CORRELATES OF PROTECTION ASSOCIATED WITH YERSINIA PESTIS F1-LICKM AND LCRV-LICKM VACCINATIONS: PROTECTION AGAINST LETHAL CHALLENGE AND POTENTIAL T CELL SPECIFIC IMMUNITY

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Masters of Science in Biological Sciences

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**ABSTRACT**

*Yersinia pestis* is a Gram-negative, facultative anaerobe that is the etiological agent of pneumonic plague. *Y. pestis* is categorized a category A select agent by the CDC, and despite over a hundred years of research an effective, safe vaccine protecting against the pneumonic form of infection has yet to be developed. Previous live vaccines using attenuated *Y. pestis* have been deemed unsafe for general use as it is highly reactogenic in healthy adults, and formalin killed whole-cell vaccines fail to protect against the pneumonic form of infection. Here, we investigate a novel plague vaccine using F1 and LcrV antigens fused to the carrier protein lichenase (LicKM), a thermostable enzyme from *Clostridium thermocellum* in a BALB/c mouse challenge model using the attenuated *Y. pestis* KIM D27 strain. Using this model, we determined the vaccine-generated parameters of protective efficacy against an intranasal challenge of *Y. pestis* KIM D27. Serum from vaccinated mice, adoptively transferred into naïve mice, conferred protection against lethal challenge. Removal of effector antibodies through Protein G column treatment eliminated this protection. Additionally, IL-2 was produced from vaccine-generated memory CD4+ T cells upon restimulation *in vitro* with vaccine proteins. Finally, adoptive transfer of F1 and LcrV specific T cells from vaccinated mice to naïve mice protected against lethal challenge. Taken together, we have demonstrated vaccine-generated humoral and cell-mediated protection against lethal challenge.
Also, we developed a novel tool to specifically study the T cell response to *Yersinia pestis* infection. This immunological tool, a pKKOVA plasmid which encodes full length ovalbumin, was transformed into two attenuated strains of *Y. pestis* KIM D27 and KIM 10. Using transgenic mice with T cell receptors specific for peptide residues within ovalbumin (OT-I and OT-II), we were able to evaluate the ability of *Y. pestis* transformed with plasmid to stimulate T cells. We determined through this model that *Y. pestis* KIM D27pKKOVA was able to stimulate OT-I, MHC I restricted, CD8+ T cells *in vitro* through upregulation of memory T cell surface markers CD44, CD25, and CD27. KIM D27pKKOVA was also able to stimulate OT-II, MHC II restricted, CD4+ T cells but to a much lesser extent. KIM 10pKKOVA was able to stimulate OT-I, CD8+ T cells which showed upregulation of CD44 and CD27, but was unable to stimulate any OT-II, CD4+ T cells. These results confirm the development of a novel tool allowing us to track T cell populations that change specifically in response to *Yersinia pestis* infection.
Chapter 1

INTRODUCTION

1.1 *Yersinia pestis* Infection and Disease Formation

This project’s main objective is to evaluate the mammalian response to *Yersinia pestis* infection, using two methods. The first uses a novel F1 and LcrV plague vaccine administered in mouse models to evaluate its protective efficacy against aerosolized plague challenge using an attenuated strain of *Y. pestis*. Plague vaccine means of protection was also evaluated by performing adoptive serotherapy into naïve mice, as well as evaluating the phenotype of harvested T cells from vaccinated mice.

The second method involved generation of recombinant *Y. pestis* strains which express full length ovalbumin. The generation of these strains allowed for monitoring of T cell response to *Y. pestis* infection in an *in vitro* model using OT-I and OT-II T cells, which have T cell receptors restricted to specific peptide residues in ovalbumin in the context of MHC I, and MHC II, respectively.

1.1.1 *Y. pestis* Plagues

*Yersinia pestis* is a Gram-negative, nonmotile, facultative anaerobic bacillus that is the causative agent of plague. At least three *Y. pestis* pandemics have occurred throughout history, resulting in an estimated 200 million deaths, the most famous of these, the Black Death, occurred during the fourteenth century (Perry, 1997). This organism infects order Rodentia, including squirrels, prairie dogs, and rats. Fleas bite...
infected rodents, becoming the intermediate for human transmission (Perry, 1997). The disease resulting from infection with *Y. pestis* has three forms; bubonic, pneumonic, and septicemic plague. Bubonic plague, the most common form of infection, includes symptoms such as lethargy, fever, and formation of characteristic “buboes” or swollen lymph nodes (Perry, 1997). Secondary pneumonic or septicemic plague results from progression of bubonic infection with organism entry into the blood stream, infecting secondary organs.

Upon entering the circulatory system, septicemic plague exhibits symptoms similar to other Gram-negative septicemias, invading secondary organs such as the liver, lungs, and spleen. Secondary pneumonic plague occurs upon entry of the organisms into the lungs, allowing for pneumonic infection. Human-to-human transmission of this organism occurs through organism charged respiratory droplets (Sha, 2008). After incubation of 1-6 days, pneumonic infection progresses and symptoms arise such as fever, malaise, and productive cough (Butler, 1983). Pneumonic infection is nearly 100% fatal unless antibiotics are administered within 20 hours post infection (Butler, 1983). During 1926-2005, 13 human cases of primary pneumonic plague were reported within the United States, 3 of which were acquired from laboratory settings, and 6 from face to face contact with infected domestic cats (Gage, 1998). The World Health Organization reports between 1,000 and 3,000 cases of the plague worldwide a year, while the CDC reports 10 to 20 cases of plague in the United States, annually (Ingles, 2000).

### 1.1.2 *Y. pestis* Virulence Factors

*Y. pestis* grows optimally at 26°C, in 5% CO₂ in laboratory culture media, requiring 24 to 48 hours for colony formation (Perry, 1997). Most *Y. pestis* strains
contain three virulence plasmids; pPCP1, pCD1, and pMT1 (Table 2). Plasmid pPCP1 is 9.5kb and encodes the plasminogen activator (*pla*) gene. The plasminogen activator protein has both fibrinolytic and coagulase activities, as well as hypothesized protease roles that may cleave fibrin deposits (Berry, 1997). The pCD1 plasmid is 75kb and contains the low-calcium response stimulon (LCRS) which encodes the V antigen (LcrV) as well as the *Yersinia* outer membrane proteins called (Yops) (Berry, 1997). This LCRS is highly conserved, expressed *in vitro* within eukaryotic hosts when calcium levels are low, as well as *in vivo* at 37°C, and is critical for full *Y. pestis* virulence in humans (Berry, 1997). Yops are a collection of proteins involved in the *Y. pestis* type III secretion system (T3SS), which is a bacterial structure allowing for extracellular bacteria to infiltrate host cells. This mechanism occurs through a needle-like formation known as an injectisome which allows for shuttling of bacterial effector proteins into host cells. The Yop effector proteins generally function to inhibit of host immune cell functions, such as phagocytosis, oxidative burst, and NFκB signaling inhibition (Cornelis, 2002). LCRS includes but is no limited to Yop proteins YopB, YopD, YopH, YopE, and YopJ. YopB and YopD compose the functional structure of the injectisome, while protein LcrV (V antigen) is believed to be the protruding needle of the injectisome. YopH is considered one of the most potent Yops, and is a tyrosine phosphatase which is hypothesized to inhibit tyrosine phosphorylated proteins within host cells. Inhibition of these proteins through YopH leads to inhibition of inflammatory signaling, such as preventing the release of TNFα (necessary for oxidative burst in phagocytic cells) and prevention of phagocytosis (Puerta Maria, 2009). YopJ suppresses immune cell release of TNFα (Zauberman, 2006). YopE is a guanine triphosphatase-activating protein, and its role is also hypothesized to be
impairment of phagocytosis and oxidative burst. These effector roles result in an impaired ability of phagocytic cells to not only phagocytize, but also destroy engulfed \textit{Y. pestis} (Viboud, 2005).

The third plasmid, pMT1, is 100kb and encodes \textit{caf1}, the F1 capsular protein (Berry, 1997). This protein is upregulated at $37^\circ\text{C}$, and produces a gel-like capsule material that surrounds the organism. F1 capsular protein is necessary for the bacteria’s resistance to phagocytosis, although fully virulence F1 negative strains of \textit{Y. pestis} have been identified (Friedlander, 1995).

All fully virulent strains of \textit{Y. pestis} contain a 102kb chromosomal locus, the \textit{pgm} locus, which is lacking in both KIM D27 and KIM 10 \textit{Y. pestis} strains. The \textit{pgm} locus is surrounded by insertion sequence (IS100) repeats, which contributes to its spontaneous deletion through homologous recombination, rendering isolates attenuated (Berry 1997). The \textit{pgm} locus encodes the siderophore yersiniabactin, the \textit{hms} locus, and \textit{ripA} and \textit{ripB}. Yersiniabactin, can remove iron from transferrin, lactoferrin, and ferritin, the three major iron binding proteins found in mammals (Kutyrev, 1992). As iron is chelated by mammals to limit pathogen infiltration and thus, difficult for pathogens to acquire, \textit{pgm}-negative \textit{Y. pestis} strains are considered attenuated (Perry, 1997). The \textit{hms} locus is also encoded on \textit{pgm}, and is required for hemin storage on the bacterial outer membrane. Hemin storage is believed to act as a reservoir for iron storage within the mammalian host, and necessary for \textit{Y. pestis} survival in the flea host (Perry, 1997). Recently, two genes within the \textit{pgm} locus, \textit{ripA} and \textit{ripB} were discovered to be necessary for replication within activated macrophages. Activated macrophages readily produce bacteriocidal nitric oxide (NO), and \textit{pgm}-positive strains of \textit{Y. pestis} demonstrate a reduction in NO within activated
macrophages *in vitro*. Mutants of *ripA* and *ripB* lose this ability to reduce NO levels (Pujol, 2005).

Although *Y. pestis* *pgm*-negative strains can attain full virulence with the simultaneous injection of hemin, ferrous sulfate, or ferrous chloride, the loss of the *pgm* locus in both KIM D27 and KIM 10 strains removes the organisms from the CDC category A select agent list (Berry, 2007). *Y. pestis* strain KIM D27 contains all three virulence plasmids; pCD1, pMT1, and pPCP1, while *Y. pestis* KIM 10 only contains the pMT1 plasmid.

*Y. pestis* has been shown to inhabit an intracellular niche early in mammalian infection, and later propagates extracellularly (Berry, 1997). There is a noted enhanced ability to propagate *in vivo* during the pneumonic form of infection, which is largely attributed to the expression of anti-phagocytic and immune cell evasion virulence factors upregulated at 37°C in mammalian hosts. Virulence components relevant to our study are the F1 capsular protein (*caf1*) and LcrV. These proteins are unexpressed at the flea body temperature of 26°C (Perry, 1997). *In vivo*, studies have shown that within three to five hours after introduction in a mammalian host, the pathogen becomes resistant to phagocytosis by both monocytes and polymorphonuclear leukocytes, likely through the upregulation of *caf1*, leading to enhanced dissemination and possible evasion of the host’s immune system (Sha, 2008).
### Yersinia pestis Strains

<table>
<thead>
<tr>
<th>Locus</th>
<th>KIM D27</th>
<th>KIM D28</th>
<th>KIM caf-</th>
<th>KIM 6+</th>
<th>KIM 10</th>
<th>KIM 10+</th>
<th>KIM D27pKK OVA</th>
<th>KIM 10pKK OVA</th>
</tr>
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<tbody>
<tr>
<td>pgm locus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCD1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pPCP1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pMT1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>caf1 (F1)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LcrV</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<td>pKK OVA</td>
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Table 1. *Y. pestis* strains with their corresponding plasmids and gene contents. A (+) sign indicates the presence of plasmid or gene within the strain, while (-) indicates the absence of element.

1.1.3 *Y. pestis* Vaccine History

Although limited in frequency and location, the occurrence of outbreaks of *Y. pestis* continues to be a health issue worldwide. Therefore, understanding its pathogenesis and dissemination remains essential for effective vaccine development. As increases in the number of cases are detected in countries such as China, India, Zambia, Algeria, Madagascar, and the Democratic Republic of Congo, the World Health Organization has categorized *Y. pestis* as a re-emerging infectious disease.
Global warming and the spread of fleas and rodents to previously uninhabited areas is a substantial factor in dissemination, as well (Parmenter, 1999). Currently, the necessity for an effective vaccine is driven by the threat of aerosolization and weaponization of *Y. pestis*, coupled with the discovery and growth of antibiotic-resistant strains (Galimand, 2006). The use of *Y. pestis* as a bioterrorist agent is not novel; it is noted as the first biological warfare agent, when *Y. pestis* infected dead corpses were catapulted into enemy territories during the 14th century (Smiley, 2008). In modern times, the WHO has hypothesized that, in a worst case scenario, if 50 kg of *Y. pestis* bacilli were released into a city of 5 million, 36,000 individuals would succumb to plague infection (Health Aspects of Chemical and Biological Weapons, 1970). In previous years, the Soviet Union developed quantities of *Y. pestis* suitable for weaponization, and in 1995, a man in Ohio was arrested under suspicious reasons for acquiring *Y. pestis* via mail, leading to antiterrorist legislation changes (Inglesby, 2000).

The struggle to develop an effective vaccine against plague has a long history, as well. In the early 20th century, rodents vaccinated with live attenuated *pgm*-negative *Y. pestis* demonstrated protection against both the bubonic and pneumonic form of the plague, and also demonstrated effectiveness in humans through vaccinations in Indonesia, Vietnam, and Madagascar. Unfortunately, the retention of virulence in nonhuman primate studies, and debilitating malaise and fever upon vaccination in healthy adults, eliminates this type of vaccine for use worldwide (Meyer, 1974). During the Vietnam War, formalin killed whole-cell *Y. pestis* was used by the United States to protect soldiers from bubonic plague. It was found previously to cause severe reaction in healthy adults, including crippling malaise,
fever, and swollen lymph nodes (Meyer, 1974). This vaccine regimen is no longer used as it causes adverse reactions, requires multiple boosters for efficacy, and does not protect against the pneumonic form of infection in nonhuman primates (Meyer, 1974). Despite the evidence of a continued worldwide issue, a safe and effective vaccine against the plague does not exist, even after nearly a century of research and prototypes. Currently, mouse models are the first step utilized to evaluate the effectiveness of any new *Y. pestis* vaccine.

### 1.1.4 Current Vaccine Goals

Based on dangerous post-vaccination symptoms from attenuated strains and the lack of protection against pneumonic infection when using formalin-killed *Y. pestis*, new vaccine strategies are required. Current vaccine goals focus on vaccine priming against two virulence factors mentioned earlier, the F1 capsular protein (*caf1*) and the LcrV antigen. Purified F1 capsular protein subunit vaccines protect rodents against fully virulent *Y. pestis* intranasal and subcutaneous challenges, and adoptive transfer of serum from F1 vaccinated rabbits to naïve rabbits confers protection from lethal challenge (Baker, 1952). However, the discovery of virulent F1-negative *Y. pestis* strains eliminates F1-only subunit vaccines as a viable vaccine option. Recombinant LcrV vaccination has been shown to protect mice from intranasal challenges of F1-positive and negative *Y. pestis* strains (Anderson, 1996). Adoptive transfer of LcrV specific monoclonal and polyclonal antibodies also protects mice from intranasal challenge (Une, 1984). The protective efficacy of LcrV alone is well documented, however *Y. pestis* can express LcrV variants, meaning that LcrV-only vaccines may not fully protect against a widespread, plague challenge. It has been
stated that the presence of both F1 and LcrV in a single plague vaccine, “may present as a more difficult challenge for bioterrorists to circumvent” (Smiley, 2008).

F1 and LcrV fusion proteins (rF1V) demonstrate protection against pulmonary challenges with *Y. pestis* in mouse models (Anderson, 1998). However, in work presented by Pitt et al. entitled “Animal Models and Correlates of Protection from Plague Vaccines,” it was noted that these fusion vaccines fail protect Cynomologus Macaques against lethal intranasal *Y. pestis* challenge, to protect African Green monkeys (Pitt, 2004). Numerous strategies to overcome this obstacle and improve vaccine efficacy have been employed, including genetically altering the F1 and LcrV antigens, such as deletion of a putative immunosuppressive region of LcrV (DeBord, 2006). Furthermore, live vectors for delivery have been tested, such as *Salmonella* strains that express F1 and LcrV delivered orally, that has shown efficacy in mice against lethal intranasal challenge with *Y. pestis* (Yang, 2007). Viral vectors expressing LcrV also demonstrate protection in mice against intranasal *Y. pestis* challenge (Chiuchiolo, 2006). F1 and LcrV administered with flagellin as an adjuvant also demonstrates protection against intranasal challenge in Cynomologus Macaques (Honko, 2006).

As another vaccine strategy, F1 and LcrV virulence factors have been cloned separately into lichenase (LicKM), a thermostable enzyme from *Clostridium thermocellum* and engineered and expressed in *Nicotiana benthamiana* plants (Musiychuk, 2006). LicKM as a carrier molecule for vaccine antigens has demonstrated effectiveness in anti-cancer vaccines and protection against a lethal challenge of *Bacillus anthracis* (Chichester, 2007, Massa, 2007). F1 and LcrV fused to the same LicKM molecule has been also been tested. This double fusion of F1 and
LcrV to LicKM has proven effective in protecting Cynomologus Macaques against lethal *Y. pestis* challenge (Chichester, 2009). Serum from these vaccinated non-human primates contain high levels of F1 and LcrV-specific IgG antibodies compared to non-vaccinated control groups. Additionally, IgG subtypes IgG1 and IgG2 were monitored for F1 and LcrV specificity. IgG1 antibodies are indicative of a Th2 response, which indicates that this vaccine can mount strong humoral immunity. IgG2 is indicative of a Th1 response, specifically antibody-dependent cell-mediated cytotoxicity, meaning this vaccine is also able to stimulate cell mediated immunity (Mountford, 1994). Generating a robust response from both cell mediated and humoral immunity seems to be critical in efficacy of a plague vaccine.

The protective efficacy of other plague vaccines, including F1-LicKM plus LcrV-LicKM, is being tested using mouse models. Most vaccine trials are first challenged using pgm-negative; non-select agent strains of *Y. pestis*, so it is reasonable to conclude that if mice survive lethal challenge with an attenuated strain, research may move forward in testing the effectiveness of the vaccine with a fully virulent strain, and possibly move into non-human primate studies. The use of mice as a model host for *Y. pestis* plague vaccination studies is relevant in that vaccine protectiveness generally carries over in nonhuman primates, and provides a useful tool to study to immunological effects of the vaccine (Smiley, 2008).

### 1.1.5 *Y. pestis* and the Innate Immune System

Regardless of vaccine status, the first line of a host’s immune defense is the innate response. The development of antigen specific immune cells, the ultimate goal of vaccines, requires the assistance of innate immunity. The innate immune system
consists of phagocytic cells such as macrophages, neutrophils, and dendritic cells which nonspecifically engulf microorganisms, such as bacteria, and fungi (Medzhitov, 1997). It is also the responsibility of the innate system to “present” the antigens that they have engulfed and digested to lymphocytes as the beginning of the adaptive immune response.

It has been hypothesized that *Y. pestis* is able to infiltrate the host’s cells by avoiding the immune response and its first line of defense. In bubonic infections, the organism must transition from the flea host body temperature of 26°C, to mammalian host body temperature of 37°C (Perry, 1997). *Y. pestis* uses an early intracellular niche (generally macrophages) to upregulate microbial anti-phagocytic virulence factors transcribed at 37°C to enable growth extracellularly in mammals (Viboud, 2005). In fact, the *ripA* gene located within the *pgm* locus in *Y. pestis* is deemed critical for replication and survival in activated macrophages, *in vitro* (Pujol, 2005). This latent period is bypassed in primary pneumonic plague, as infection from person to person does not require the flea host, and therefore, production of virulence factors expressed at 37°C is maintained, leading to rapid dissemination and fatality in mammals.

In pneumonic infection, evasion of phagocytosis by *Y. pestis* is aided by the upregulation of the T3SS encoded on plasmid pCD1 (Viboud, 2005). Targeting neutrophils, macrophage, and dendritic cells *in vivo*, the T3SS has the ability to shuttle effector Yops prohibiting these cells’ ability to release pro-inflammatory cytokines, to mobilize cytoskeletal arrangements, and can even induce apoptosis, thus inhibiting phagocytosis. Repression of pro-inflammatory cytokines by Yops and the T3SS contributes to a delayed inflammatory response, prohibiting white blood cell (WBC)
recruitment (Viboud, 2005). In addition, lipopolysaccharide (LPS) associated with Y. pestis changes from highly inflammatory hexa-acylated lipid A to weakly inflammatory tetra-acylated lipid A upon transition from a 26°C host to a 37°C host (Montminy, 2006). LPS, specifically hexa-acylated lipid A on the surface of Y. pestis, activates toll-like receptors (TLRs) on the surface of eukaryotic immune system cells, which activates host immune defenses including pro-inflammatory cytokines such as TNFα and IL-6 (Takeda, 2003).

1.1.6 Y. pestis and the Adaptive Immune System: Humoral Immunity

The adaptive leg of the immune system is necessary for antigen specific immunity (Alberts, 2002). Cells of the innate system play a large role in adaptive immunity as they produce cytokines and chemokines, resulting in recruitment of immune cells to the area of infection. Production of cytokines will lead to a milieu tailored towards the response; either extracellular humoral immunity (Th2) or intracellular cell mediated immunity (Th1) (Alberts, 2002).

Humoral immunity is used by the mammalian system to defend against extracellular pathogens. This can occur through the presentation of foreign invaders by antigen presenting cells (APCs), and the presentation of these antigens on major histocompatibility complex (MHC II) molecules to CD4 T cells, in T cell-dependent B cell activation. From here, CD4 T cells are then able to activate antigen specific B cells, or plasma cells, which can produce antigen-specific antibodies (Alberts, 2002). Antigen-specific antibodies are used to neutralize extracellular pathogens, induce apoptosis of infected host cells, or bind to extracellular pathogens and assist in phagocytosis (Slifka, 1997). The complement system is also a necessary
component in the eukaryotic immune system, and assists in antibody mediated protection. The complement system aids in the targeting, recruitment, and engulfment of pathogens by phagocytic cells, as well as inducing osmotic lysis through formation of a membrane attack complex, which forms a pore in the pathogenic cell membrane (Alberts, 2002). T cell independent means of B cell stimulation can also occur, where antibody production requires macrophages to present the same antigen in multiple ways to a B cell, causing cross linking of antibodies on the B cell surface (Alberts, 2002).

Initial antigen presentation by APCs and a rapid antigen-specific response leading to the generation of long lasting memory B and T cells is the ultimate goal of vaccination (Alberts, 2002). To combat Y. pestis effectively, the immune system requires both specific antibodies and cell mediated immunity. The passive transfer of Y. pestis specific antibodies to naïve rodents has been demonstrated numerous times to protect against lethal Y. pestis challenge (Green, 1999). Coupled with inhabiting an extracellular niche during infection, the focus of Y. pestis vaccines has been humoral immunity. Typically, high vaccine specific antibody titers correlate with vaccine efficacy (Orenstein, 1985). However, in a work presented by Pitt et al. entitled “Animal Models and Correlates of Protection from Plague Vaccines,” it was noted that high LcrV and F1 antibody titers do not always correlate with vaccine efficacy against plague challenges in non-human primates (Pitt, 2004). In fact, it was stated that F1 and LcrV subunit vaccination protected Cynomologus Macaques, but not African green monkeys, despite corresponding high LcrV and F1-specific, IgG antibodies. Therefore, it is apparent that targeting of the humoral response is not sufficient for full protective efficacy of a subunit LcrV and F1 plague vaccine in nonhuman primates.
1.1.7 *Y. pestis* and the Adaptive Immune System: Cell Mediated Immunity

The search for an effective plague vaccine, and data demonstrating plague deaths even in the presence of high antibody titers, has shifted some focus of plague vaccine development towards cell mediated immunity. Cell mediated immunity is composed mainly of Th1 CD4 T cells and cytotoxic CD8 T cells.

Cytotoxic CD8 T cells effectively kill a host cell infected with an intracellular organism, with and without the assistance of antigen presentation by APCs. Cytotoxic T cells recognize antigens presented in the context of MHC I molecules (Alberts, 2002). Antigen-specific T cells may also release pro-inflammatory cytokines that activate other cells, such as macrophages, to engulf and destroy infected host cells (Alberts, 2002). As previously stated, *Y. pestis* inhabits an intracellular niche, primarily in macrophage early in infection, but activating macrophages *in vitro* with cytokines INFγ and TNFα restricts the organism’s ability to grow. (Lukaszewski, 2005). Inducible nitric oxide (iNOS) is transcribed in a cell upon stimulation with INFγ and TNFα as a means of destroying intracellular pathogens (Chan, 2000). In transgenic mice with reduced IFNγ production, LcrV and F1 vaccine protection against bubonic plague is impaired (Elvin, 2004). Supporting the antimicrobial importance of these cytokines in combating infection, *Y. pestis* itself produces RipA, a protein that antagonize reactive nitrogen *in vitro*, neutralizing its ability to eliminate organisms (Pujol, 2005).

Relevant vaccine work with pgm-negative strains of *Y. pestis* supports a T cell mediated role in priming the immune system for combating pneumonic infection, as well. When vaccinated with a pgm-negative, live-attenuated *Y. pestis*, guinea pigs and
nonhuman primates survive lethal challenge, despite the absence of high antibody titers (Pitt, 2004). Additionally, live attenuated vaccinations are associated with T cell mediated immunity, indicating that T cells may be playing a role in conferring protection.

A critical study in 2005 established that vaccination of μMT (B cell deficient) mice with pgm-negative Y. pestis, coupled with post-exposure serum, protects mice from lethal Y. pestis challenge (Parent, 2005). This protection is eliminated upon reduction of CD4 and CD8 T cells, IFNγ, and TNFα (Parent, 2005). Similarly, vaccination with F1 and LcrV in STAT-4 deficient mice, transgenic mice deficient in a Th1 response, eliminates protection from plague challenge (Elvin, 2004).

When T cells are activated by antigen presentation, they can develop into memory T cells. The induction and maintenance of memory cells is required for long term immunity to specific pathogens. This is the goal of vaccines (Ahmed, 1996). Therefore, exploration of memory T cell production upon plague vaccination will lead to further insight on T cells and their role in conferring vaccine-mediated protection. Observation of interleukin-2 (IL-2) production upon vaccination would lead to insight about the role of T cells in vaccination, as IL-2 is primarily produced by activated memory T cells during clonal expansion, or the rapid division and growth of antigen specific T cells, in response to antigen-specific stimulation (Taniguchi, 1993). We can also observe changes in memory T cell markers upon vaccination, including CD25 which is the α chain of the IL-2 receptor, and CD62L and CD44, cell surface markers implicated with lymphocyte homing, expansion, and maturation (Taniguchi, 1993, Bachmann, 2005, Vachon, 2006). Changes in these cell surface makers will add to the investigation of memory T cell production in response to vaccination.
1.2 OT-I and OT-II Transgenic T Cell Receptors to Monitor Responses to Bacterial Infection

Attempts to identify a dominant T cell epitope generated in response to *Y. pestis* infection have not been successful. A tool previously used to study T cell-mediated response against other bacterial pathogens is the use of transgenic T cells with TCRs exclusive to OT-I and OT-II epitopes, peptide resides found on chicken ovalbumin (OVA). This immunological tool utilizes the high-copy plasmid, pKKOVA, which contains a gene to fully produce ovalbumin, and is readily electroporated into numerous bacteria species (Takahashi, 1995). Once the bacteria is transformed, the recombinant full length OVA is expressed (Luu, 2006). Infection of APCs, such as dendritic cells, with pKKOVA recombinant bacteria, leads to OVA production and digestion into peptides, where in OT-I and OT-II peptides are presented and can stimulate OVA-specific T cells derived from transgenic mice C57BL/6-Tg(TeraTerb)1100Mjb/J (OT-I mice) and B6, Cg-Tg(TeraTerb)425Cbn/J (OT-II mice). OT-I mice have CD8 T cells with T cell receptors (TCRs) designed to recognize OVA residues 257-264, in the context of MHC I, while OT-II mice have CD4 T cells TCRs designed to recognize peptide residues 323-339 on OVA in the context of MHC II molecules. By insertion of pKKOVA plasmid into *Y. pestis*, we can examine T cells that specifically recognize an antigen expressed by our recombinant *Y. pestis* strains. It is reasonable to conclude, then, that we can analyze the T cell response skewed by the presence of *Y. pestis* infection, which allows for monitoring changes in cell activation and expression of memory T cell markers. We are able to explore T cell changes upon interaction with bone marrow-derived dendritic cells (BMDCs) infected with *Y. pestis* KIM D27 and *Y. pestis* KIM 10. Additionally, we hope to monitor *in vivo* T cell changes upon *Y. pestis* pKKOVA
infection. Currently, this tool will allow for the investigation of CD44, a cell surface marker upregulated in mature T cell stimulation to antigen (Gerberick, 1997), CD25, the α chain of the IL-2 receptor upregulated upon T cell stimulation (Taniguchi, 1993), and CD27, a cell surface marker necessary for T cell clonal expansion, also upregulated upon T cell stimulation (Hendriks, 2000).

1.3 *Y. pestis* Infection and Means of Death

Upon *Y. pestis* infection, mice exposed to high doses of intranasal organism succumb to pneumonia or septicemia. Literature has identified major organ failure after intranasal challenge of *Y. pestis* in mouse models, including invasion of the spleen within 36 hours after intranasal challenge in mice (Inglesbry, 2002). At 0 hours post inoculation with fully virulent isolates, blood measurements of total protein, as well as electrolytes increase and bicarbonate decreases, which is indicative of failing kidney and liver function, dehydration, and edema. These blood chemistry changes correlated with altered behavior and appearance commonly found in *Y. pestis* infected mice. These symptoms may include hunching, matted fur, lethargy, and eventual death, supporting that blood changes correspond with *Y. pestis* disease progression in mice. As early as 12 hours post intranasal infection, histological samples of infected mice showed a significant increase in polymorphonuclear (PMN) cells within the lungs. At 60 hours post infection the greatest evidences of bacterial invasion and infection in the lungs is detected, including increasing numbers of PMN cells, extensive pulmonary edema, bronchopneumonia, and bacterial clusters in areas of necrotic tissue (Inglesbry, 2002).

Septicemia also contributes to cause of death in intranasal challenged mice. Basic physiological events are the same in *Y. pestis* sepsis as compared to other
Enterobacteriaceae Gram-negative sepsis. Recruitment of white blood cells (WBCs) to areas of infection occurs, initiating an immense inflammatory response through interaction of lipopolysaccharide (LPS) with toll-like receptors on the eukaryotic host’s cells’ surfaces (Cohen, 2002). When infection becomes systemic, the recruitment of such WBCs causes a proportional release of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and platelet-activating factor. This results in an overwhelming systemic inflammatory response, weakening vessel integrity and diminishing tissue function (Astiz, 1995). Increased hyperpermeability of blood vessels allows fluids, electrolytes, and cells once impermeable to blood vessel walls to now freely move out of the blood stream. The loss of total blood volume results in a decrease circulating volume and venous return. There is a decrease in total peripheral resistance, due to vasodilation from proinflammatory cytokines, histamine from mast cells, and NO release from hypoxic tissues (Inglesby, 2000). Decreased urine output, light headedness, and rapid heartbeat are directly related to hypovolemia associated with septicemia (Inglesby, 2000). To compensate for hypoxic tissues, loss in total peripheral resistance and decrease venous return, the heart rate increases to supply the body with oxygen and nutrients.

Because of the noted severe edema and organism presence within the lungs of infected animals, mice succumb to intranasal infection by multifocal exudative bronchopneumonia along with septicemia, hemorrhage, and necrosis (Finegold, 1968). Comprehension of Y. pestis infection and rodent means of death during infection is important in understanding its pathogenesis in BALB/c mice.
Chapter 2

MATERIALS AND METHODS

2.1 Bacteria, Cell Culture Conditions, and Media

All experiments utilized *Yersinia pestis* KIM D27 or KIM 10 strains. KIM D27 and KIM 10 are laboratory isolated strains mutated from wild type KIM5, isolated from a pneumonic plague infection in Iran in 1961 (Une, 1984). The wild-type strain contains three virulence plasmids, pCD1, pPCP1, and pMT1. KIM D27 contains these three plasmids, but is considered a nonselect agent due to a spontaneous deletion of the 102kb *pgm* region, a locus required by the organism for iron storage and acquisition (Une, 1984). KIM 10 is a *Y. pestis* strain missing the *pgm* locus, as well as virulence plasmids pCD1 and pPCP1, thus missing the LcrV (Low-calcium response V) protein.

Organisms utilized for dendritic cell infections were cultured overnight in Heart Infusion Broth (HIB) (BD Biosciences, San Diego, CA) at 26°C. *Y. pestis* KIM D27 and KIM 10 transformed with pKKOVA were grown in HIB as describe but with the addition of 100μg/ml of ampicillin. Cultures were grown to appropriate concentrations by measuring OD$_{600}$ and plating for colony forming units (CFU) determination.
2.2 Testing the Protective Efficacy of the Novel F1-LicKM plus LcrV-LicKM Vaccine

2.2.1 Priming and Boosting of BALB/c Mice Using F1-LicKM and LcrV-LicKM Adsorbed onto Alhydrogel

6-10 week old, female BALB/c mice (Harlan Laboratories, Indianapolis, IN) were used for all vaccination studies. Vaccines containing *Y. pestis* proteins caf1 (F1) and LcrV were formulated as previously described (Musiychuk, 2007). F1 and LcrV were fused separately into the carrier molecule LicKM, a thermostable enzyme from *Clostridium thermocellum* and expressed in *Nicotiana benthamiana* [Musiychuk, 2007] (Fraunhofer USA, Fraunhofer Center for Molecular Biotechnology, Newark, DE). Alhydrogel was used as an adjuvant at a final concentration of 0.3%. Mice were primed intramuscularly on Day 0 and boosted on Day 21 using one of the following concentrations of each vaccine component (F1 and LcrV) 25 μg, 10 μg, 5 μg, 1 μg, or 0.2 μg. Serum was collected for analysis of IgG titer by ELISA before priming at Day 0, before boosting at Day 21, and before challenge on Day 35.

2.2.2 *Y. pestis* KIM D27 Challenge Dose Preparation

Freezer stocks of *Y. pestis* KIM D27 in 10% glycerol were thawed and centrifuged for 10 minutes at 10,000 rpm. Supernatants were removed and cells were washed in 1mL of Phosphate Buffered Solution (PBS) (Cellgro®, Manasas, VA). Final challenge doses were 1x10^6 organisms in 30 μL based on previously performed growth curves unless otherwise indicated. All infectious challenge dose concentrations were confirmed through colony forming units (CFU) on blood agar plates.
2.2.3 Challenge of Vaccinated BALB/c Mice

Mice vaccinated with F1-LicKM and LcrV-LicKM were challenged on study Day 35, mice and boosted were used for challenge. Primed and boosted mice (3-5 mice per group) were challenged intranasally with $1 \times 10^6$ Y. pestis KIM D27 as described above. Mice were monitored for health and survival for 14 days post infection. Once mice demonstrated symptoms of disease (hunched stance, matted fur, squinting eyes, and general lethargy) they were monitored every 8 hours and sacrificed using a double kill method if moribund.

2.2.4 Adoptive Serotherapy from Vaccinated into Naïve Mice

After priming and boosting as previously described, vaccinated mice were sacrificed on Day 35 and blood was collected via cardiac puncture. Blood samples were spun to remove cells, and stored at -80°C until use. To remove IgG from serum, Recombinant Protein G Agarose “ Protein G” (Exalpha Biologicals, Inc., Maynard, MA) was used. Protein G was first thoroughly washed with PBS to remove sodium azide preservative. “Treated” serum was incubated for 8 hours with Protein G beads on a rotator at 4°C. Protein G beads were separated from serum by centrifugation with a 0.45μm Corning® Costar® Spin-X® plastic centrifuge tube filters (Sigma-Aldrich, St. Louis, MO), at which point serum was ready for transfer to naïve mice. For serum transfers, PBS diluted serum was transferred by intraperitoneal route into naïve BALB/c mice in a final volume of 50 μL. To determine the lowest protective volumes of vaccinated serum, serum was diluted according to the following: 40μl serum with 10μl PBS, 30μl serum with 20μl PBS, 20μl serum with 30μl PBS, and 10μl with 40μl PBS.
2.2.5 CD4 T Cell Isolation and Culture for Ex Vivo Production of IL-2

On Day 35 post prime, mouse spleens and lymph nodes were harvested for in vitro T cell stimulation assays. Red blood cells (RBCs) were lysed using 0.5 mL per mouse of RBC Lysing Buffer (Sigma-Aldrich, St. Louis, MO). Fcγ receptors were blocked (Fc block) by adding 25 μg of monoclonal antibody clone 4.2G2 and cells were incubated for 15 min on ice. T cell isolation was performed using CD4 L3T4 microbeads (Miltenyi Biotec, Cologne, Germany). Cell and bead mixtures were centrifuged, the supernatant was removed, and the pellet was resuspended in 1ml of Dulbecco’s Modification of Eagle’s Medium (DMEM) (Fisher Scientific, Pittsburgh, PA) with 10% GemCell™ U.S. Original Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Gemini Bio-Products, West Sacramento, CA), 25mM HEPES (Invitrogen, Carlsbad, CA), 10mM non-essential amino acids (Invitrogen, Carlsbad, CA), and 1mM sodium pyruvate (Invitrogen, Carlsbad, CA), and 55 μM beta-mercaptoethanol (Sigma-Products, St. Louis, MO) (cDMEM). Cell preparations were filtered through nylon mesh into a 50 mL conical tube and isolated T cells were collected using 25 LS MACS separation columns (Miltenyi Biotec, Cologne, Germany) per manufacturer’s instructions. Cells were centrifuged and resuspended in cDMEM at a final concentration of 2x10^6 cells/mL.

2.2.6 Antigen Presenting Cell (APC) Harvest and Preparation for Ex Vivo T Cell Stimulation

6 to 10 week old male BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were used for all in vitro experiments. Spleens were mashed through nylon mesh in PBS, centrifuged, and red blood cells were lysed using RBC Lysing Buffer. Cells were then rinsed in, centrifuged, and resuspended in cDMEM with 0.5mg/ml Mitomycin C from Streptomyces caespitosus (Sigma-Aldrich, St. Louis, MO) and
incubated for 33 minutes at 37°C. Cells were washed three times with cDMEM and resuspended in cDMEM at 2x10^7 cells/mL.

### 2.2.7 Ex Vivo Stimulation of CD4 T Cells with Protein

CD4 T cells at 2x10^6 cells/ml from each vaccinated group were combined with 2x10^7 cells/mL APCs, a final ratio of 10 APCs to 1 T cell. CD4 T cells were stimulated with 100 μg/ml of protein, either LicKM, F1-LicKM, LcrV-LicKM, or LcrV-LicKM plus F1-LicKM. Ovalbumin Egg White was used as a negative control (Worthington Biochemical Corporation, Lakewood, NJ) and anti-mouse CD3ε as a positive control (clone 145-2C11, eBioscience, San Diego, CA). T cell cultures were incubated at 37°C for 24 hours, at which time supernatant was collected and stored at -20°C until use. IL-2 production was measured in the supernatant using the BD OptEIA™ Mouse IL-2 ELISA Set (BD Bioscience, San Diego, CA).

### 2.2.8 T Cell Harvest and Culture for Characterization of T Cell Response to Vaccination and Restimulation In Vitro

T cell and APC cultures were setup as described previously achieving a final ratio of 10 APCs to 1 T cell and allowed to incubate with LcrV-LicKM plus F1-LicKM vaccine proteins at 100μg/ml for 72 hours. Cells were removed from culture and Fcγ receptors were blocked by treatment with MAb clone 2.4G2. Cells were stained with mouse antibodies against CD4, CD62L, CD44, CD8, CD25, CD62L, and CD44 to assess cellular changes associated with T cell memory. Live/dead cell discrimination was performed using propidium iodide (P.I.) (Invitrogen, Carlsbad, CA). Only live T cells were analyzed for changes in cell surface marker expression using Accuri C6 Flow Cytometer (Accuri Cytometers, Inc, Ann Arbor, MI) and data
analysis was performed using CFlow Plus software (Accuri Cytometers, Inc, Ann Arbor, MI).

2.3 Utilizing OT-I and OT-II Transgenic Mice and Transformed \textit{Y. pestis} KIM D27 and KIM10 Strains with pKKOVA to Investigate Cellular Response to \textit{Y. pestis} Infection

2.3.1 Bone Marrow Derived Dendritic Cell (BMDC) Infections

BMDCs were cultured from male 6 to 10 week old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). For bone marrow harvest, mice were sacrificed and femurs and tibias were removed. Bones were flushed with cDMEM using a 26-gauge needle and syringe and run through a 100 μM cell strainer before growing in cDMEM containing 1% penicillin/streptomycin (MP Biomedicals, LLC, Solon, OH) and 20ng/ml Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (PeproTech Inc, Rocky Hill, NJ) for 8 days. BMDCs were fed with fresh media on Day 3 and 5, and 7. BMDCs were cultured in cDMEM and grown at 37°C in 5% CO₂. On Day 8, BMDCs and OT-I and OT-II T cell cultures were collected and resuspended by flushing the back of the flask three times with 20 mL of cDMEM taken from the flask. Dendritic cells were infected with a multiplicity of infection (MOI) of 10:1 with \textit{Y. pestis} KIM D27, \textit{Y. pestis} KIM D27pkkOVA, \textit{Y. pestis} KIM 10 or \textit{Y. pestis} KIM 10pKKOVA for all experiments with OT-I and OT-II transgenic mice T cells.

2.3.2 \textit{Escherichia coli} DH5α Transformation with pKKOVA Plasmid

\textit{E. coli} DH5α colonies were transformed with plasmid pKKOVA using transformation solution (Bio-Rad Laboratories, Hercules, CA) (kindly donated from Subash Sad, Ph.D. from the NRC-Institute for Biological Sciences) on CloneSaver™ Card (Whatman, Maidstone, Kent, United Kingdom). Plasmid recovery was first
performed by placing the CloneSaver™ card into in 200µl TE Buffer (10mM Tris-HCl (Ambion®, Austin, TX) 0.1mM EDTA (Ambion®, Austin, TX) pH 8.0) and vortexing for 5 minutes at room temperature. This solution (1.25 µg plasmid) was used for the transformation. The transformation solution was plated onto Luria Bertani broth (LB) (Difco, Lawrence, KS) and agar (Difco, Lawrence, KS) containing 100 µg/mL Ampicillin (Amp) (Fisher BioReagents, Fair Lawn, NJ) as pKKOVA plasmid encodes Ampicillin resistance. Plates were incubated at 37°C for 24 hours and colonies with Amp resistance (DH5αpKKOVA) were selected for using patch plating on LB, agar, and Amp plates.

2.3.3  *Y. pestis* KIM D27 and KIM 10 Strains Transformed with pKKOVA

Overnight cultures of *Y. pestis* KIM D27 or KIM 10 in HIB were grown for 8 hours at 28°C. Culture was diluted 1:50 in fresh HIB and allowed to grow for another 3 hours at 28°C OD₆₀₀ reading were taken for confirmation of Log phase growth. Cells were then harvested through centrifugation (4000xg for 15 minutes at 4°C) and resuspended in 5 mL of ice cold molecular grade water (Cellgro®, Manasas, VA). Next, 100 µL of *Y. pestis* in water mixture was added to an electroporation cuvette (Bio-Rad Laboratories, Hercules, CA). Freezer stocks of pKKOVA plasmid, extracted through QIAprep™ Spin Miniprep Kit (Qiagen, Hilden, Germany) from *E. coli* DH5α transformed with pKKOVA plasmid supplied 4 µL (500 ng/µL) of plasmid prep solution that was then added to the electroporation cuvettes. The two were mixed gently by swirling and the cuvette was given a single pulse at 2.5 kV using an Electroporator 2510 (Eppendorf, Hamburg, Germany). Fresh HIB was added to the pulsed cuvette, and the entire mixture was allowed to grow at 28°C for 1 hour, before plating 200 µL onto Luria Bertani (LB) (Difco, Lawrence, KS) plates containing 100
μg/mL Amp. Amp resistant colonies were harvested via patch plating, and 10% glycerol freezer stocks were generated.

2.3.4 **Confirmation of Y. pestis KIM D27pKKOVA and KIM 10pKKOVA**

Stocks of transformed *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA were grown in overnight cultures with 100 μg/ml Amp at 28°C for 8 hours. Plasmids from the organism were isolated using QIAprep™ Spin Miniprep Kit (Qiagen, Hilden, Germany) and plasmid size and presence was determined through agarose gel electrophoresis of samples to confirm the presence of plasmid at 5.7kb. A 0.8% agarose (Sigma-Aldrich, St. Louis, MO) gel was made using a 1X solution of Tris-Borate solution (Sigma-Aldrich, St. Louis, MO) with 5 μL of ethidium bromide (Amersham Pharmacia Biotech AB, Fairfield, CT). Samples were compared to a Lambda DNA HindIII Marker (Promega, Madison, WI) and run at 80V (BioRad, Hercules, CA).

2.3.5 **Confirmation of Resident Plasmids in Y. pestis KIM D27 and KIM 10 After pKKOVA Plasmid Transformation**

Polymerase chain reaction (PCR) was used for amplification and confirmation of genes found on each of the three resident plasmids in *Y. pestis* KIM D27 and the one resident plasmid in KIM 10 (Table 2). DNA was isolated from each transformed strain using a DNeasy™ Blood and Tissue Kit (Qiagen, Hercules, CA). Primers (Integrated DNA Technologies, Coralville, Iowa) targeting genes encoded on each plasmid were used and fragments amplified using HotStartTaq™ Master Mix (Qiagen, Hercules, CA) in a DNA Engine® Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The thermocycler was programmed for the following cycles; 95°C for 15 minutes to activate the HotStartTaq™ Master Mix Kit, followed by 25 cycles at 94°C
for 1 minute, 50°C for 2 minutes, and 72°C for 3 minutes, ending with a final cycle of 7 minutes at 72°C. The presence of each plasmid was confirmed using agarose gel electrophoresis as described previously.

2.3.6 Determination of OVA Expression in Transformed *Y. pestis* KIM D27pKKOVA and KIM 10pKKOVA

Overnight cultures of transformed *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA were grown at 28°C in HIB with 100 μg/mL of Amp to an OD₆₀₀ correlating to approximately 10⁸ CFU/mL. Organisms were pelleted and resuspended in 1ml PBS, whereupon they were sonicated using a Sonicator™ (Heat Systems-Ultrasonics Inc., Newtown, CT) as per manufacturer’s instructions. Lysates were pelleted through centrifugation and the supernatant was subjected to ammonium sulfate precipitation (Fisher Scientific, Pittsburg, PA) overnight at -20°C. The pellet was then dissolved in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and heated in a thermomixer (Eppendorf, Hamburg, Germany) at 99°C for 5 minutes. Samples were then loaded on a 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad Laboratories, Hercules, CA) for PAGE and western blot confirmation of OVA production. Gels were equilibrated first by a 30 minute soak in towbin buffer (0.025M Tris (Ambion®, Austin, TX), 0.192M glycine (Fisher Scientific, Pittsburg, PA), 20% methanol). Blotting paper and a 0.2 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) were also soaked in towbin buffer for 15 minutes. PVDF membrane blotting occurred using a Hoefer TE 70 semi-dry transfer unit (GE Health Systems, Waukesha, WI) for 35 minutes at approximately 8mA/cm² (total area of membrane). Membranes were then blocked overnight at room temperature in 3% bovine serum albumin (BSA) (Akron Biotech, Boca Raton, FL) in Tris-buffered saline
(50mM Tris (Ambion\textsuperscript{®}, Austin, TX), 150mM NaCl (Fisher Scientific, Pittsburg, PA), and 0.05% Tween\textsuperscript{®} 20 (TBST) (Sigma-Aldrich, St. Louis, MO), pH 7.6). Membranes were washed thoroughly with TBST, and incubated for 1 hour with a mouse monoclonal anti-ovalbumin antibody (Abcam, Cambridge, MA) at a 1:2000 dilution per manufacturer’s instructions in TBST with 3% BSA. Membranes were thoroughly washed again with TBST, followed by incubation with secondary anti-mouse IgG horseradish peroxidase-linked antibody for one hour (Cell Signaling Technology\textsuperscript{®}, Beverly, MA). The membrane was washed thoroughly again with TBST and incubated with lumilight substrate for one minute-followed by exposure to X-ray film for 10-15 seconds. OVA expression was detected at 45kDa as compared to a standard biotinylated protein marker (Cell Signaling Technology\textsuperscript{®}, Beverly, MA). OVA control was detected at concentrations as low as 125 ng/mL.

2.3.7 BMDC Cultures and Antigen Presentation for Stimulating Cells from OT-I and OT-II Transgenic Mice

BMDCs were harvested and grown as previously described. On Day 7, BMDCs were resuspended at 3x10\textsuperscript{4} cells/mL and infected at an MOI of 10:1 (10 bacteria to every 1 dendritic cell) with overnight cultures of \textit{Y. pestis} KIM D27 or KIM 10 grown in HIB, or KIM D27pKKOVA or KIM 10pKKOVA in HIB containing 100 \textmu g/mL of Amp. \textit{Y. pestis} and BMDCs were incubated in cDMEM without antibiotics for 4 hours at 37\textdegree C in 5% CO\textsubscript{2}. After 4 hours, 50 \textmu g/mL of Gentamycin Sulfate (Gemini Bio Products, West Sacramento, CA) was added to the cell culture. The next day CD4 and CD8 OVA specific T cells were isolated from 6 to 10 week old male C57BL/6-Tg (TeraTerb) 1100Mjb/J (OT-I) and B6.Cg-Tg(TeraTerb)425Cbn/J (OT-II) mice (Jackson Laboratory, Bar Harbor, ME). OT-I have transgenic T cell
receptors designed specifically to recognize chicken ovalbumin residues 257-264, and were used to study the role of CD8+ T cells to antigen presentation, while OT-II mice are CD4+ MHC Class II I-A\(^2\) restricted for chicken ovalbumin residues 323-339 (Jackson Laboratories, Bar Harbor, ME). Spleens and lymph nodes from the mice were filtered through a 100 μm cell strainer and added to Y. pestis infected dendritic cell flasks at an MOI of 1:10 (1 BMDC for every 10 T cells). Cell cultures were incubated for 9 days in cDMEM with IL-2 at 10 ng/mL added for the first 7 days of culture. Cells were then centrifuged and analyzed using FACS flow cytometry as previously described.

<table>
<thead>
<tr>
<th>Resident Plasmid</th>
<th>Gene encoded</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMT1</td>
<td>caf1</td>
<td>forward  5’-ATA CTG CAG ATG AAA AAA ATC AGT TCC-3’&lt;br&gt;reverse  5’-ATA AAG CTT TTA TTG GTT AGA TAC GGT-3’</td>
</tr>
<tr>
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<td>repA1</td>
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<tr>
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<td>forward  5’-ATC TTA CTT TCC GTG AGA A-3’&lt;br&gt;reverse  5’-CTT GGA TGT TGA GCT TCC TA-3’</td>
</tr>
</tbody>
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Table 2. Primers used to detect Y. pestis resident plasmids
Chapter 3

RESULTS

3.1 Vaccination of BALB/c Mice with F1-LicKM and LcrV-LicKM with Alhydrogel Confers Complete Protection Against a Lethal Challenge with \( Y. pestis \) KIM D27 Strain

3.1.1 BALB/c Mice are Susceptible to an Intranasal Challenge with a Lethal Dose of \( Y. pestis \) KIM D27

Naïve BALB/c mice (n=5) were challenged with an intranasal, lethal dose of \( 1 \times 10^6 \) \( Y. pestis \) D27 (25 times the LD\(_{50}\)) and monitored for health and survival. All naive mice succumbed to infection by Day 7 post-challenge (Figure 1). Mice are thought to succumb to infection to lethal intranasal challenges of \( Y. pestis \) KIM D27 through bronchopneumonia (Lathem, 2005). Evaluation of this challenge dose confirmed effective dosing and method of challenge delivery of the KIM D27 strain in mice.

3.1.2 F1-LicKM and LcrV-LicKM Adsorbed on Alhydrogel Confers Protection Against a Lethal \( Y. pestis \) KIM D27 Intranasal Challenge

Groups of BALB/c mice (n=4) were vaccinated on study Day 0 and 21, and then challenged intranasally with \( Y. pestis \) KIM D27 strain. Mice vaccinated with 25 \( \mu \)g of LcrV-LicKM had a 75% survival, while 25 \( \mu \)g of F1-LicKM alone or the combination of F1-LicKM and LcrV-LicKM conferred 100% protection after lethal challenge (25 LD\(_{50}\)). A control group of adjuvant (Alhydrogel) alone did not protect against lethal challenge (Figure 2). Vaccination with F1 alone has been shown
previously to confer full protection against lethal *Y. pestis* KIM D27 challenges (Smiley, 2008), however the presence of virulent F1 negative strains (Perry, 1997) and the greater efficacy of a bi-valent vaccination system (Smiley, 2008) suggests that F1 and LcrV in combination would be a more effective vaccine. We concluded that this bi-valent F1-LicKM plus LcrV-LicKM in conjunction with Alhydrogel is effective against lethal *Y. pestis* KIM D27 challenge.

Our next goal was to determine the minimum effective dose of F1-LicKM and LcrV-LicKM that confers full protection against a lethal *Y. pestis* KIM D27 challenge. Doses were titrated from 25 μg of each component to 1 μg F1-LicKM plus LcrV-LicKM. Following a prime and boost regiment, groups of mice (*n*=5) received a lethal *Y. pestis* KIM D27 intranasal challenge (25 LD$_{50}$) (Figure 3). Interestingly, we found that vaccination with as little as 1 μg of each component conferred full protection against a lethal challenge of 25 LD$_{50}$. Additional titration of the dose down to 0.2 μg of F1-LicKM and LcrV-LicKM also showed protection (Figure 5). Control groups (*n*=10) include vaccination with Alhydrogel alone, LicKM carrier protein alone, and a complete naïve group (Figure 5). All control groups succumbed to infection by Day 7 post-challenge, while all vaccinated groups, including the low dose group vaccinated with 0.2 μg F1-LicKM and LcrV-LicKM were fully protected from a lethal KIM D27 challenge (Figure 5).

### 3.1.3 Determination of the Limit of Protection of the F1-LicKM plus LcrV-LicKM Vaccine Using a Range of LD$_{50}$ Challenge Doses

To determine the maximum protective efficacy of a 1 μg or a 0.2 μg dose of F1-LicKM plus LcrV-LicKM, BALB/c mice (*n*=5) were challenged with increasing LD$_{50}$ challenge doses (25 LD$_{50}$, 250 LD$_{50}$, 500 LD$_{50}$ and 1000 LD$_{50}$) of *Y. pestis* KIM.
D27 strain and monitored for health and survival. While 100% of mice challenged with 25 LD$_{50}$ dose survived as expected (Figure 4A), surprisingly 80% of vaccinated mice challenged at 250 LD$_{50}$ survived the 14 day monitoring period (Figure 4B). Vaccinated groups challenged with 500 LD$_{50}$ also survived challenge, with 60% of mice vaccinated with 0.2 μg F1-LicKM plus LcrV-LicKM, and 40% of 1μg F1-LicKM plus LcrV-LicKM surviving (Figure 4C). Lastly, both F1-LicKM and LcrV-LicKM at 1 μg and 0.2 μg groups challenged with 1000 LD$_{50}$ did not survive, with 60% of mice dying within 24 hours (Figure 4D). This rapid death is not explained by septicemia or pneumonia, as is the expected cause of death with *Y. pestis* KIM D27 strain intranasal challenge and is seen by Day 7 in all other challenged groups. Instead, perhaps the rapid death is due from endotoxin induced sepsis of lipopolysaccharide (LPS) overload (Haimovitz Friedman, 1997).

### 3.2 Determining the F1-LicKM plus LcrV-LicKM Vaccination Correlates of Protection

#### 3.2.1 Serum from Mice Vaccinated with 1 μg of F1-LicKM plus LcrV-LicKM Protects Naïve Mice from a Lethal *Y. pestis* KIM D27 Challenge

Groups of BALB/c mice (n=8) were vaccinated with 1 μg of each vaccine component and half the group was challenged with 25 LD$_{50}$ of KIM D27 to confirm vaccine efficacy (Figure 5). The other half was sacrificed and serum was collected through cardiac puncture (Figure 5). Serum collected from the 1 μg F1-LicKM plus LcrV-LicKM vaccinated mice was then transferred into naïve mice via an intraperitoneal injection in a total volume of 50 μL prior to Day 1 challenge with lethal dose (25 LD$_{50}$) of *Y. pestis* KIM D27. All groups that received serum from vaccinated mice survived challenge, and we determined that 10 μL of serum was
required to fully protect from a lethal challenge (25 LD$_{50}$) (Figure 6). To confirm this protection is antibody mediated, serum from vaccinated mice was treated with Recombinant Protein G agarose beads to remove effector IgG antibodies prior to adoptive transfer. This method effectively removes IgG antibodies, resulting in lack of protection for those mice given Protein G treated serum (Figure 7). This corresponds with previous F1 and LcrV vaccine work, wherein protective antibodies are hypothesized to be mediating vaccine mediated protection (Jawetz, 1944).

3.3 Cell Mediated Immunity Plays a Role in Vaccine Protection: T Cells Harvested from Vaccinated BALB/c Mice Produce Increased Levels of IL-2

To elucidate the role of cell mediated immunity in vaccine protection, lymph nodes and spleens were harvested from groups of vaccinated mice shown to survive challenge (Figure 5). An experimental design is shown in Figure 8. Harvested cells were either used for CD4 T isolation and an IL-2 ELISA (Figure 9), or for in vitro restimulation with F1-LicKM plus LcrV-LicKM proteins for assessment of T cell memory markers (Figures 11, 12) or grown in culture for adoptive transfer to naïve mice (Figure 13).

For IL-2 production, isolated CD4 T cells were restimulated ex vivo for 24 hours with F1-LicKM plus LcrV-LicKM proteins. IL-2 production was measured in the cell supernatant, as IL-2 is necessary for expansion and induction of memory T cells (Cantrell, 1984). Cell cultures containing T cells from 1 μg and 0.2 μg F1-LicKM plus LcrV-LicKM vaccinated mice had increased levels of IL-2 as compared to unstimulated cells from the same vaccinated group (Figure 9). T cells from 1 μg F1-LicKM plus LcrV-LicKM vaccinated mice produced an average of 51.5 pg/ml IL-2 when stimulated with vaccine proteins F1-LicKM plus LcrV-LicKM in vitro,
compared to an average of 8.4 pg/ml IL-2 produced from unstimulated T cells.
Likewise, T cells from 0.2μg F1-LicKM and LcrV-LicKM vaccinated mice produced
117.6 pg/ml IL-2 upon stimulation with vaccine proteins F1-LicKM and LcrV-LicKM
in vitro, compared to an average of 10.8 pg/ml IL-2 produced from unstimulated cells.
Unexpectedly, T cells from mice vaccinated with LicKM in the absence of F1 and
LcrV produced significant levels of IL-2 (an average of 164.7 pg/ml) upon
restimulation with the LicKM protein and vaccine proteins F1-LicKM and LcrV-
LicKM as compared to unstimulated cells (an average of 14.2 pg/ml) (Figure 9). T
cells from control groups vaccinated with Alhydrogel and saline or naïve mice did not
have statistically significant increases in IL-2 production as compared to unstimulated
cells. These results indicate that F1-LicKM and LcrV-LicKM vaccination produce
antigen-specific CD4 memory T cells, and that LicKM alone is able to induce specific
T cells that respond to restimulation.
Harvested cells were also restimulated with F1-LicKM and LcrV-LicKM
vaccine proteins and cultured for 72 hours after which assessment of memory T cell
surface markers was performed using flow cytometry (Figure 10). We observed both
restimulated CD4+ (Figure 11B) and CD8+ (Figure 12B) cells from mice vaccinated
with 1 μg F1-LicKM plus LcrV-LicKM, showed an increased population of cells
expressing CD25 cell surface marker as compared to control naïve groups and those
that received Alhydrogel only. F1-LicKM plus LcrV-LicKM groups display an 18.1%
of total CD4+ cells expressing increased levels of CD25, and 17.2% of CD8+ cells
expressing increased levels CD25. As the α chain of the IL-2 receptor, upregulation of
CD25 is indicative of memory T cell activation (Bitegye, 2002). Next we observed
down modulation of the cell surface marker CD62L, a cell surface marker that down
modulates to induce mobilization of T cells away from lymph nodes to sites of infection upon antigen presentation (Berstein, 2008). Down modulation of CD62L cell populations is observed in both CD4+ and CD8+ from F1-LicKM plus LcrV-LicKM vaccinated cells restimulated in vitro. CD4+ T cells displayed a 20.3% population with low levels of expression of CD62L (Figure 11D) and CD8+ T cells showed a 35.2% population of cell expressing low levels of CD62L (Figure 12D). The percentage of cells expressing low levels of CD62L in both CD4+ and CD8+ cells is increased in cells from vaccinated mice compared to control naïve groups.

Interestingly, cells harvested from LicKM only vaccinated mice and stimulated in vitro with LicKM protein, have down modulation CD62L in both CD4+ and CD8+ T cells as compared to control naïve groups. CD4+ cells have a 19.8% population of cell expressing low CD62L (Figure 11D), while CD8+ cells have a 25.7% population of cells expressing low CD62L (Figure 12D). This immunogenic property of LicKM carrier protein corresponds with high levels of IL-2 production shown in the IL-2 ELISA data (Figure 9).

We did not observe a notable increase in P.I. negative CD4 (Figure 11A) or CD8 (Figure 12A) cell populations, nor was there notable change in the expression of CD44 in both CD4+ (Figure 11C) and CD8+ (Figure 12C) cell populations.

Restimulated T cells grown in culture were adoptively transferred into naïve mice to determine if T cells alone could confer protection against a lethal Y. pestis KIM D27 challenge. T cells stimulated in vitro with F1-LicKM plus LcrV-LicKM vaccine proteins were transferred invenously into groups of naïve mice (n=3), followed by a lethal (25 LD50) Y. pestis KIM D27 challenge the next day. Although it is pilot data that will be repeated in the future, these results indicate that 75% of naïve
mice given T cell are able to survive a lethal (25 LD$_{50}$) KIM D27 challenge (Figure 13). Transfer of naïve T cells stimulated in vitro with anti-CD3, a universal T cell activator, leads to only a 33% survival rate in lethally challenged mice.

3.4 *Y. pestis* KIM D27 and KIM 10 Strains Transformed with Plasmid pKKOVA Activates OT-I and OT-II T Cells upon Stimulation

3.4.1 *Y. pestis* KIM D27 and KIM 10 are Successfully Transformed with pKKOVA Plasmid

Transformants of *Y. pestis* KIM D27 and KIM 10 with plasmid pKKOVA were isolated using Ampicillin (Amp) resistance. Plasmid presence was confirmed by a band size of 5,700 bp in both KIM D27pKKOVA (Figure 13A) and KIM 10pKKOVA on an agarose gel (Figure 12B) (Takahashi, 1994). As the process of transforming a novel plasmid into a bacterial strain containing resident plasmids can lead to the loss of resident plasmids (Couturier, 1988), we next wanted to confirm the presence of the three virulence plasmids of *Y. pestis* KIM D27 (pMT1, pCD1, pPCP1) and one resident plasmid of KIM 10 (pMT1). First we determined that all plasmids are from different incompatibility groups. Next, DNA was isolated, and genes encoded on each resident plasmid were amplified using PCR from overnight cultures of the transformants (Table 2). PCR on a gene from each plasmid repA1 from pMT1, cafI from pCD1, and pla from pPCP1 from KIM D27pKKOVA (Table 2) were confirmed at 831bp, 531bp, and 480bp, respectively (Figure 14). The gene cafI from pMT1 from *Y. pestis* KIM D27pkkOVA was confirmed at 831bp (Figure 14).

As transgenic OT-I and OT-II have T cell receptors specific for peptide residues within the full length ovalbumin protein, it is essential that our KIM D27pKKOVA and KIM 10pKKOVA transformants express full length ovalbumin.
Western blot confirmed full length ovalbumin expression using an anti-ovalbumin monoclonal antibody, documenting an ovalbumin band at 45kDa (Figure 15) (Luu, 2006).

3.4.2 Infection of BMDCs with *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA Activates T Cells Specific for OT-I

BMDCs infected with *Y. pestis* KIM D27pKKOVA encoding full length ovalbumin were able to stimulate T cell surface markers on OT-I CD8+ T cells. Harvested from OT-I transgenic mice, these T cells have MHC-I TCRs designed to recognize OVA residues 257-264. Both *Y. pestis* KIM D27pKKOVA and KIM 10pKKOVA were used for this protocol as KIM D27 contains virulence factors found on all three plasmids and has the ability to kill cells upon infection (Perry, 1997). Even though *Y. pestis* KIM 10 is lacking all but one virulence plasmid, it is still able to infect BMDCs successfully. We used this strain as a control for possible antigen presenting cell death by KIM D27.

Control groups of BMDCs include 1) ovalbumin protein, 2) an OT-I specific peptide, 3) OT-I T and BMDCs only, were used to confirm the ability of the OT-I T cells to be stimulated. OT-I peptide exposed to BMDCs resulted in an increase in surface expression of CD44, CD25, and CD27 on OT-I CD8+ T cells, thus confirming the ability of the OT-I T cells to be stimulated *in vitro* upon presentation of this antigen. BMDCs infected with *Y. pestis* KIM D27pKKOVA were able to increase cell populations of CD44 (Figure 16C), CD25 (Figure 17C), and CD27 (Figure 18C) compared to control groups of uninfected and ovalbumin only BMDCs. These results reveal population shifts towards increased expression of these cell surface markers compared to BMDCs infected with untransformed *Y. pestis* D27. The upregulation of
these cell surface markers suggests stimulation of mature T cell, or antigen specific T cells (Hendriks, 2000, Bitegye, 2002, Vachon, 2006).

BMDCs infected with *Y. pestis* KIM 10pKKOVA were only able to induce an increase in CD44 (Figure 16B) and CD27 (Figure 18B) on OT-I CD8+ T cells. BMDCs infected with KIM 10pKKOVA are unable to induce increases in CD25 expression in OT-I CD8+ T cells (Figure 17B). In spite of this, it appears that BMDCs infected with KIM 10pKKOVA induce an increase in expression of mature T cell surface markers to a certain extent. OT-I CD8+ T cells incubated with BMDCs infected with *Y. pestis* KIM D27pKKOVA and *Y. pestis* KIM 10pKKOVA showed no changes in cell surface markers CD62L or CD43 (data not shown), which are down regulated upon T cell stimulation (Tong, 2004, Bachman, 2005).

OT-II T cells with T cell receptors specific for OT-II peptide residues 323-339 on full length OVA (OT-II peptide), also display an increase in T cell surface marker expression upon stimulation with BMDCs infected with transformed *Y. pestis* KIM D27pKKOVA as compared to KIM D27, with increases in expression of CD44 (Figure 19C), slightly increases in CD25 (Figure 20C), and increases in CD27 (Figure 21C). Control groups of OT-II T cells incubated with BMDCs and full length OVA or OT-II peptide did not induce any changes in expression of all three of these cell surface markers (Figure 19-21A). We continue to investigate the lack of response to the OT-II peptide with OT-II CD4+ T cells.

BMDCs infected with *Y. pestis* KIM 10pKKOVA only induce minor increase in expression of CD25 (Figure 20B) in OT-II CD4+ T cells as compared to BMDCs infected with KIM 10. There is no observable change in OT-II CD4+ T cell population expressing CD44 (Figure 19B) or CD27 (Figure 21B). BMDCs infected
with *Y. pestis* KIM 10pKKOVA demonstrated no notable difference in cell populations expressing CD62L or CD43 cell surface marker (data not shown).
Figure 1. **BALB/c mice succumb to an intranasal challenge dose of *Y. pestis* KIM D27 strain.** Naïve 6 to 10 week old female BALB/c mice were challenged intranasally with $1 \times 10^6$ *Y. pestis* KIM D27 in 30 μL of PBS and monitored for health and survival for 7 days. Mice were monitored for signs of infection such as matted fur, squinting eyes, and a hunched stance. All moribound animals were sacrificed using a double kill method. $n=5$
Figure 2. **F1-LicKM and LcrV-LicKM on Alhydrogel protects BALB/c mice against lethal *Y. pestis* KIM D27 challenge.** Mice were primed, boosted, and challenged as described previously. An Alhydrogel only group (0.3%) was used as a control. Both F1-LicKM and LcrV-LicKM were administered at 25μg with 0.3% of Alhydrogel. Mice were monitored for health and survival for 14 days post challenge. *n*=4.
Figure 3. **Dose titration of F1-LicKM and LcrV-LicKM.** BALB/c mice were primed, boosted and challenged as described previously. An adjuvant Alhydrogel group (0.3%) was used as a control. All other groups were given the indicated dose of F1-LicKM and LcrV-LicKM with 0.3% of Alhydrogel. Mice were monitored for health and survival for 14 days post challenge. n=5.
A. 1 μg F1+V, 25LD₅₀
   △ 0.2 μg F1+V, 25LD₅₀
   ▽ Alhydrogel, 25LD₅₀

B. 1 μg F1+V, 25LD₅₀
   Δ 0.2 μg F1+V, 25LD₅₀
   ▽ Alhydrogel, 25LD₅₀
Figure 4. **Protection of vaccinated mice following a challenge dose escalation.**
BALB/c mice were primed, boosted, and challenged as described previously. Adjuvant (Alhydrogel, 0.3%) only was used in a control group. Mice were vaccinated with either 1 μg or 0.2 μg of each component of the plague vaccine and monitored for health and survival for 14 days post challenge. \( n=5. \)
Figure 5. Vaccination with 1 μg F1-LicKM plus LcrV-LicKM confers protection against a lethal KIM D27 challenge. BALB/c mice were primed, boosted and challenged as described previously. Control groups received 25 μg of LicKM and Alhydrogel, saline and Alhydrogel, or no treatment. F1-LicKM and LcrV-LicKM vaccinated groups were given both components at the indicated dose on 0.3% Alhydrogel. Mice were monitored for health and survival for 14 days post challenge. n=10.
Adoptive serotherapy from F1-LicKM and LcrV-LicKM vaccinated mice into naïve mice protects from lethal challenge. Serum from vaccinated mice was adoptively transferred into naïve mice at the indicated volumes one day prior to challenge with 25 LD$_{50}$ Y. pestis KIM D27 strain. Mice were monitored for health and survival for 14 days post challenge. Represents duplicate experiments, $n=9$. 

Figure 6.
Figure 7. **Removal of IgG antibodies eliminates vaccine-generated protective efficacy.** Serum from 1 ug F1-LicKM plus LcrV-LicKM vaccinated mice were harvested as described previously. Serum was incubated with Protein G agarose beads to remove IgG antibodies and later transferred intraperitoneally into naïve mice. The following day mice were challenged with 25 LD50 of *Y. pestis* KIM D27 strain. Mice were monitored for health and survival for 14 days post-challenge. Represents duplicate experiments, *n*=10.
Figure 8. **T cells harvested from F1-LicKM plus LcrV-LicKM vaccinated mice.**

To determine the role of cell mediated immunity in vaccine mediated protection, vaccinated mice were either challenged (Figure 5) or sacrificed on Day 35. Spleens and lymph nodes were harvested from the sacrificed animals for T cells. Harvested T cells were used in an IL-2 ELISA (Figure 9) or for *in vitro* stimulation followed by adoptive T cell transfer into naïve mice (Figure 10).
Figure 9. **F1-LicKM plus LcrV-LicKM vaccine generates IL-2 producing CD4 T cells.** CD4 T cells isolated from vaccinated mice were incubated for 24 hours with 100 μg/mL of proteins LicKM, F1-LicKM, LcrV-LicKM, or F1-LicKM plus LcrV-LicKM. The protein ovalbumin (OVA) or the universal T cell activator anti-CD3 served as controls. Supernatant was collected after 24 hours and an IL-2 ELISA was performed. Data is representative of results from two different experiments.
Figure 10. **Flow cytometric analysis.** All analyzed samples were first visualized through side scatter vs. forward scatter (A). Cells were further gated for live cells only, by gating on propidium iodide (P.I.) negative cells (B). We then selected for CD4+ cells only (C). Cells that appeared to be lymphocytes (A), are alive (B), and are CD4+ (C) were then analyzed for changes in expression of specific T cell markers (D), with CD44 cell surface marker shown as an example.
Figure 11. **CD4+ T cells express increase in levels of memory T cell surface markers.** To determine if the F1-LicKM plus LcrV-LicKM vaccine generated memory T cell phenotypes, FACS analysis was performed. Green lines indicate T cells from Alhydrogel vaccinated mice with no *in vitro* stimulation, orange lines indicate T cells from naïve mice with no *in vitro* stimulation, blue lines indicate T cells from LicKM vaccinated mice with LicKM stimulation *in vitro*, and read lines indicate T cells from F1-LicKM and LcrV-LicKM vaccinated mice with F1-LicKM and LcrV-LicKM restimulation *in vitro*. T cells from F1-LicKM plus LcrV-LicKM immunized mice show no increase in expression of CD4+ cells in P.I. negative (living) T cells (A), but do have increased expression of CD25 in the P.I. negative fraction (B). Also, in F1-LicKM plus LcrV-LicKM vaccinated mice, there is no change in the expression levels of CD44 in P.I. negative CD4+ T cells (C), but the P.I negative, CD4+ lymphocytes demonstrate a down modulation of CD62L (D).
Figure 12. **CD8+ T cells express increased levels of memory T cell surface markers.** To determine if the F1-LicKM and LcrV-LicKM vaccine generated memory T cell phenotypes, FACS analysis was performed. Green lines indicate T cells from Alhydrogel vaccinated mice with no *in vitro* stimulation, orange lines indicate T cells from naïve mice with no *in vitro* stimulation, blue lines indicate T cells from LicKM vaccinated mice with LicKM restimulation *in vitro*, and read lines indicate T cells from F1-LicKM plus LcrV-LicKM vaccinated mice with F1-LicKM and LcrV-LicKM stimulation *in vitro*. T cells from mice vaccinated with F1-LicKM plus LcrV-LicKM show no increase in expression of CD8 in the P.I. negative (living) cells (A), but P.I. negative CD8+ T cells show an increased expression of CD25 in this same group (B). T cells from F1-LicKM plus LcrV-LicKM vaccinated mice also do not show increase in expression of CD44 in P.I. negative, CD8+ cells (C), but do show down modulation of CD62L (D).
Figure 13. F1-LicKM plus LcrV-LicKM stimulated T cells protect against lethal challenge. Naïve T cells were stimulated with αCD3, while T cells from F1-LicKM plus LcrV-LicKM vaccinated mice were stimulated with 100 μg/mL of F1-LicKM and LcrV-LicKM protein. T cells were then adoptively transferred to naïve mice and challenged the following day with 25 LD$_{50}$ of Y. pestis KIM D27. Mice were monitored for health and survival for 14 days post challenge. n=3.
Figure 14. **Y. pestis** KIM D27 and KIM 10 transformed with pKKOVA plasmid and retention of resident plasmids. *Y. pestis* KIM D27pKKOVA (A) or KIM10pKKOVA (B) were grown overnight in HIB and plasmids were purified using QIAprep™ Spin Miniprep Kit. PCR was performed to confirm pKKOVA plasmid location at 5.7kb using agarose gel electrophoresis. Wild type *Y. pestis* KIM D27 contains plasmids pMT1, pCD1, and pPCP1 encoding genes *cafl*, *repA1*, and *pla*, respectively. Wild type *Y. pestis* KIM10 contains only plasmid pMT1 encoding gene *cafl*. The presence of all resident plasmids was confirmed after successful pKKOVA transformation.
Figure 15. *Y. pestis* KIM D27 and KIM 10 transformed with pKKOVA produce ovalbumin. *Y. pestis* KIM D27 or KIM10 transformed with pKKOVA was grown to a concentration of $10^8$ CFU/ml overnight in HIB at 26°C. Organisms were sonicated and the supernatant was subject to ammonium sulfate precipitation overnight at -20°C. The pellet was heated for 5 minutes at 99°C in Laemmeli sample buffer and loaded into a 10% SDS-PAGE gel. The gel was blotted onto a nitrocellulose membrane, blocked with 3% BSA in TBST and incubated with a mouse anti-ovalbumin monoclonal antibody. Anti-mouse HRP-conjugated tag was used as a secondary antibody. The membrane was incubated with lumilight substrate for one minute and exposed to X-ray film for 10-15 seconds.
Figure 16. *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA stimulates CD44 cell surface marker expression in CD8+ OT-I T cells. Culture of BMDCs and OT-I CD8+ T cells incubated with OT-I peptide demonstrate increase expression of CD44 as compared to cell cultures with BMDCs and OT-I T cells only and BMDCs with OT-I T cells and full length OVA (A). BMDCs infected with *Y. pestis* KIM10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-I T cells with BMDCs infected with KIM 10 pKKOVA (B) and KIM D27pKKOVA (C) showed a marked increase in CD44 expression compared to wild type counterparts (KIM D27 and KIM 10, respectively).
Figure 17. Y. pestis KIM D27pKKOVA stimulates CD25 expression on CD8+ OT-I T cells. Culture incubations of BMDCs and OT-I T CD8+ cells incubated with OT-I peptide demonstrate increase expression of CD25 as compared to cell cultures with BMDCs and OT-I T cells only and BMDCs with OT-I T cells and full length OVA (A). BMDCs were infected with Y. pestis KIM 10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-I T cells with BMDCs infected with KIM 10pKKOVA did not show an increase in expression of cell surface marker CD25 compared to those infected with KIM D27 only (B). OT-I T cells cultured with BMDCs infected with KIM D27pKKOVA showed a marked increase in cell surface marker expression of CD25 compared to those incubated with KIM D27 only (C).
Figure 18. *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA stimulates CD44 cell surface marker expression on CD8+ OT-I T cells. Culture of BMDCs and OT-I T CD8+ cells with OT-I peptide demonstrate increase expression of CD27 as compared to cultures with BMDCs and OT-I T cells only, and BMDCs with OT-I T cells with full length OVA (A). BMDCs were infected with *Y. pestis* KIM 10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-I T cells cultured with BMDCs infected with KIM 10pKKOVA (B) and KIM D27pKKOVA (C) and shows a marked increase in CD expression compared to wild type counterparts (KIM 10 and KIM D27, respectively).
Figure 19. *Y. pestis* KIM D27pKKOVA or KIM 10pKKOVA do not stimulate increased CD44 on CD4+ OT-II T cells. Cultures of BMDCs and OT-II T CD4+ cells with OT-II peptide do not demonstrate increase expression of CD27 as compared to BMDCs and OT-II T cells only, and BMDCs with OT-II T cells and full length OVA (A). BMDCs were infected with *Y. pestis* KIM 10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-II cells cultured with BMDCs infected with KIM 10pKKOVA (B) do not show increases in CD44 expression compared to BMDCs infected with KIM 10 only. OT-II T cells cultured with BMDCs infected with KIM D27pKKOVA (C) show slight increases in CD44 expression compared to BMDCs infected with KIM D27.
Figure 20. *Y. pestis* KIM D27pKKOVA stimulates CD25 expression in CD4+ OT-II T cells. Cultures of BMDCs and OT-II CD4+ T cells only, and BMDCs with OT-II T cells incubated with full length OVA show no marked expression of CD25 cell surface marker (A). BMDCs were infected with *Y. pestis* KIM 10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-II T cells cultured with BMDCs infected with KIM 10pKKOVA (B) do not show increases in CD25 expression compared to BMDCs infected with KIM 10 only. OT-II T cells cultured with BMDCs infected with KIM D27pKKOVA (C) have slight increases in CD25 expression compared to BMDCs infected with KIM D27.
Figure 21. *Y. pestis* KIM D27pKKOVA or KIM 10pkkOVA do not stimulate expression of CD27 on CD4+ OT-II T cells. Culture of BMDCs and OT-II T cells only, BMDCs and OT-II CD4+ T cells incubated with full length OVA, and BMDCs and OT-II T cells with OT-II peptide show no marked increase expression of CD25 cell surface maker (A). BMDCs were infected with *Y. pestis* KIM 10 or KIM 10pKKOVA (B) or KIM D27 or KIM D27pKKOVA (C). OT-II T cells cultured with BMDCs infected with or KIM 10pKKOVA (B) or KIM D27pKKOVA (C) show no significant increase in CD25 expression compared to their wild type counterparts (KIM 10 and KIM D27, respectively).
4.1 Recombinant F1-LicKM and LcrV-LicKM Produced in Nicotiana benthamiana Plants

Here, we have explored the protective efficacy of a novel F1-LicKM plus LcrV-LicKM plague vaccine in BALB/c mice. The vaccine proteins are fused to LicKM carrier protein, and expressed in *Nicotiana benthamiana*, a tobacco plant derivative. Expression of recombinant proteins in plant systems has many advantages for production of vaccines for mammalian use. Primarily, plants offer a low-cost means of protein expression and are considered safe for mammalian use as the risk of harboring mammalian pathogens in plants is low (Yusibov, 2010). Traditional transient expression system in plants, however, has one main short-coming. That is low level expression of target proteins. To circumvent this, a launch vector system was designed which is composed of a plant viral vector from the tobacco mosaic virus (TMV) incorporated into a binary plasmid of *Agrobacterium* (Yusibov, 2010).

Vacuum infiltration introduces the *Agrobacterium* into the intercellular spaces of *Nicotiana benthamiana* plants (Musiychuk, 2006). Once infiltrated, single stranded DNA copies of viral vector sequences and their target is inserted into the plant cells where they are amplified and translated into large quantities of target protein within the plant. This launch vector system has been used to produce target antigens for *Bacillus anthracis*, influenza virus, *Plasmodium falciparum*, *Trypanosoma brucei*, measles virus, as well as, *Yersinia pestis* described in this study (Yusibov, 2010).

The carrier molecule, lichenase (LicKM), is a thermostable β-1,3-1,4-glucanase from *Clostridium thermocellum* and was used to enhanced expression and
stability of the F1 plus LcrV vaccine in this launch vector system. Lichenase is an enzyme that cleaves β-1,4 linkages adjacent to β-1,3 bonds in glucans, which compose 70% of the cell wall in plants such as barley. Lichenases are used by plant-inhabiting bacteria including *Rhizobium meliloti*, *Cellvibrio mixtus*, *Ruminococcus flavefaciens*, and *Clostridium thermocellum* (Chen, 1997). LicKM can be fused to large and complex proteins and retains its enzymatic activity at high temperatures making it an ideal carrier protein for vaccine antigens. Enzymatic activity retention at temperatures as high as 65°C can assist with vaccine protein purification as a quick heat treatment removes up to 50% of plant proteins (Musiychuk, 2006). In addition, it may provide thermostability to the target proteins fused to it, a decided advantage for development of vaccines for developing countries. This ability to carry large and complex proteins is advantageous for a *Y. pestis* vaccine as multiple LcrV variants exist and the ability of LicKM to incorporate a large range of proteins in both size and composition may be vital for protection against a weaponized strain of *Y. pestis*.

Data reported here shows that a F1-LicKM plus LcrV-LicKM plague vaccine, administered in range of protein concentrations, coupled with the adjuvant Alhydrogel, can provide protective efficacy in BALB/c mice against a lethal *Y. pestis* KIM D27 intranasal challenge.

### 4.2 F1-LicKM and LcrV-LicKM Correlates of Protective Efficacy

When testing a new vaccine, understanding vaccine limitations and optimum vaccine parameters is critical. In our study, we conducted a number of experiments to better understand the limitations of our *Y. pestis* model in BALB/c mice. After establishing that BALB/c mice are, in fact, susceptible to a lethal intranasal challenge of *Y. pestis* KIM D27 strain (Figure 1) and that F1-LicKM and LcrV-LicKM in
combination provided challenge protection in the model (Figure 2), we performed a titration study to determine the minimum dose of F1-LicKM plus LcrV-LicKM that would provide protection. Results showed that concentrations as low as 1 μg and 0.2 μg of F1-LicKM plus LcrV-LicKM conferred full protection against a lethal KIM D27 challenge (Figures 3 and 5). Additionally, we determined that mice are protected from 25 LD50, 250 LD50, and 500 LD50 challenge doses of KIM D27. Mice vaccinated with 1 μg or 0.2 μg F1-LicKM plus LcrV-LicKM vaccine had an 80% survival rate after challenge with 250 LD50 (Figure 4B) and up to a 60% survival rate after challenge with 500 LD50 of Y. pestis KIM D27 (Figure 4C). Mice were not protected against 1000 LD50 and death may be the result of overwhelming LPS concentrations (Haimovitz Friedman, 1997).

After the inclusion of multiple controls, including an Alhydrogel only group, a LicKM plus Alhydrogel group, and a naïve group, we determined that 1 μg F1-LicKM and LcrV-LicKM adsorbed onto Alhydrogel was the optimal dose to achieve protective efficacy in this BALB/c mouse model of lethal Y. pestis KIM D27 challenge (Figure 5). This concentration and formulation were used for the duration of the studies.

4.3 Determining Immunological Means of F1-LicKM and LcrV-LicKM Vaccine Protection

4.3.1 Humoral Immunity: F1-LicKM and LcrV-LicKM Specific Antibody Protection

Previously, the protective efficacy of F1 and LcrV fused separately to LicKM molecules as in this study, and F1 and LcrV fused together to a single LicKM molecule was tested in Cynomologus Macaques (Lyons, 2007, Chichester, 2009).
Mett et al. demonstrated that the two antigens fused separately to LicKM not only protect nonhuman primates against an aerosolized challenge of 100 LD$_{50}$ of *Y. pestis*, but also that immunization produces robust LcrV and F1 specific IgG antibodies titers upon vaccination. Chichester et al. established that LicKM-F1-LcrV double fusion is also effective in protection against fully virulent *Y. pestis* at 100 LD$_{50}$ (Chichester, 2009). This formulation also produced a robust antigen-specific IgG antibody response, with IgG1 as the predominant isotype generated. IgG1 is associated with a Th2 response, supporting the classical aim of vaccines generating a robust humoral response to protect against plague infection (Mountford, 1994).

Vaccines for plague have primarily focused on humoral immunity, and multiple studies have demonstrated that the passive transfer of F1-and LcrV-specific antibodies into naïve animals can produce protection against lethal *Y. pestis* challenges (Anderson, 1997). To this point, we have shown that the passive transfer of serum from mice vaccinated with 1μg F1-LicKM plus LcrV-LicKM confers full protection against a lethal *Y. pestis* KIM D27 intranasal challenge (Figure 6) and that removal of antigen-specific IgG antibodies by treating serum with a Protein G column eliminates this protective efficacy (Figure 7). However, at least two studies have shown that high pre-challenge antibody titers after vaccination with F1 and LcrV do not always correlate with full protection against *Y. pestis* aerosolized challenges in non-human primates (Williamson, 2007, Bashaw, 2007). Furthermore, F1 and LcrV vaccinated nonhuman primates have succumbed to challenge with *Y. pestis* even with high F1 and LcrV specific antibody titers (Bashaw, 2007, Pitt, 2004).

The exact role of antigen-specific antibodies in preventing *Y. pestis* infection is still controversial. Serum from vaccinated mice does not possess the ability to lyse or
destroy *Y. pestis* bacilli *in vitro or in vivo* in the presence of phagocytic cells and bacilli are resistant to complement-mediated destruction (Jawetz, 1944, Barta 2008). It is possible that vaccine specific antibodies, specifically that of LcrV, protects through assistance in opsonization. This hypothesis was demonstrated *in vitro* where LcrV-specific antibodies were shown to assist neutrophils and macrophages to phagocytose *Y. pestis* (Weeks, 2002, Cowan, 2005).

As LcrV is the hypothesized protruding needle in the T3SS used by *Y. pestis* to shuttle effector Yops into phagocytic cells, it is also possible that LcrV-specific antibodies neutralize this process. These effector Yops not only suppress bacteriocidal effects of macrophages, but can induce apoptosis thereby contributing to *Y. pestis* dissemination in the host through phagocytic cell evasion (Weeks, 2002). LcrV-specific antibodies have been shown to suppress this Yops translocation and macrophage apoptosis (Cowan, 2005, Bashaw, 2007). In this role, LcrV-specific antibodies eliminate, or at least suppress, the ability of *Y. pestis* to evade the host immune system through destruction of phagocytic cells that engulf the bacillus. It is likely that antibody mediated protection requires the assistance of phagocytic cells and cell mediated immunity. For instance, mice deficient in bacteriocidal nitric oxide synthase 2 (NOS2), IFNγ, and TNFα impairs serum mediated protection against *Y. pestis* lethal challenge (Parent, 2006).

4.3.2 T Cells’ Role in Conferring Protection in F1-LicKM and LcrV-LicKM Vaccination

As previously stated, T cells likely play a significant role in mediating vaccine protection. We know that *Y. pestis* inhabits an intracellular niche early in infection, and high antibody titers against LcrV and F1 do not always confer protection in
nonhuman primates, therefore the study of the role T cells play in vaccine protection is essential.

Here, we investigated the production of IL-2 from isolated CD4+ T cells from our F1-LicKM plus LcrV-LicKM vaccinated mice (Figure 9). CD4+ T cells are necessary in adaptive immunity and recognize processed epitopes on MHC II molecules by APCs. In vaccines, antigen specific CD4+ T cells are necessary to confer protection and for long term protection mediated by humoral immunity (Ahmed, 1996). CD4+ T cells also assist in affinity maturation of antibodies and the maturation of antibodies with progressively higher affinity for a specific pathogen (Ahmed, 1996). It has also been shown that effective F1 and LcrV vaccines prime specific CD4+ T cells and here we explored this potential role for the F1-LicKM plus LcrV-LicKM vaccine (Leary, 1995, Oyston, 1995, Williamson, 1995). We monitored IL-2 production by CD4+ T cells from vaccinated mice, as IL-2 production is indicative of antigen-specific clonal expansion upon T cell activation and the production of memory T cells (Minami, 1993). This clonal expansion is induced by specific MHC and antigen presentation by T cell receptors. By restimulating F1-LicKM plus LcrV-LicKM vaccine primed CD4+ T cells in vitro with vaccine proteins and monitoring changes in IL-2 production in stimulated cells compared to unstimulated T cells, we have determine that vaccination results in F1 and LcrV specific, mature CD4+ T cells.

To further explore the CD4+ phenotype generated upon F1-LicKM plus LcrV-LicKM vaccination, we evaluated the change in cell surface markers on live, CD4+ T cells upon restimulation in vitro with vaccine proteins for 72 hours. Restimulation in vitro in this manner mimics the T cell phenotype generated upon pathogen encounter
in vivo, generating an antigen-specific memory T cell response to vaccine antigens. We noted an increase in CD25+ CD4+ T cells (Figure 11B) and a decrease in CD62L+ CD4+ T cells (Figure 11D) from F1-LicKM plus LcrV-LicKM vaccinated mice restimulated in vitro as compared to control groups. As CD62L is down modulated, and CD25 is up regulated upon activation and generation of a memory T cell, it can be concluded we are inducing memory T cell production upon vaccination (Taniguchi, 1993, Berstein, 2008). We do not see an obvious increase in CD44+ CD4+ T cells (Figure 11C), a cell surface marker also upregulated upon T cell stimulation, as ligation of CD44 by low molecular weight hyaluronan (an extracellular matrix protein expressed in areas of infection) stimulates IL-2 production by T cells (Vachon, 2006). Although vaccination does not induce the upregulation of this marker, we believe vaccination is generating a clear trend towards CD4+ memory T cell response coupled with IL-2 production (Figure 9).

CD4+ T cells also play an essential role in cell mediated immunity and priming of cell mediated immunity during vaccination may be essential for plague vaccine protection in nonhuman primates. One hypothesized role for CD4+ T cells in vaccine protection is the production of Th1 cytokines in priming phagocytic cells for engulfment and destruction of Y. pestis (Smiley, 2008). Priming macrophages with IFNγ and TNFα, produced by mature T cells, limits the organism’s ability to replicate readily in macrophages (Lukasewski, 2005). When vaccinated with F1 and LcrV, STAT-4 knock-out mice, which are deficient in Th1 cytokine response, fail to be protected from plague challenge, supporting a cell mediated role in vaccine protection (Elvin, 2004). Th1 cytokines also facilitate the development of memory CD8+ T cells, which can quickly recognize and assist in destroying infected cells (Ahmed,
CD8+ T cells do not yet have a clearly described role in LcrV and F1 vaccine-mediated protection. We therefore wanted to study the induction of memory CD8+ T cells from F1-LicKM plus LcrV-LicKM mice restimulated \textit{in vitro} with vaccine proteins. CD8+ T cells from vaccinated mice display down modulation of CD62L (Figure 12D) and increased expression of CD25 (Figure 12B), which are the same cell surface marker changes observed in CD4+ T cells from F1-LicKM plus LcrV-LicKM vaccinated mice. We can therefore assume that vaccination with F1-LicKM plus LcrV-LicKM is able to generate cell populations that upregulate these cell surface markers, indicative of memory T cell production, even in CD8+ T cell populations.

There are several works that implicate cell mediated immunity is necessary for complete vaccine-mediated protection against pneumonic plague. In an important study, Parent et al. demonstrated that antibody-mediated protection against pneumonic plague is only successful if the host is able to produce IFN\(\gamma\), TNF\(\alpha\), and NOS2 (Parent, 2006). Vaccines that prime T cells to produce IFN\(\gamma\) and TNF\(\alpha\) may fulfill the missing link for successful pneumonic plague protection. Furthermore, vaccination of B cell-deficient, \(\mu\)MT mice with live \textit{Y. pestis} KIM D27 with minimally protective post-exposure serotherapy fully protects mice against a lethal pneumonic plague challenge (Parent, 2005). Likewise, when IFN\(\gamma\), TNF\(\alpha\), or both CD4+ and CD8+ T cells are depleted, vaccine-mediated protection is lost. T cells from \textit{Y. pestis} KIM D27 vaccinated \(\mu\)MT mice expanded \textit{in vitro} confer some protection from lethal challenge when transferred to naïve mice, and vaccination of C57BL/6 mice with attenuated \textit{Y. pestis} can prime T cells that confer protection in naïve mice without expansion \textit{in vitro} (Parent, 2005, Philipovskiy, 2007). It has also been shown that CD8+ T cells from vaccinated mice adoptively transferred into naïve mice protect more effectively than
CD4+ T cells against lethal pneumonic plague infection in mice, however, the addition of CD4+ cells to adoptively transferred CD8+ cells appears to add to protection against lethal *Y. pestis* challenge (Philipovskiy, 2007). This finding indicates a possible role of T cell cross-talk in mediating pneumonic plague vaccine protection.

From this data we further hypothesized that primed T cells from F1-LicKM plus LcrV-LicKM vaccinated mice can be sufficient to confer protection against lethal *Y. pestis* KIM D27 challenge. Although preliminary, a majority (75%) of naive mice that received adoptively transferred *in vitro* stimulated T cells from vaccinated mice survived lethal *Y. pestis* KIM D27 infection (Figure 13). Naïve T cells stimulated *in vitro* with universal T cell stimulator αCD3 conferred minimal protection against lethal challenge (25%). It is unlikely that T cells alone are responsible for conferring protection against pneumonic plague, however we believe the data support that they play a major role and are necessary for effective pneumonic plague protection.

While the exact role T cells play in mediating F1 and LcrV vaccine protection is not yet known, the production of antigen specific mature T cells through F1-LicKM plus LcrV-LicKM vaccination appears important. More than likely, both humoral and cell mediated immunity priming is necessary to fight *Y. pestis* pneumonic plague infection. A potential mechanism could be that F1 and LcrV specific antibodies facilitate opsonization and eventual phagocytosis of *Y. pestis* by phagocytic cells, while vaccine specific T cells prime these phagocytic cells through IFNγ and TNFα activation (Smiley, 2008). The priming of phagocytic cells facilitates an upregulation of antimicrobial reactive oxygen species, which we know *Y. pestis* encounters upon infection of macrophage (Pujol, 2005). Whatever the case may be, we provide a comprehensive evaluation of an effective, yet novel plant-produced F1 and LcrV
vaccine in a mouse model *Y. pestis* infection, and elucidate the role of both cell mediated and humoral immunity in conferring protection against a lethal *Y. pestis* KIM D27 pneumonic plague challenge.

### 4.4 Immunogenicity of LicKM Carrier Protein

Interestingly, we found that CD4+ T cells from the LicKM only vaccinated control group are able to respond to *in vitro* stimulation by producing IL-2 and showing changes in cell surface markers associated with memory phenotypes. LicKM generates immunogenicity in CD4+ T cells harvested from vaccinated mice in restimulated, *in vitro* conditions. Not only did CD4+ T cells from LicKM-only vaccinated mice generate a significant IL-2 response, indicative of memory T cell production, when restimulated with LicKM protein, but also in response to restimulation with F1-LicKM and LcrV-LicKM vaccine proteins (Figure 10). Therefore we analyzed the memory T cell surface markers from LicKM-only vaccinated mice, restimulated for 72 hours with LicKM protein. CD4+ (Figure 11D) and CD8+ (Figure 12D) T cells display down modulated CD62L which demonstrates antigen specific stimulation of T cells. As LicKM is a foreign protein, it may have immunogenic properties when introduced into mammalian systems (Ahmed, 1996). Undoubtedly, the ability of LicKM to induce an immune response and the role it may plan in vaccine immunogenicity should be further evaluated.

### 4.5 Evaluating *Y. pestis* T Cell Stimulation *In Vitro* Using the Immunological Tool, Plasmid pKKOVA, and OVA-Specific CD4+ and CD8+ T Cells

The generation of memory lymphocytes is a multistep process requiring several cell adhesion and costimulatory markers. Some of these markers, such as L-selectin (CD62L) are down modulated upon T cell activation, while others (CD44,
CD25, CD27) are upregulated upon T cell activation. The ability of *Y. pestis* to induce an activated T cell phenotype can be critical in understanding the immune response and role of T cells in plague infection. Here, we use transformed *Y. pestis* KIM D27 and KIM 10 with an ovalbumin encoding pKKOVA plasmid to study the ability of *Y. pestis* to stimulate ovalbumin specific OT-I and OT-II T cells. To our knowledge, the formulation of a transgenic *Y. pestis* strain encoding pKKOVA plasmid is novel. This plasmid has been used to study the ability of other intracellular bacteria to stimulate T cell responses, including *Salmonella typhimurium* and *Listeria monocytogenes* (Luu, 2010, Bueno, 2007). We have confirmed the retention of all resident plasmids after pKKOVA transformation of *Y. pestis* (Figure 14) and confirmed the expression of full length ovalbumin in the transformants (Figure 15). These two steps are essential for proper usage of this type of immunological tool (Luu, 2010). The loss of resident virulence plasmids through the transformation process would alter true implications of the study, as loss of resident plasmids and virulence genes would affect the ability of *Y. pestis* to stimulate the host’s response (Perry, 1997). Loss of resident plasmids would also impact the ability of *Y. pestis* to inhabit its intracellular niche, causing loss of organism presentation through MHC molecules and thus loss of the ability to stimulate OT-I and OT-II T cells. Likewise, ovalbumin expression by transformed *Y. pestis* is necessary as engulfment of the transgenic *Y. pestis* species should lead to antigen presentation of ovalbumin or OT-I and OT-II peptide fragments through MHC molecules to stimulate OT-I and OT-II T cells.

4.5.1 The Ability of *Y. pestis* pKKOVA to Stimulate OT-I T Cells *In Vitro*

It is evident that *Y. pestis* KIM D27/pKKOVA is able to stimulate OT-I T cells *in vitro*, presumably through antigen presentation of ovalbumin peptides by KIM
D27pKKOVA infected dendritic cells. Control groups of OT-I peptide only were also able to induce activated T cell phenotypes through cell surface marker upregulation. CD25+ CD8+ T cells are implicated as long-term memory T cells in old-age (Herndler-Brandstetter, 2005) with robust response to IL-2 \textit{in vitro}. As CD25 is the $\alpha$ chain of the IL-2 receptor, and IL-2 is necessary for memory T cell expansion and formation, the high expression of IL-2 receptor on CD8+ T cells implies that \textit{Y. pestis} is able to induce memory CD8+ T cell formation (Oba, 2009). Furthermore, induction of CD44+ CD8+ T cells is also indicative of memory T cells with CD44 upregulated upon T cell stimulation (Yamada, 2001). Finally, we observed increased expression of CD27 in CD8+ T cells. As CD27 is implicated in memory T cell formation as well and its upregulation has been shown in long term memory T cells, also demonstrates memory T cell formation. CD27, a TNF family receptor, is necessary for clonal expansion upon antigen presentation to T cells. In fact, CD27 deficient mice have impaired abilities in T cell clonal expansion (Henriks, 2000). Although we do not see any changes in expression of CD62L (generally down modulated in stimulated T cells) or CD43 (upregulated in stimulated T cells) it is clear that we have generated a transformed \textit{Y. pestis} strain, \textit{Y. pestis} KIM D27pKKOVA, that is able to stimulate OT-I T cells \textit{in vitro}. This capability is critical in further understanding the role of cell mediated immunity during \textit{Y. pestis} infection. From here, we can gain further insight on the organism’s ability to stimulate and generate memory CD8+ T cells, and how this can be implemented to understand host response to infection or for alternative treatments for plague. As previously stated, Th1 cytokines such as IFN$\gamma$ and TNF$\alpha$ contribute greatly to the immune systems defense against plague, and pre-priming this leg of the immune system could assist \textit{Y. pestis} treatment or prophylactic measures.
The ability of *Y. pestis* to stimulate CD8+ T cells is further supported by a recent work by Chen et al. that described *Y. pestis* YopE as the dominant epitope recognized by CD8+ T cells *in vitro*. CD8+ T cells harvested from mice vaccinated with live-attenuated *Y. pestis* not only recognize YopE *in vitro*, but vaccination of naïve mice with YopE fully protected mice from lethal pulmonary challenge (Chen, 2011). This finding is critical for the future of *Y. pestis* vaccines, and recognition of a CD8+ T cell epitope may further our understanding of the immunogenicity of *Y. pestis* infection.

We also examined the ability of *Y. pestis* KIM 10pKKOVA to induce a CD8+ T cell memory phenotype. We used this strain of *Y. pestis* as more virulent *Y. pestis* KIM D27 can destroy the phagocytic cells using its T3SS encoded on plasmid pCD1 (Perry, 1997). KIM 10 is lacking plasmid pCD1 and use of this transformed strain may help explain any lack of result in T cell stimulation with KIM D27pKKOVA, as phagocytic cells may have been destroyed. However, it appears that this lack of virulence altered the *Y. pestis* immunogenicity due to the lack of the two virulence plasmids, pCD1 and pPCP1 (Table 1). While KIM 10pKKOVA generated minor phenotypic differences in OT-I T cells, it was not as robust as what was seen with KIM D27pKKOVA.

### 4.5.2 Exploring the Ability of *Y. pestis* KIM D27pKKOVA to Stimulate OT-II T Cells

The role of CD4+ T cell and humoral immunity in combating *Y. pestis* infections has been recently established. In order to further study and understand this complicated response, we used transformed *Y. pestis* D27pKKOVA to study these stimulated T cells. Interestingly, control groups of OT-II peptide with BMDCs and
OT-II T cells failed to produce any changes in memory T cell surface markers CD44 (Figure 19), CD25 (Figure 20), or CD27 (Figure 21). As we know that *Y. pestis* KIM 10 and KIM D27 can infect BMDCs adequately, the lack of response from control groups may be explained by improper OT-II peptide presentation on MHC II molecules on the surface of the BMDCs and we are currently investigating this result. Antigens are processed by antigen presenting cells once exogenous proteins are engulfed. The pathogen is then degraded into small peptide fragments when the phagosome fuses with a lysosome. MHC II α and β receptor chains are synthesized in the endoplasmic reticulum (ER), and transported via the Golgi apparatus to the phagolysosome. Once there, exogenous proteins associate with MHC II receptors chains and are transported to the surface of the APC for antigen presentation (Gene, 2012). Since MHC II loads primarily from the phagolysosome, it is possible that *Y. pestis* KIM D27pKKOVA expressing ovalbumin was not seen because of improper presentation on a MHC II molecule for recognition by OT-II T cells. No changes in expression of CD44 (Figure 19), CD25 (Figure 20), and CD27 (Figure 21) were observed in OT-II T cells incubated with BMDCs infected with KIM 10pKKOVA, as well. We did see a dramatic increase in CD25+, CD4+ T cells when stimulated with KIM D27pKKOVA compared to wild-type KIM D27 (Figure 20), indicative of a stimulated T cells (Oba, 2009). Without functioning control groups, however, little conclusions can be drawn from these results. Further work needs to be conducting on the aptitude of transformant KIM D27pKKOVA and KIM 10pKKOVA to stimulate OT-II T cells.
REFERENCES


following exposure to aerosols of Pasteurella pestis. American Journal of Pathology, 53(1), 99-&.


Yersinia pestis protects mice against plague. *Infection and Immunity*, 63(8), 2854-2858.


Appendix

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE FORM

Title of Protocol: Yersinia infection of mice

AUP Number: 1166-2012-2

Principal Investigator: Michelle A. Parent

Common Name: C57BL/6

Genus Species: Mus musculus

Pain Category: (please mark one)

<table>
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<th>Description</th>
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<tr>
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<td>Breeding or holding where NO research is conducted</td>
</tr>
<tr>
<td>C</td>
<td>Procedure involving momentary or no pain or distress</td>
</tr>
<tr>
<td>D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
</tr>
<tr>
<td>E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
</tr>
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Date of Approval: 3/14/2012