the expression of CXCL1 and CXCL5 are also increased in the wounds of Ad5ClfA/Ad5SdrD mice. I expect that immunohistochemistry or FACS would show increased neutrophil recruitment to the wounds of Ad5ClfA/Ad5SdrD animals, consistent with the increased expression of these neutrophil recruiting chemokines. Neutrophils are critical for immunity against *S. aureus* infections, therefore I expect that a key mechanism of vaccine mediated protection is enhanced neutrophil mediated bacterial clearance due to increased IL-17 and downstream chemokine production.

Consistent with the IgG profiles seen following immunization, MRSA infected mice that have been previously immunized with Ad5ClfA/Ad5SdrD contain anti-ClfA antibodies that are

primarily IgG2b and IgG2c. This is in contrast to mice immunized with recombinant proteins, which contain mainly IgG1 antibodies against ClfA. IgG2 isotypes in mice are known to have higher binding affinity for FCγR, which I expected would result in greater uptake of bacteria by phagocytic cells. My OPA data show that this is indeed the case, as serum from Ad5ClfA/Ad5SdrD immunized mice mediates significantly greater bacterial uptake compared to serum from control or protein immunized mice. These data support my theory that Ad vaccine mediated protection involves increased bacterial clearance by neutrophils. The data further suggest that in addition to increased neutrophil recruitment, vaccine mediated protection may be due to the generation of antibodies with increased opsonophagocytic capabilities. While the OPA data show that serum from Ad5ClfA/Ad5SdrD immunized animals mediates greater bacterial uptake, additional experiments are needed to confirm that this also corresponds with killing of internalized bacterial.

In our immunization and challenge experiments, mice immunized with Ad5ClfA/Ad5SdrD generate titers of anti-ClfA antibodies that are comparable to the titers seen in protein immunized mice. While protein immunized mice also generate anti-SdrD antibodies, no anti-SdrD antibodies were detected in Ad5ClfA/Ad5SdrD immunized mice. The immune response to Ad5SdrD immunization has not yet been evaluated, therefore it is possible that SdrD expressed from our Ad vector is not as immunogenic as ClfA. It is also possible that the transgene is not getting expressed properly in mice, however *in vitro* data show that SdrD is expressed following Ad5SdrD infection of 293□5 cells.

Transgene Immunity Induced by Immunization with Ad5PF Vectors

FliC has been used as an adjuvant both by co-administering FliC with vaccine antigens and by creating FliC fusion proteins with antigen. FliC has been shown to increase activation of

DCs, T cells and to promote B cell class switching (Bennett et al. 2015). Our lab has generated Ad5 vectors that display the 35 C terminal amino acids from FliC on the surface of the capsid. FliC activates the NLRC4 inflammasome, which can then cleave pro-IL-1□ and pro-IL-18 into forms that are secreted from the cell. Since these cytokines are involved in directing T cell differentiation toward T_H17 and T_H1 subsets, respectively, I hypothesized that vectors displaying FliC would enhance the differentiation of naïve T cells into T_H17 and T_H1 subsets. Although FliC is displayed on the viral capsid and the vaccine antigen is delivered as a transgene, I hypothesized that the increased presence of IL-1□ and IL-18 in the local milieu would enhance the differentiation of T_H17 and T_H1 subsets not only to the viral capsid but also to the transgene.

Animals immunized with Ad5PFgfp showed significantly greater IL-17 and IFNγ responses than animals immunized with unmodified Ad5 (Ad5gfp). Importantly, the increased cytokine responses were seen not only to the viral capsid but also to the transgene GFP. These data show that FliC displayed on the viral capsid is able to act as an adjuvant for vaccine antigens delivered as transgenes. The ELISA data show increased IL-17 and IFNγ responses which suggest that the antigen specific T_H17 and T_H1 responses are increased, however it is possible that other cell types are responsible for the increased cytokine responses. Ad very potently induced $CD8^+$ T cell responses, so it is possible that $CD8^+$ rather than $CD4^+$ T cells are responsible for the increased IL-17 and IFNγ responses that we see. In the future I could use FACs and intracellular cytokine staining to determine the phenotype of cells expressing IL-17 or IFNγ following restimulation with antigen.

In addition to performing ELISA assays, I also measured mRNA expression in the draining lymph nodes of immunized animals. q-RT-PCR data support the findings from the ELISA assays, showing that the lymph nodes from Ad5PFgfp immunized animals have

increased expression of IL-17, IFNγ and IL-21 mRNA. IL-21 is produced by T_H17 cells as well as T_{FH} cells and plays a critical role in supporting B cell differentiation and production of high affinity class switched antibodies (Spolski and Leonard 2010). Therefore, it is likely that the Ad5PFgfp vector is able to augment both T and B cell responses to vaccine antigens and give rise to antibody responses that have increased effector functions.

Ad5PF as a MRSA Vaccine Vector

Mice immunized with Ad5PFgfp vectors show increased T_H17 and T_H1 responses to antigens expressed as transgenes. T_H17 and T_H1 responses have been shown to mediate protection against *S. aureus* infection. Additionally, data from our immunization and challenge experiments suggest that one mechanism of Ad vector mediated protection is through increased IL-17 production in response to *S. aureus* antigens. I hypothesized that due to the ability of Ad5PF vectors to more potently induce T cell responses, and specifically IL-17 responses, these vectors would provide greater protection against MRSA infection compared to Ad5 vectors.

Since we have not yet purified Ad5PF vectors expressing *S. aureus* antigens as transgenes I used Ad5PF vectors expressing GFP as a model transgene to perform immunization and challenge experiments. To more closely mimic a vector expressing a bacterial antigen, I also used *S. aureus* strains that express GFP. In these strains GFP would not be acting as a virulence factor, nor would it mediate immune evasion or invasion of new tissues as ClfA does, but it would allow antigen specific T cells induced during immunization to encounter their antigen and mediate effector functions.

Initially, we performed challenge experiments using a non-MRSA strain of *S. aureus*, RN4220gfp. This strain is less virulent than the MRSA strain USA300 and therefore would allow us to more easily detect protective effects afforded by Ad5PF vectors. While neither PBS

nor Ad5gfp immunized animals were protected, Ad5PFgfp immunized animals had reduced bacterial titers in their wounds. Splenocytes from these mice showed increased IL-17 responses to GFP, which is consistent with the data from immunization experiments. Given the promising results from the RN4220gfp challenge experiments, we repeated the immunization and challenge experiments using USA300gfp. Again, Ad5PFgfp immunized mice had significantly reduced bacterial titers and increased IL-17 responses.

These data suggest that the increased IL-17 response induced by Ad5PF vectors are capable of inducing an immune environment that provides greater protection against cutaneous infection than immunization with Ad5 vectors. In the future, Ad5PF vectors expressing MRSA antigens should be used to determine the ability of these vectors to provide protection against USA300 infection. I expect that immunization with Ad5PFClfA/Ad5PFSdrD would provide enhanced protection over that seen in response to Ad5ClfA/Ad5SdrD immunization. Given that ClfA plays a role in *S. aureus* pathogenesis, whereas GFP does not, I would expect that immune responses directed at ClfA would provide greater protection since they would likely inhibit the pathogenic effects of ClfA.

If Ad5PF vectors are to be used as vaccine candidates, it will be necessary to more thoroughly assess the mechanisms involved in vaccine mediated protection. I hypothesize that these vectors are inducing greater T_H17 and T_H1 responses, and ELISA data showing increased IL-17 and IFNγ responses support this theory. However, it remains to be definitively shown that T_H17 and T_H1 cells are the cell types responsible for the increased cytokine response. Challenge experiments using Ad5ClfA/Ad5SdrD suggest that increased IL-17 responses mediate increased neutrophil recruitment and induce antibodies that are more capable of mediating opsonophagocytosis. Ad5PF vectors induce even greater IL-17 responses than Ad5 vectors.

Therefore, I expect that the enhanced protection seen following immunization with Ad5PF is due to increased IL-17 driven neutrophil recruitment and opsonophagocytosis.

Concluding Remarks

This work explores how Ad5 vectors can be used to generate protective immune responses to vaccine antigen. Ad vectors are ideal adjuvants because they potently activate both the innate and adaptive immune response, and vectors can be modified to encode vaccine antigens as transgenes. We generated Ad5 vectors expressing MRSA antigens and found that these vectors are able to induce both T and B cell responses to antigen. Specifically, Ad5 vectors induced both IL-17 and IFNγ responses to antigen that presumably come from antigen specific T_H17 and T_H1 cells. Immunization with a combination of these vectors, Ad5ClfA/Ad5SdrD, provided protection against cutaneous MRSA challenge. Current *S. aureus* vaccine strategies, mainly consisting of protein in Alum immunization, have proven unsuccessful in clinical trials. Importantly, Ad5ClfA/Ad5SdrD immunization provided greater protection than protein immunization, suggesting that Ad vectors represent a novel approach to generating a protective MRSA vaccine.

T cell responses, and T_H17 produced IL-17 in particular, have been shown to be critical in mediating protection against cutaneous *S. aureus* infections. In order to augment Ad5 mediated protection, we generated modified Ad5 vectors displaying a peptide from the bacterial protein FliC that more potently induce IL-17 responses to transgene antigens. These vectors, Ad5PF, afforded greater protection from cutaneous infection with two different *S. aureus* strains than unmodified Ad5 vectors. Our studies using Ad5PF vectors suggest that it acts as a T_H17 adjuvant and that Ad5PF vectors expressing *S. aureus* antigens would be ideal vaccine candidates.

The experiments described in this work begin to explore the mechanism of Ad5 mediated protection against cutaneous MRSA infections. Our data show that protection correlates with increased expression of IL-17, which is known to stimulate production of neutrophil recruiting chemokines. Unsurprisingly, protection also correlated with expression of neutrophil recruiting chemokines CXCL1 and CXCL5. One mechanism by which phagocytic cells, including neutrophils, mediate *S. aureus* clearance is through opsonophagocytosis. Serum from Ad5ClfA/Ad5SdrD immunized mice contained antibodies with high binding affinity for FCγRs and mediated the greatest opsonophagocytosis of *S. aureus* compared to other immunization groups. From our data, we propose a possible model in which Ad5 vectors induce increased production of IL-17 and downstream chemokines leading to enhanced neutrophil recruitment to the site of infection. Upon recruitment to the site of infection, vaccine induced IgG2 antibodies against *S. aureus* surface proteins mediate opsonophagocytosis and clearance of *S. aureus*.

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