STUDIES ON THE DEVELOPMENT OF A BACILLUS ENDOSPORE-BASED MICROPARTICLE VACCINE PLATFORM

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By

CURTIS JOHN PRITZL

Dr. Daniel E. Hassett, Dissertation Mentor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled:

STUDIES ON THE USE OF *BACILLUS THURINGIENSIS* ENDOSPORES AS A MICROPARTICLE VACCINE PLATFORM

Presented by Curtis John Pritzl,

A candidate for the degree of doctor of philosophy and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Daniel E. Hassett

Dr. Charles R. Brown

Dr. David Lee

Dr. Habib Zaghouani

Dr. George Stewart

Dr. Mark Daniels

Dedication

First, to my parents, Lawrence and Sheila for instilling the importance of education from the beginning and allowing me to go out and find it wherever I needed to, and supporting me in every way possible throughout it all

Next to Dan, for not only being an outstanding mentor but also a great friend

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Thank you all, this document only has one name on it, but it is really the result of work by everyone named here

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ABSTRACT

The rapid and safe immunization of populations is critical in response to emerging infectious diseases or biological attacks. Currently, many subunit vaccines are insufficiently immunogenic to protect against pathogen invasion, therefore novel adjuvants are required. The antigen display on Bacillus endospore system provides a unique antigen delivery system with inherent adjuvant properties that is suitable for both parenteral and noninvasive delivery routes of immunization. This dissertation describes the development of this novel antigen display system. In addition, initial immunogenicity studies were performed utilizing the model antigen β-galactosidase. Furthermore, this study demonstrates that UV-irradiated *B. thuringiensis* spores elicit potent innate immune responses from murine, bone marrow-derived dendritic cells. Finally, the protective capacity of this vaccine platform was investigated using the low calcium response V antigen (LcrV), a dominant antigen of *Yersinia pestis.* LcrV was efficiently displayed on the surface of biotinylated *Bacillus thuringiensis* spores. Mice immunized with spore-displayed LcrV rapidly develop high-titer systemic IgG antibodies and were protected from a lethal intranasal plague challenge. These data imply that the spore-displayed antigen system is a potent adjuvanted microparticle delivery system that is suitable for parenteral or mucosal immunizations against emerging infectious diseases and potential biological weapons.

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HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DAPI	4',6-diamidino-2-phenylindole
ANOVA	analysis of variance
BCG	Bacillus Calmette-Guérin
BclA	Bacillus collagen-like protein A
Bti	*Bacillus thuringiensis* strain: israelensis
BGAL	beta-galactosidase
BMDC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
BHI	brain heart infusion
CpG-ODN	cytosine-phosphate-guanine motif containing oligodeoxynucleotides
^{o}C	degrees celsius
K_D	dissociation constant
ELISA	enzyme-linked immunosorbant assay
EDTA	ethylenediaminetetraacetic acid
F1	factor one antigen
FITC	fluoroscein isothiocyanate
HIB	heart infusion broth
HRP	horseradish peroxidase
HIV	human immunodeficiency virus
HCl	hydrochloric acid
iNOS	inducible nitric oxide sythase
IFN-γ	interferon-gamma
IL-10	interleukin-ten
IL-12	interleukin-twelve
IL-1β	interleukin-one beta
IL-6	interleukin-six
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton
LPS	lipopolysaccharide
LcrV	low calcium response V antigen
LB	Luria-Bertani
Lamp-1	lysosome-associated membrane protein one
MHC I	major histocompatibility class one
MFI	mean fluorescence intensity
µg	microgram
µl	microlitier
µM	micromolar

mg	milligram
ml	milliliter
mls	milliliters
mM	millimolar
M	molar
MOI	multiplicity of infection
nm	nanometer
NAV	NeutrAvidin
N	normal
SATA	N-Succinimidyl-S-acetylthioacetate
OD	optical density
OPD	ortho-phenylenediamine
OVA	ovalbumin
PBS	phosphate buffered saline
PE	phycoerythrin
PE-Cy5	phycoerythrin cyanin five
PAGE	polyacrylamide gel electrophoresis
rpm	revolutions per minute
7-AAD	seven-aminoactinomycin D
SDS	sodium dodecyl sulfate
SAV	streptavidin
TBS	tris buffered saline
TNF-α	tumor necrosis factor alpha
TIP-DC	tumor necrosis factor alpha and inducible nitric oxide synthase producing dendritic cells
UV	ultraviolet
V	volts
x g	times gravity
pg	picograms
TH1	T-helper 1
TH2	T-helper 2
IFN-β	interferon-beta

Chapter I: Literature Review

A. The Immunological Basis of Vaccination

The development of effective vaccines against pathogens has been a major contributor to the improved quality of life in the modern world. Diseases such as rabies, smallpox, measles, rubella, and more recently, influenza have threatened populations throughout history. With the advent of vaccines, one disease, smallpox, has was completely eliminated [1]. In addition, the World Health Organization has targeted poliovirus for global eradication a worldwide vaccination program [2]. Vaccinations also protect millions of people against diseases that have not yet been eradicated including measles, hepatitis B, and Varicella-Zoster viruses [3-6].

Although current vaccines have provided a great deal of protection against many infectious diseases, there are, as yet, still unmet needs. First, safe, efficacious, cost-effective vaccines are not yet available for many diseases such as human immunodeficiency virus (HIV), rotaviruses, tuberculosis, and plague. Second, medical professionals and supplies such as sterile needles and syringes are required for the administration of most vaccines, and this reduces participation in vaccine programs due to the cost and inconvenience of doctor visits [7].

The goal of any vaccine platform is the generation of effective, long-lasting immunological memory. In general, vaccines provide protection against disease by inducing immune responses in the recipient that are able to quickly and decisively respond to the infection. Immunization exposes the immune system to antigens from the pathogen, and primes humoral and cell-mediated immune

responses without causing severe disease. Following immunization; these primary immune responses induced by vaccine antigens contract over time leaving behind a small population of memory cells that can quickly respond to subsequent pathogen encounters.

Types of Vaccines

All vaccines currently in use today can be divided into two broad categories: live attenuated and nonreplicating. The major difference between these two types of vaccines is the ability of the vaccine to replicate in the host. Live-attenuated vaccines replicate within the recipient, while the nonreplicating vaccines do not. Because of this characteristic, these two types of vaccines may induce different immune responses; however, both live attenuated and nonreplicating vaccines are used to successfully prevent disease.

Live Attenuated Vaccines

Live attenuated vaccines consist of either a bacteria or virus that has been cultured *in vitro* or genetically altered in such a way to reduce its capacity to cause disease. Live attenuated vaccines replicate within the host and are able to rapidly induce long lasting cellular and humoral immune responses. In addition these vaccines do not typically require additional adjuvants or frequent boosting doses. Examples of live-attenuated virus vaccines include the vaccines against measles, mumps, rubella, and varicella viruses. The Bacillus Calmette Guérin (BCG) vaccine against tuberculosis and the typhoid vaccine against *Salmonella thyphi* are examples of live-attenuated bacterial vaccines. Because live-attenuated vaccines replicate, there is a concern for that they could cause

disease in certain patients [8-9]. Thus, the use of live-attenuated vaccines is often precluded for use in immunocompromised individuals, such as transplant recipients, pregnant women, the elderly, and the very young. In addition, these vaccines require refrigeration, which increases the costs of vaccine production, transportation, and storage.

Nonreplicating Vaccines

In contrast to live-attenuated vaccines, non-replicating vaccines, by definition, cannot replicate within the recipient. Non-replicating vaccines are generally considered to be safer alternatives than live-attenuated vaccines, because there is little chance for pathogenicity. Examples of nonreplicating vaccines include the parenteral influenza virus vaccine, the Salk polio vaccine, and the vaccine against hepatitis A. These vaccines consist of formalin or heat inactivated virus or bacteria. Non-replicating vaccines also include subunit and toxoid vaccines, which are comprised of specific antigens derived from the pathogen of interest. Examples of subunit vaccines include the tetanus vaccine which contains the tetanus toxoid, and the pertussis vaccine, which contains a mixture of inactivated pertussis toxin, filamentuous hemagluttinin, and pertactin proteins [10-11]. Although non-replicating vaccines are much safer, the immune response generated by these vaccines is often less durable and requires frequent boosting doses in order to maintain protective levels of immunity. In addition, non-replicating vaccines often fail to elicit strong cell-mediated immune responses, such as the induction of cytotoxic, CD8 T-cells.

Adjuvants

Most non-replicating vaccines are unable to generate strong, long-lasting immune responses on their own. Therefore, most of these vaccines are formulated with additional compounds, or adjuvants, designed to enhance the immune response to the immunizing agent. The United States Food and Drug Administration does not approve adjuvants for general use. Instead, it approves vaccine-adjuvant formulations. Currently, the adjuvants contained in human-approved formulations include: aluminum salts (alum); the adjuvant system ASO4, which contains alum and monophosphorylated lipid A (MPL); and MF59, a squalene oil and water emulsion.

All adjuvants work by stimulating innate immune defenses, but not all utilize the same mechanisms for immunopotentiation. For example, alum has been used for many years and the exact mechanism of action has yet to be fully understood. It has been demonstrated that the presence of alum creates antigen depots, or sites of antigen persistence that prolong the exposure of the immune system to the antigen [12]. In addition, alum activates inflammatory dendritic cells by inducing the cellular release of uric acid, which in turn, induces interleukin-1β production [13]. The adjuvant system ASO4 also contains alum, but it also utilizes MPL, a form of lipopolysaccharide which activates toll-like receptor 4 (TLR4). TLR4 activation by MPL has leads to the upregulation of inflammatory cytokines and markers of dendritic cell activation, such as major histocompatibility complex class II, CD80, and CD86 [14]. The adjuvant MF59, on the other hand, activates macrophages and monocytes to produce

granulocyte and monocyte chemo-attractants such as CCL2, CCL3, CCL4, and CXCL8, which act to increase the pool of professional antigen presenting cells [15-16]. Because these adjuvants enhance immune responses via independent pathways, it is possible that multiple adjuvants could be used concurrently to obtain synergistic effects.

In addition to those adjuvants currently approved for use, multiple experimental adjuvants based on other pathogen-associated molecular patterns (PAMPs) are being investigated. Oligodeoxynucleotides containing unmethylated cytosine-phosphate-guanine nucleotide motifs can mimic microbial DNA and not only activate antigen presenting cells, but also to increase humoral responses to co-administered vaccine antigens [17-22]. Polyinosinic:polycytidylic acid (polyI:C) simulates the presence of single stranded viral RNA in cells, and has adjuvant properties such as inducing dendritic cell activation and improving cell mediated immune responses to co-administered antigens [23-27].

Modern Vaccines and Rational Vaccine Design

Traditional methods of vaccine design have led to the development of many successful vaccines. However, all of these vaccines have been developed empirically, through trial and error. There are a large number of diseases, such as, HIV and malaria, for which no effective vaccine is available. The need for novel, efficacious vaccines has led to the development of experimental vaccine strategies, which include: plasmid DNA based vaccines; fusion protein vaccines; and microparticle-based vaccine delivery systems, all of which employ a rational vaccine design rather than empirical trials [28-33]. Many of these vaccines are

designed to be both safe and capable of inducing long lasting memory immune responses [34-36]. In addition, some vaccine platforms have been specifically formulated to target professional antigen presenting cells. This targeting of the antigen to the antigen presenting cell, in some cases, results in more efficient antigen presentation and therefore, more effective immune responses [37-38]

Mucosal Vaccines

The potential use of mucosal vaccine administration would be extremely beneficial because this method would not only reduce the costs associated with vaccination, but also the burden on medical care workers and improve patient compliance [7]. Vaccines given intranasally or orally have the additional benefit of generating a mucosal immune response. Because many pathogens gain entry to the body through mucosal surfaces, these vaccines are increasingly appealing and multiple studies have explored novel ways of generating effective mucosal immunity [39-41]. Currently the only vaccines approved by the United States Food and Drug Administration for mucosal administration are the FluMist®, an intranasal vaccine against influenza and RotaRix ®, an oral vaccine against rotavirus. Additional mucosal vaccines are approved for use in countries other than the United States, including vaccines against poliovirus, cholera, and typhoid. Although both oral and intranasal vaccines utilize unique strategies for delivery, the exact mechanisms involved in the development of mucosal immune responses are not yet fully understood. The stimulation of potent and durable mucosal immune responses has proven to be difficult.

Microparticle vaccines

Microparticle delivery systems for antigens have been developed to circumvent many vaccine-associated problems. Moreover, microparticles have been used successfully for both oral and intranasal immunizations [42-44]. Microparticles allow for the stable display or encapsulation of multiple copies of the vaccine antigen, which is optimal for the activation of antigen-specific B-cells [45-47]. The size of the microparticle (1-3 μm) is similar to the size of many pathogens. Because professional antigen presenting cells continually survey the body for pathogens, this size resemblance encourages antigen presenting cells to engulf the vaccine particles [48-49]. Enhanced uptake increases cell mediated responses to the displayed antigens [50-52]. Finally, microparticle vaccine platforms allow for the inclusion of additional molecules that can act as adjuvants. Since these molecules are physically associated with both the particle and the antigen, they can act directly on the target antigen presenting cell and avoid the unnecessary activation of nearby antigen presenting cells that have not encountered the antigen.

Microparticles used in vaccine delivery have been developed from materials such as poly (lactic/glycolic) acid (PLGA), latex beads, and gold particles, or were derived from biological materials such as chitosan, lipids, bacteria-like particles, virus-like particles, or bacterial spores, including those from the Bacillus genus of bacteria [39, 53-62]. These microparticle vaccines are an attractive alternative to traditional vaccines strategies because they can deliver both the vaccine antigen or antigens along with additional molecules such as adjuvants to the antigen presenting cell [63-64]. Currently, no microparticle based vaccines

are approved for human use. However, a great deal of research has been devoted to the development of novel vaccines using this platform. Experimental vaccines against diptheria, HIV, and certain types of tumors have been reported. These vaccines were demonstrated to elicit not only high titer, protective antibody responses, but also cell-mediated immune responses [65-67].

B. Biology of Bacillus Endospores

Notable members of the Bacillus genus include *Bacillus subtilis, Bacillus thuringiensis, Bacillus cereus,* and *Bacillus anthracis.* Morphologically, these bacteria are endospore-forming, gram-positive rods that are ubiquitous in the environment. *B. anthracis* and *B. cereus* are also incidental pathogens of humans.

A large amount of work has been done on understanding *B. anthracis* because it causes anthrax, a fatal disease of humans and livestock. *B. subtilis* has been widely studied as a model for prokaryotic gene regulation, DNA replication, and sporulation [68-73]. The pathogenicity of *B. thuringiensis* in insects has been studied because it is a commonly used bioinsecticide. *B. cereus* has been examined due to its ability to cause food-borne illnesses, most notably an acute, self-limiting gastrointestinal disease and it is also an occasional wound contaminant [74]. Because of this research, there is a tremendous body of knowledge on the life cycle of the Bacillus bacteria, as well as both sporulation and the structure of the spores.

All members of the Bacillus genus form endospores. These endospores allow the bacteria to survive long periods of unfavorable environmental

conditions, such as nutrient starvation or temperature extremes [75-77]. The process of sporulation involves asymmetric cell division during which only one copy of the genome is segregated to a cell pole and becomes engulfed by an asymmetric cell division event. The segregated copy of the genome-containing cell compartment becomes the core of the developing endospore [78-79]. The endospore core is surrounded by a thick layer of peptidoglycans termed the cortex. The coat layer is then formed around the cortex [80]. Finally, some Bacillus species also produce a loose balloon-like layer called the exosporium which encompasses the entire spore particle [81].

The coat layer is the major contributor to the durability of the spores under harsh environmental conditions [77, 82]. This layer forms a shell structure around the cortex consisting of cross-linked proteins. The overall protein composition of the coat has been well characterized and is comprised of more than 70 different proteins, with both structural (CotB and CotC) and enzymatic functions (LipC and SodA) [83-86]. These proteins protect the underlying layers from enzymes that could digest the peptidoglycan of the cortex such as lysozyme and from harsh chemicals such as ozone, hypochlorite, and chlorine dioxide that may damage the DNA contained within the core [87-89].

The exosporium is the outermost layer of *B. anthracis*, *B. cereus,* and *B. thuringiensis* spores, while spores from *B. subtilis,* do not have an exosporium. The exosporium is composed of proteins and carbohydrates that come together to form two major structures, a basal layer and a hair-like nap [90]. The predominant protein of the exosporium is Bacillus collagen-like protein of

anthracis (BclA), which is thought to be responsible for the hair-like projections on the outermost surface of the spore [91]. Although the exosporium layer does not appear to play a role in virulence or environmental resistance *in vitro,* it does provide a hydrophobic surface that enhances spore adhesion [92-93]. In addition, the BclA protein is involved in the recognition of host cells by *B. anthracis* spores [94].

C. Bacillus Endospores as Tools for Vaccine Development

B. subtilis has been used as a protein expression system to generate protein antigens for vaccine research [95-98]. *B. subtilis* is a gram positive bacterium that lacks an outer membrane. which reduces the costs and complication of protein expression. Unlike *E. coli,* secreted proteins produced by *B. subtilis* are exported directly into the culture medium [99]. Furthermore, *B. subtilis* is not pathogenic for humans and is generally regarded as safe. Using this expression system, vaccines against the *B. anthracis* protective antigen (PA), pneumolysin from *Streptococus pneumonia,* and pertussis toxin subunits from *Bordatella pertussis* have been produced [100-102]. In all of these cases, *B. subtilis* was used solely to generate the vaccine antigens.

Since bacterial spores are extremely heat-stable, cost effective to produce, and inherently immunogenic, recombinant spore vaccines have been considered as viable alternatives to other carrier vaccine systems. Although the initial spore vaccine was developed by expressing a component of the iota toxin from *Clostridium perfringens* under the control of the toxin promoter in *B. anthracis* [103], the majority of spore-based vaccines have been developed using *B.*

subtilis. The genomic organization, expression, and localization of spore coat proteins in *B. subtilis* are well understood. This knowledge has allowed for the expression of vaccine antigens fused to coat proteins that are then localized to the spore surface.

The display of heterologous antigens on the surface of *B. subtilis* endospores was initially described by Isticato *et al.* [104]. In that study, DNA encoding the 459 amino acid C-terminal fragment of the tetanus toxin was cloned into the open reading frame encoding CotB, an outer coat protein of *B. subtilis.* This study first demonstrated that the toxin fragment was successfully expressed on the surface of the endospores by flow cytometry. Quantitative dot-plot analysis of extracted spore coat proteins determined the amount of toxin displayed on the spore surface to be approximately $1.5x10^3$ molecules of toxin per spore. Furthermore the subcutaneous immunization of Balb/c mice with recombinant, germination-competent spores elicited detectable, tetanus toxin-specific IgG responses [104]. These data indicated that the recombinant spore vaccine systems may have some promise as an alternative vaccine platform.

In a follow-up report by the same group, oral administration of tetanus toxin-expressing recombinant spores was shown to elicit tetanus-toxin specific antibody responses. In these experiments, recombinant, tetanus-toxin expressing spores were administered orally to groups of Balb/c and C57BL/6 mice in two doses 4 weeks apart. Each dose consisted of three separate intragastric administrations of $1.5x10^{10}$ live, recombinant spores. Finally, a subcutaneous boosting dose consisting of soluble tetanus toxin fragment C was

administered 8 weeks following the initial priming dose. This immunization schedule resulted in significant levels of tetanus toxin-specific fecal IgA and serum IgG responses compared to immunizations with non-recombinant *B. subtilis* spores; however, no comparisons with other vaccination strategies were reported, therefore no conclusions regarding the efficacy of this platform can be made. This study was the first to characterize IgG isotype responses in serum following immunizations with a spore-based vaccine. Serum levels of tetanus toxin-specific IgG antibodies in both C57BL/6 and Balb/c mice were dominated by the IgG1 responses. However, detectable levels of toxin-specific IgG2a and IgG2b were observed [105]. These two studies suggested that recombinant, orally administered spores may provide a safe vaccine carrier platform capable of generating protective antigen-specific antibody responses.

A second system for the heterologous display of antigens on the surface of *B. subtilis* was developed by fusing a protein of interest to another spore coat protein, CotC. Recombinant spores expressing either the fragment C of tetanus toxin or the β subunit of the *Escherichia coli* heat labile toxin fused to the *B. subtilis* CotC protein were produced. Quantitation of these proteins in spore coat extract preparations revealed that this system resulted in similar antigen densities on the spore surface as the CotB fusion system (approximately $1.5x10^3$ molecules per spore). Oral immunizations with live, recombinant spores resulted in the production of both mucosal and systemic antibody responses that, in the case of the tetanus toxin producing spores, were similar to those observed with CotB fusion recombinant spores system. Interestingly, recombinant spores

containing CotC fused to *E. coli* heat labile toxin elicited IgG responses that were dominated by IgG2a, compared to the IgG1-dominated responses observed with the tetanus toxin-containing spores [106]. These data suggested that the nature of the displayed antigens had an impact on the immune responses to the vaccine preparation.

Further studies were conducted to determine the immunogenicity of both recombinant spores and vegetative *B. subtilis* bacteria following oral inoculations [107]. Following a single oral dose of spores, detectable levels of spore- and vegetative bacteria-specific IgG have be observed in serum, whereas no *B. subtilis*-specific antibodies were detected after multiple oral doses with vegetative bacteria. This suggested that the spore form of the bacteria was much more efficient at stimulating the immune system following oral delivery than the vegetative bacteria. It was also observed that the initial spore-specific IgG response was dominated by IgG2a antibodies. Over time and with multiple vaccine doses, the IgG2a response waned and IgG1 antibodies came to dominate the response. Various cytokine mRNAs were determined in the spleens, livers, mesenteric lymph nodes, and submandibular glands of mice that had received a single oral inoculation with spores and only interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) were detected. As expected, the levels of cytokine mRNA peaked within the first 100 hours after inoculation in the submandibular glands and mesenteric lymph nodes, whereas in the spleens and livers, these levels increased between 100 and 300 hours post inoculation [107]. The levels of IFN-γ corresponded to the strong IgG2a responses observed

following the single oral inoculation of spores, as both indicate a T-helper 1 (TH1) CD4 T-cell response.

All of the studies described up to this point have utilized live, germination-competent, recombinant *B. subtilis* spores that were able to generate immune responses specific for the recombinant antigen *in vivo*. In a study by Mauriello *et al.,* germination-deficient, recombinant spores expressing tetanus toxin fragment C were used to immunize mice. They demonstrated that the germination deficient, tetanus toxin-displaying spores elicited similar levels of cellular immunity compared to the germination competent recombinant spores expressing the same protein [108]. Thus, immune responses elicited by recombinant *B. subtilis* spores were independent of a need for bacterial germination or replication. However, This study did not report the levels of antibody responses specific to the tetanus toxin. Thus, the effects of bacterial germination or replication on the production of antigen-specific antibodies following spored based vaccination are unknown.

Further studies have been conducted using the recombinant *B. subtilis* spore vaccine systems. Additional antigens used include the protective antigen of *B. anthracis* (PA), the tegumental protein of *Clonorchis sinensis*, the alpha toxoid of *Clostridium perfringens,* and the glutathione S-transferase of the trematode *Schistosoma japonicum* [109-111]. Each of these vaccine formulations proved to be immunogenic following oral or intranasal immunization of rats or mice. A vaccine specific for the *E. coli* distinct colonization factor/I fimbriae was also used in a heterologous prime-boost vaccine study with a DNA vaccine. The prime-

boost schedule induced significantly greater immune responses in recipient mice compared to plasmid DNA or recombinant spore vaccines alone [112].

Most of the work using recombinant spores has focused on eliciting antigen-specific antibody responses against recombinant bacterial or parasitic antigens. The first antiviral vaccine developed using a recombinant *B. subtilis* spore system was specific for the VP28 protein of white spot syndrome virus (WSSV), which is considered to be one of the most serious viral pathogens of shrimp and a major concern of shrimp industry [113]. In these experiments, crayfish were fed pellets containing recombinant spores and vegetative *B. subtilis* bacteria expressing VP28. Three, 14, or 28 days following feeding, the crayfish were challenged with WSSV. Those animals that had received either recombinant, vegetative bacteria or spores from recombinant bacteria had significantly lower mortalities following viral challenge compared to crayfish immunized with vector alone [113].

The second antiviral vaccine was targeted against the VP6 protein of murine and bovine rotaviruses [114]. Rotavirus are considered to be a major cause of severe diarrhea in young children, therefore a safe, effective vaccine would be tremendously beneficial worldwide. Mice were immunized intranasally with either recombinant *B. subtilis* spores or vegetative bacteria displaying VP6 from either murine rotavirus or bovine rotavirus. The mice were given subsequent boosts two and four weeks later. These vaccines were formulated with or without adjuvants that consisted of either cholera toxin or mutant *E. coli* heat labile toxin. Mice that had received recombinant spores alone demonstrated detectable levels of VP6-specific serum IgG and fecal IgA responses, and mice that had received

spores formulated with adjuvants demonstrated the highest levels of rotavirus-specific antibodies. Mice immunized with the spore-displayed antigen formulated with the mutant heat labile toxin were almost completely protected from rotavirus challenge, indicating that vaccination was able to induce protective immunity. In the presence of adjuvant, the IgG isotype responses were dominated by IgG2a, whereas in the absence of the adjuvant, the responses were dominated by IgG1 [114]. These data reinforced the idea that the nature of the displayed antigen influences the IgG isotype responses. Furthermore, the presence of an additional adjuvant could alter the immune responses elicited by recombinant spore vaccines.

While most of the literature regarding recombinant spore vaccines have dealt with the induction of pathogen-specific humoral immune response, several studies have also focused on the ability of the spores to act as an adjuvant to improve not only CD4 T-cell responses, but also enhance the cross presentation of vaccine antigens to CD8 T-cells. In a series of experiments by D'Apice *et al*., recombinant *B. subtilis* spores displaying CD4 epitope peptides from human immunodeficiency virus (HIV) fused with the CotC protein were generated. When these recombinant spores were used to elicit responses from CD4 T-cells *in vitro*, moderate levels of proliferation were observed [115]. Although this system was not as efficient as other self-adjuvanting vaccine platforms, such as the E2 core of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*, the combination of the recombinant spores' ability to elicit humoral immune responses following mucosal administration and the ability to

induce CD4 T-cells, warrant further investigations into the use of Bacillus spores as a vaccine platform.

B. subtilis spores can also act as adjuvants to soluble, co-administered antigens that are not displayed on the spore surface. The Klavinskis group demonstrated that antibody responses to fragment C of tetanus toxin were increased following intranasal co-immunization with live *B. subtilis* spores compared to soluble antigen alone. Furthermore, when both spores and tetanus toxin were given subcutaneously, both CD4 and CD8 T-cell responses specific for tetanus toxin were dramatically increased compared to tetanus toxin alone or tetanus toxin formulated with the adjuvant alum. Similar findings were observed when spores were co-introduced with the model antigen chicken ovalbumin [116]. These results indicate that spores act to not only boost antigen-specific antibody responses, but also cell-mediated immunity and can enhance the cross presentation of exogenous antigens to CD8 T-cells [117].

Collectively, these data suggest that Bacillus spores are not only efficient carriers for vaccine antigens, but also possess an inherent adjuvant property that enhances the immune responses to both displayed and soluble antigens. However, there are several limitations of the current recombinant spore vaccine system. Although *B. subtilis* has been used to express many recombinant antigens, transformed plasmids are not stable in the bacterial cell. Therefore, chromosomal integration is required for efficient protein production [118]. This requirement adds a further layer of complexity to the development of novel vaccines. In addition, post-translational modifications can be critical in vaccine-

specific immune responses [119]. Antibodies specific for discontinuous epitopes or sugar moieties can be essential in protective immune responses. Viral proteins in particular that are expressed in prokaryotic cells often have different tertiary structures and glycosylation patterns compared to the native proteins expressed in eukaryotic cells [120]. Finally, in the recombinant spore-display system, the choice of vaccine antigens is limited to the proteins that can not only be expressed by Bacillus bacteria, but also to proteins that can be efficiently localized to the spore surface.

D. The Antigen Display on Bacillus Endospore System

The purpose of the studies described in this dissertation was to generate and evaluate the immunogenicity of a novel self-adjuvanted microparticle vaccine platform based on the display of heterologous antigens on the surface of non-replicating *B. thuringiensis* endospores. The basis of these studies was to capitalize on the benefits of recombinant spore vaccine systems while addressing the primary challenges described above. In contrast to those studies which used spores from recombinant bacteria, this system utilizes the strong interaction between biotin and streptavidin to display antigens or other molecules on the outermost surface of the spores. This system circumvents the issues associated with expressing antigens in recombinant Bacillus bacteria and instead allows for the use of recombinant proteins derived from the most efficient expression systems available for the antigen in question. Due to the modular nature of the linkage chemistry, this system also theoretically allows for the display of multiple antigens on a single spore. In addition to protein antigens, this

system could allow for the display of other molecules as well. Examples include small molecule antigens, such as haptens, biologically active enzymes that could alter the chemistry of the microenvironment, antibodies that target the spores to specific cell types, and molecular adjuvants such as CpG-containing oligodeoxynucleotides (Figure 1).

The decision to use the biotin-streptavidin linkage chemistry was based primarily on the strong interaction between biotin and streptavidin molecules. This linkage is well described in the literature, and has often been used to label antibodies for *in vitro* detection systems or to immobilize molecules onto solid substrates [121-122]. The dissociation constant (K_d) of the biotin-streptavidin interaction has been reported to be 10^{-15} M and is considered to be the strongest non-covalent chemical interaction known. Along with the high binding affinity of biotin and streptavidin, the ease of use of both biotin and streptavidin were major factors in the choice of linkage chemistries. Commercial forms of pre-activated biotin and avidin derivatives were readily available from various vendors. As demonstrated by this study, bacterial spores can be readily biotinylated with a commercial, pre-activated form of biotin and recombinant antigens expressed in *E. coli* can be efficiently linked to a commercial avidin derivative. Because the activated form of biotin reacts with primary amine groups on proteins, biotin is displayed on the surface of the spores at a very high density. Although a variety of other linkage chemistries were available, the biotin-streptavidin system was thought to be ideal for initial trials of this novel system.

Another important consideration in the development of this system was the species of Bacillus spores to use. Although a majority of the studies on recombinant spores as vaccine vehicles utilized *B. subtilis* spores, these spores do not have an exosporium layer. Proteins of the exosporium layer have been shown to be immunogenic and therefore may provide additional adjuvant properties to the vaccine platform [123-124]. The exosporium layer is composed of the hair-like nap which greatly increases the surface area of the spore. This increase in surface area was thought to provide additional binding partners for the activated biotin, therefore increasing the density of displayed antigen.

Three prominent members of the Bacillus genus produce exosporium-containing spores: *B. anthracis, B. cereus, and B. thuringiensis. B. anthracis* and *B. cereus* were not considered for use due to their pathogenic potentials in both humans and livestock. *B. thuringiensis* however, has been used as a natural bioinsecticide for nearly 60 years and is generally thought to be safe for humans [125-126]. To further ensure that this platform was safe a toxin-negative strain of *B. thuringiensis* was used. As an additional safety precaution all spore preparations were completely inactivated by high dose ultraviolet irradiation prior to biotinylation.

The data reported in this dissertation demonstrate that biotinylated *B. thuringiensis* spores can efficiently bind and display streptavidin-linked proteins, fluorochromes, or bioactive enzymes. To gain insight into the adjuvanticity of the system, the effect of spore exposure on dendritic cells *in vitro* was also examined. In addition, subcutaneous or intranasal immunization with spores that

displayed either the model antigen β-galactosidase, or a recombinant antigen from *Yersinia pestis* elicted antigen-specific serum antibody responses in mice. Furthermore, the plague-specific antibody responses neutralize *Yersinia pestis* bacteria *in vitro* and can protect mice in high dose intranasal plague challenge studies. Collectively, these data suggests that the antigen display on Bacillus endospores system has promise as a vaccine platform and warrants further investigations on the system to improve antigenicity as well as explore other applications for the high density display of heterologous molecules on the spore surface.

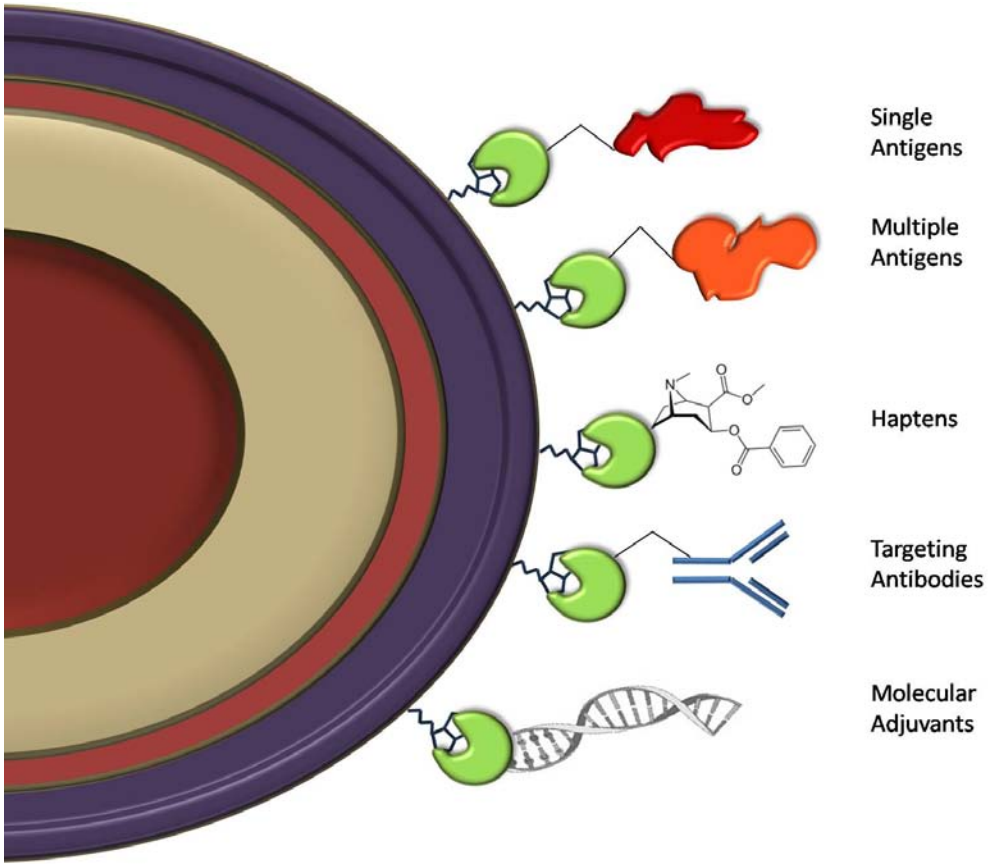

Figure 1. The Antigen Display on Bacillus Endospore System. Biotinylated Bacillus endospores can serve as an efficient microparticle-based platform for the linkage of a variety of avidin-conjugated molecules including: antigens, haptens, cell-type specific antibodies, and molecular adjuvants.

Chapter II: Materials and Methods

A. Reagents

1. **Bacillus thuringiensis endospore preparation:** A plasmid-negative strain of *Bacillus thuringiensis* (serovariant *israelensis*, Cacillus Genetic Stock Center 4Q7*)* was used in all experiments. The bacteria were maintained on brain-heart infusion (BHI) agar plates or as glycerol stocks at -80^{o}C. To prepare spores, a single colony of *B. thuringiensis* was used to inoculate 5 mls of BHI broth and the bacteria were grown with agitation at 37^{o}C overnight. The stationary phase bacteria were diluted in BHI broth and plated on nutrient agar plates at 30^{o}C. Bacteria were allowed to undergo sporulation for four days, after which, free spores were swabbed from the surface of the plates and suspended in sterile distilled water. Spores were purified of cell debris by three washes in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2) and centrifugation for 10 minutes at 10,000 x g. The final spore preparations were examined microscopically for the absence of vegetative cells and stored at 4^{o}C in distilled water.
2. ***Bacillus thuringiensis* endospore inactivation:** Spores were diluted in sterile 1X PBS to a concentration of 1x10^{9} per ml and inactivated by exposure to 2x10^{7} μJ/cm^{2} of ultraviolet radiation in an HL-2000 HybriLinker (UVP LLC, Upland, CA). Following inactivation, the spores were washed three times in distilled water and stored at 4^{o}C until use. Complete inactivation of spore preparations was confirmed by plating 100

μl aliquots of UV-irradiated spores onto BHI agar plates, incubating them at 37^{o}C for 48 hours, and monitoring for lack of colony formation.

3. **Recombinant Low Calcium Response V antigen (LcrV): expression and purification:** The prokaryotic expression plasmid pET20b containing an open reading frame encoding the LcrV protein and an N-terminal 6X histidine tag (pET20b-LcrV) was kindly provided by Dr. Hanni Lee-Lewis and Dr. Deborah Anderson. Chemically competent *E. coli* (BL21 Star™, Invitrogen) were transformed with 1 μg of plasmid DNA by co-incubating bacteria and plasmid DNA for 20 minutes on ice, followed by a 30 second heat shock at 42^{o}C, then incubating again on ice for 2 minutes. Sterile Luria-Bertani (LB) broth was added to final volume of 1 ml. Cultures were grown for 1 hour at 37^{o}C then plated onto LB agar plates containing 0.1 mg/ml ampicillin and incubated overnight at 37^{o}C. Transformants were obtained the following day and protein production was screened by inducing 10 ml cultures. Log phase cultures were induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and grown for an additional four hours at 37^{o}C. Large scale protein inductions were carried out as follows. Starter bacterial cultures (10 ml) were grown overnight at 37^{o}C in LB broth + 0.1mg/ml ampicillin with agitation (200 rpm). The following day, 250 ml cultures were inoculated with the starter culture and grown to log phase ($OD_{600\ nm}$ = 0.8 – 1.0). When the cultures reached log phase, 1 M IPTG was added to a final concentration of 0.1 mM and the culture was allowed to grow for an

additional four hours. Following the incubation, the bacterial cells were harvested by centrifugation for 20 minutes at 6,000 xg. Soluble lysate was obtained by resuspending the bacterial pellet in 1X binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) containing 0.1 mg/ml lysozyme. Samples were frozen and thawed three times (-80^{o}C, 25^{o}C), then sonicated for 30 seconds (Fisher Scientific Sonic Dismembrator, Model 100, setting = 6, output power approximately = 18 – 20). Soluble and insoluble fractions were separated by centrifugation at 15,000 x g for 20 minutes. Recombinant LcrV protein was purified from the soluble bacterial lysate using a commercial Ni^{2+} chromatography purification kit (His-Bind, EMD Biosciences) according to the manufacturer's protocol.

4. Antibodies

Anti-LcrV (BA-5): A mouse monoclonal antibody specific for recombinant LcrV protein from *Yersinia pestis* was provided by Dr. Deborah Anderson, University of Missouri.

Anti-Bcl-A polyclonal rabbit serum: A rabbit polyclonal antibody specific for recombainant Bacillus collagen-like protein A from *Bacillus anthracis* was provided by Dr. George Stewart, University of Missouri.

CD11c-PE: clone HL3: An Armenian hamster IgG1 monoclonal antibody specific for the mouse CD11c protein. This antibody was chemically conjugated to phycoerythrin (PE) was purchased from BD Biosciences, San Jose, CA.

Mouse MHC Class II (I-A/I-E)-FITC: clone M5/114.15.2: A rat IgG2b monoclonal antibody specific for the murine I-A and I-E major histocompatibility complex II molecules. This antibody was chemically conjugated to fluorescein isothiocyanate (FITC) was purchased from eBioscience, San Diego, CA.

CD80-PE-Cy5: clone 16-10A1: An Armenian hamster IgG monoclonal antibody specific for the murine CD80 protein. This antibody was chemically conjugated to phycoerythrin-cyanin 5 tandem dye (PE-Cy5) was purchased from eBioscience.

CD86-PE-Cy5: clone GL1: A rat IgG2a monoclonal antibody specific for the murine CD86 protein. This antibody was chemically conjugated to phycoerythrin-cyanin 5 tandem dye (PE-Cy5) was purchased from eBioscience.

Goat Anti-Mouse Total IgG-horseradish peroxidase (HRP) H + L Chain Specific: Polyclonal antibodies consisting of pooled antisera from goats hyperimmunized with mouse IgG paraproteins, chemically conjugated to HRP. This antibody reacts with the heavy and light chains of mouse IgG1, IgG2a, IgG2b and IgG3, and with the light chains of mouse IgM and IgA as demonstrated by ELISA and flow cytometry. This antisera was purchased from Southern Biotech, Birmingham, AL.

Goat Anti-Mouse IgG1-HRP: Polyclonal antibodies consisting of pooled antisera from goats hyperimmunized with mouse IgG1 paraproteins, chemically conjugated to HRP. This antibody reacts with the heavy chain

of mouse IgG1 as demonstrated by ELISA and flow cytometry and was purchased from Southern Biotech.

Goat Anti-Mouse IgG2a-HRP: Polyclonal antibodies consisting of pooled antisera from goats hyperimmunized with mouse IgG2a paraproteins. These antisera were chemically conjugated to HRP. These antisera react with the heavy chain of mouse IgG2a as demonstrated. This reagent was purchased from Southern Biotech.

Goat Anti-Rabbit Total IgG-HRP: Polyclonal antibodies consisiting of pooled antisera from goat hyperimmunized with normal rabbit IgG. These antisera were chemically conjugated to HRP. This antibody reacts with the heavy chain of rabbit IgG. This reagent was purchased from Southern Biotech.

Lysosomal associated membrane protein-1 (Lamp-1)-FITC: Rat IgG2a monoclonal antibody specific for mouse Lamp-1 (CD107a) molecules chemically. This antibody was conjugated to fluorescein isothiocyanate (FITC) and was purchased from Southern Biotech.

5. Streptavidin-linked enzymes

Streptavidin-linked β-galactosidase: Streptavidin from *Streptomyces avidinii* conjugated to β-galactosidase grade VIII was purchased from Sigma-Aldrich, St. Louis, MO.

Streptavidin-linked peroxidase: Streptavidin from *Streptomyces avidinii* conjugated to Type VI peroxidase was purchased from Sigma-Aldrich.

6. **Streptavidin-linked fluorochromes**

Streptavidin-fluorescein isothiocyanate (SAV-FITC): Streptavidin from *Streptomyces avidinii* chemically conjugated to multiple fluorescein isothiocyanate molecules was purchased from eBioscience.

Streptavidin-phycoerythrin (SAV-PE): Streptavidin from *Streptomyces avidinii* chemically conjugated to phycoerythrin molecules was purchased from eBioscience.

Streptavidin-phycoerythrin-cyanin 5 (PE-Cy5): Streptavidin from *Streptomyces avidinii* chemically conjugated to multiple phycoerythrin-cyanin 5 tandem dye molecules was purchased from eBioscience.

7. **Biotin-linked enzymes**

Biotin-linked horseradish peroxidase: Biotin molecules chemically conjugated to horseradish peroxidase. Purchased from Pierce (Thermo-Fisher), Rockford, IL

B. Methodologies

1. **Immunization of mice with unmodified *Bacillus thuringiensis* endospores:** Four to six week old Balb/c female mice were immunized subcutaneously between the shoulder blades with $1x10^9$ spores suspended in 100 μl 1X PBS (pH 7.2). Concurrently, control mice were immunized with 100 μl 1X PBS (pH7.2) alone in the same manner.
2. **Enzyme-linked immunosorbent assay (ELISA) to determine spore-specific antibody titers:** Ninety-six well Microtest ELISA plates (BD

Bioscience) were coated overnight at 4^{o}C with 5x10^{5} *B. thuringiensis* spores diluted in carbonate buffer (0.05 M sodium carbonate-bicarbonate, pH 9.8). The unbound antigen was removed and the plates were blocked with 1X PBS + 3% bovine serum albumin (BSA) for 1 hour at room temperature, then washed three times with ELISA wash (1X PBS [pH 7.4] + 0.05% Tween 20). Plates were incubated for 1 hour with twofold serial dilutions of test sera diluted in 1X PBS + 3% BSA, washed three times with ELISA wash buffe,r and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies specific for mouse IgG (Southern Biotechnology) diluted 1/5,000 in 1X PBS + 3% BSA. The plates were then washed three times in ELISA wash and 100 μl of a solution containing 0.5 mg/ml o-phenylenediamine dihydrochloride (OPD, Thermo-Fisher), 52 mM citric acid, 989 mM dibasic sodium phosphate and 0.01% hydrogen peroxide was added to each well. The reaction was allowed to proceed for 60 minutes and then stopped by the addition of 100 μl of 1 N HCl to each well, after which the absorbance at 492 nm (OD_{492}) was recorded using a Spectramax plus microplate reader running Softmax pro version 5.2 sofware (Molecular Devices Inc, Sunnyvale CA). Each serum sample was assayed in duplicate and the endpoint titer was determined as the reciprocal of the lowest dilution which yielded an OD_{492} value of three standard deviations above the mean optical density of control wells incubated in the presence of serum from nonimmune mice.

3. **Biotinylation of Bacillus endospores:** $1x10^9$ UV-inactivated endospores were incubated in the presence of 2 mM EZ-Link Sulfo-NHS-LC-LC-biotin (Thermo-Fisher) in 1 mL PBS (pH 8.0) for 16 hours at room temperature while rocking. The reaction was quenched by sequential washing with 100 mM glycine in PBS (pH 7.2). The biotinylated spores were resuspended in sterile PBS at a final concentration of $1x10^9$ spores/ml and stored at 4^oC until use.
4. **Western blotting of biotinylated Bacillus spores:** Non-biotinylated and biotinylated *B. thuringiensis* endospores were boiled in tris-glycine buffer (25 mM Tris·HCl, 192 mM glycine) containing 8 M urea, 20% glycerol, and 1% sodium dodecyl sulfate (SDS). The proteins were then separated by polyacrylamide gel electrophoresis (PAGE) on a 4-20% Tris-Glycine SDS-PAGE gel, and transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA). Biotin was detected with a streptavidin-conjugated horseradish peroxidase (SAV-HRP, Sigma Aldrich, 1:25,000 diluted in Starting Block TBS Blocking Buffer (Thermo-Fisher)). A rabbit polyclonal antiserum specific for the *Bacillus anthracis* collagen-like protein A (BclA, 1:25,000 diluted in Starting Block TBS Blocking Buffer [Thermo-Fisher]) and an anti-rabbit IgG-specific secondary antibody reagent (Southern Biotech, 1:10,000 in Starting Block TBS Blocking Buffer [Thermo-Fisher]) were used to identify the *B. thuringiensis* BclA protein [127]. All blots were developed using an enhanced chemiluminiscence

detection system (Thermo-Fisher) according to the manufacturer's instructions.

5. **Display of streptavidin-linked fluorochromes:** Untreated or biotinylated Bacillus endospores ($1x10^8$) were reacted with varying concentrations of streptavidin-conjugated fluorescein isothe iocyanate (SAV-FITC, eBioscience) for 30 minutes in 100 μl of PBS at 4^oC. Unbound fluorochrome was removed by sequentially washing the spores three times in 1 ml of PBS at 4^oC. Spores were then resuspended in PBS + 4% formaldehyde and fluorescence intensity in the FL-1 channel was measured on a BD FACScan. The data was analyzed with WINMDI software ver. 2.8 (Joseph Trotter, 2000).
6. **Estimation of antigen density on the spore surface:** To calculate the maximum quantity of avidinated antigen that can be bound to biotinylated spores, dose titration experiments ranging from 0 - 10 μg/$1x10^8$ of SAV-FITC was performed. In each of three separate trials, the maximal mean fluorescence intensity observed and the molecular mass of SAV-FITC (52,800 daltons) was used to determine the number of SAV-linked molecules displayed on the surface of the spore.
7. **Conjugation of spores to streptavidin-linked β-galactosidase (SAV-β-Gal):** Spores ($1x10^{10}$ spores/ml) were incubated with 20 μg of SAV-β-Gal for 30 minutes in 1X PBS (pH 7.2) for 30 minutes at room temperature with constant rocking. Unbound SAV-β-Gal was removed with five sequential washes and centrifugation at 10,000 x g in 1X PBS (pH 7.2).

8. **Enzymatic activity associated with endospores displaying streptavidin-linked enzymes:** Untreated or biotinylated Bacillus endospores ($1x10^8$) were incubated in the presence or absence of streptavidin-linked β-galactosidase (6 μg) for 30 minutes at room temperature in 100 μl volumes. Excess streptavidin-linked enzyme was removed by sequentially washing the spores three times in 1 ml of PBS at 4^oC. Spores conjugated to β-galactosidase or horseradish peroxidase were resuspended in 1 ml of PBS and 5 μl aliquots were assayed in triplicate for enzymatic activity. β-galactosidase and horseradish peroxidase activities were determined spectrophotometrically with a Spectramax plus microplate reader running Softmax pro version 5.2 sofware (Molecular Devices, Inc). The spectrophotometric β-galactosidase and horseradish peroxidase assays monitored the hydrolysis of o-nitrophenyl-β-galactoside at 420 nm, or the oxidation of o-phenylenediamine dihydrochloride at 450 nm, respectively, for 60 minutes using previously described methods [128-129].
9. **Immunization of mice with spores displaying β-galactosidase:** Eight to 10 week old C57BL/6 mice were used in all immunization experiments. Mice were immunized subcutaneously with 20 μg of streptavidin β-galactosidase (Sigma-Aldrich) mixed with $2.5x10^8$ biotinylated or non-biotinylated *B. thuringiensis* endospores, or 20 μg of streptavidin-β-galactosidase alone. All vaccine preparations were formulated in saline and a 100 μl volume was injected subcutaneously between the shoulder

blades using a 26 1/2 gauge needle fitted to a 1 ml syringe. All animals were boosted at 2 weeks post-primary immunization with the same dose and form of vaccine. Serum was obtained from peripheral blood collected at -1, 14, and 28 days post primary immunization. Serum samples were stored at -20^{o}C until use.

10. **ELISA to determine β-galactosidase-specific antibody titers:** Ninety-six well Microtest ELISA plates (BD Bioscience) were coated overnight at 4^{o}C with 2 μg of a soluble extract of *E. coli* cells expressing β-galactosidase diluted in carbonate buffer (0.05 M carbonate-bicarbonate, pH 9.8). The unbound antigen was removed and the plates were blocked with 1X PBS + 3% BSA for 1 hour at room temperature. The plates were washed three times with ELISA wash (1X PBS (pH 7.4) + 0.05% Tween 20). Plates were incubated for 1 hour with twofold serial dilutions of test sera diluted in 1X PBS + 3% BSA, washed three times (as above) and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies to either mouse IgG, IgG1, or IgG2a (Southern Biotech) diluted 1/5,000, 1/2,500, and 1/2,500 respectively in 1X PBS + 3% BSA. The plates were washed three times in ELISA wash and 100 μl of a solution containing 0.5 mg/ml o-phenylenediamine dihydrochloride (OPD, Thermo-Fisher), 52 mM citric acid, 989 mM dibasic sodium phosphate and 0.01% hydrogen peroxide was added to each well. The reaction was allowed to proceed for 60 minutes and then stopped by the addition of 100 μl of 1 N HCl to each well, after which the absorbance value at 492 nm was

recorded. Each serum sample was assayed in duplicate and the endpoint titer was expressed as the reciprocal of the lowest dilution which yielded an OD492 value of three standard deviations above the mean optical density of control wells incubated in the presence of serum from nonimmune mice.

11. **Isolation of bone marrow-derived dendritic cells (BMDC's):** Bone marrow–derived dendritic cells (BMDC's) were cultured using modifications to a previously described protocol [130]. Bone marrow cells from female C57BL/6 mice were harvested and red blood cells were lysed in ammonium chloride lysis buffer (0.16 M NH_4Cl, 0.17 M Tris·HCl). Cells were cultured for six days in 6-well plates (Nunc) at a concentration of $1x10^6$ cells per ml in 3 ml RPMI + 5% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, 10 mM HEPES, and 20 ng/ml granulocyte−macrophage colony-stimulating factor (GM-CSF, Biolegend, San Diego, CA). The medium was replaced on day three. Nonadherent cells were harvested after six days of culture. Dendritic cells were identified by flow cytometry with a PE-labeled antibody specific for CD11c (BD Bioscience). Typically, cultures yielded a population of cells of which, 90% – 98% were CD11C+ dendritic cells.

12. **Modified Giemsa staining:** BMDC's ($5x10^5$ cells/well) were co-incubated with or without *Bacillus thuringiensis* spores at a spore:cell ratio of 10:1 in 1 ml of RPMI + 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, and 10 mM HEPES for 24 hours in one well

of a 24 well dish then applied to glass slides using the cytospin technique, dried, and fixed in methanol. For staining, slides were incubated in Diff-Quick solution I for 30 seconds and counterstained in Diff-Quick solution II for 30 seconds.

13. **Immunofluoresence microscopy to determine spore uptake:** BMDC's ($5x10^5$ cells/ml) were allowed to adhere onto poly-L-lysine-coated cover slips in RPMI + 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, and 10 mM HEPES for 24 hours then incubated with or without streptavidin-phycoerythrin-labeled spores (2 μg/$1x10^8$ spores) for 2 hours, thoroughly washed with PBS, and fixed in 1X PBS containing 2% formaldehyde. Cells were permeabilized with 0.05% saponin in 1X PBS and stained with a LAMP-1 (clone 1D4B) antibody (10 μg/ml, 100 μl volume, BD Bioscience). Nuclei were visualized using a solution of 1X PBS + 3 μM DAPI immediately prior to microscopy. Micrographs were acquired on a Nikon E600 microscope with a 100X objective and analyzed using Olympus DP2-BSW software (ver. 2.1).

14. **Dendritic cell phagocytosis of spores:** BMDC's ($5x10^5$) were pretreated for 30 minutes at either 4^oC or at 37^oC in RPMI + 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, and 10 mM HEPES with or without 10 μM cytochalasin D (Sigma, St. Louis MO). Cells were then exposed to streptavidin-FITC labeled spores (6 μg/$1x10^8$ spores, spore:cell ratio = 20) for 2 hours in the presence or absence of

inhibitors, then washed extensively with 1X PBS + 5% FBS. The presence or absence of the phagocytosed spores was determined by measuring the fluorescence in the FL-1 channel by flow cytometry on a BD FACscan. Cells incubated in the absence of spores at 37^{o}C served as controls for nonspecific autofluorescence.

15. **Stimulation of BMDC's:** Day 6 BMDC's ($5x10^5$ cells) were cultured for 24, 48, or 72 hours in RPMI supplemented with 10% FBS, 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, 10 mM HEPES. Cells were cultured in the presence or absence of lipopolysaccharide (LPS, 0154B, Sigma, St Louis, MO) at 1 μg/ml or UV-inactivated *Bacillus thuringiensis* endospores (spore to cell ratios of 2.5, 5, or 10). Cells incubated in media alone served as negative controls. Each experimental condition was set up in triplicate. At the various time points, nonadherent cells were harvested for phenotypic analyses and culture supernatants were harvested for cytokine and soluble nitrite measurements. Each supernatant was centrifuged at 10,000 x g for 10 minutes to remove cellular debris, aliquoted, and stored at -80^{o}C until use.

16. **Upregulation of dendritic cell activation markers:** BMDC's were stimulated and harvested as described above. The expression of major histocompatibility complex II (MHCII), CD80, and CD86 were determined by flow cytometry. Live cells were gated by forward and side scatter profiles. Dendritic cells were then identified using a phycoerythrin-labeled CD11c-specific antibody (BD Bioscience). MHC II expression was

assessed by staining with a fluorescein isothiocyanate conjugated monoclonal antibody (eBioscience). CD80 and CD86 were identified using specific antibodies conjugated to phycoerythrin-cyanin 5 (PE-Cy5). All samples were acquired on a BD FACscan and analyzed with Summit Software (v. 5.0, Dako).

17. **Phagocytosis and pinocytosis assays:** Stimulated and nonstimulated dendritic cells were harvested and seeded in a 96 well plate ($2.5x10^5$ cells/well). The cells were then pre-incubated at 37^oC or 4^oC for 30 minutes in RPMI + 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 20 μg/ml gentamicin sulfate, and 10 mM HEPES. Phagocytic activity was determined by measuring the uptake of pHrhodo-labeled *E. coli* (Invitrogen) particles at a concentration of 20 particles per cell. Cells pre-incubated at 4^oC and incubated with *E. coli* particles at 4^oC served as negative controls. The cells were allowed to phagocytose the labeled particles for 1 hour at either 37^oC or 4^oC, washed five times with 1X PBS + 5% FBS and fixed in cold 2% formaldehyde in 1X PBS. Fifteen thousand live-gated events were acquired on a BD FACScan and fluorescence was measured in the FL-2 channel. Endocytic activity was determined by incubating dendritic cells with 0.5 mg/ml of FITC-labeled bovine serum albumin (FITC-BSA) for 1 hour at either 4^oC or 37^oC. After washing and fixing in 2% formaldehyde in 1X PBS, 15,000 live-gated events were acquired on a BD FACScan and fluorescence was measured in the FL-1 channel.

18. **Dendritic cell viability assay:** The viability of BMDC's following *B. thuringiensis* exposure was determined by flow cytometry using 7-amino-actinomycin D (7-AAD, Sigma Aldrich). Cells were incubated with spores at spore:cell ratios of 10:1 and 20:1 for 0, 24, 48, and 72 hours. Cells incubated in media alone served as negative controls; while cells incubated with *E. coli* (strain 0157) LPS (1μg/ml) served as positive controls. All cells were stimulated and harvested as described above. Following harvest, the cells were resuspended in 1X PBS + 5% FBS containing 5 μg/ml 7-AAD and fluorescence was measured in the FL-3 channel on a BD-FACscan. To determine the percentage of viable cells, the percentage of 7-AAD positive cells was subtracted from 100%.

19. **Measurement of nitric oxide synthase activity:** Soluble nitrite levels in the supernatants of spore-stimulated BMDC cultures were measured according to previously published methods [131] as an indication of nitric oxide activity. Briefly, 50 µl of supernatant was mixed with 100 µl of Greiss reagent (2% (w/v) sulfanilamide in 10% (v/v) o-phosphoric acid and 0.2%, (w/v) N-(1-napthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in a 96-well plate and incubated for 15 min at room temperature. The absorbance at 570 nm was then measured using a Spectramax plus microplate reader running Softmax pro version 5.2 sofware (Molecular Devices Inc, Sunnyvale CA). The quantification of nitrite in each sample was determined by comparison to sodium nitrite standards (0-35 μM).

20. **Measurement of cytokine protein levels:** The concentration of the cytokines interleukin 1β (IL-1β), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 12 (IL-12), and interleukin 10 (IL-10) were determined using commercially available ELISA assays according to the manufacturer's protocols (eBioscience). Supernatants used in the TNFα assay were diluted 1:10, those used in the IL-6 assay were diluted 1:100 to provide readings within the linear range of the standard curve provided. Supernatants used in all other assays were not diluted.
21. **Conjugation of LcrV to NeutrAvidin protein:** Initially, sulfhydryl groups were added to LcrV by reacting 5 mg of the recombinant LcrV protein in 1X PBS (pH 7.4) with 2 mg of N-Succinimidyl-S-acetylthioacetate (SATA) dissolved in dimethylformamide (Pierce) for 30 minutes at room temperature. Sulfhydryl groups were activated by incubation with 5 mg hydroxylamine-HCl. Free SATA was removed by filtration centrifugation (Amico Ultra 3,000 MWCO, Millipore). NeutrAvidin protein was conjugated to 5 mg of SATA-modified LcrV by co-incubation with 5 mg of maleimide-activated NeutrAvidin protein (Pierce) for 12 hours in maleimide conjugation buffer (100 mM sodium phosphate, 5-10 mM EDTA, pH 7.6). Following conjugation, the buffer was exchanged to 1X PBS (pH 7.4) + 5% glycerol by filtration centrifugation (Amicon Ultra 3,000 MWCO, Millipore). The avidinated protein was aliquoted and stored at 4^{o}C until use.
22. **Western blotting of LcrV-NeutrAvidin conjugates:** Conjugation of purified, recombinant LcrV protein to NeutrAvidin was determined by

western blotting. LcrV and NeutrAvidin-conjugated LcrV proteins were boiled and resolved on nonreducing 10% Tris-Glycine SDS-PAGE gels, then transferred to a polyvinyl difluoride (PVDF) membranes (Millipore) overnight at 30 V in 25 mM Tris·HCl, 192 mM glycine, and 20% methanol. The presence of the avidin protein was detected with a biotin-conjugated horseradish peroxidase (biotin-HRP, Thermo-Fisher, 1:25,000). A mouse monoclonal antibody specific for LcrV (BA-5, 1:25,000, diluted in Starting Block TBS Blocking Buffer [Thermo-Fisher]) and an anti-mouse IgG-specific secondary antibody (Jackson Immunoresearch), 1:5,000, diluted in Starting Block TBS Blocking Buffer [Thermo-Fisher]) were used to identify the LcrV protein [132]. All blots were developed using an enhanced chemiluminiscence detection system according to the manufacturer's instructions (Thermo-Fisher).

23. **Determination of LcrV on the spore surface by flow cytometry:** $1x10^8$ UV-irradiated biotinylated *B. thuringiensis* endospores were incubated in the presence or absence of 2 μg NeutrAvidin-conjugated LcrV protein for 30 minutes at 4^oC. Endospores were washed three times with 3% BSA in 1X PBS, and then incubated with 5 μg of LcrV-specific mouse monoclonal antibody. The spores were thoroughly washed and incubated with FITC conjugated goat anti-mouse IgG (Southern Biotech). Background controls, including only the FITC-labeled secondary antibody were included in each experiment. Immediately prior to flow cytometry, spores were fixed with

4% formaldehyde in 1X PBS and fluorescence intensity in the FL-1 channel was measured on a BD FACscan.

24. **Immunization of mice with spore-displayed LcrV:** Eight to 10 week old female C57BL/6 mice were used in all immunization experiments. Mice were immunized either subcutaneously or intranasally with *B. thuringiensis* spores alone, 30 μg of NeutrAvidin-conjugated LcrV (NAV-LcrV) displayed on $5x10^8$ biotinylated *B. thuringiensis* endospores, or an equi-molar amount of recombinant, purified LcrV protein (11 μg). For subcutaneous immunizations, all vaccine preparations were formulated in 1X PBS (pH 7.4) and a 100 μl volume was injected subcutaneously between the shoulder blades using a 26 1/2 gauge needle fitted to a 1 ml syringe. All animals were boosted at 2 weeks post-primary immunization with the same dose and form of vaccine. Serum samples were obtained from peripheral blood collected at -1, 14, and 28 days post primary immunization. Serum samples were stored at -20°C until use. For intranasal immunizations, 5 μl of the vaccine preparation formulated in either 1X PBS (pH 7.4) or 1% Alhydrogel® were administered drop-wise into each nare of isofluorane-anesthetized mice (total of 10 μl per mouse per dose). All animals were boosted at 2 and 4 weeks post-primary immunization with the same dose and formulation of vaccine. Serum was obtained from peripheral blood collected at -1, 14, 28, and 42 days post primary immunization. Serum samples were stored at -20°C until use.

For *Yersinia pestis* survival challenge, all mice were rested for an additional 4 weeks following the last dose of vaccine prior to challenge.

25. **ELISA to determine LcrV-specific antibody titers:** Ninety-six well Microtest ELISA plates (BD Bioscience) were coated overnight at 4^{o}C with 10 ng of recombinant, purified LcrV protein diluted in carbonate buffer (pH 9.8). The unbound antigen was removed and the plates were blocked with 1X PBS + 3% BSA for 1 hour at room temperature, then washed three times with ELISA wash (1X PBS (pH 7.4) + 0.05% Tween 20). Plates were incubated for 1 hour with twofold serial dilutions of test sera diluted in 1X PBS + 3% BSA, washed three times and incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies to either mouse IgG, IgG1, or IgG2a (Southern Biotechnology) diluted 1/5,000, 1/2,500, and 1/2,500 in 1X PBS + 3% BSA, respectively. The plates were washed three times in ELISA wash and 100 μl of a solution containing 0.5 mg/ml o-phenylenediamine dihydrochloride (OPD, Thermo-Fisher), 52 mM citric acid, 989 mM dibasic sodium phosphate and 0.01% hydrogen peroxide was added to each well. The reaction was allowed to proceed for 60 minutes and then stopped by the addition of 100 μl of 1 N HCl to each well, after which the absorbance value at 492 nm was recorded. Each serum sample was assayed in duplicate and the endpoint titer was expressed as the reciprocal of the lowest dilution which yielded an OD_{492} value of three standard deviations above the mean optical density of control wells incubated in the presence of serum from nonimmune mice.

26. *In vitro* neutralization assays: Antibody blocking of the type three secretion system by the inhibition of caspase-3 activation was used to determine if serum samples from immunized mice were able to neutralize *Yersinia pestis in vitro*. RAW 264.7 macrophages (approximately 1 x 10^6 cells/well) were plated in a 12-well culture dish in Dulbecco's modified Eagle's medium (DMEM) + 5% FBS at a confluence of 80 to 90%. Overnight cultures of *Y. pestis* (strain KIM D27) were diluted in heart infusion broth so that the absorbance value at 600 nm (OD_{600}) was 0.05. Cultures were then incubated at 28°C for 2 hours, followed by 1 hour of incubation at 37°C. One ml of bacteria was centrifuged, washed with 1X PBS, and resuspended in DMEM plus 5% FBS. Serum samples (diluted 1:100) or an equal volume of PBS were preincubated with 50 µl bacteria in DMEM plus 5% FBS at 37°C in a total volume of 100 µl. The preincubated cultures were then added to macrophages (at a multiplicity of infection of 10:1), and the plate was spun at 450 rpm (40 x g) for 5 min. Infected cells were allowed to incubate at 37°C for 3.5 hours. Cells were scraped off the plate and washed with 1X PBS. Cells were then lysed by freezing and thawing (-80^{o}C, room temperature). Activated caspase-3 was detected using the EnzChek caspase-3 assay according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The *in vitro* neutralization assays were kindly carried out by Nicholas Eisele, Department of Veterinary Pathobiology University of Missouri.

27. *Yersinia pestis* infection and survival studies: Groups of 10 C57BL/6 mice were immunized intranasally as described above. Ten weeks post primary immunization; mice were challenged with *Yersinia pestis* strain Colorado 92 (CO92). Bacterial cultures were plated on heart infusion agar (HIA) supplemented with 0.005% Congo Red and 0.2% galactose to verify the presence of the pigmentation locus. Pigmented, isolated colonies were then inoculated into heart infusion broth (HIB) supplemented with 2.5 mM $CaCl_2$ and grown for 18 to 24 hours at 37°C, followed by dilution to the desired dose in sterile 1X PBS pH 7.2. Groups of mice were challenged with either 9,000 or 74,000 colony forming units (CFU) in 0.02 ml, which correspond to 30 or 250 50% lethal doses (LD_{50}), respectively. All animals were lightly anesthetized by isoflurane inhalation prior to intranasal infection with *Y. pestis* CO92. Animals were observed for recovery from anesthesia and returned to housing. All animals were monitored daily for weight loss and twice daily for survival. The challenge assays were kindly carried out by Nicholas Eisele and Dr. Hanni Lee Lewis, Department of Veterinary Pathobiology University of Missouri.

Chapter III: The Development of the Spore-Displayed Antigen System

A. Rationale

Endospores from members of the genus Bacillus exhibit many of the attributes of an ideal vaccine. They are inexpensive to produce, as well as highly thermostable. Killed spores from two members of the genus, *B. subtilis* and *B. anthracis*, have been previously shown to efficiently prime both systemic and mucosal immune responses [62, 133-135]. A formulation of viable spores from a capsule-negative strain of *B. anthracis* is currently used as a veterinary vaccine against anthrax [136].

In 2001, studies by Isticato et al. demonstrated that genetically-modified *B. subtilis* spores could be engineered to display fragment C of the tetanus toxin on their surface by fusing the gene encoding tetanus toxin with the gene encoding CotB, a spore coat protein [104]. Additional studies using the same vector-antigen system and an oral-parenteral prime boost approach have since demonstrated that orally administered recombinant spores can elicit systemic IgG responses as well as mucosal IgA's specific for the recombinant antigen. Oral vaccination with recombinant spores can also protect against lethal tetanus intoxication in a mouse model [62]. Using a similar approach, a bivalent vaccine consisting of spores expressing the fragment C of tetanus toxin and the β subunit of *E. coli* heat labile toxin has also been shown to be immunogenic in mice after oral administration of live, recombinant *B. subtilis* spores [106]. More recently, *B. subtilis* spores expressing the *Clostridium perfringens* alpha toxoid have been shown to elicit neutralizing antibodies

which protect against alpha toxin intoxication [110]. Although promising results have been obtained with live recombinant spore vaccines, to date this approach has only been reported with a limited number of antigens. This may be due to difficulties in expressing non-bacterial proteins in Bacillus or the low level of recombinant fusion proteins present on the spore surface.

The experiments reported herein describe a vaccination approach which takes advantage of the immunopotentiating properties of spores by directly coupling antigenic proteins of interest to the outer surface of inactivated bacterial endospores. This method has been designed to be a broadly applicable approach to immune intervention. Unlike the aforementioned recombinant spore-based vaccines, this system does not involve the genetic manipulation of the bacteria. Instead, the spores function as both biodegradable microparticles on which to display antigens to the immune system, and as potent adjuvants to enhance immune responses directed against the surface bound antigen. Foreign antigens are linked to the spore surface by the high-affinity interaction between biotin and streptavidin. Using biotinylated spores and avidinated antigens, spores stably displaying single or multiple biologically active antigens have been successfully produced. Furthermore, these experiments document that systemic delivery of a monovalent vaccine composed of biotinylated spores displaying avidinated β-galactosidase can prime high-titer anti-β galactosidase serum antibody responses in mice.

B. Results

1. Killed *Bacillus thuringiensis* endospores elicit systemic IgG responses after subcutaneous administration

Previous reports have suggested that live endospores of both *B. subtilis* and *B. anthracis* are highly immunogenic [62, 133-135, 137]. To test the feasibility of using spores from the related *B. thuringiensis* as a microparticle carrier for heterologous antigens, the ability of killed *B. thuringiensis* spores to prime spore-specific IgG responses was examined. To do this, groups of C57BL/6 mice were immunized subcutaneously with $1x10^9$ killed *B. thuringiensis* endospores (n=4) or sham immunized with saline (n=3). Each animal was then boosted two weeks later using the same dose and route. Two and four weeks after the initial dose of spores, spore-specific serum IgG titers were measured by ELISA. As shown in Figure 2A, subcutaneous vaccination with killed spores led to the production of high titer, spore-specific IgG responses in sera of all spore-immunized mice. As expected, no responses were detected in the control group of animals immunized with saline. Spore-specific IgG titers detected at week two ranged from 5,000 to 10,000 (geometric mean titer of 7,071). Antibody titers increased substantially two weeks after the second dose of spores (Student's t test, p= 0.0012), and ranged from 1,600,000 to 3,200,000 (geometric mean titer of 1,902,731). An analysis of spore-specific IgG1 and IgG2a antibodies primed by killed spores is

shown in Figure 2B. These data revealed an almost equal distribution of IgG1 and IgG2a after a single dose of spores. Although both the IgG1 and IgG2a titers increased significantly after the boost, the responses at four weeks post primary vaccination were dominated by antibodies of the IgG1 isotype.

It should be noted that intact and inactivated spores were used as antigen in the ELISAs thus, serum antibodies were recognizing determinants present on the outer surface of the spores rather than internal proteins. This raised the possibility that if spores could be coated with a heterologous antigen, it may be possible to use the immunogenic properties of the killed spores to promote high-titer antibody responses to the surface displayed-antigen.

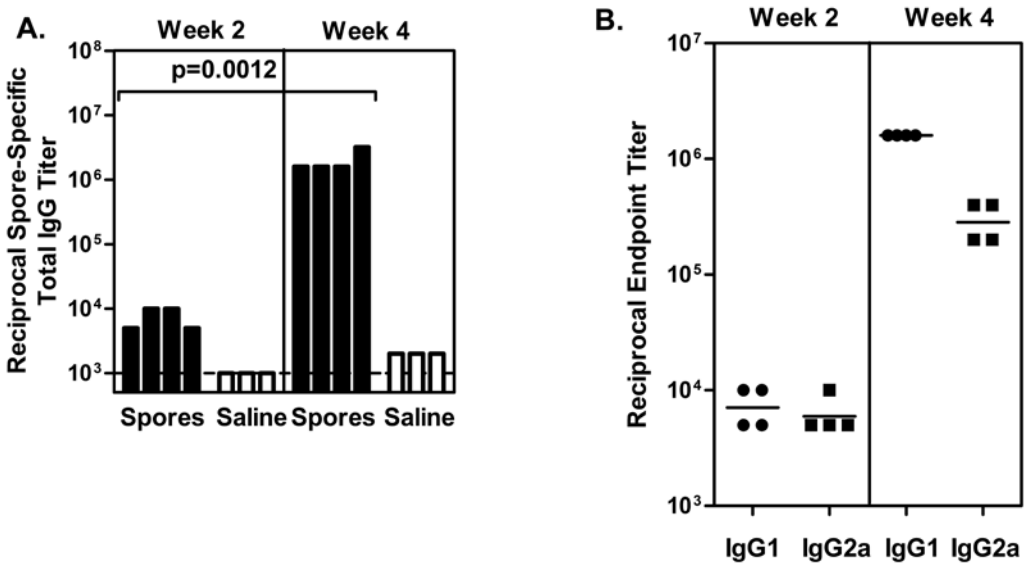

Figure 2. The induction of humoral responses following immunization with killed *B. thuringiensis* endospores.

(A) Groups of C57BL/6 mice were immunized subcutaneously with $1x10^9$ UV-irradiated *B. thuringiensis* endospores (solid bars, n=4) or saline (open bars, n=3) and boosted two weeks later. At two and four weeks post primary immunization, anti-spore IgG titers in serum were measured by ELISA. Each bar represents the results of a single mouse, and the limit of detection in the ELISA (1:1000) is represented by the dashed horizontal line. (B) Spore-specific IgG1 (closed circles) and IgG2a (closed squares) titers in sera from the same spore-immunized mice shown in panel A. The solid horizontal line in each plot represents the geometric mean isotype titer at each time point. This experiment was repeated twice and representative data from a single experiment are shown.

2. *In vitro* biotinylation of *B. thuringiensis* endospores

To efficiently display heterologous antigens on the outer surface of spores, the strong and specific interaction between streptavidin and biotin ($K_d = 10^{-15}$ M) was used. Killed spores were labeled *in vitro* with an N-hydroxysulfosuccinimide ester of biotin (NHS-biotin). This reagent reacts with surface-exposed primary amine groups to form stable amide bonds between biotin and the target protein [138]. To confirm that spores can be efficiently biotinylated, and to identify the molecular weights of spore proteins that are accessible to NHS-biotin, biotinylated and non-biotinylated spores were subjected to SDS-PAGE under reducing and denaturing conditions, blotted onto PVDF membranes, and probed with a streptavidin-horseradish peroxidase conjugate (Figure 3). Non-biotinylated spores failed to react with the streptavidin conjugate, whereas two dominant bands of molecular weights of approximately 50 kDa and 150 kDa were detectable in the biotinylated spore sample. To establish that the exosporium was the target of biotinylation, the same two spore preparations were resolved on the same gel, and western blotted using polyclonal antibodies specific for the Bacillus collagen like protein A (BclA). The BclA protein is a dominant antigen of the exosporium layer. As shown in Figure 3, two bands of similar molecular weights (50 and 150 kDa) were detected. This suggests that the biotinylation reaction is selectively targeting the exosporium layer.

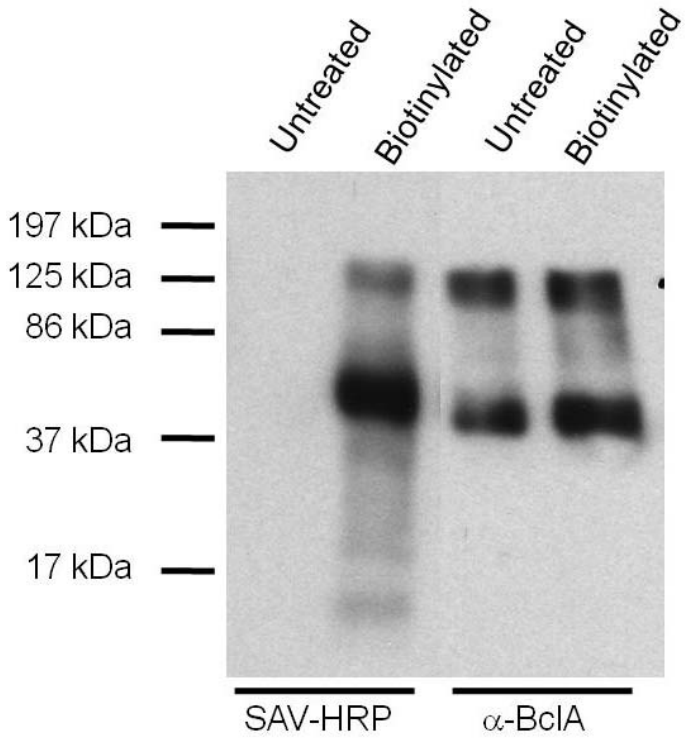

Figure 3. Biotinylation of *Bacillus thuringiensis* endospores.

Non-biotinylated (Untreated, lanes 1 and 3) and biotinylated (lanes 2 and 4) *Bacillus thuringiensis* endospore were subjected to Western blotting using a streptavidin horseradish peroxidase conjugate (lanes 1 and 2), or a rabbit polyclonal antibody against BclA (lanes 3 and 4).

3. Multivalent spore preparations can be synthesized by mixing spores conjugated to individual antigens, or by linking multiple antigens to a single spore

Using flow cytometry, it was next determined if biotinylation altered the structure of the spore and more importantly, if biotinylated spores could successfully display an avidinated antigen. There was no detectable difference in the forward and side scatter characteristics between nonbiotinylated and biotinylated spores (Figure 4A), demonstrating that the *in vitro* biotinylation reaction did not grossly alter the size or structure of the spores.

Fluorescently labeled streptavidin conjugates were used to determine if biotinylated spores could successfully capture avidinated antigens in solution and stably display them on the spore surface. Biotinylated spores were incubated separately with streptavidin-fluorescein (SAV-FITC) or streptavidin-phycoerythrin-Cy5 (SAV-PECy5), washed extensively, mixed, and analyzed by flow cytometry (Figure 4B upper right panel). Alternatively, biotinylated spores were incubated with both conjugates simultaneously (Figure 4B lower right panel). To demonstrate the specificity of the interaction between the streptavidin conjugates and biotinylated spores, non-biotinylated spores were subjected to the same binding reactions and washing conditions (Figure 4B upper and lower left panels). Non-biotinylated spores exhibited no detectable fluorescence,

demonstrating that stable association of an avidinated antigen with the spore surface was via it's interaction with biotin.

Two populations of mono-fluorescent spores were readily detectable when biotinylated spores were labeled with single conjugates and then mixed. Thus, once the avidinated antigen is associated with biotin on the spore surface it is highly stable. A minor population (<5%) of dual fluorescent spores was detectable in this experiment. Because the mean fluorescence intensity (MFI) of these spores is equivalent to the MFI of each of the monovalent populations, it is possible that these spores represent doublets, i.e. two spores which are stuck together. When incubated simultaneously with both conjugates, 99% of the biotinylated spores exhibited a dual fluorescent pattern. Thus, polyvalent vaccines can be easily created by either mixing separate preparations of monovalent spores or by incubating spores simultaneously with multiple antigens.

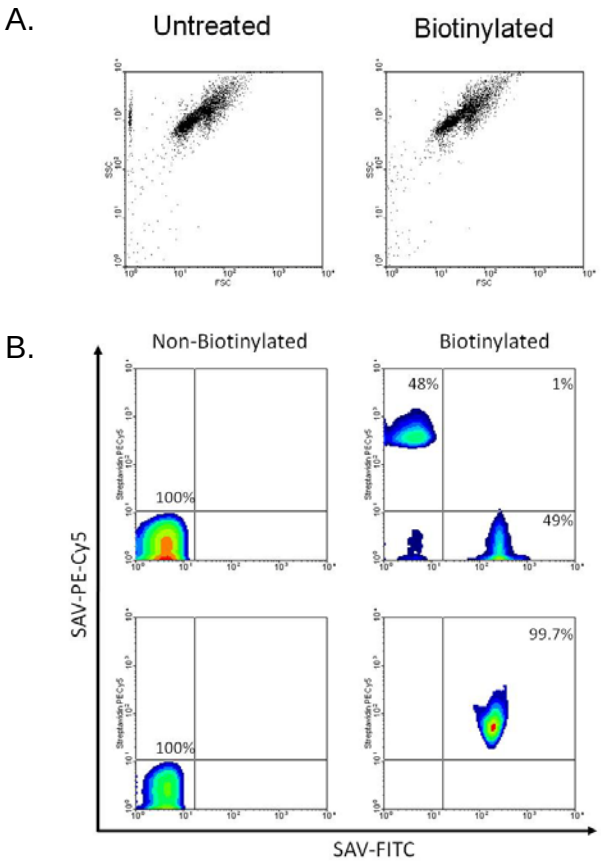

Figure 4. Surface biotinylation results in spores which efficiently capture avidinated antigens.

(A) The forward and side scatter patterns of untreated (left panel) and biotinylated (right panel) spores were assessed by flow cytometry. (B) Nonbiotinylated (left panels) or biotinylated (right panels) endospores were incubated with streptavidin-linked FITC and streptavidin linked PE-Cy5 individually, then mixed in equivalent numbers (top panels), or incubated with both streptavidin-linked FITC and PE-Cy5 simultaneously (bottom panels). The fluorescence of PE-Cy5 (x axis) and FITC (y axis) bound to the spores was then measured by flow cytometry. The representative data shown were repeated three times with similar results

4. The display of heterologous molecules requires both biotin and streptavidin

To determine if the display of avidin-linked heterologous molecules on the surface of spores was dependent on the presence of biotin, biotinylated and nonbiotinylated *Bacillus thuringiensis* endospores were incubated with ovalbumin that was labeled with FITC, then conjugated to NeutrAvidin (NAV-OVA-FITC). The presence of FITC on the spore surface was then determined by flow cytometry. As shown in Figure 5, when biotinylated endospores were incubated with NAV-OVA-FITC, an increase in the fluorescence of the spores was observed (purple histogram). Conversely, when nonbiotinylated spores were incubated with NAV-OVA-FITC, no fluorescence was detected (open histogram). Next, to determine if streptavidin was needed to link antigens to the surface of biotinylated spores, FITC-labeled ovalbumin (OVA-FITC) was incubated with biotinylated and nonbiotinylated spores (Figure 5, bottom panel) and the fluorescence of the spores was again determined by flow cytometry. The data in Figure 5 demonstrate that regardless of the biotinylation status of the spores, incubation with a nonavidinated antigen did not result in detectable fluorescence. Thus, the display of antigens on the surface the spores requires the presence of biotin on the spore surface and avidination of the target antigen.

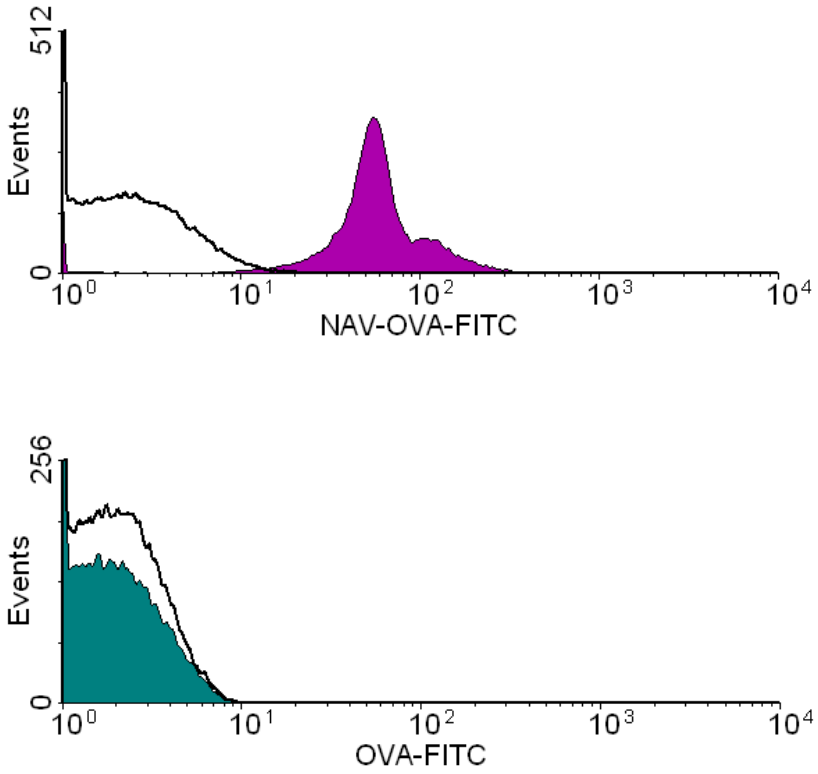

Figure 5. Both biotin and a streptavidin are required for the display of molecules on the surface of spores.

(Top panel) Biotinylated (filled histogram) or non-biotinylated (open histogram) spores were incubated with fluorescein isothiocyanate (FITC)-labeled ovalbumin that was conjugated to NeutrAvidin. (Bottom panel) Biotinylated (filled histogram) or non-biotinylated (open histogram) spores were incubated with FITC-labeled ovalbumin. Fluorescence in the FL-1 channel was determined by flow cytometry on a BD FACScan.

5. Creation of multivalent spores stably expressing heterologous antigens in their native conformation

In many instances, successful vaccination is dependent upon the development of neutralizing antibodies which recognize three dimensional structural determinants on the surface of the target antigen. For these cases, the target antigen needs to be presented to the immune system in its native conformation. Two recombinant antigens, *E. coli* β-galactosidase and horseradish peroxidase, were used to determine if biotinylated spores could be used to display heterologous antigens in their native context. To create monovalent spores, biotinylated spores were incubated separately with the single antigens, streptavidin-β-galactosidase (B spores+BGAL) or streptavidin-horseradish peroxidase (B spores+HRP). To create divalent spores, biotinylated spores were incubated with both antigens simultaneously (B spores+BGAL+HRP). Biotinylated spores incubated in the absence of either antigen (B spores) were used as negative controls. Non-biotinylated spores incubated concurrently with both enzymes (NB spores+BGAL+HRP) were used to assess nonspecific interactions between the avidinated enzymes and the spore surface. After a 30 minute binding reaction, all spores were washed extensively and aliquots of each were assayed kinetically for horseradish peroxidase (Figure 6 upper panel) or β-galactosidase (Figure 6 lower panel) enzyme activity.

Biotinylated spores exhibited no endogenous β-galactosidase or peroxidase activity. Non-biotinylated spores incubated with both enzymes displayed barely detectable peroxidase activity and no discernible β-galactosidase activity. When biotinylated spores were incubated with both antigens simultaneously, the spores exhibited high levels of both peroxidase and β-galactosidase activities. Similarly, spores incubated with individual enzymes displayed high levels of the appropriate enzymatic activity. These results confirm the flow cytometry results presented in Figure 4B in that biotinylated spores can capture and display multiple antigens on their surface, and extend them to include the display of enzymes in their native conformation.

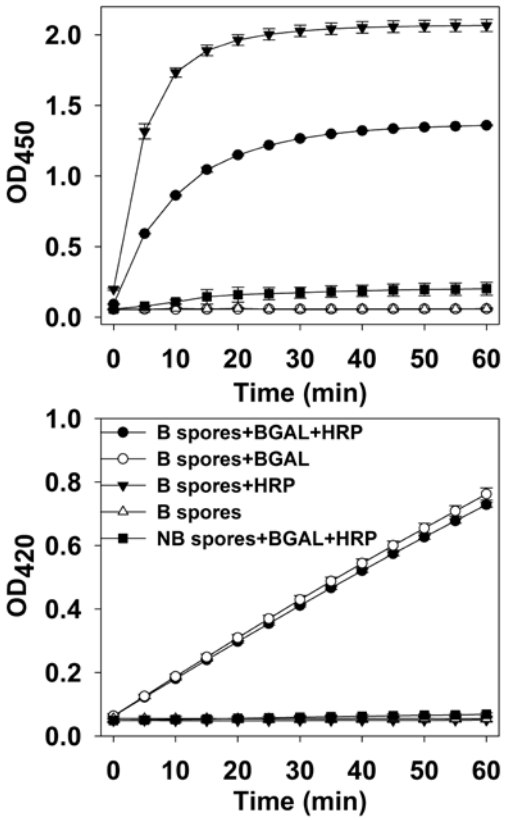

Figure 6. Spore-displayed antigens retain their enzymatic activities.

Biotinylated spores were incubated concurrently with streptavidin-linked β-galactosidase and streptavidin-linked horseradish peroxidase (closed circles). Biotinylated spores were also incubated individually with either streptavidin-linked β-galactosidase (open circles), streptavidin-linked horseradish peroxidase (inverted triangles), or in binding buffer alone (open triangles). Nonbiotinylated control spores were incubated simultaneously with both enzymes (closed squares). Spores were then assayed kinetically for horseradish peroxidase (upper panel) or β-galactosidase (lower panel) activity as described in the *Materials and Methods*. Representative data from three independent experiments are shown. The data used for this figure were generated by Matthew Kaiser and Dr. Daniel Hassett.

6. Quantification of the binding capacity of biotinylated spores

In order to determine how much antigen could be physically associated with each spore, dose titration experiments were performed using SAV-FITC. A fixed amount of killed, biotinylated spores ($1x10^8$) were incubated with increasing concentrations of SAV-FITC, washed extensively, and the MFI of SAV-FITC bound to the spores was determined by flow cytometry. The average MFI value among triplicate samples incubated with 180 picomoles of SAV-FITC (10 μg) was set at 100%, and the results obtained at lower concentrations of SAV-FITC were all expressed as a percentage of this value (Figure 7). Because the conjugate was equally distributed among all the spores at low concentrations of SAV-FITC, only background fluorescence could be detected at concentrations of less than 10 picomoles per reaction. However, a linear increase in the MFI was observed as the concentration of SAV-FITC in the binding reaction was increased from 20 to 110 picomoles. Maximal fluorescence values were observed at concentrations at or above 110 picomoles (6 μg) of SAV-FITC per $1x10^8$ spores. Taking into account the molecular weight of SAV-FITC, and the number of spores in the binding reaction, 110 picomoles of SAV-FITC bound to $1x10^8$ spores translates into 1.1 attomoles of SAV-FITC per spore, or approximately 686,000 molecules of SAV-FITC displayed on the surface of each spore.

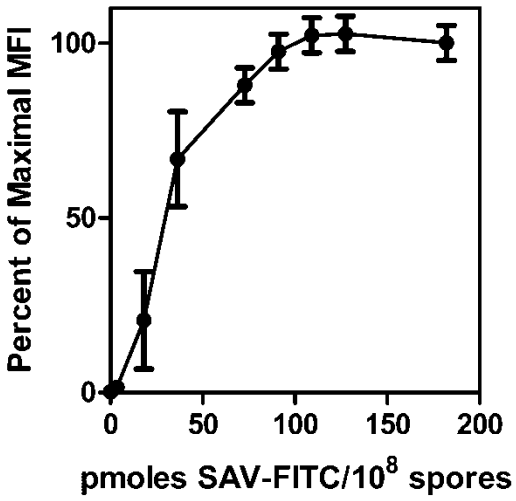

Figure 7. Estimation of antigen density on the surface of biotinylated spores.

UV inactivated biotinylated spores were incubated with increasing concentrations of streptavidin FITC (SAV-FITC). Unbound SAV-FITC was removed by thorough washing and the spores were fixed. Fluorescence intensities were measured by flow cytometry on a BD FACScan.

7. Heterologous antigens displayed on the spore surface elicit high titer antibody responses following systemic administration

The ability of monovalent spores to elicit humoral immune responses specific for the spore-displayed antigen was determined using the model antigen β-galactosidase. Groups of C57BL/6 mice were immunized subcutaneously with streptavidinated β-galactosidase linked to the surface of biotinylated spores (Biotinylated spores+SAV-β-Gal, n=8). Control mice were immunized either with streptavidinated β-galactosidase alone (SAV-β-GAL, n=8), or streptavidinated β-galactosidase mixed with non-biotinylated spores (Spores+SAV-β-GAL, n=7). Two weeks after the initial priming dose, all animals were boosted with the same dose and form of antigen. Serum IgG titers were measured by ELISA on serum collected at two (Figure 8A) and four (Figure 8B) weeks after the primary immunization. At two weeks post immunization, seven out of eight mice immunized either with biotinylated spores displaying SAV-β-galactosidase, or with SAV-β-galactosidase protein alone produced detectable serum IgG antibodies directed against β-galactosidase. However, after a single administration of antigen there was no statistically significant difference in IgG titers among animals which received SAV-β-galactosidase coupled to spores compared to those which received SAV-β-galactosidase alone (Mann Whitney, p= 0.0998). At this time, only one mouse immunized with SAV-β-galactosidase mixed with nonbiotinylated spores had responded to the immunogen.

At four weeks post immunization, two weeks after the boost, all mice immunized with biotinylated spores displaying SAV-β-galactosidase responded, and the endpoint IgG titers ranged from 4,000 to 64,000 with a geometric mean titer of 27,000. At this same time point, antibody responses were also detected in all mice immunized with SAV-β-GAL alone. The IgG titers in this group ranged from 4,000-16,000 (geometric mean = 8,700). The data at week four revealed that coupling the antigen to the spore led to a statistically significant increase in IgG titers compared to immunization with protein alone (Mann Whitney, p= 0.015). Although only one mouse immunized with a mixture of nonbiotinylated spores and SAV-β-galactosidase seroconverted at week two, all mice in this group developed detectable IgG responses after the boost with titers ranging from 2,000 to 8,000. The differences among groups receiving biotinylated and nonbiotinylated spores +SAV-β-GAL were highly significant (Mann Whitney, p=0.004). Comparing the reciprocal endpoint titers at week four among all mice that responded to the vaccines revealed that coupling the antigen directly to the spore surface via biotin and streptavidin resulted in a seven fold increase in the geometric mean serum antibody titer compared to just mixing the soluble, avidinated antigen with killed *B. thuringiensis* spores and a fourfold increase in the geometric mean titer compared to protein alone. These results indicate that directly displaying the antigen on the spore surface results in more efficient

recognition of the antigen by the immune system, and a more robust antibody response after systemic immunization.

To further characterize the immune response to antigens linked to the spore surface, the serum IgG1 and IgG2a titers to β-galactosidase in all immunized mice at weeks two (Figure 8C) and four (Figure 8D) were also measured. In all cases, the β-galactosidase specific responses were dominated by IgG1 antibodies. No IgG2a was detectable at two weeks post immunization in any of the responding mice. However, anti-β-galactosidase IgG2a antibodies were evident two weeks after boosting. This is consistent with previously published reports with *B. subtilis* and *B. anthracis* that have documented a mixed T helper type 2 dominated response following vaccination with recombinant spores [106, 134].

It is important to note that robust antibody responses to the spore displayed antigen did not require the addition of any adjuvant. Thus, the spores are acting not only as an efficient means of delivering antigen to the immune system, but they also function as a potent adjuvant to enhance humoral responses against a heterologous protein present on the spore surface.

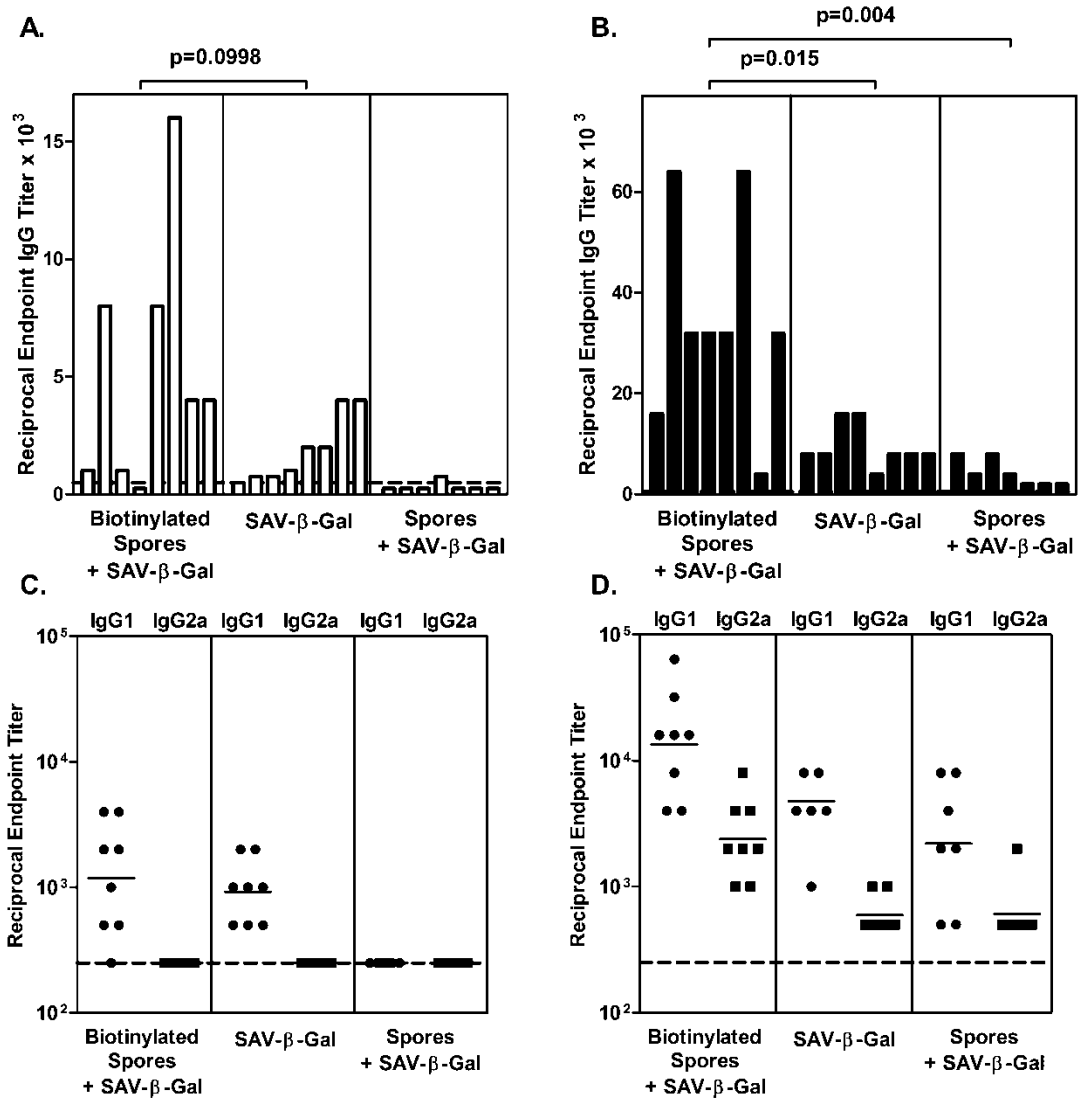
A.
p=0.0998
Reciprocal Endpoint IgG Titer x 10^3
15
10
5
0
Biotinylated Spores + SAV-β-Gal
SAV-β-Gal
Spores + SAV-β-Gal
B.
p=0.004
p=0.015
Reciprocal Endpoint IgG Titer x 10^3
60
40
20
0
Biotinylated Spores + SAV-β-Gal
SAV-β-Gal
Spores + SAV-β-Gal
C.
IgG1 IgG2a IgG1 IgG2a IgG1 IgG2a
Reciprocal Endpoint Titer
10^5
10^4
10^3
10^2
Biotinylated Spores + SAV-β-Gal
SAV-β-Gal
Spores + SAV-β-Gal
D.
IgG1 IgG2a IgG1 IgG2a IgG1 IgG2a
Reciprocal Endpoint Titer
10^5
10^4
10^3
10^2
Biotinylated Spores + SAV-β-Gal
SAV-β-Gal
Spores + SAV-β-Gal

Figure 8. Systemic administration of spores results in high titer antigen-specific serum antibody responses.

Individual C57BL/6 mice were immunized subcutaneously with either 20 μg of streptavidin-β-galactosidase bound to biotinylated spores (Biotinylated Spores+SAV-β-Gal, n=8), 20 μg of streptavidin β-galactosidase alone (SAV-β-Gal, n=8), or 20 μg of streptavidin-β-galactosidase mixed with nonbiotinylated spores (Spores + SAV-β-Gal, n=7). All mice were boosted two weeks later with the same dose and form of antigen. Anti-β-galactosidase serum IgG titers in individual mice were measured at two (A) and four (B) weeks post immunization. Anti-β-galactosidase serum IgG1 and IgG2a titers were measured by ELISA in the same seropositive mice shown in panels A and B at two (C) and four (D) weeks post immunization. The dotted lines in each panel reflect the limit of detection in the ELISAs which were 1:500 in panels A and B and 1:250 in panels C and D.

8. Antibody responses elicited against the spore and streptavidin molecules in addition to the displayed β-galactosidase

Immunizations with recombinant spore-based vaccines as well as other microparticle vaccines have demonstrated that antibodies are raised not only against the displayed antigen, but also the carrier particle. ELISA assays were used to determine if immunization with spore-displayed β-galactosidase also generated spore and streptavidin-specific antibodies. The β-galactosidase, streptavidin, and spore-specific IgG antibodies were measured in sera from mice immunized with two doses of streptavidin-linked β-galactosidase displayed on biotinylated spores (Figure 9). Sera from control mice immunized with streptavidin-linked β-galactosidase alone, biotinylated spores alone, or streptavidin-linked β-galactosidase mixed with non-biotinylated spores were also examined.

Sera from mice immunized with streptavidin-linked β-galactosidase displayed on the surface of biotinylated spores (Bspores + SAV-Bgal) contained IgG antibodies specific for not only β-galactosidase, but also against both streptavidin and spore determinants (Figure 9, black bars). Similarly, mice immunized with streptavidin-linked β-galactosidase mixed with non-biotinylated spores (NBspores + SAVBgal) also had detectable antibody titers specific for all three antigens (Figure 9, striped bars). As expected mice immunized with streptavidin-linked β-galactosidase alone (SAVBgal Alone) displayed IgG antibody titers specific for both β-galactosidase and streptavidin but not spores (Figure 9, gray bars). Mice

immunized with spores alone only had antibodies specific for the spores in the ELISA assay (Figure 9, white bars). As before, significant differences in the β-galactosidase-specific IgG titers were observed between antibody responses in mice immunized with β-galactosidase linked to the surface of spores and those immunized with streptavidin-linked β-galactosidase alone ($p \leq 0.05$, ANOVA). Similarly, significant differences were also observed in the β-galactosidase-specific titers between mice that received spore-displayed β-galactosidase and mice that antigen mixed with non-biotinylated spores ($p \leq 0.05$, ANOVA). However, regardless of the biotinylation state of the spores used for the immunization, or the presence of β-galactosidase antigen, no significant differences were observed among spore-specific serum IgG titers ($p > 0.05$ Bonferroni's Multiple Comparison Test). Similarly, no significant differences were observed in the streptavidin-specific serum IgG titers among mice that had received streptavidin-linked antigen, regardless of the spore co-administration or display ($p > 0.05$ Bonferroni's Multiple Comparison Test).

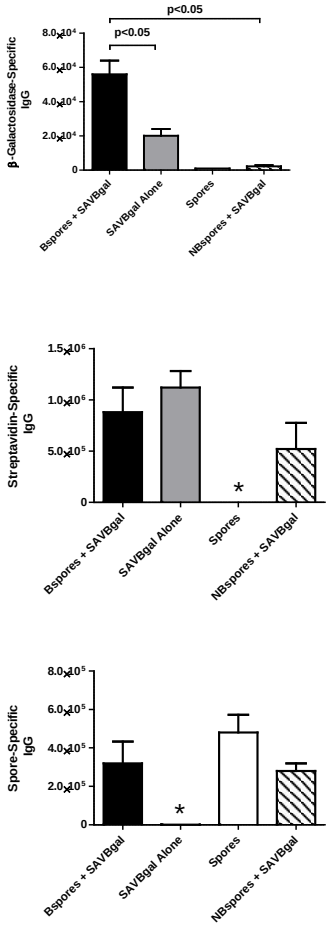

Figure 9 Antibodies are elicited against the spores and streptavidin molecules in addition to displayed β-galactosidase.

Groups of C57BL/6 mice (n=4) were immunized subcutaneously with two doses of either 20 μg of streptavidin-β-galactosidase bound to biotinylated spores (Biotinylated Spores+SAV-β-Gal), 20 μg of streptavidin β-galactosidase alone (SAV-β-Gal), or 20 μg of streptavidin-β-galactosidase mixed with nonbiotinylated spores (Spores + SAV-β-Gal). Anti-β-galactosidase, streptavidin, and spore serum IgG titers in individual mice were measured at four weeks post immunization.

C. Discussion

The noncovalent interaction between biotin and streptavidin has been the basis for many procedures that require the formation of specific linkages between biological macromolecules [139-141]. Due to the high affinity of this interaction, the binding of the vitamin biotin to avidin or streptavidin is essentially irreversible. This interaction was utilized to attempt to produce a highly flexible, biodegradable, microparticle-based vaccine platform using inactivated Bacillus endospores. This study is the first to use a streptavidin biotin linkage system to tether heterologous antigens to a bacterial spore for use as a vaccine. All Bacillus spores are composed of an inner core of nucleoprotein surrounded by a thick cortex and a spore coat. *B. anthracis*, *B. cereus*, *and B. thuringiensis* spores contain an additional outermost layer termed the exosporium. The external face of the exosporium consists of proteinaceous projections termed the “hair-like” nap [142]. Unlike members of the *Bacillus cereus* group, *B. subtilis* does not produce an exosporium [80, 143]. In developing an efficient method to physically link proteins of interest to the spore surface, the increased surface area offered by the “hair-like” nap might permit a higher density of antigen-display than would be possible on the spore coat alone.

A toxin-negative strain of *B. thuringiensis,* an insect pathogen that is widely used as a safe alternative to chemical insecticides [144], was chosen for this vaccine platform. Both *B. anthracis* and *B. cereus* were

not considered for use because both bacterial strains can cause disease in humans and animals, Toxin-producing *B. thuringiensis* spores have been used as bioinsecticides for over ninety years, and are generally considered to have little to no detrimental impact on human or animal health [145]. Despite their perceived safety, an added precaution was taken by developing the vaccine platform using a toxin-negative bacterial strain. As a further safety measure, the spores were inactivated prior to biotinylation using ultraviolet radiation. Even though the spores were unable to successfully replicate, the data in Figure 2 demonstrate that they are still able to elicit potent humoral immune responses following systemic administration. The induction of high levels of IgG1 and IgG2a also indicated that killed spores are able to activate cell-mediated immune responses as well.

Using a water soluble ester of biotin (NHS-biotin), *B. thuringiensis* spores were successfully biotinylated. Although over 10 proteins have been identified in the exosporium layer of *Bacillus* species, western blotting results suggest that the primary target of NHS-biotin is most likely a single protein of 50 kDa that forms a stable trimer on the spore surface, or two separate proteins of 50 and 150 kDa. A possible candidate antigen is the Bacillus collagen-like protein A. This heavily glycosylated protein is a major component of the exosporium and is present on the spore surface as a trimer [146].

As hypothesized, biotinylated spores acted as highly efficient microparticle platforms on which to stably display avidinated antigens. Flow cytometric analyses revealed that biotinylated spores can successfully display single or multiple antigens on the spore surface, simply by controlling which avidinated antigens are included in the binding reaction. The ease with which streptavidinated antigens can be produced and then coupled to the spore surface provides a potential strategy for rapidly responding to highly mutable infectious agents, such as influenza or human immunodeficiency virus. As new antigenic variants arise in the population, protective proteins containing the corresponding mutations can be produced *in vitro* and coupled to the spore surface. In addition, data demonstrating the stable coupling of biologically active enzymes to spores demonstrates that the same linkage chemistry can be used to create spores with novel biological properties. This could potentially be exploited in a vaccine platform by linking additional immune response modifiers such as cytokines, chemokines, or cell-targeting antibodies to the spore surface in addition to the immunogen.

A number of published reports have described genetic methods for producing recombinant spores displaying heterologous proteins on the spore surface [104, 106, 115, 135]. Isticato *et al.* have estimated that approximately 1,500 molecules of recombinant CotB-tetanus fusion proteins are present on the spore coat of recombinant *B. subtilis* spores expressing the tetanus antigen [104]. Using biotinylated *B. thuringiensis*

spores, approximately 600,000 molecules of an avidinated antigen can be accommodated on the spore surface. This represents a 400-fold increase in the number of antigen molecules present on the exosporium surface using the streptavidin biotin linkage method compared to fusion constructs utilizing a spore coat targeting vector. Thus, the method described in this report is expected to be able to achieve a much higher antigen density than the currently described recombinant spore-vaccine systems. The increased concentration of antigen on the surface of spores would lead to more efficient cross-linking of B cell receptors, and an increased availability of antigen for MHC I and II presentation. However, differences in the bacterial species and antigens used, as well as the dose of spores and route of administration in this study and in other reports with recombinant *B. subtilis* spores prohibit a direct comparison of the available immunological data at this time.

As “proof of concept” the systemic administration of killed avidinated spores displaying the model antigen β-galactosidase revealed that spores displaying a single heterologous antigen can safely and reproducibly elicit robust humoral responses *in vivo*. After a single boost, antibody titers to spore displayed β-galactosidase were substantially higher than when the same protein was mixed with non-biotinylated spores or when the native antigen was administered alone. The poor responses observed among mice immunized with streptavidin-β-galactosidase and non-biotinylated spores were somewhat surprising as previous work by the Klavinskis

laboratory has shown that when mixed with soluble proteins, *B. subtilis* spores can act as potent immune-stimulating adjuvants *in vivo* [116]. One possibility that deserves further study is that there may be differences in the host response to spores from different *Bacillus* species. In addition, the infectious status of the spores may also exert quantitative and qualitative effects on the immune response to heterologous antigens. The irradiated spores used in this study are incapable of replicating. Therefore, the killed spores may stimulate less vigorous responses from the innate immune system than replication competent spores. Although killed spores will undoubtedly be safer for use in immunocompromised individuals such as the elderly and the very young, further studies are necessary to determine if viable spores are more potent vaccine vehicles than the killed spores used here.

Another important finding in this study was that strong immune responses were raised against the spores and the streptavidin linker molecules in addition to β-galactosidase. This phenomenon was expected due to the strong immunogenicity of both the spores and streptavidin. Pre-existing immunity to carriers has been shown to have a deleterious effect on multiple immunizations utilizing the same platform [147-148]. Although a second dose did lead to enhanced antigen-specific antibody production, the presence of carrier-specific immunity may have implications on subsequent immunizations with other spore-displayed

antigens. Further experiments should be carried out to further investigate these findings.

In conclusion, these data demonstrate that the interaction of streptavidin with biotin can be used to efficiently display large quantities of foreign antigens on the surface of killed *B. thuringiensis* spores. These modified spores function as efficient vehicles for the systemic delivery of antigens, and result in the priming of high-titer antigen-specific B cell responses. Due to the flexibility and ease with which new vaccines can be formulated, chemically modified spores displaying heterologous antigens may prove to be a useful addition to our vaccine armamentarium.

Chapter IV: Immunostimulatory Effects of UV-Inactivated *Bacillus thuringiensis* Endospores on Murine Bone Marrow-Derived Dendritic Cells

A. Rationale

Most of the data on the effects of *Bacillus* spores on the innate immune system has resulted from studies with *Bacillus anthracis*, the causative agent of anthrax. Although primarily a pathogen of ruminants, anthrax spores are infectious to humans. When spores of *Bacillus anthracis* gain access to the body they rapidly germinate into encapsulated vegetative cells which produce two dipartite exotoxins that are responsible for the high morbidity and mortality associated with anthrax disease [149-151]. Previous immunological studies with anthrax spores have focused primarily on either the effects of the two dipartite bacterial exotoxins, the bacterial capsule, or outgrowth-competent forms of *Bacillus anthracis* spores. Thus, there is very little information available on how the host's immune system responds to the spores alone [152-159].

Previous work has shown that recombinant spores from apathogenic strains of *Bacillus anthracis* and *Bacillus subtilis* can prime mucosal and systemic immune responses in small animal models and provide protection against certain toxins and pathogens [105, 109-111, 113, 133-135, 160-164]. Inactivated spores from *Bacillus subtilis* have also been shown to function as efficient adjuvants *in vivo* by potentiating antibody and CD4+ T cell responses to a co-delivered soluble antigen [116]. In addition, the data described in Chapter 3 indicate that *Bacillus thuringiensis* spores also work as efficient vaccine delivery vehicles.

However, the nature of the adjuvant effect induced by Bacillus spores is currently unknown.

Most adjuvants are thought to work by stimulating cells of the innate immune system [165-168]. Dendritic cells are particularly important in this regard as they have the capacity to regulate the development of adaptive B and T cell responses by stimulating the activation and differentiation of CD4+ and CD8+ T cells into antigen experienced effector cells [169-174]. In the absence of overt inflammation, myeloid dendritic cells exist in tissues in an immature, quiescent state where they participate in suppressing responses to self antigens [172]. Upon encountering products of microbial metabolism or other “danger” signals immature dendritic cells undergo a series of maturational steps in which they upregulate major histocompatability (MHC) and costimulatory molecules, begin secreting immunostimulatory cytokines and chemokines, and migrate to secondary lymphoid organs where they present antigen to naïve T cells. The physiochemical nature of the maturational trigger can have a profound effect on the type of acquired immunity which results in response to infection or vaccination. A clearer understanding of the biological consequences of the interactions between immature dendritic cells and complex microbial structures like *Bacillus* endospores is important not only for the development of more effective spore-based vaccines but also for understanding the initial host response to mammalian pathogens of the genra such as *Bacillus anthracis* and *Bacillus cereus*.

To understand how dendritic cells respond to spores, these experiments focused on spores from a toxin-negative strain of the dipteran pathogen *Bacillus thuringiensis*. In an effort to clearly discriminate between responses to the spores versus responses to germinating spores or vegetative cells all spore preparations were inactivated by exposure to high dose ultraviolet radiation. Inactivated spores were readily phagocytosed by immature bone marrow derived dendritic cells and this led to cellular maturation and the production of a complex array of inflammatory mediators including TNF-α, IL-6, IL-12, IL-10 and inducible nitric oxide synthase. These data demonstrate that killed *Bacillus* endospores can stimulate the functional maturation of myeloid dendritic cells into mature, cytokine secreting, antigen presenting cells.

B. Results

1. Bone marrow-derived dendritic cells efficiently phagocytize *Bacillus thuringiensis* endospores

Previous data suggest that live spores of *B. anthracis* and *B. cereus* are rapidly engulfed by both macrophages and dendritic cells [94, 137, 175-179]. To assess the ability of immature dendritic cells to phagocytose inactivated spores of the related *B. thuringiensis*, cells were exposed to biotinylated spores which had been surface labeled with streptavidin-phycoerythrin (SAV-PE). Dendritic cells were exposed to 20 spores per cell for 2 hours at 37^{o}C, fixed, permeabilized, and incubated with an antibody directed against lysosomal associated membrane protein type 1 (LAMP-1) and the nuclear dye DAPI. The cells were then analyzed by fluorescence microscopy (Figure 10A). PE-labeled spores were clearly evident within bone marrow-derived dendritic cells (BMDCs) by 2 hours post exposure and some spores co-localized with LAMP-1. To confirm that dendritic cells were phagocytosing the spores, the uptake of FITC labeled spores at 4^{o}C or in the presence of cytochalasin D, both of which inhibit phagocytosis, was assessed by flow cytometry [180-182]. As expected, incubation at 4^{o}C (Figure 10B) or inclusion of cytochalasin D (Figure 10C) significantly reduced the uptake of killed spores. Collectively, these results are consistent with the hypothesis that immature dendritic cells actively phagocytose killed *B. thuringiensis* spores into phagosomal vesicles which then mature into late endosomes and lysosomes.

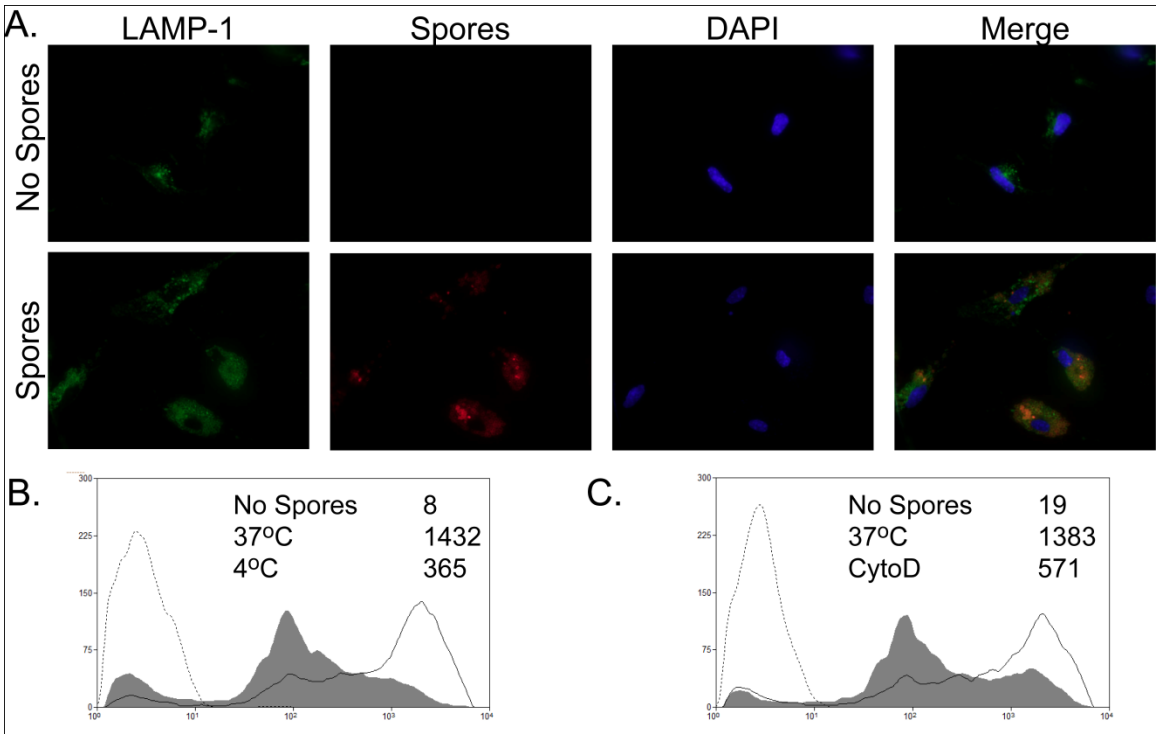

Figure 10. Uptake of *Bacillus thuringiensis* endospores *in vitro*.

(A) Dendritic cells were incubated with (Spores) or without (No spores) biotinylated spores labeled with streptavidin-PE (red) for 2 hours at a spore:cell ratio of 20:1, fixed in 2% formaldehyde, and stained with a FITC-labeled antibody specific for LAMP-1 (green) and the nuclear dye DAPI (blue) (top panels,100X magnification). (B) Dendritic cells were incubated with FITC-labeled spores, at a spore to cell ratio of 20:1 at 37^{o}C (thick-lined histogram) or at 4^{o}C (gray histogram) for 1 hour. (C) Dendritic cells were incubated with FITC-labeled spores for 1 hour in the presence (gray histogram) or absence (thick-lined histogram) of cytochalasin D (10 µM, CytoD). Dotted line histograms in all panels represent negative control cells incubated in the absence of FITC-labeled spores at 37^{o}C. The mean fluorescent intensities (MFI) are shown for each histogram.

2. Killed Bacillus endospores activate bone marrow-derived dendritic cells

The functional maturation of dendritic cells leads to the coordinated upregulation of a variety of cell surface proteins including, major histocompatability class II (MHC II) molecules and the T cell costimulatory molecules CD80 and CD86 [183]. To determine if killed *B. thuringiensis* spores are capable of stimulating dendritic cell maturation, flow cytometry was used to assess MHC II, CD80, and CD86 levels on the surface of BMDCs that had been stimulated for 48 hours with 10 spores per cell. Unstimulated dendritic cells, represented by the open histograms in Figure 11, expressed modest levels of MHC II (MFI of 562) and low levels of CD80 (MFI of 188) and CD86 (MFI of 61). The expression of all three markers was increased among dendritic cells exposed to spores (closed histograms in Figure 11). In the experiment shown, which is representative of three such performed, MHC II levels were increased 1.6 fold (MFI of 907) whereas CD80 (MFI of 562), and CD86 (MFI of 2132) expression was increased 3 and 35 fold respectively. Thus, killed spores are able to stimulate the upregulation of MHC II, CD80, and CD86.

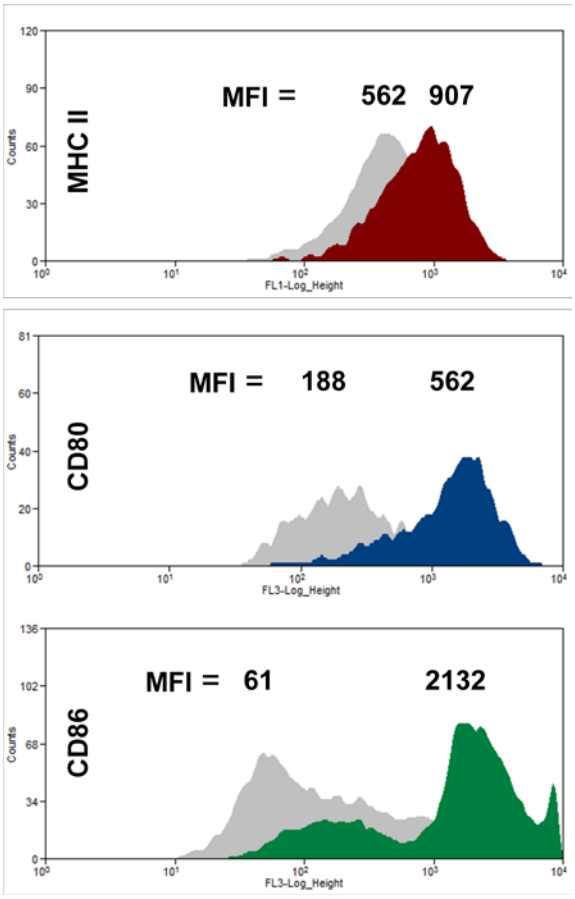

Figure 11. Upregulation of maturation markers on bone marrow-derived dendritic cells following incubation with killed spores.

Dendritic cells were incubated in the absence (gray histograms) or presence (colored histograms) of *Bacillus thuringiensis* spores at a spore to cell ratio of 10:1. After 48 hours the cells were harvested and stained for MHC II (upper panel), and either CD80 (middle panel), or CD86 (lower panel). All samples were gated on live, CD11c+ cells. The mean fluorescence intensities (MFI) of both nonstimulated and spore stimulated cells are shown in each panel. The experiment was independently conducted three times and data from a representative experiment is shown.

3. Inactivated Bacillus endospores stimulate dendritic cell survival

The effect of killed spores on dendritic cell viability was evaluated by measuring the exclusion of the vital dye 7-aminoactinomycin D. For these experiments BMDCs were cultured for 24, 48 or 72 hours in medium alone, or with concentrations of killed *B. thuringiensis* spores corresponding to 20 spores/cell. Cells stimulated with *E. coli* lipopolysaccharide (LPS, 1 μg/ml) served as positive controls for this assay, as LPS promotes BMDC maturation and survival [183-184]. As shown in Figure 12, 24 hours after initiating the cultures the percentage of live cells was similar among cells cultured in medium alone (77%), LPS (80%), or spores (86%). A gradual and steady decline in the proportion of live cells was observed among BMDCs cultured in medium alone with the percentage of live cells dropping from 77% at 24 hours to 48% at 72 hours. The addition of 20 spores/cell to the cultures increased the survival of dendritic cells at both 48 (77% $p=0.004$) and 72 (76%, $p=0.0001$) hours compared to cells incubated in medium alone. These data suggest that killed spores can promote the survival of immature bone marrow-derived dendritic cells *in vitro*.

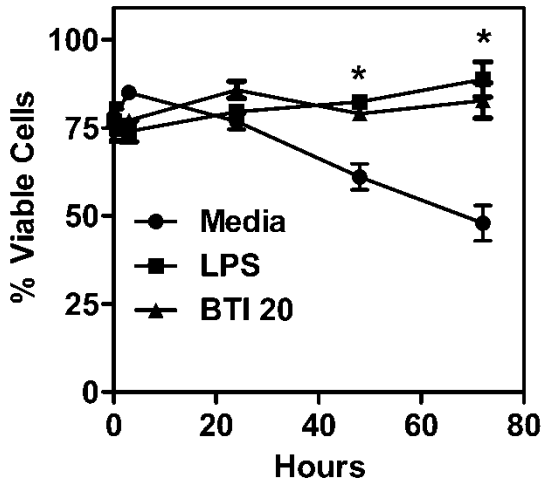

Figure 12. Killed Bacillus endospores promote dendritic cell survival. Dendritic cells were cultured in media alone (closed circles), LPS (closed squares), or UV inactivated *Bacillus* spores at a ratio of 20 (closed triangles) or 10 (inverted triangles) spores per cell. After 24, 48, or 72 hours, cells were harvested and cell viability was determined by flow cytometry after staining with 7-aminoactinomycin D. Asterisks indicate timepoints at which the percentages of viable cells were significantly different ($p < 0.05$, ANOVA)

4. Spore stimulated dendritic cells exhibit reduced phagocytic capacity

Dendritic cells are designed to capture antigens in the periphery and transport them to local lymph nodes for perusal by naïve T cells. Immature DCs are characterized by high phagocytic and endocytic activity and low level expression of the cell surface receptors necessary for T cell activation [185-186]. However, during cellular maturation their ability to phagocytose particulate antigens decreases [185-186]. Because exposure to killed *B. thuringiensis* spores can increase the expression of both MHC II, and costimulatory molecules, the effect of spore exposure on the phagocytic and endocytic activities of BMDCs were next examined as additional markers of BMDC maturation. Phagocytosis was assayed using fluorescently labeled *E. coli* particles, whereas endocytosis was assayed using soluble fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA). Immature BMDCs were cultured in media alone, LPS, or stimulated with 20 killed *B. thuringiensis* spores per cell. Seventy-two hours later the cells were then incubated with pHrhodo-labeled *E. coli* particles (20 particles per cell) or FITC-BSA (0.5 mg/ml) for 2 hours at either 37^{o}C or 4^{o}C, fixed and analyzed by flow cytometry (Figure 13). As expected, unstimulated BMDCs incubated at 37^{o}C with either *E. coli* or FITC-BSA were highly fluorescent, indicating that they were actively phagocytosing the *E. coli* and endocytosing the soluble BSA. The uptake of bacteria and soluble protein was reduced by incubation of non-activated

BMDCs with *E. coli* particles or FITC-BSA at 4^{o}C. In contrast, spore-stimulated BMDCs exhibited reduced fluorescence at 37^{o}C compared to the unstimulated cells, demonstrating that prior exposure to killed spores reduces the ability of BMDCs to phagocytose particulate antigens. Stimulation with LPS or spores did not result in a detectable decrease in the uptake of BSA, implying that the ability of these cells to take up certain soluble antigens may not be adversely affected by their maturational status.

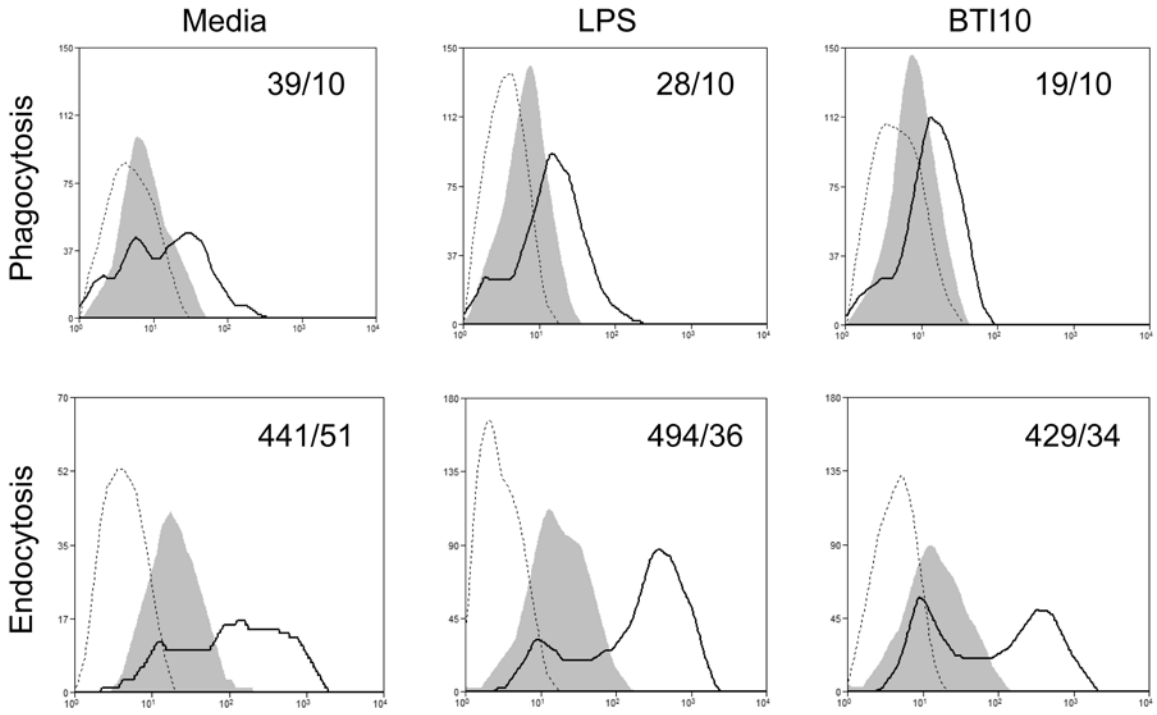

Figure 13. Spore-exposed dendritic cells exhibit reduced phagocytic capacity *in vitro*.

Dendritic cells were cultured in media alone, LPS (1 µg/ml), or killed Bacillus spores (10 spores/cell) for 72 hours and then assayed for phagocytosis (pHrhodo *E. coli*, top panels) or endocytosis (FITC-BSA, bottom panels) activity as described in the *Materials and Methods*. Dotted line histograms in each panel represent negative control cells incubated in the absence of pHrhodo *E. coli* or FITC-BSA. The thick lined and gray histograms represent samples incubated at 37^{o}C and 4^{o}C respectively. The mean fluorescence intensity values at 37^{o}C and 4^{o}C are shown in each panel. Data shown is representative of three independent experiments performed.

5. Spore-stimulated dendritic cells produce pro- and anti-inflammatory cytokines in a dose-dependent manner

Activated dendritic cells are capable of producing a wide variety of both pro- and anti-inflammatory mediators. The specific pattern of cytokines and chemokines produced by dendritic cells has a direct impact on the type of acquired immune response that will develop *in vivo* [185-186]. Therefore, the cytokine profiles of spore-exposed BMDCs were characterized. In these experiments BMDCs were cultured with 2.5, 5, or 10 spores/cell and cytokines secreted in the supernatant were measured 24 hours later. Using quantitative enzyme linked immunosorbant assays the concentration of the proinflammatory cytokines TNF-α, IL-6, IL-12p70, and IL-1β, as well as the anti-inflammatory cytokine IL-10, were analyzed (Figure 14). With the exception of small amounts of IL-10 (31 +/- 22 pg/10^6 cells) none of the cytokines were produced by BMDCs cultured in media alone. Within 24 hours of exposure to killed spores, dendritic cells secreted TNF-α, IL-6, IL-12p70, and IL-10 but not IL-1β. At an MOI of 10 the most abundant cytokine was IL-6 (1,139 +/- 85 pg/10^6 cells) followed by TNF-α (900 +/- 49 pg/10^6 cells). At this spore to cell ratio IL-12p70 (297 +/- 47 pg/10^6 cells) and IL-10 (270 +/- 33 pg/10^6 cells) levels were similar. A clear dose-response relationship was evident among spore stimulated BMDCs with respect to the production of IL-6, TNF-α and IL-12. In contrast, IL-10 levels remained fairly constant regardless of the spore to

cell ratio and ranged between 269 +/- 33 pg/10^6 cells at an MOI of 10 to 190 +/- 41 pg/10^6 cells at an MOI of 2.5.

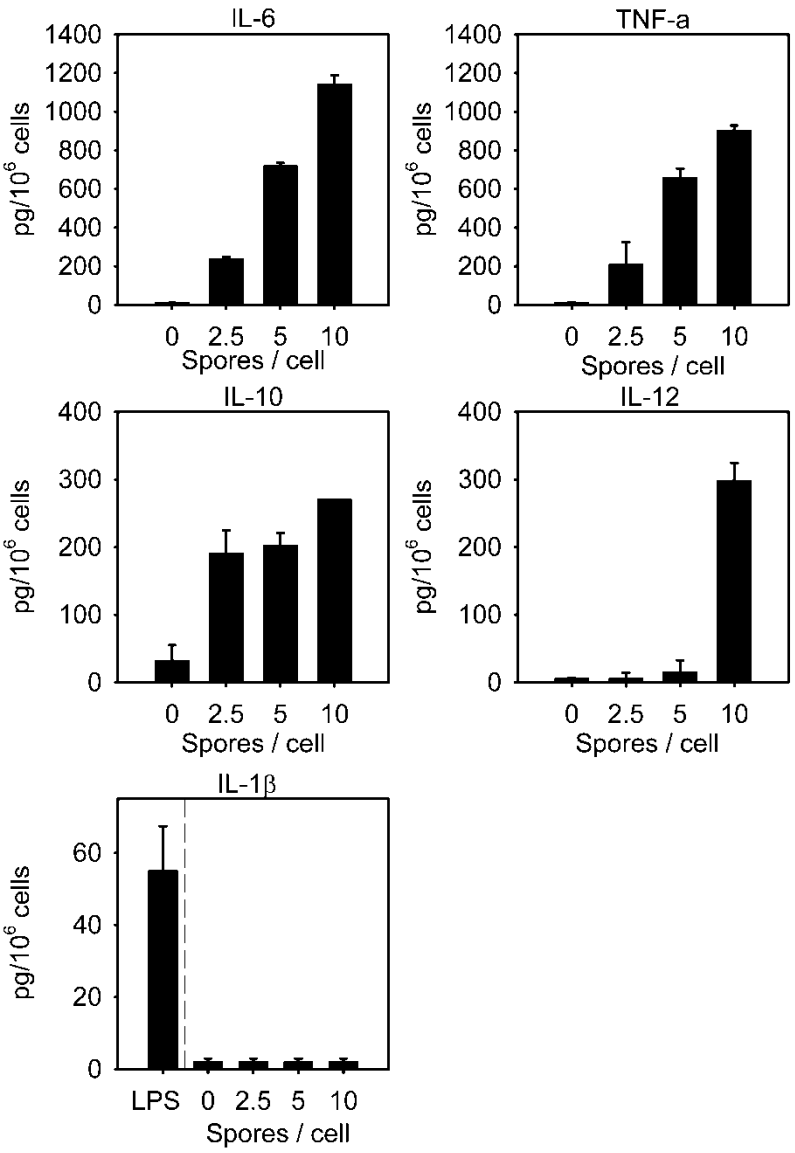

Figure 14. Production of pro- and anti-inflammatory cytokines by bone marrow-derived dendritic cells following co-incubation with killed spores.

Dendritic cells were cultured at a concentration of $5x10^5$ cells/ml with 0, 2.5, 5, or 10 *Bacillus thuringiensis* spores per cell for 24 hours and then the levels of IL-12, IL-10, IL-6, TNF-α, and IL-1β in the supernatants were determined by ELISA. The data used in this figure were generated by Carla Bermudez.

6. Temporal expression of pro- and anti-inflammatory cytokines

To gain a clearer understanding of how killed spores affected the temporal regulation of cytokine induction in BMDCs, cytokine concentrations were measured at 6, 24, 48, and 72 hours post-spore exposure. To directly compare the results at different timepoints, the quantity of individual cytokines present at 6, 48, and 72 hours were normalized to the amount measured at 24 hours post spore exposure (Figure 15). Near maximal levels of IL-10 were detected within 6 hours after spore exposure, and then remained constant over the course of the next three days. A large proportion of IL-6 was also detected within the first 6 hours of stimulation, but unlike IL-10, the amount of IL-6 in the cultures continued to increase from 24 to 72 hours. Although TNF-α and IL-12 were produced more slowly, maximum concentration of both of these cytokines was also observed in supernatants collected at 24 hours post-spore exposure. Thus, within 6 hours BMDCs produced all four cytokines and the cytokine was detectable for at least 72 hours.

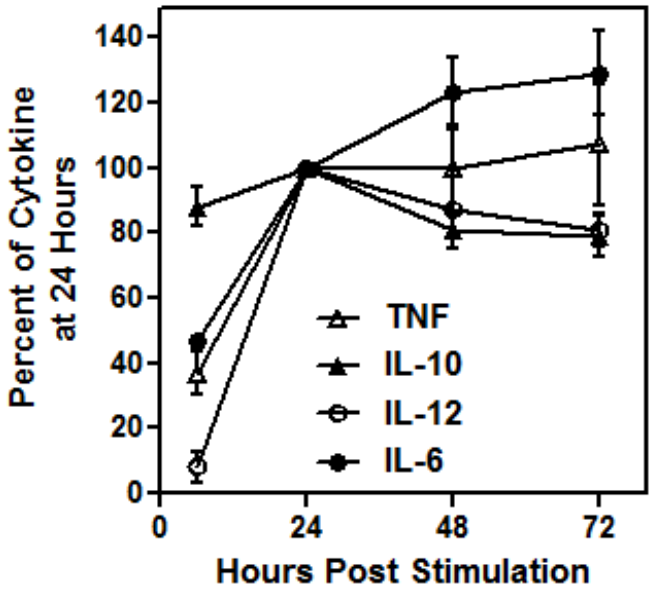

Figure 15. Temporal regulation of cytokine production by dendritic cells exposed to killed *B. thuringiensis* spores.

Dendritic cells were cultured at a concentration of $5x10^5$ cells/ml with 10 spores per cell and the levels of IL-6 (closed circles), IL-10 (closed triangles), IL-12 (open circles), and TNF-α (open triangles), were determined by ELISA at 6, 24, 48 and 72 hours post spore-exposure

7. Spore-stimulated dendritic cells produce reactive oxygen species

Following exposure to killed spores, BMDCs exhibited a mature phenotype, expressed elevated levels of CD80, CD86, and MHC II molecules, had a reduced capacity to phagocytose particulate antigens, and produced significant quantities of TNF-α and low levels of IL-10. This phenotype is reminiscent of tumor necrosis factor alpha- inducible nitric oxide synthase producing DCs (TIP-DCs), a subpopulation of bone marrow-derived monocytes that have been reported to be essential for eliminating certain intracellular bacterial and parasitic pathogens [187-188]. To determine if *B. thuringiensis* spores also stimulated the production of inducible nitric oxide synthase (iNOS), nitrite levels were measured over the course of 48 hours in supernatants from spore stimulated BMDC cultures (MOI of 10). As shown in Figure 16, by 12 hours post-exposure, a significant increase in the concentration of nitrite was detected in spore stimulated cultures compared to cells incubated in medium alone ($p= 0.001$). Nitrite levels continued to accumulate over the course of the experiment indicating that the BMDCs were actively producing nitric oxide. Therefore, killed spores also are capable of inducing the sustained production of reactive nitrogen species.

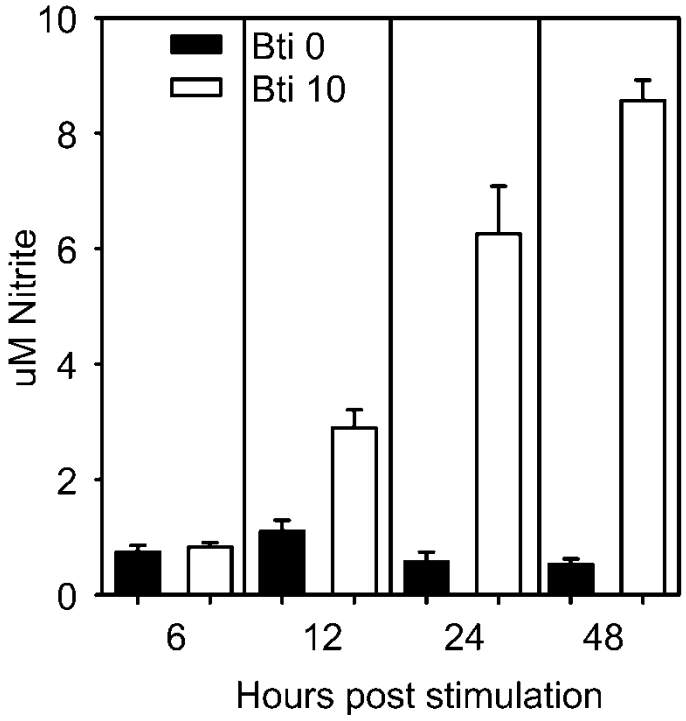

Figure 16. Induction of inducible nitric oxide synthase by dendritic cells following co-incubation with killed spores.

Dendritic cells were cultured in media alone (Bti 0, white bars) or with UV inactivated Bacillus spores at a spore to cell ratio of 10 (Bti 10, black bars). After 6, 12, 24, or 48 hours, supernatants were harvested and iNOS enzyme activity was determined by measuring soluble nitrite levels in cell supernatants as described in Chapter II. Data shown is representative of five independent experiments. The data used in this figure were generated by Dan Hassett

C. Discussion

The results presented in this Chapter show that killed spores from the insect pathogen, *Bacillus thuringiensis*, can directly modulate the function of bone marrow-derived dendritic cells. Immature dendritic cells rapidly phagocytosed spores, and exposure to non-replicating spores resulted in decreased phagocytic activity, as well as a substantial increase in the cell surface expression of MHC II, and the costimulatory molecules CD80 and CD86. UV-inactivated spores were also able to stimulate dendritic cell survival *in vitro*. All of these data suggest that killed spores are capable of driving dendritic cell maturation. Although there have been a number of previous publications that have attempted to dissect the functional consequences of Bacillus spore exposure on monocyte and dendritic cell physiology, these are the first data documenting innate immune responses triggered by *Bacillus thuringiensis* spores [152-153, 157, 159, 189].

While phagocytosis was decreased after spore stimulation, somewhat surprisingly, there was no detectable decrease in the ability of spore treated BMDCs to take up soluble BSA. Dendritic cells acquire external antigens via a number of distinct mechanisms including macropinocytosis, receptor mediated endocytosis and phagocytosis [190]. Murine and human dendritic cells are thought to downregulate both phagocytosis and endocytosis as they mature. However, recent work using murine cells has shown that certain receptor-mediated endocytic pathways are intact in mature myeloid dendritic cells [191]. This is important in that it may

partially explain the mechanism behind data from the Klavinskis laboratory in which heat-killed *B. subtilis* spores were able to stimulate antigen specific T cell responses to co-delivered soluble antigens. In this study, the investigators showed that antigen-specific CD4+ T cell responses to tetanus toxoid could be increased by including killed spores as an adjuvant. In addition, the inclusion of heat killed *B. subtilis* spores also resulted in the effective crosspresentation of soluble chicken ovalbumin to naïve CD8+ T cells [116]. These data would suggest that killed spores are acting to enhance dendritic cell maturation and cytokine production while still allowing the cells to capture external antigens in solution. Because this study involved pre-sensitizing BMDCs with spores prior to examining endocytosis, further studies are warranted to determine how efficiently these BMDCs present soluble antigens to T cells.

In addition to promoting cell survival and upregulating the machinery necessary to efficiently present antigens to naïve T cells, *B. thuringiensis* spores also stimulated the production of a number of important inflammatory mediators. Spore-stimulated dendritic cells secreted TNF-α, IL-6, IL-10, and IL-12, as well as nitric oxide in a dose and time dependent manner. While killed spores were efficient at driving dendritic cell maturation and cytokine production there was no evidence that UV inactivated spores exerted any toxic effects on dendritic cells.

Due to the fact that it is an occasional pathogen of man and a potential bioweapon, most of the available literature on innate immune response to

Bacillus has focused on spores from the ruminant pathogen *Bacillus anthracis*. Genetic analyses of members of the group 1 bacilli which include *Bacillus anthracis*, *B. cereus*, and *B. thuringiensis* have revealed a high degree of interrelatedness, a fact which has led to the proposal that they should all be classified as a single species [192-193]. Although direct comparison of the results reported here with the available data on anthrax spores is difficult due to the differences in the bacteria and experimental models used, common themes have emerged from these studies. Using a germination defective, non-toxin producing strain of *Bacillus anthracis* Basu et al. reported that macrophages exposed to non-replicating spores produce IL-6, TNF-α, IL-1β, and nitric oxide [194]. Later studies by the same laboratory showed that activation of caspase 1 and production of IL-1β was crucial for defense against *B. anthracis* [195]. Live, germination compentent, anthrax spores also induced the production of IL-1β, TNF-α, IL-6, and IL-12 from human dendritic cells [189]. The data presented here would suggest that production of TNF-α, IL-6, IL-12, and nitric oxide by dendritic cells does not require spore germination or bacterial outgrowth.

B. thuringiensis strains are natural pathogens of insects and have been used commercially for over 50 years as environmentally-safe biological control agents. Strains pathogenic for insects produce one or more exotoxins during sporulation that are then packaged into extracellular, parasporal crystalline bodies. In this study, a strain that does not produce parasporal bodies or any known exotoxin was used. In addition, to clarify

what effects *B. thuringiensis* spores exert on the innate immune system, irradiated spores which are incapable of replicating *in vitro* were used. By taking these precautions, the ability of the spore itself to drive dendritic cell maturation and cytokine production was evaluated, rather than the effect of a bacterially derived toxin or the outgrowth of vegetative bacteria. Epidemiological evidence suggests that even exotoxin producing *B. thuringiensis* strains are non-pathogenic in mammals. However, the data presented here reveals that spores from these bacteria can elicit strong inflammatory reactions from innate immune cells and therefore appropriate caution should be used when working with this species. Furthermore, these data also imply that killed *B. thuringiensis* spores may be useful as non-toxic vaccine adjuvants to stimulate dendritic cell responses *in vivo*.

Chapter V: Development of a Prototype Vaccine Against *Yersinia pestis*

A. Rationale

In the previous chapters, UV-irradiated Bacillus endospores have been shown to be potent inducers of both innate and antibody responses that were specific for both the spores and antigens displayed on the spore-surface. Therefore, this system has the potential to provide an excellent platform for vaccine development. However, the true measure of vaccine efficacy is the ability to elicit sufficient immune responses in the host to protect against an infectious challenge. In this chapter, a prototype vaccine against *Yersinia pestis* was developed and the immune responses to this vaccine were characterized.

Yersinia pestis is a gram negative, rod shaped bacterium that belongs to the Enterobacteriaceae family. The disease caused by the bacterium manifests in three distinct pathologies: bubonic, pneumonic, or septicemic plague. Although, large outbreaks are uncommon today, there are still endemic areas in Africa, Asia, and the Americas [196]. Furthermore, *Yersinia pestis* has been weaponized, and the threat of bioterrorism has led to the need for quick-response treatments and vaccines [197-198]. Although there is no effective vaccine against an aerosol plague attack currently available to the public, much research has been focused on developing a plague vaccine. These efforts have included both killed whole cell and subunit vaccines [33, 37, 41, 199-209].

The low-calcium response V antigen (LcrV), and the Fraction 1 antigen (F1) have been identified as highly immunogenic, and therefore, good

potential targets for vaccine development [201-202]. Both F1 and LcrV have been demonstrated to be virulence factors during infection, and antibodies specific for these proteins may interfere with bacterial evasion mechanisms and regulation of the bacteria's type III secretion system [132].

Multiple vaccination and adjuvant strategies have been attempted using these *Y. pestis* proteins. Initially, these proteins were partially purified from the supernatant of a *Yersinia pestis* culture or from recombinant *Escherichia coli* and used as immunogens [201-202]. It was demonstrated that mice immunized intramuscularly with combinations of these antigens or a fusion protein containing both antigens in the presence of additional adjuvants, such as Alhydrogel® or cholera toxin, were protected from *Y. pestis* challenge [33]. Furthermore, it was shown that survival during challenge directly correlated with high F1 or LcrV-specific IgG1 titers in the serum [210].

Other routes of immunization with the F1-V fusion vaccine have also been investigated. Intranasal immunizations were shown to elicit LcrV-specific IgG1 antibody titers in both bronchoalveolar lavage fluid and in serum [207, 211]. Additionally, an α-DEC-205-LcrV fusion protein, which targets the LcrV protein specifically to DEC-205-expressing dendritic cells, has been developed for use as a plague vaccine. This vaccine was shown to elicit improved T-helper 1 (TH1) CD4 T-cell responses and IgG2a antibodies when compared to the F1-LcrV subunit vaccine alone [37].

Other vaccination strategies have included an interleukin-12 (IL-12) - coexpressing DNA vaccine, which was able to protect against *Y. pestis* infection equally well as the subunit vaccine [212], and a recombinant adenovirus expressing the LcrV antigen, which also induced sufficient antibodies to confer protection against intranasal bacterial challenge [213].

Collectively, these data suggested that a vaccine that can induce sufficient levels of LcrV or F1-specific antibodies will protect against *Y. pestis* challenge, making this bacterium an excellent model to evaluate the efficacy of spore-displayed antigens as a vaccine platform. In these experiments, purified, recombinant LcrV protein was conjugated to the commercially available streptavidin derivative, NeutrAvidin and displayed on the surface of UV-irradiated biotinylated *Bacillus thuringiensis* endospores to generate a vaccine against *Y. pestis*. Mice immunized subcutaneously with spore-displayed LcrV developed high-titer, LcrV-specific IgG antibodies in the serum that were able to neutralize *Y. pestis in vitro*. Similarly, mice immunized with multiple intranasal doses of spore-displayed LcrV also developed high titer antigen-specific IgG1, and IgG2a responses. Furthermore, intranasally-immunized mice were protected against a stringent, pneumonic *Y. pestis* challenge.

B. Results

1. Purification, avidination, and display of LcrV on the surface of Bacillus endospores

2. His-tagged LcrV protein was expressed in recombinant *E. coli* and purified using Ni^{2+} labeled beads. Following purification, the protein was linked to the commercially available streptavidin derivative NeutrAvidin using activated maleimide (Figure 17). To verify successful avidination, LcrV alone (Untreated, lanes 1 and 3) or the LcrV-NeutrAvidin conjugate (NeutrAvidin, lanes 2 and 4) were resolved by non-reducing SDS-PAGE and subjected to western blotting. Recombinant LcrV protein was identified using the LcrV-specific monoclonal antibody BA-5 (lanes 1 and 2). The western blots revealed two dominant bands corresponding to the molecular weight of monomeric and dimeric forms of LcrV (Figure 17, lane 1) and three dominant bands with molecular weights that corresponded to two NeutrAvidin-conjugated LcrV molecules and the LcrV dimer (Figure 17, lane 2). The smallest band in the avidinated sample does not correspond to the size of LcrV or avidinated forms of the protein and is most likely a degradation product resulting from the sample manipulation required for the avidination process (Figure 17, lane 4). The presence of NeutrAvidin was demonstrated by western blotting with a biotin-linked horse radish peroxidase enzyme (HRP, lanes 3 and 4). The biotin-HRP did not react with any proteins in the untreated sample (Figure 17, lane 3) but did react with two species in the NeutrAvidin-treated sample. The size

of the high molecular weight species is similar to the high molecular weight species that reacted with the LcrV-specific monoclonal antibody (Figure 17, lane 4 compared to lane 2). The molecular weight of the minor species observed in this lane corresponds to the molecular weight of free NeutrAvidin molecules. This is likely residual NeutrAvidin that was not conjugated to the LcrV protein. These data suggest that the LcrV protein has been stably linked to NeutrAvidin.

Next, flow cytometry was used to determine if the NeutrAvidin-linked LcrV protein could be efficiently displayed on the surface of biotinylated spores. Biotinylated, UV-irradiated *Bacillus thuringiensis* endospores ($1x10^8$) were incubated in the presence (filled histogram) or absence (open histogram) of avidinated LcrV protein. LcrV was detected using the mouse monoclonal antibody BA-5. Bound BA-5 was then detected using a mouse-specific IgG-specific antibody linked to fluorescein isothiocyanate (FITC). The data in Figure 18 demonstrate that 65% of the spores reacted with the LcrV-specific antibody, which indicates the presence of LcrV bound to the spore surface. These data suggest that NeutrAvidin-linked LcrV can be successfully displayed on the spore surface.

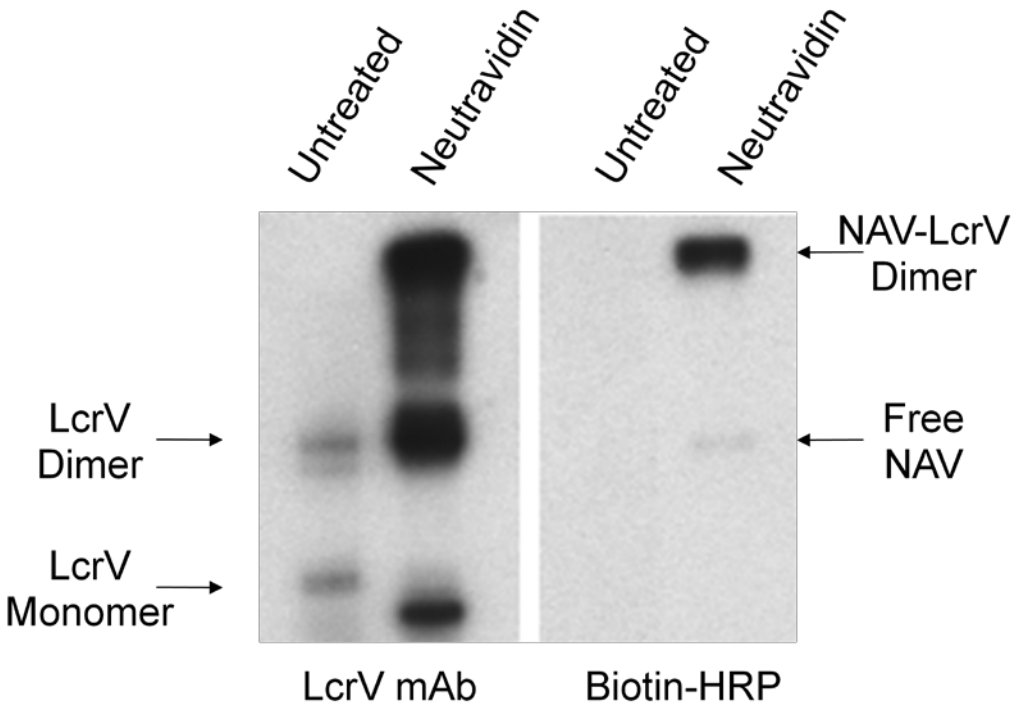

Figure 17. Avidination of recombinant LcrV Protein

Non-avidinated (lanes 1 and 3) and avidinated (lanes 2 and 4) samples of LcrV were subjected to Western blotting using an LcrV-specific mouse monoclonal antibody (BA-5, lanes 1 and 2) or biotin-linked horseradish peroxidase (Biotin-HRP, lanes 3 and 4).

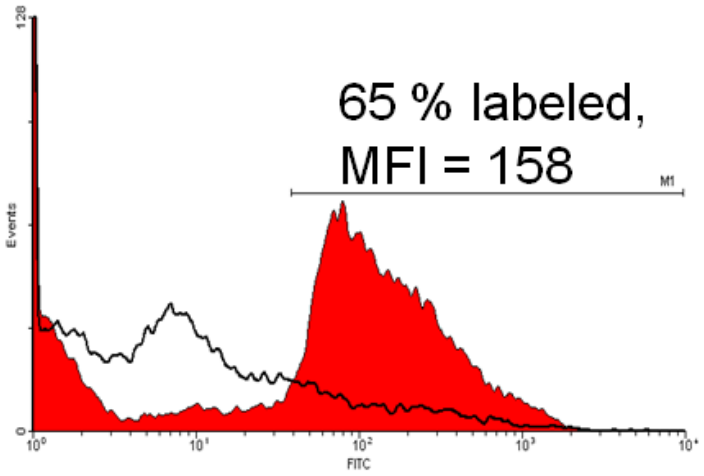

Figure 18. Efficient display of LcrV on the surface of biotinylated spores. *Bacillus thuringiensis* spores were incubated in the presence (filled histogram) or absence (open histogram) of NeutrAvidin-linked LcrV protein. LcrV bound to the spore surface was detected with an LcrV-specific monoclonal antibody (BA-5) and a FITC-conjugated, anti-mouse IgG secondary antibody. Fluorescence in the FL-1 channel was visualized on a BD FACScan.

3. Subcutaneous immunization with spore-displayed LcrV results in high titer antibody responses that can be increased by boosting

To determine if the spore-displayed LcrV antigen was able to induce LcrV-specific antibody responses, groups of six C57BL/6 mice were immunized subcutaneously with $5x10^8$ biotinylated, UV-inactivated *B. thuringiensis* endospores linked to 30 μg of NeutrAvidin-conjugated LcrV protein. A group of three control mice were immunized with spores alone. Two weeks following the priming dose, all mice were bled and serum levels of LcrV-specific IgG were determined using enzyme-linked immunosorbent assay (ELISA) (Figure 19, red bars). All six mice that received the avidinated LcrV displayed on the surface of biotinylated spores developed, LcrV-specific serum IgG responses that ranged from 15,000 to 190,000, with a geometric mean titer of 105,000. The control mice immunized with spores alone did not display detectable LcrV-specific IgG titers (Figure 19, red bars). All mice were then given a second subcutaneous dose of either spore-displayed LcrV or spores alone. Two weeks following the boosting dose, serum levels of LcrV-specific IgG antibodies were again measured by ELISA (Figure 19, white bars).The second subcutaneous dose of spore-displayed LcrV dramatically increased the LcrV-specific response to 625,000 to 2,400,000 with a geometric mean titer of 1,840,000 (Figure 19, white bars). Some background reactivity was observed in the spore-immunized mice

following the boost, most likely due to naturally occurring antibodies present in the sera that cross-reacted with the LcrV antigen preparation.

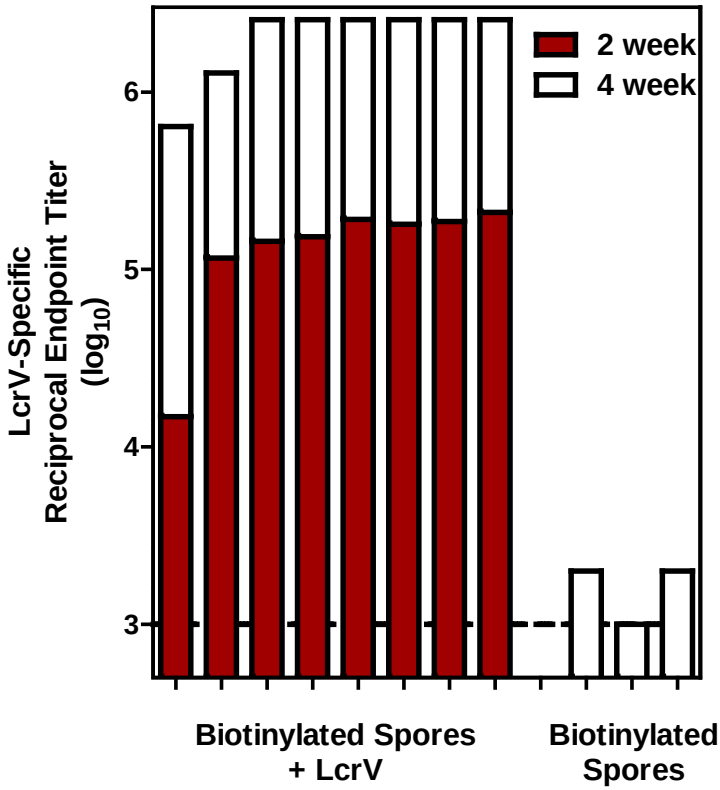

Figure 19. High titer, LcrV-specific IgG levels in the serum of mice immunized with spores displaying LcrV.

Female C57BL/6 mice were immunized subcutaneously with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV and boosted two weeks later. A group of three female C57BL/6 mice were given $5x10^8$ biotinylated spores alone as negative controls. At two weeks (red bars) and four weeks (white bars) post immunization, LcrV-specific IgG titers in serum were measured by ELISA. The dashed line indicates the limit of detection in both assays (1:1000).

4. Mixed IgG1/IgG2a responses are observed after subcutaneous vaccination with spore-displayed LcrV

To further characterize the systemic IgG antibody responses and to also gain insight into the CD4 T cell responses elicited by the spore-displayed LcrV protein, the levels of systemic LcrV-specific IgG1 and IgG2a were assessed at two and four weeks post-immunization. Typically, high IgG1 to IgG2a ratios indicate T-helper 2 dominated responses that are thought to be required for immunity to extracellular pathogens such as bacteria. In contrast, low IgG1 to IgG2a ratios are characteristic of T-helper 1 dominated responses involved in the clearance of intracellular pathogens.

Two weeks following the immunization, the IgG1 titers in mice immunized with spore-displayed LcrV ranged from 16,000 to 110,000 with a geometric mean titer of 64,000, while the IgG2a titers ranged from 2,000 to 16,000 with a mean geometric titer of 6,000 (Figure 20, left panel). A subcutaneous boosting dose dramatically increased the levels of both isotypes and increased the ratio of IgG1 to IgG2a. Two weeks following the second administration of vaccine, the IgG1 serum titers ranged from 1:64,000 to 1:2,600,000 with a geometric mean titer of 1:1,100,000, while the IgG2a titers ranged from 1:40,000 to 1:80,000 with a geometric mean titer of 1:62,000 (Figure 20, right panel). Although IgG2a antibodies were detectable in all cases, IgG1 dominated the responses (2 week

IgG1:IgG2a = 12.7, 4 week IgG1:1gG2a = 32.5). Previous data on *Y. pestis* vaccines suggest that high IgG1 titers correlate to protection against bacterial infection [210]. Thus, these data suggest that this vaccine platform may defend the recipient against plague challenge.

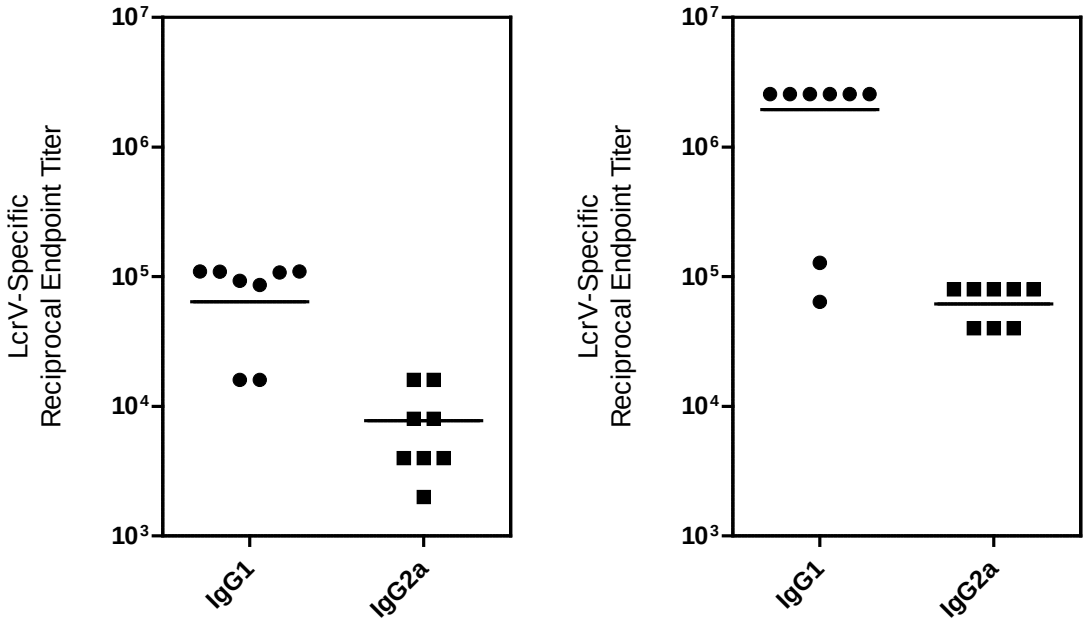

Figure 20. Mixed IgG1 and IgG2a isotype responses in mice subcutaneously immunized with spore-displayed LcrV.

Groups of 8 mice were immunized subcutaneously with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV and boosted two weeks later. At two weeks (left panel) and four weeks (right panel) post primary immunization, LcrV-specific IgG1 (circles) and IgG2a (squares) titers in serum were measured by ELISA. Solid lines indicate the geometric mean titer in each group. The limit of detection in both assays was 1:1000.

5. Serum from immunized mice neutralizes *Yersinia pestis in vitro*

The data presented above demonstrate that mice immunized with spore-displayed LcrV protein generate high titer serum IgG responses. However, for the vaccine to be considered effective, the antibodies generated by the immunization must be able to neutralize *Y. pestis* bacteria. When *Y. pestis* encounters host cells such as macrophages, the bacteria inject virulence factors using the type III secretion system. These virulence factors induce the activity of caspase-3 in the host cell [214]. Antibodies that block the type III secretion system have been demonstrated to prevent the transfer of these virulence factors, and thereby reduce host cell caspase-3 activity [132]. Therefore, the induction of macrophage caspase-3 was used to determine the ability of serum from immunized mice to neutralize the bacteria *in vitro.*

Y. pestis (strain KIM D27) cultures were first pre-incubated with pooled serum samples from mice immunized with spore-displayed LcrV (n=3), spores alone (n=3), or nonimmune sera (n=3). Control bacterial cultures were incubated in PBS only. The pre-incubated bacterial samples were then co-cultured with RAW 264.7 macrophage cultures for 3.5 hours. The macrophages were harvested and the level of caspase-3 induction was measured using a substrate that fluoresces when cleaved by caspase-3.

As shown in Figure 21, serum from non-immunized (gray bar) or spore-immunized mice (green bar) did not reduce the level of caspase-3 induction compared to the PBS-treated bacteria controls (white bar). Pre-

incubation of the bacteria with serum from mice immunized with spore-displayed LcrV (red bar) however, reduced the amount of caspase-3 induction in the infected macrophages by 25% compared to untreated bacteria ($p=0.0345$, ANOVA). These data indicate that the serum samples contain LcrV-specific antibodies capable of neutralizing *Y. pestis* bacteria *in vitro*.

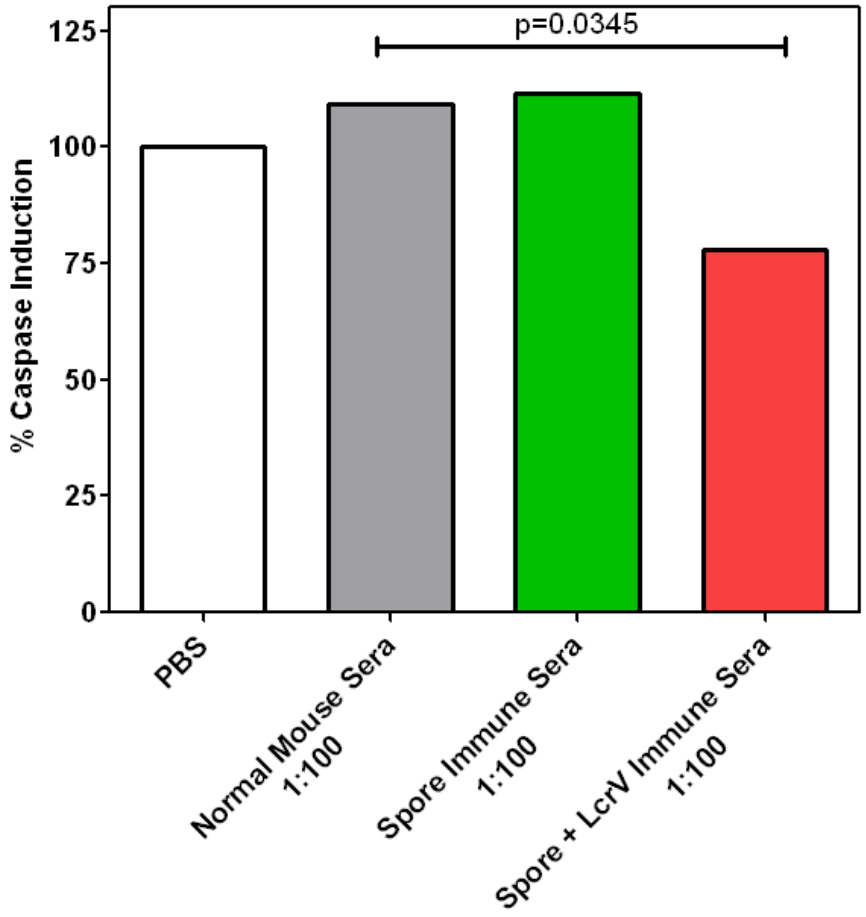

Figure 21. LcrV-immune sera reduces the infectivity of *Y. pestis* in cultured murine macrophages.

Y. pestis (KIM D27) was pre-incubated with PBS (white bar), pooled sera from non-immunized mice (gray bar), sera from mice immunized with spores alone (green bar), or sera from mice immunized with spores displaying LcrV (red bar). The bacteria were then used infect RAW 264.7 cells. Caspase-3 activation in the cultured cells following infection, a measure of bacterial-induced apoptosis, was measured by EnzChek Caspase-3 Induction Assay (Invitrogen).

6. Pilot studies indicate that two intranasal immunizations are sufficient to induce high levels of LcrV-specific IgG in the serum

Alternative, noninvasive routes of immunization, such as intranasal administration, that can be administered without the use of a needle and syringe would allow for the rapid distribution of vaccines in times of crisis. Mucosally administered vaccines would also reduce the cost of routine vaccinations. Spores from other members of the *Bacillus* genus, such as *B. anthracis*, can infect via the nasal route [215]. This attribute makes the spore platform an attractive vehicle to administer antigens as intranasal vaccines.

A series of pilot experiments were undertaken to determine if the intranasal administration of antigens displayed on *B. thuringiensis* spores were sufficiently immunogenic to elicit serum antibody responses. Groups of eight C57BL/6 mice were immunized with $5x10^8$ spores displaying 30 μg recombinant, purified LcrV protein. Groups of three control mice were immunized concurrently with $5x10^8$ spores alone. Two weeks following the initial dose, LcrV-specific IgG titers were determined in sera obtained from peripheral blood. As expected from data on other intranasal vaccines [216-217], the initial dose of vaccine did not elicit detectable levels of serum IgG (data not shown). Therefore, all mice were given a second intranasal dose of either spore-displayed LcrV or spores alone. Two weeks following the boosting dose, serum was isolated from peripheral blood and LcrV-specific total IgG levels were determined by

ELISA. Two weeks following the second dose, LcrV-specific serum IgG antibodies were detected (Figure 22, blue bars) with titers ranging from 1:130,000 to 1:320,000 with a geometric mean titer of 1:150,000. As expected, no LcrV-specific IgG was detected in mice immunized with spores alone (Figure 22, white bars). These data indicate that intranasal immunization with spore-displayed LcrV can elicit antigen-specific antibody responses in the serum of recipients.

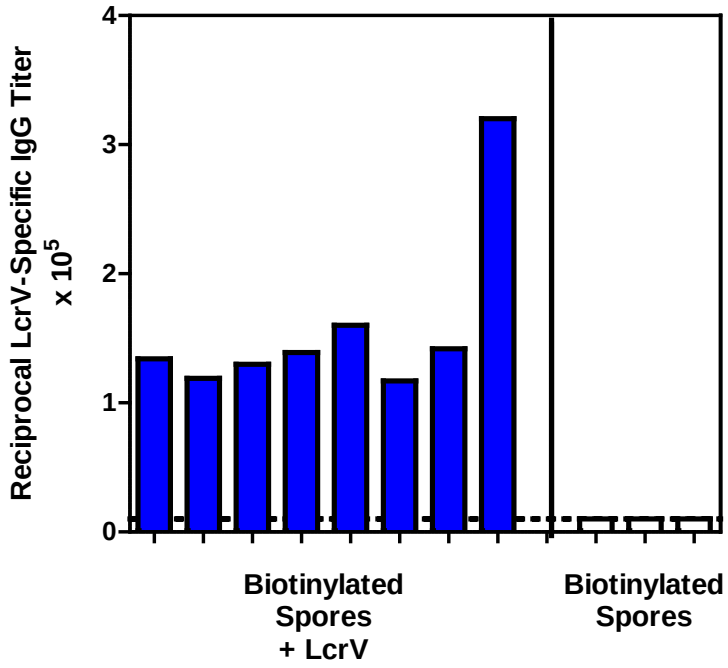

Figure 22. Two Intranasal doses of spore-displayed LcrV elicits high-titer LcrV-specific antibodies in the serum.

Groups of 8 C57BL/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV and boosted two weeks later (filled bars). A group of three mice immunized with $5x10^8$ biotinylated spores alone served as negative controls (open bars). At four weeks post immunization, anti-LcrV IgG titers in serum were measured by ELISA. The dashed line indicates the limit of detection in the ELISA (1:250).

7. Intranasal administration of spore-displayed LcrV elicits similar systemic IgG responses to adjuvanted LcrV protein.

The prototype *Yersinia* pestis vaccine consisting of spore-displayed LcrV antigen has been demonstrated to elicit antigen-specific serum antibody responses in the pilot subcutaneous and intranasal immunizations described above. However, these pilot studies were not designed to directly compare the antibody responses of mice immunized with spore-displayed LcrV to mice immunized with a more conventional vaccine, such as soluble LcrV protein. Additionally, previous LcrV vaccines have included adjuvants such as Alhydrogel® [206]. Therefore, in these experiments, LcrV-specific antibody responses in the serum of mice immunized with spore-displayed LcrV were compared to the responses in mice concurrently immunized with soluble LcrV protein and soluble protein formulated with the adjuvant Alhydrogel®. For comparison, groups of mice were also immunized with spore-displayed LcrV formulated with Alhydrogel®.

Groups of C57BL/6 mice were given three intranasal doses of various vaccine formulations two weeks apart. The first group of mice received spores linked to 30 μg NeurtrAvidin-conjugated LcrV formulated in PBS (n=10). A second group was immunized with the same preparation of spore-displayed LcrV formulated in 1% Alhydrogel® (n=10). To compare the immunogenicity of the spore-displayed LcrV to soluble protein, a group of mice received three doses of an equimolar amount (11 μg) of

nonavidinated LcrV protein in the absence of spores (n=10). To control for the effect of Alhydrogel®, a final group was immunized with the same LcrV protein formulated in 1% Alhydrogel® (n=10). Additional groups of control mice were immunized with either spores suspended in PBS or PBS alone (n=10) as described in *Materials and Methods*.

The serum levels of LcrV-specific IgG antibodies were quantified by ELISA in serum obtained from vaccinated mice two weeks following the final dose of vaccine. The series of immunizations was performed in duplicate at separate times with separate groups of mice. The data described below include the pooled data obtained from both experimental repetitions.

Eighteen of the 20 mice immunized with three doses of LcrV protein displayed on the spore surface responded with serum titers of LcrV-specific IgG. The titers in these mice ranged from 800,000 to 12,800,000 with a geometric mean titer of 3,800,000 (Figure 23). The two mice that were considered non-responders had titers below the limit of detection of the assay (10,000). Three doses of LcrV protein in the absence of spores elicited similar titers in 20 out of 20 vaccinated mice ($p>0.05$, Bonferrori one-way ANOVA). The serum levels of total IgG in these mice ranged from 800,000 to 12,800,000 with a geometric mean titer of 2,800,000 (Figure 23). All 20 mice immunized with LcrV protein formulated with Alhydrogel® also responded and had similar levels of serum IgG titers which ranged from 800,000 to 10,240,000 with a geometric mean titer of

3,800,000 as shown in Figure 23. Interestingly, only 15 of the 20 mice that received spore-displayed LcrV in the presence of Alhydrogel® responded to the vaccine. These mice had lower serum LcrV-specific IgG titers. The levels of IgG in these mice ranged from 10,000 to 6,400,000 with a geometric mean titer of 725,000. As expected, control mice immunized with PBS or spores alone had no detectable LcrV-specific IgG antibodies (data not shown).

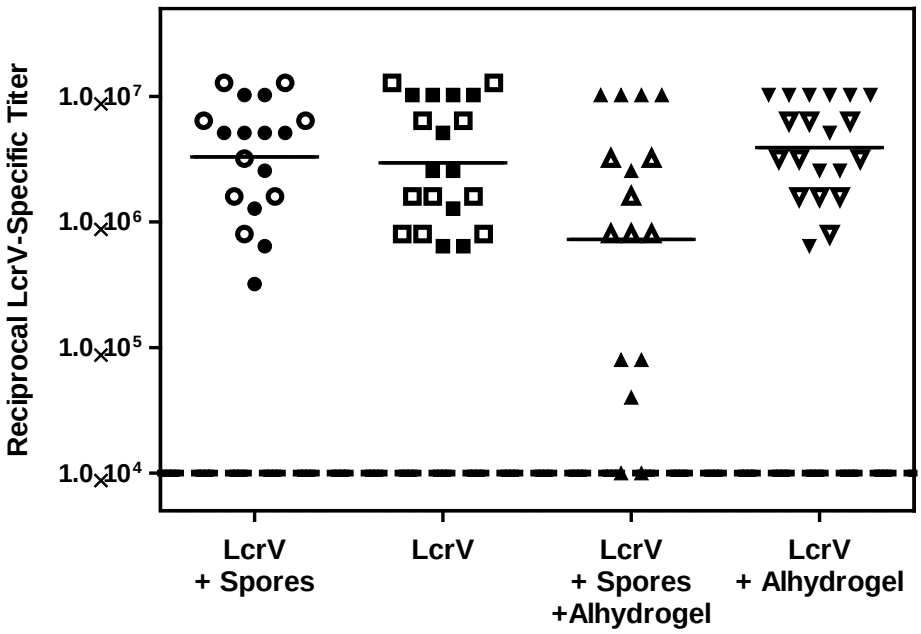

Figure 23. High titer serum IgG levels following three intranasal doses of LcrV-based vaccines.

Groups of 10 female C57Bl/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV (circles), 19 μg LcrV protein alone (squares), spore-displayed LcrV formulated with 1% Alhydrogel® (triangles) or LcrV protein formulated with 1% Alhydrogel® (inverted triangles). Mice were boosted two additional times with the same dose and vaccine formulation at two week intervals. Two weeks following the third vaccination, LcrV-specific IgG titers in serum were measured by ELISA. Solid lines indicate the geometric mean titers. The limit of detection in these assays was 10,000. The experiment was carried out two different times and the data were pooled. Open and closed symbols indicate the data from the different repetitions.

8. Spore-displayed LcrV increases LcrV-specific IgG2a following intranasal administration.

The serum levels of IgG antibodies previously observed in the immunized mice were further characterized to determine the isotype composition. Serum samples that were collected two weeks following the second boosting dose (6 weeks post-primary immunization) were assayed by ELISA to quantify the amount of LcrV-specific IgG1 and IgG2a antibodies (Figure 24). Mice immunized with spore-displayed LcrV exhibited high titer antigen-specific IgG1 and IgG2a antibodies that ranged from 50,000 to 800,000 and 32,000 to 128,000 respectively (Figure 24). Mice immunized with LcrV protein alone also developed similar high titer IgG1 antibodies, but significantly lower IgG2a titers compared to spore-displayed LcrV ($p \leq 0.05$, Bonferroni's Multiple Comparison Test). The IgG1 titers in this group of mice ranged from 100,000 to 3,200,000, while the IgG2a antibody titers ranged from 4,000 to 64,000 (Figure 24). Mice immunized with soluble LcrV with Alhydrogel® displayed similar isotype responses to soluble LcrV alone. The addition of Alhydrogel® to spore-displayed LcrV did not significantly alter IgG1 titers, but significantly decreased the LcrV-specific IgG2a responses ($p \leq 0.05$, Bonferroni's Multiple Comparison Test).

Consistent with other studies of LcrV-based vaccines, all the IgG antibody responses were dominated by IgG1 [205-207, 211] (Table 1). However, mice immunized with spore-displayed LcrV in the absence of

Alhydrogel® had higher significantly higher IgG2a responses than the mice immunized with LcrV alone or the recombinant protein formulated with Alhydrogel® (Table 1, $p \leq 0.05$, Bonferroni's Multiple Comparison Test).

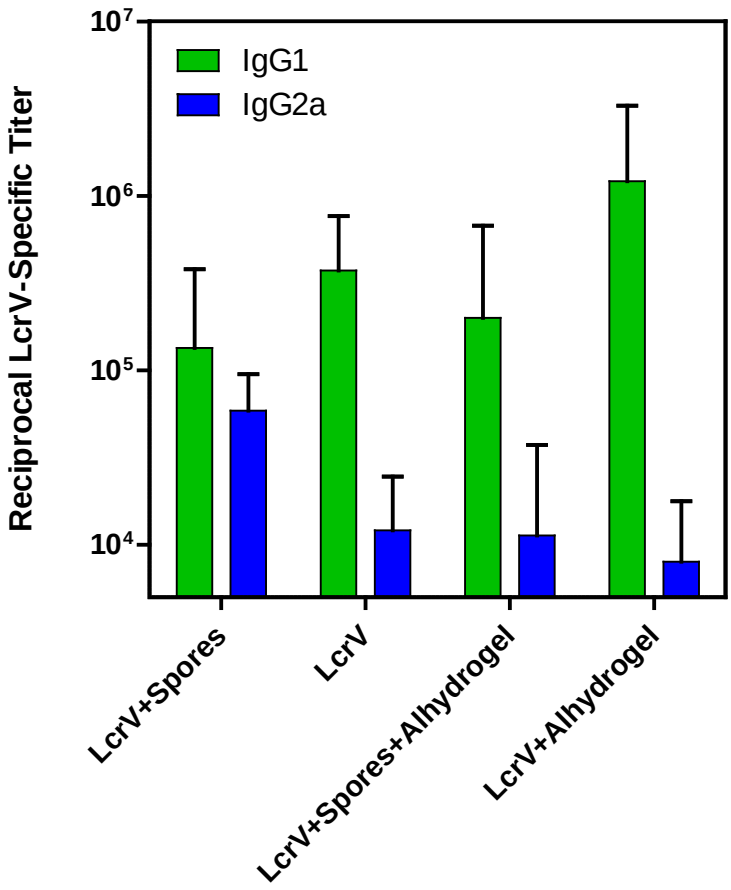

Figure 24. Intranasal immunizations elicit IgG1-dominated antibody responses.

Groups of 10 female C57Bl/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying NeutrAvidin-labeled LcrV, an equimolar amount of LcrV protein alone, spore-displayed LcrV formulated with 1% Alhydrogel® or LcrV protein formulated with 1% Alhydrogel®. Mice were boosted two additional times with the same dose and formulation at two week intervals. Two weeks following the third vaccination, LcrV-specific IgG1 (green bars) and IgG2a (blue bars) titers in serum were measured by ELISA. Limits of detection: IgG1 = 1:5,000 and IgG2a = 1:1,000.

Table 1: Systemic antibody responses of LcrV-immunized mice

Treatment	Responders	Geometric Mean IgG	Geometric Mean IgG1	Geometric Mean IgG2a	IgG1:IgG2a
Spores + LcrV	18/20	$3.4x10^6$	$1.9x10^5$	$5.5x10^4$	2.6
LcrV	20/20	$3.0x10^6$	$6.7x10^5$	$1.2x10^4$	58.3
Spores + LcrV + Alhydrogel	16/20	$9.5x10^5$	$1.9x10^5$	$1.3x10^4$	18.9
LcrV + Alhydrogel	20/20	$4.2x10^6$	$1.5x10^6$	$7.2x10^3$	233.9

9. Intranasal immunization protects mice from an intranasal *Yersinia pestis* challenge

The most stringent test of any vaccine or vaccine platform is its ability to protect against a lethal challenge. In these series of experiments, the vaccine was designed to protect against a lethal *Y. pestis* challenge. Groups of 10 mice were vaccinated with three intranasal doses of spores displaying 30 μg avidinated LcrV formulated in PBS or 1% Alhydrogel®. Groups of mice immunized with an equimolar amount of nonavidinated LcrV formulated in PBS or 1% Alhydrogel® were used to compare the spore platform to a more traditional vaccine formulation. Control mice were immunized with spores in PBS or PBS alone as described in *Materials and Methods*. Four weeks after the second boosting dose (third of three immunizations), all mice were infected intranasally with 9,000 colony forming units (CFU) (30 LD_{50}) *Y. pestis* (strain CO92) and monitored for survival for 14 days. It is important to note that mice with undetectable LcrV-specific IgG titers at 6 weeks post-immunization were considered non-responders to the vaccine and not included in the challenge study.

The PBS-immunized control mice (n=10) were not protected and succumbed to pneumonic infection within five days. Similarly, ninety percent of the mice immunized with spores alone had also died by day five (n=10). As shown in Figure 25, mice immunized with LcrV antigen displayed on the spore surface had the highest survival rate among all of

the groups examined (n=8, 62.5% survival, p=0.003, Mantel-Cox). Similarly, sixty percent of the mice immunized with LcrV protein formulated with 1% Alhydrogel (n=10, p=0.0002, Mantel-Cox) also survived the plague challenge. Mice immunized with LcrV protein alone were also well protected (n=10, 50% survival p=0.0111, Mantel-Cox). In contrast, the addition of 1% Alhydrogel® to spore-displayed LcrV appeared to resulted in diminished protection (n=6, p=0.7055 Mantel-Cox). The results of this experiment suggest that spore-displayed LcrV without Alhydrogel® is a viable platform for the administration of pathogen-derived antigens.

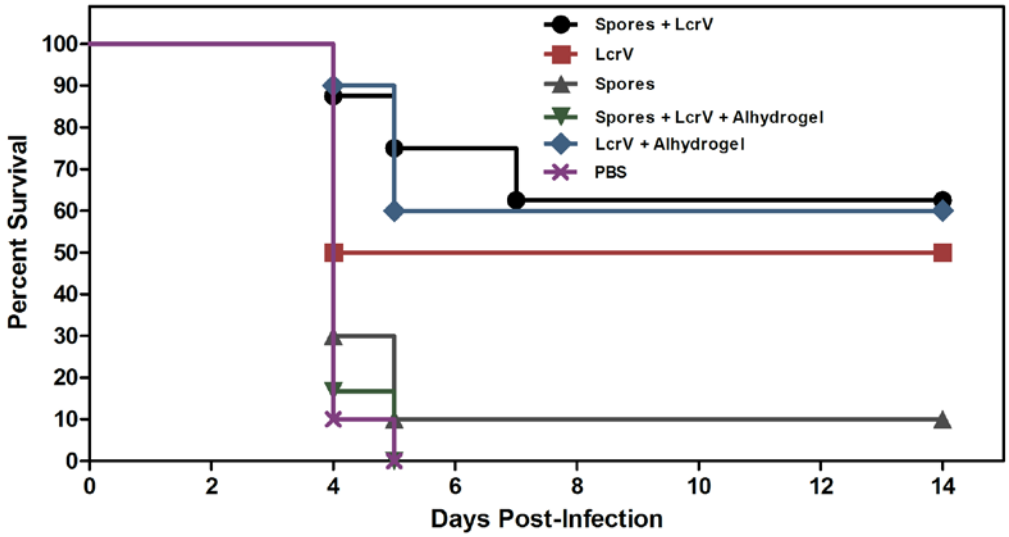

Figure 25. Intranasal immunizations protect mice from a low-dose, intranasal *Y. pestis* challenge.

Groups of 10 female C57Bl/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV (N=8, black circles), 19 μg LcrV protein alone (N=10, red squares), spore-displayed LcrV formulated with 1% Alhydrogel® (N=6, green inverted triangles), or LcrV protein formulated with 1% Alhydrogel® (N=10, blue diamonds). Control mice were immunized with either $5x10^8$ spores alone (N=10, gray triangles) or PBS (N=10, violet X's) Mice were boosted two additional times with the same dose and formulation at two week intervals. Four weeks after the final dose, all mice were challenged intranasally with *Yersinia pestis* (9,000 CFU).

To further evaluate the protective efficacy of this prototype plague vaccine, groups of mice were immunized as above, but infected intranasally with a more stringent challenge dose of bacteria (74,000 CFU, 250 LD_{50}). As before, survival of immunized mice was monitored for 14 days. When the immunized mice were infected with this very high dose of *Yersinia pestis,* all control mice died or were euthanized within the first four days of the challenge. As shown in Figure 26, immunization with LcrV displayed on the spore surface again resulted in significant survival when compared to the controls (30%, $p<0.0001$, Mantel-Cox). In addition, a significant proportion of mice immunized with LcrV alone and with LcrV formulated with Alhydrogel® also survived the intranasal *Yersinia pestis* challenge (30%, $p=0.0118$ and 70%, $p=0.0006$, Mantel-Cox, respectively). In contrast to the low-dose *Y. pestis* challenge described above, a significant number of mice immunized with spore-displayed LcrV formulated with Alhydrogel® survived the challenge (30%, $p=0.0043$, Mantel-Cox). However the addition of the adjuvant did not improve the survival of these mice compared to immunizations of spore-displayed LcrV without Alhydrogel® ($p=0.4674$, Mantel-Cox).

Collectively, these data suggest that intranasal immunization with LcrV displayed on the surface of *B. thuringiensis* endospores generates sufficient memory responses to protect against an exceedingly stringent dose (250 LD_{50}) of *Y. pestis* that is as effective as immunizations with protein formulated with the conventional adjuvant Alhydrogel®.

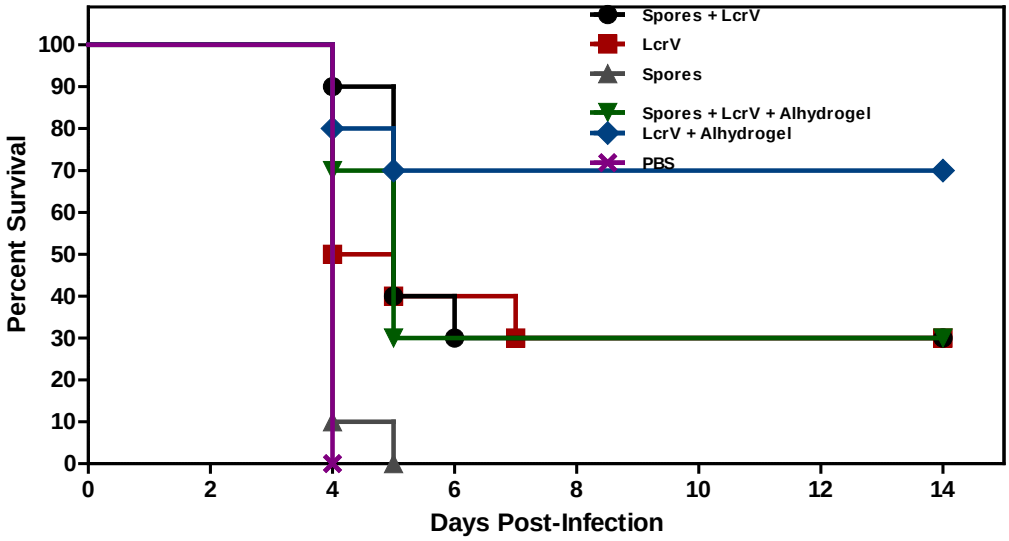

Figure 26. Intranasal immunizations protect mice from a high-dose, intranasal *Y. pestis* challenge.

Groups of 10 female C57Bl/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying 30 μg of NeutrAvidin-labeled LcrV (N=10, black circles), 19 μg LcrV protein alone (N=10, red squares), spore-displayed LcrV formulated with 1% Alhydrogel® (N=10, green inverted triangles), or LcrV protein formulated with 1% Alhydrogel® (N=10, blue diamonds). Control mice were immunized with either $5x10^8$ spores alone (gray triangles) or PBS (N=10, violet X's) Mice were boosted two additional times with the same dose and formulation at two week intervals. Four weeks after the final dose, all mice were challenged intranasally with *Yersinia pestis* (74,000 CFU).

Although the prototype vaccine consisting of spore-displayed LcrV did not appear to significantly improve the overall survival of infected mice compared to LcrV protein alone, it does however appear to increase both the median survival time and mean time to death in recipient mice. Protein displayed on the spore surface increased the median survival from 4 days to 9.7 days in the low dose study, and from 4 days to 5 days in the high dose study. In addition, immunizations with the LcrV antigen linked to the spore surface allowed the challenged mice to survive significantly longer. When challenged with the low dose of bacteria, the mean time to death of mice immunized with spore-displayed antigen was 5.3 days compared to the mean time to death of 4 days in the mice that received soluble protein alone ($p \leq 0.05$, Tukey's multiple comparison test). A similar trend was also observed when mice were challenged with a high bacterial dose. The mean time to death was increased from 4.6 days in mice receiving protein alone to 5 days in mice receiving spore-displayed protein. These values were calculated from both the high dose and low dose infection experiments and the data are summarized in Table 2.

Table 2: Survival of LcrV-immunized mice

Treatment	Responders	*Yersinia pesitis* dose (CFU)	No. of Survivors	Median Survival (Days)	Mean Time to Death (Days)
Spores + LcrV	8/10	9,000	5/8	*	5.3
LcrV	10/10	9,000	5/10	9	4.0
Spores + LcrV + Alhydrogel	6/10	9,000	0/6	4	4.2
LcrV + Alhydrogel	10/10	9,000	6/10	*	4.8
Spores + LcrV	10/10	74,000	3/10	5	5.0
LcrV	10/10	74,000	3/10	4.5	4.6
Spores + LcrV + Alhydrogel	10/10	74,000	3/10	5	4.6
LcrV + Alhydrogel	10/10	74,000	7/10	*	4.3

*Median survival cannot be calculated when the total population does not fall below 50%

C. Discussion

Previous vaccines against *Y. pestis,* the causative agent of plaque, have evolved from killed bacteria, to subunit vaccines, to recombinant fusion vaccines, and finally to DNA vaccines [33, 41, 201-202, 205, 209, 212]. From this literature, it is evident that vaccines directed against the low calcium response V antigen (LcrV) are excellent targets for the inhibition of bacterial infection and prevention of disease [201-202]. In the absence of any additional adjuvants, LcrV is an extraordinarily immunogenic protein [203-204]. The purpose of this study was twofold. The first was to utilize the antigen display on Bacillus endospores platform to further enhance the immunogenicity of the LcrV antigen and develop an improved, nonreplicating vaccine against *Y. pestis* and the second was to determine the efficacy of this vaccine when delivered intranasally.

Recombinant LcrV protein was successfully expressed and purified from *E. coli*. It was then conjugated to commercially available NeutrAvidin and displayed on the surface of biotinylated *B. thuringiensis* endospores. Subcutaneous immunization with this material, without any additional adjuvants, generated high titer LcrV-specific antibody responses in C57BL/6 mice (Figure 19). In addition, intranasal immunization also yielded serum IgG antibodies specific for LcrV (Figure 22). However, the intranasal immunizations with vaccines consisting of spore-displayed LcrV, equimolar amounts of soluble LcrV protein, and the protein formulated in Alhydrogel®, resulted in similar LcrV-specific serum IgG

titers. This is likely due to the immunogenicity of LcrV. The quantity of LcrV protein used in these studies was substantially higher (11 μg of nonavidinated LcrV protein and 30 μg of NAV-LcrV) than the doses used in previous studies, which ranged from 1 μg to 5 μg [19, 207]. The differences between the serum IgG titers of mice immunized with LcrV protein alone and LcrV formulated with Alhydrogel® were also insignificant ($p=0.4767$, Student's t-test), indicating that the immunopotentiating effect of the aluminum-based adjuvant was not detectable. It is possible that the amount of protein used in these experiments saturated the immune response and a plateau effect was achieved. Because of this effect, the any potential differences because of adjuvant or Bacillus spores display were undetectable.

To further characterize the immune responses to spore-displayed antigens, IgG1 and IgG2a were also quantified in the serum of the immunized mice. Previous studies have correlated the titer of IgG1 to protection against bacterial infection [210]. In addition, these data also provide insight into the type of CD4+ T-cell responses elicited by the spore-based vaccine platform [218]. Immunizations with recombinant LcrV protein have been well documented to primarily elicit IgG1 antibodies in the serum [205-207, 211]. Similar results were also observed in this study. Furthermore, the addition of Alhydrogel® has been previously shown to increase the IgG1 responses to subunit vaccines [219-220]. Other studies have shown that formulation of vaccines with *Bacillus*

subtilis spores can increase TH1 CD4 T-cell responses and leads to an increase in the serum levels of IgG2a [116, 163]. When mice were immunized with spore-displayed LcrV antigen, the levels of IgG1 dominated the response; however the levels of IgG2a were significantly increased. This phenomenon was also observed in previous studies with *B. thuringiensis* displaying β-galactosidase (Chapter III). This increase of IgG2a was not observed when the spores displaying LcrV were formulated in 1% Alhydrogel® which was likely due to the strong IgG1 polarizing effect of the adjuvant [219-220]. In all vaccine preparations, the IgG isotype data suggest that the LcrV protein immunizations primarily elicit a TH2-type CD4 T-cell response. However, spore-displayed LcrV appears to be inducing a mixed TH1/TH2 response. Further studies to characterize the cytokines that are produced by the CD4 T-cells would yield more conclusive evidence of the type of CD4 T-cells generated by the different vaccines.

Surprisingly, the LcrV-specific serum IgG titers observed in mice immunized with spore-displayed LcrV formulated with 1% Alhydrogel® were much lower than those in mice that had received protein alone, or protein on the surface of spores. A major concern with this group was the intranasal administration of a viscous adjuvant, combined with a viscous vaccine preparation. It is possible that the gelatinous nature of this preparation prevented the reliable administration of the vaccine past the nasal passage and into the lung mucosa where it would be most

efficacious. Instead, it is thought that the material was swallowed by the mice and entered the gastrointestinal tract. This is a problem that has been encountered in other intranasal delivery systems. It has been shown previously that only about 10-30% of an intranasally administered drug preparation actually reaches the lung and the rest is either exhaled or swallowed upon recovery from anesthesia [221-222]. From a practical point of view, the spore-displayed LcrV formulated with Alhydrogel® was quite difficult to administer intranasally and the immunized mice were observed swallowing upon recovery from anesthesia. To determine if the observed differences are due to this difficult method of delivery, the serum responses to the various vaccine preparations need be evaluated following a more reliable delivery method, such as subcutaneous or intramuscular administration.

Immunizations with even low levels of LcrV protein in the presence of additional adjuvants have been observed to be sufficient to prevent mice from succumbing to a lethal *Y. pestis* infection [200-201, 208]. This protection has been attributed to high levels of vaccine-induced IgG1 antibodies in the serum [210]. When groups of vaccinated mice were infected with 9,000 CFU (30 LD_{50}) of *Y. pestis*, the mice immunized with spore-displayed LcrV had the highest rate of survival (5/8, 62.5%), whereas 60% (6/10) of the mice immunized with LcrV protein in the presence of Alhydrogel®, and only 50% (5/10) of the LcrV-immunized mice were able to survive the infection (Figure 25, Table 1). These data

suggest that the display of LcrV on the surface of spores does improve the immunogenicity of the subunit vaccine.

To further characterize the efficacy of the spore-based vaccine platform, groups of similarly immunized mice were infected with a much more stringent dose consisting of 74,000 CFU or 250 LD_{50} of *Y. pestis* bacteria. In this experiment, no significant differences were observed in the survival of the mice that received spore-displayed LcrV compared to mice immunized with the LcrV protein alone or LcrV protein formulated with Alhydrogel® (Figure 26).

An interesting observation during the course of these experiments was that the mice immunized with spore-displayed LcrV that died following a lethal challenge survived significantly longer than mice immunized with either LcrV alone or LcrV in Alhydrogel® (5.3 days compared to 4.0 and 4.8 days respectively, $p \leq 0.05$, Tukey's multiple comparison test). This is only a slight increase, but considering the high dose of pathogenic bacteria, this vaccine may prolong the onset of disease sufficiently to allow an infected individual to obtain further clinical treatment, and survive. In one challenge experiment, the mice immunized with spore-displayed LcrV formulated with Alhydrogel® were not well protected. This correlated to lower titers of LcrV-specific antibodies and was most likely due to the unreliability of vaccine administration as discussed above.

In conclusion, these experiments demonstrated not only that the recombinant LcrV antigen from *Y. pestis* could be efficiently displayed on

the surface of killed *B. thuringiensis* spores, but also that either subcutaneous or mucosal immunizations with these spores generated strong, LcrV-specific, serum IgG responses. In addition, the delivery of three successive doses of the vaccine elicited levels of immunity that could protect the mice from a lethal plague challenge. Collectively, these data indicated that the generation of a prototype plague vaccine was successful. However, further studies on this vaccine preparation are warranted. The scope of these analyses did not focus on the mucosal immune responses generated after single or multiple doses of the vaccine. Protection against intranasal inoculation with *Y. pestis* has not been shown to be dependent on mucosal immunity. However, the potential to generate IgA antibody responses in the mucosa would be beneficial in the rational development of vaccines specific to other pathogens such as influenza, herpesviruses, and human immunodeficiency virus [223-225]. Also, although this study did characterize the IgG1 and IgG2a responses to the different vaccine formulations to gain some insight into the CD4 T-cell responses, actual CD4 T-cell responses were not examined because of technical limitations. These experiments may also provide insight on the differences observed between the two different challenge doses. Another important aspect that must be examined with this system is the response to intranasal administration of spore-displayed LcrV formulated with Alhydrogel®. In these studies, the response to this formulation was very poor, and the speculation is that this is due to the unreliability of

administration because of the viscosity of the vaccine preparation. Although Alhydrogel® is the most commonly used adjuvant; it is not the only one available. Currently, novel adjuvants such as double-stranded RNA (polyI:C) and CpG-containing oligodeoxynucleotides are being explored as alternatives [19, 37]. These adjuvants may be better suited than Alhydrogel® for intranasal delivery. The following chapter describes a collection of preliminary investigations into the efficacy of the spore-based LcrV vaccine formulated with CpG-containing oligodeoxynucleotides.

Chapter VI: The Effect of CpG-Containing Oligodeoxynucleotides on the Immune Response to Spore-Displayed Antigens

A. Rationale

As discussed in Chapter III, the avidin and biotin linkage can be used to display heterologous antigens on the surface of *Bacillus* spores. However, this system can also be used to link additional molecules that can modulate the immune responses to spore-displayed antigens. These immune stimulating adjuvants could be used to increase humoral and/or cell-mediated immune responses. In addition, adjuvants may also be used to specifically alter the type of the immune response to the displayed antigen.

Short synthetic CpG-ODN have been extensively studied as molecular adjuvants [226-230]. CpG-ODN molecules are composed of unmethylated, single stranded DNA molecules that contain a cytosine (C) residue followed by a guanine (G) residue. These short synthetic immune responses DNA molecules resemble bacterial DNA that is present during bacterial infections [18]. CpG-ODN are classified as pathogen-associated molecular patterns (PAMPs) that are recognized by toll-like receptors and stimulate innate immune responses. The DNA residues flanking the CpG motif have been shown to dramatically affect the quality of the innate immune responses [231]. All CpG-ODN are recognized by toll-like receptor 9 (TLR9) and act as immune stimulants that boost either antigenic-specific B and T-cell responses [18, 232]. In addition, certain CpG-ODN have been demonstrated to induce T-helper type 1 (TH1) responses when co-administered with vaccine preparations [232].

In previous chapters, mice immunized with spore-displayed antigens have developed antigen-specific serum IgG responses. These data indicate T-helper type 2 (TH2) responses based on high serum levels of antigen-specific IgG1 antibodies compared to IgG2a. The experiments described in this chapter were designed to determine if the immune responses to spore-displayed LcrV can be modified by a CpG-ODN (CpG 1826) that was co-administered with the vaccine preparation, rather than displayed on the spore surface. The data presented below demonstrate that CpG-ODN can significantly increase the LcrV-specific antibody responses following subcutaneous or intranasal administration. Interestingly, although the adjuvant increased antibody responses, it did not alter the ratio of serum IgG1 to IgG2a. This information suggests that the inclusion of CpG-ODN did not affect the quality of the CD4 T-cell responses.

B. Results

1. The addition of CpG-containing oligodeoxynucleotides dramatically increases systemic IgG responses to spore-displayed LcrV

To initially determine if the addition of CpG-ODN had a boosting effect on spore-displayed LcrV, groups of six mice were immunized subcutaneously with $5x10^8$ biotinylated *Bacillus thuringiensis* spores displaying 30 µg of NeutrAvidin-conjugated LcrV antigen formulated with or without 25 µg of a synthetic CpG-ODN (CpG 1826

TCCATGACGTTCCTGACGTT). Two weeks following the immunization, all mice were bled and given a second subcutaneous dose of spore-displayed LcrV again formulated with or without the CpG-ODN. Two weeks following the second dose of the spore-based vaccine, all mice were again bled to determine serum levels of LcrV-specific IgG.

The levels of LcrV-specific IgG in the serum at two and four weeks post-immunization were determined by ELISA. At two weeks post-immunization, mice that received spore-displayed LcrV in the absence of CpG-ODN had serum titers of LcrV-specific IgG that ranged from 60,000 to 640,000 (Figure 27). Mice that had received spore-displayed LcrV formulated with CpG-ODN had significantly higher titers ranging from 2,600,000 to 5,100,000 (Figure 27, $p=0.0002$, Student's t-test).

Similar results were observed two weeks following the second dose of the vaccine formulations. At this time point, both groups of mice had increased levels of LcrV-specific serum IgG antibodies. However, the group of mice that received the CpG-ODN in addition to spore-displayed LcrV again had significantly higher LcrV-specific IgG titers compared to the mice boosted in the absence of the Toll-like receptor ligand ($p=0.031$, Student's t-test). These data demonstrate that the addition of CpG-ODN has a significant adjuvant effect on the responses to the spore-displayed antigen platform.

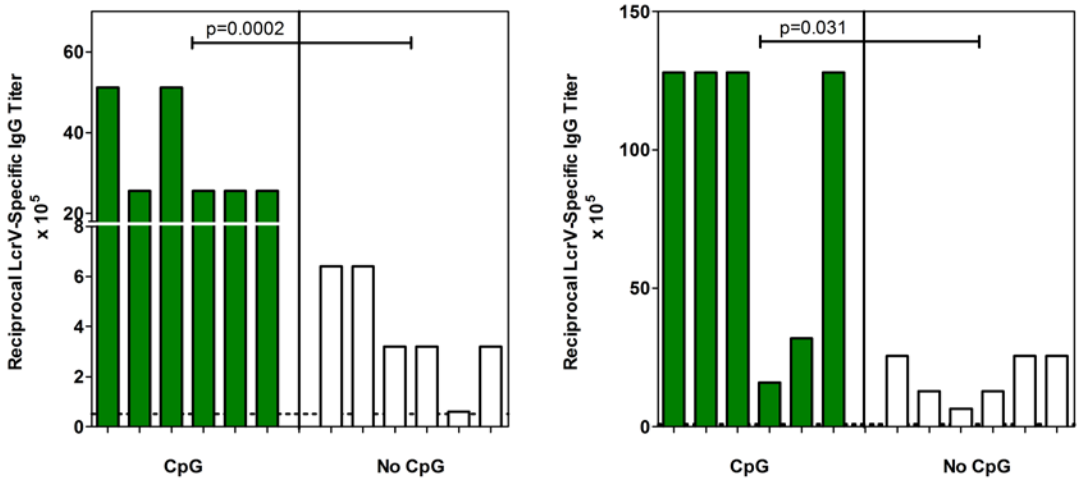

Figure 27. Formulation of spore-displayed CpG-ODN enhances LcrV-specific IgG responses following subcutaneous immunization

Groups of 6 female C57Bl/6 mice were immunized subcutaneously with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying NeutrAvidin-labeled LcrV formulated with (green bars) or without (white bars) 25 µg CpG-ODN. Two weeks following the immunization all mice were bled and serum levels of LcrV-specific total IgG antibodies were determined by ELISA (left panel). All mice were then given a second subcutaneous immunization with the same formulation of vaccine. Two weeks following the second administration all mice were again bled and serum levels of LcrV-specific total IgG antibodies were again determined by ELISA (right panel). Dotted lines indicated the limit of detection (1:500) in each assay.

2. The inclusion of CpG boosts, but does not alter the IgG isotype responses to spore-displayed LcrV

The addition of CpG-ODN to the spore-displayed LcrV had a significant effect on the levels of LcrV-specific IgG in the recipient mice. Because CpG-ODN have also been described to have a TH1-polarizing effect on CD4 T-cell responses, it was also important to determine the effect of CPG-ODN on the T-helper responses in mice immunized with spore-displayed LcrV. As before, the levels of IgG1 and IgG2a were used as indicators of TH1 and TH2 responses respectively. The levels of IgG1 and IgG2a were examined by ELISA in the serum of same mice two weeks following the initial immunization. As shown in Figure 28, at this time point, the addition of CpG-ODN to the vaccine preparation appeared to have a significant, positive effect on the serum levels of both LcrV-specific IgG1 and IgG2a. The mean IgG1 titer in the mice that received the vaccine with CpG-ODN was 16 times higher than the mice that were immunized without the adjuvant, 1,600,000 compared to 100,000 (Figure 28, open and closed circles, p=0.0002, Student's t-test). A similar phenomenon was also observed in the serum levels of LcrV-specific IgG2a antibodies. Five of the six mice immunized with spore-displayed LcrV in the absence of CpG-ODN had detectable IgG2a antibodies two weeks following the initial immunization with a geometric mean titer of 8,700. However when CpG-ODN were included with the vaccine

preparation, the titers were significantly increased to 142,000 (Figure 28, open and closed squares, p=0.004 Student's t-test).

Although the addition of CpG-ODN to the vaccine formulation significantly affected the levels of both IgG1 and IgG2a that were specific for the displayed antigen, the additional adjuvant did not appear to alter the T-helper type responses. In the mice that were immunized with spore-displayed LcrV without CpG-ODN, the mean ratio of IgG1 to IgG2a was determined to be 11.6. Similarly, the mean ratio of IgG1 and IgG2a in mice that were co-administered CpG-ODN with the spore-based vaccine was determined to be 11.3. These data suggest that the addition of CpG-ODN did not significantly affect the type of CD4 T-cell responses elicited by the spores (p=0.4, Student's t-test).

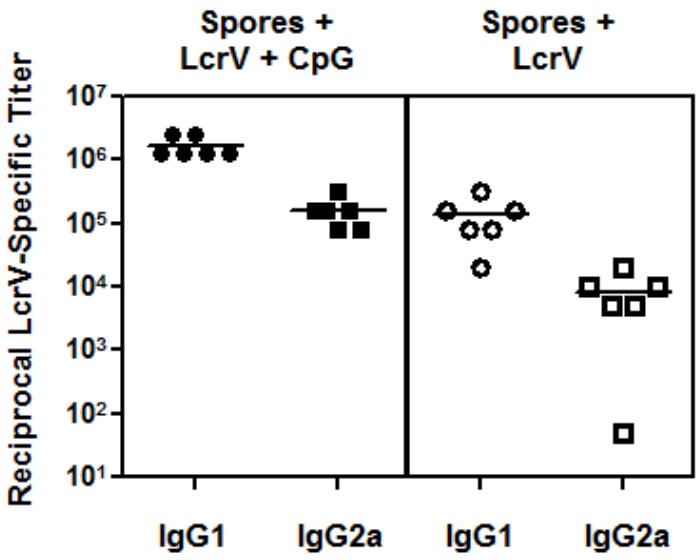

Figure 28. The addition of CpG-ODN to spore-displayed LcrV enhances both antigen-specific IgG1 and IgG2a, but does not alter the ratio of IgG1 to IgG2a.

Groups of 6 female C57BL/6 mice were immunized with spore-displayed LcrV formulated with (filled symbols) or without (open symbols) 25 µg CpG-ODN. Two weeks following the immunization all mice were bled and the titers of LcrV-specific IgG1 (circles) and IgG2a (squares) were determined by ELISA.

3. CpG-ODN co-administration results in detectable levels of LcrV-specific IgG after a single intranasal vaccination that are further increased following subsequent vaccine doses

Collectively, the goal of these experiments was to develop a microparticle vaccine that can be administered intranasally. Because the responses to intranasal vaccines can be drastically different from vaccines administered parenterally, it was important to determine the effect of CpG-ODN when it was co-administered intranasally with spore-displayed LcrV antigen. In these experiments, groups of 6 C57BL/6 mice were immunized intranasally with $5x10^8$ spores displaying 30 µg of NAV-LcrV formulated with or without 25 µg CpG-ODN. Two weeks following the initial dose, all mice were bled and boosted with the same dosage and formulation of the vaccine. Two weeks following the second dose all mice were again bled and serum titers of LcrV-specific IgG were determined by ELISA.

As shown in Figure 29, only one mouse immunized without the addition of CPG-ODN had detectable levels of LcrV-specific IgG antibodies, while five of the six mice that had received the co-administered CPG-ODN had detectable titers of LcrV-specific IgG in their serum. Although these titers were much lower than those observed in mice that received parenteral immunizations, it is important to note that most intranasal vaccines do not elicit detectable IgG responses following a single immunization [216-217]. The data shown in Figure 29 indicate that

two weeks following the second dose of LcrV linked to the surface of spores, both groups of mice displayed increased LcrV-specific IgG titers. As observed in mice that received subcutaneous immunizations, the mice which received the additional CpG-ODN had significantly higher levels LcrV-specific IgG in their serum ($p=0.031$, Student's t-test).

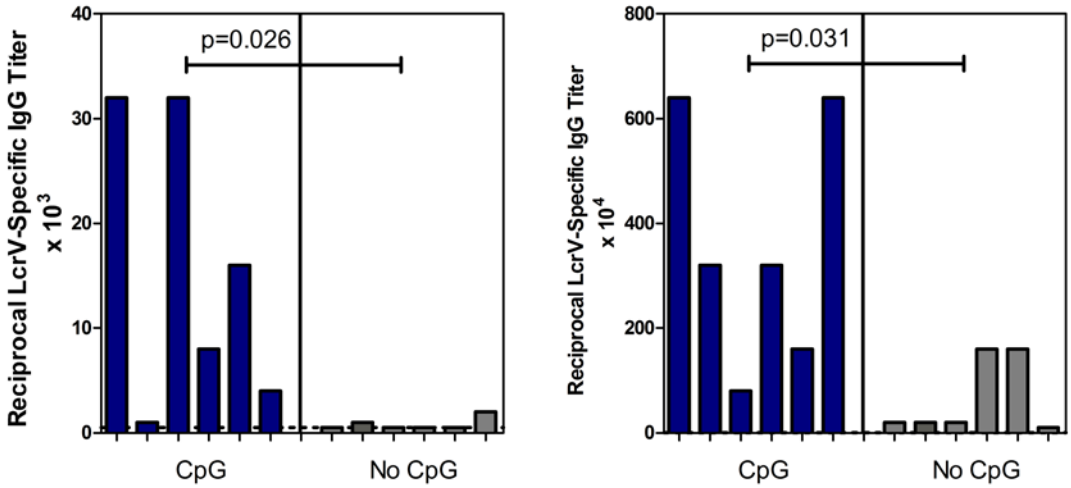

Figure 29. Formulation of spore-displayed CpG-ODN enhances LcrV-specific IgG responses following intranasal immunization

Groups of 6 female C57Bl/6 mice were immunized intranasally with $5x10^8$ UV-irradiated *B. thuringiensis* endospores displaying NeutrAvidin-labeled LcrV formulated with (blue bars) or without (gray bars) 25 µg CpG-ODN. Two weeks following the immunization all mice were bled and serum levels of LcrV-specific total IgG antibodies were determined by ELISA (left panel). All mice were then given a second intranasal immunization with the same formulation of vaccine. Two weeks following the second administration all mice were again bled and serum levels of LcrV-specific total IgG antibodies were again determined by ELISA (right panel). Dotted lines indicated the limit of detection (1:250 and 1:1000) in each assay.

C. Discussion

The purpose of the studies described in this chapter was to evaluate the LcrV-specific antibody responses when mice were immunized with spore-displayed LcrV that was administered in the presence of CpG-ODN. The use of CpG-ODN as an immune stimulant has been thoroughly investigated [226-230]. Klinman *et al.* first reported that synthetic oligodeoxynucleotides that contained sequences similar to those found in bacterial DNA could induce the production of interferon-gamma (IFN-γ) by natural killer cells [233]. Since then, the receptor for CpG-ODN has been identified as TLR9, which is expressed by a variety of cell types, most importantly for this study are both B-cells and dendritic cells [18, 232]. B-cells that are incubated in the presence of CpG-ODN mature, as shown by proliferation and the production of immunoglobulin molecules [18]. Similarly, dendritic cells exposed to CpG-ODN have been shown to upregulate major histocompatibility complex class II (MHC II), CD80, and CD40; all markers of dendritic cell activation. Furthermore, these dendritic cells also secrete interleukins 12 and 6 (IL-12, IL-6) and TNF-α [17]. The production of the IL-12 cytokine also suggests that exposure of CpG-ODN may lead to enhanced TH1-type CD4 T-cells responses.

Previously, the formulation of parenteral vaccine preparations with CpG-ODN resulted in significant increases in serum IgG responses [19]. The results of these experiments demonstrated that the addition of the CpG-ODN had a dramatic affect on the total LcrV-specific IgG antibody responses when

administered systemically. The increased antibody levels were observed at both two and four weeks post immunization (Figure 27). This phenomenon was likely due to increased B-cell proliferation because of CpG-ODN as described by Krieg *et al.* [18]. The increased maturation of professional antigen presenting cells following CpG-ODN encounters may also have contributed the activation of B-cells.

A key factor in the choice of the CpG-ODN used in this study (CpG-ODN 1826) was the ability of this molecule to induce strong TH1-type CD4 T-cell responses. It has been well documented that the addition of certain CpG-ODN to vaccine preparations induces a significant shift from IgG1 to IgG2a [231]. The increased production of TH1-type cytokines such as IFN-γ and IL-12 along with low levels of type 1 interferons have been implicated in this shift [234]. As in Chapters III and V, the ratios of IgG1 and IgG2a were used as indicators to gain some insight into the CD4 T-cell responses to the spore-based vaccines. Interestingly, although there was a significant increase in the levels of LcrV-specific IgG following CpG-ODN co-administration, no difference in the ratios of IgG1 to IgG2a were observed. The levels of these cytokines were not examined in these studies. It is possible that Bacillus endospores induce such vigorous TH2-type CD4 T-cell responses, that the amount and type of CpG-ODN that was used in this study was unable to overcome the effect of the spores.

CpG-ODN have also been used previously to adjuvant mucosal vaccines. The inclusion of CpG-ODN with an intranasal influenza vaccine was shown to

increase flu-specific antibody levels in the serum, genital tract, and saliva. The addition of CpG-ODN has also been demonstrated to increase antigen-specific IgA titers throughout the mucosa [235]. An increase in antigen-specific IgG titers was also observed in this study, in which the co-administration of CpG-ODN with intranasal immunizations of spore-displayed LcrV significantly increased the levels of LcrV-specific IgG titers. This increase was evident after a single immunization, with detectable levels of LcrV-specific IgG in all but one of the mice (Figure 29). Many vaccines, including the spore-displayed LcrV described in this study, require multiple or parenteral boosts to yield detectable levels of IgG antibodies when given intranasally [216].

It is important to note, that the CpG-ODN used in these experiments was not linked to the spores. These experiments were carried out to determine the effects of CpG-ODN on the spore-based vaccine platform. The data obtained from this study suggest that the inclusion of CpG-ODN with spore-based immunizations significantly increased the antigen-specific immune responses. These results are substantially different than that observed with intranasal immunizations with Alhydrogel® in Chapter V, in which the adjuvant had a significant negative impact on the antigen-specific immune responses. Therefore, further experiments should be conducted to not only determine the optimal method of conjugating the CpG-ODN to the spore, but also the immunological implications of linking the molecules to the spore.

Chapter VII: Conclusions and Future Directions

A. The Spore-Displayed Antigen System Compared to Recombinant Spores as Vaccine Vehicles

Collectively, the data presented in this dissertation suggest that successful vaccines can be developed using the spore-displayed antigen system. Bacterial spores are extremely stable, yet biodegradable microparticles. Because they are microbial agents, bacterial endospores are strong inducers of innate immune responses. These attributes have led to the rationale that spores can act as microparticle vaccine platforms that provide inherent adjuvant properties [62, 107]. In addition, spores from some Bacillus species have been shown specifically target professional antigen presenting cells [137, 175], delivering the vaccine directly to the cells that initiate immune responses. It has also been demonstrated that Bacillus spores can be used to deliver antigens into the cytoplasm of antigen presenting cells, therefore enhancing antigen cross presentation [163]. Moreover, several species of Bacillus are considered apathogenic and have been used as a safe alternative to chemical pesticides Moreover, *B. subtilis* spores are marketed as probiotics in some countries. There have also been a number of previous studies utilizing recombinant Bacillus spores that express vaccine antigens on the spore surface as candidate vaccines. [62, 103-107, 111, 113-114, 133-134, 163, 236].

Although recombinant spores have been used successfully to produce multiple vaccine candidates, they have their limitations. All of these systems require a certain degree of the technical expertise to generate the recombinant Bacillus bacteria. Because plasmids are usually not stable in transformed

bacterial cells, integration into the genome is required for stable maintenance of the foreign gene. Next, the recombinant spore vaccine systems are limited to the subset of antigens that can be efficiently expressed by the Bacillus cells, and many viral or eukaryotic antigens are not efficiently expressed by prokaryotes. In addition, only protein antigens can be displayed on the surface of the recombinant bacterial spores, which narrows the repertoire of the target antigens. Furthermore, because antigens are expressed in the bacterial cell, some eukaryotic cell-specific post-translational modifications that may be critical for proper recognition by the immune system are not correctly applied to the antigen [120]. Finally, expression of certain eukaryotic antigens in bacteria may affect the three-dimensional structure of the proteins, which in turn may affect the recognition of the vaccine by the immune system.

The chemical linkage of molecules to the surface of Bacillus spores could provide some solutions to the shortfalls of recombinant spore-based vaccines. The spore-based vaccine platform described in this dissertation can display molecules from any source on the spore surface. This method increases the repertoire of molecules available for use as vaccine candidate antigens. Furthermore, the ability to obtain antigens from a variety of recombinant systems ensures that the antigen will have the appropriate three-dimensional structure and post-translational modifications for recognition by the immune system. Also, due to its modular nature, this novel system allows for the generation of mono or polyvalent vaccines. In addition to linking multiple antigens, the modular nature

of this system also has the possibility to co-display adjuvant molecules that are able to locally increase or modify immune responses to the displayed antigens.

Whereas recombinant spore vaccine systems are limited to protein antigens that can be expressed by Bacillus bacteria, the data presented here demonstrate that this system can be used to quickly and efficiently label biotinylated *Bacillus thuringiensis* spores with virtually any streptavidin-linked molecule (Figures 4, 5, and 6). The data obtained in these studies also demonstrate that streptavidin-linked small molecules such as fluorochromes can be displayed on the spore surface, suggesting that the spore-displayed antigen platform may have some applications as a method to display and deliver haptens. Although the results obtained during the course of this research suggest that immune responses can be raised against heterologous molecules on the spore surface, further experiments are required to determine the efficacy of this platform as a carrier for haptens.

In addition to small molecules, these data suggest that streptavidin-linked antigens from any source, commercial or recombinant, can be displayed on the spore surface, which allows for antigens to retain the correct post-translational modifications along and their native conformations. This evidence is shown in Figure 6, in which the enzymes β-galactosidase and horseradish peroxidase retained their enzymatic activities, and therefore, their native conformations when displayed on the spore surface. In addition, purified commercial hen egg ovalbumin and purified, recombinant low calcium response V antigen (LcrV) from

Yersinia pestis were successfully linked to NeutrAvidin and displayed on the surface of biotinylated *B. thuringiensis* spores (Figures 5 and 18).

In addition to the repertoire of antigens, the amount of antigen displayed on the surface of the spores using streptavidin and biotin is remarkably different when compared to recombinant spore systems. Data obtained from titration studies with streptavidin-linked fluorescein isothiocyanate (SAV-FITC) show that streptavidin-linked molecules can be displayed at much higher densities than recombinant spore-based systems. Studies by Isticato *et al.* demonstrated that approximately 1,500 molecules of antigen can be expressed and displayed on a spore using a recombinant spore vaccine system [104]. The experiments presented here demonstrate that biotinylated spores can display approximately 686,000 molecules of SAV-FITC per spore. However, this dissertation did not perform a direct comparison of the display capacities of the two different systems. Therefore, further experiments should be conducted using recombinant *B. thuringiensis* spores that express a molecule that can be quantitated and biotinylated *B. thuringiensis* spores that display the same indicator molecule linked to streptavidin. This experiment would effectively determine which system has a better capacity for antigen display.

Another promising aspect of the spore-displayed antigen system is the ability to generate polyvalent vaccines with this platform. Two separate experiments were conducted during the course of these studies to establish that this system can support divalent antigen display. This was first shown in Figure 4 using the streptavidin-fluorochromes FITC and phycoerythrin-cyanin5 (PE-Cy5). Two

different divalent formulations were demonstrated. First, spores that were individually labeled with SAV-FITC and SAV-PE-Cy5 were pooled, each population retaining its molecular label, and second, a single population of spores was produced that was labeled simultaneously with both fluorochromes. Either system could be used to generate a bivalent vaccine. The divalent display was also demonstrated in Figure 6 with both streptavidin-linked β-galactosidase and horseradish peroxidase linked to the same population of spores. Although the prospect of developing multivalent vaccines is presented in this work, the ability of spores to elicit potent immune responses against multiple displayed antigens was not investigated here and requires further study. To this end, it may also be more beneficial to mix populations of spores displaying a single antigen to create the multivalent vaccine. Again, although the ability to create vaccines in this manner has been established here, further studies are needed to determine if vaccines generated in this manner are sufficiently immunogenic, and which method is most efficient to elicit the necessary immune responses for protection against a particular pathogen.

B. Future Improvements to the Spore-Displayed Antigen System

Although there are many benefits of this novel spore-displayed antigen system, it does have some disadvantages that may be overcome with further investigations. The primary drawback to this system is the use of biotin and streptavidin to display molecules on the surface. Biotin and streptavidin were chosen due to the well documented use of these molecules to link two different proteins together. However, streptavidin is a large molecule of approximately 53

kDa and contains some repetitive sequences which make it highly immunogenic. This has been shown to result in much higher antibody titers to the linker molecule than to the intended antigen (Figure 9). This may result in antigen competition in which the more dominant streptavidin overpowers the less dominant vaccine antigen. A number of other chemical linkages have been reported that are designed to crosslink two proteins together [237-241]. The use of small molecule linkers, which are discussed below, may circumvent this issue in the event that the streptavidin molecule is found to be a dominant antigen of the vaccine platform.

Another major consideration of this system is the placement of the linker molecules on the target antigen so that it does not interrupt critical epitopes. The maleimide activated streptavidin used here only modifies free sulfhydryl groups on amino acids. Because most of the proteins used in this study did not contain sulfhydryls, this moiety was added to free primary amine groups which occur at the amino terminus of the protein and on internal lysine residues. This process gives very little control over the location of the linker molecule on the protein, and if a lysine residue were to occur in a dominant or neutralizing epitope, it is likely this could disrupt the sequence and render the vaccine less effective.

The problems associated with the biotin and streptavidin system described above suggest that further investigations need to be carried out to identify a more appropriate method to link antigens to the surface of the spores. A number of small heterobifunctional linking molecules have been identified that may provide alternatives to biotin and streptavidin. The first alternative is a 1-Ethyl-3-[3-

dimethylaminopropyl] carbodiimide hydrochloride (EDC) which is a zero-length linker that would crosslink carboxy groups of exosporium proteins to primary amine groups on target antigens [242]. Another potential crosslinking molecule is succinimidyl-4-[N-Maleimidomethyl] cyclohexane-1-carboxy-[6-amidocaproate] (LC-SMCC) [243]. LC-SMCC is a long molecule that would crosslink primary amine groups on the exosporium proteins to a sulfhydryl group on the target antigen. LC-SMCC may provide some advantages over EDC due, in part, to its length which would allow for additional flexibility of the target antigen and make it more accessible to immune cells. Also, LC-SMCC could potentially allow for more control over the crosslinking location. Recombinant antigens could be designed in such a way that sulfhydryl-containing cysteine residues are located away from known dominant or neutralizing epitopes. This would help to prevent the disruption of critical epitopes. As neither of these linkers has been examined with this system, further experiments are necessary to determine both the densities of spore-displayed antigens achievable with these linkers and the effect of these linkers on the immunogenicity of the heterologous antigens.

Another potential limitation to the spore-displayed antigen system is the use of spores produced by *B. thuringiensis.* Previous spore-based vaccine studies used *B. subtilis* spores because this bacterium is widely accepted as a human probiotic and the well documented strategies for the cloning and expression of proteins. *B. thuringiensis* spores were chosen for this study because like *B. subtilis,* these bacteria are considered safe for humans. In addition, spores from *B. thuringiensis* contain an exosporium layer, the loose balloon-like structure that

surrounds the spores with a hair-like nap. It was postulated that this hair-like nap would increase the surface area of the spores. This increased surface area would allow for higher numbers of molecules to be displayed on the spores.

Although exosporium-containing spores were thought to be the ideal choice for use in the spore-displayed antigen system, the exosporium has been previously demonstrated to limit innate immune responses to spores from the closely related bacteria *B. anthracis* [194]. In this study, murine macrophages were co-incubated with wild-type or exosporium deficient spores from *B. anthracis*. Following the incubation, the levels of interferon-beta (IFN-β), IL-1β, TNF-α, and IL-6 mRNA were quantitated by real time PCR. Following the one hour exposure, the murine macrophages exposed to the exosporium deficient spores produced significantly higher levels of all four cytokines, suggesting that the exosporium may indeed have immunosuppressive properties [194]. It is impossible from the data presented in this dissertation to determine if either *B. thuringiensis* or *B. subtilis* spores are best suited to deliver antigens as a vaccine. Therefore, it will be important to assess the potential influence of both the exosporium and the species of spore on the immunogenicity of spore-displayed antigens.

Although this study demonstrates that immune responses are generated against the surface-displayed antigen, strong responses were also observed against both the spores and streptavidin. Strong pre-existing immune responses against a carrier molecule have been shown to reduce the efficacy of subsequent immunizations. These strong carrier-specific immune responses may also lead

to significantly decreased responses to the vaccine antigen. The data presented here demonstrate that the antibody responses to the target antigen can be boosted with subsequent immunizations with the same vaccine platform which suggest that this phenomenon may not occur. However, experiments need to be conducted in which one group of mice is pre-immunized with biotinylated *B. thuringiensis* spores, while a second is treated only with the vehicle, such as saline. Then both groups would be immunized with a spore-displayed antigen. The antigen specific titers can then be compared after the second immunization in order to determine if pre-existing immunity would reduce the efficacy of subsequent vaccinations. Similar experiments also need to be conducted to examine the effect of pre-existing immunity to streptavidin on subsequent immune responses.

C. Innate immune responses to *B. thuringiensis* spores compared to spores from other Bacilli

The data presented in Chapter IV demonstrate that *B. thuringiensis* spores are potent inducers of innate immune responses by murine bone marrow-derived dendritic cells (BMDCs). Immature BMDCs rapidly phagocytosed spores, and exposure to UV-irradiated spores resulted in decreased phagocytic activity, as well as a significant increase in the cell surface expression of MHC II and costimulatory molecules. UV-irradiated spores also increased the survival of BMDCs *in vitro*. Collectively, these data suggest that spores are capable of initiating dendritic cell maturation.

These data are the first to evaluate the innate immune responses to *B. thuringiensis*, however some investigation has been done into the responses of innate immune cells following exposure to *B. anthracis* spores due to its implication for human anthrax. The innate immune responses to *B. anthracis* spores have been examined using different cell lines and primary cells from both mice and humans. The results of each of these studies have established a pattern of cytokines that are expressed by inflammatory cells following exposure to germination competent *B. anthracis* spores. Murine macrophages and dendritic cells have been demonstrated to express TNF-α, IL-1β, and IL-6 following *in vitro* spore exposure [152]. In addition, it has been shown that spore-exposed murine splenocytes also produce IL-12 and IFN-γ *in vitro* [244]. Similarly, splenocytes from mice infected with *B. anthracis* also produce IL-6, IL-1β, TNF-α, as well as IL-12 [244].

Although the experiments performed in this study used UV-inactivated *B. thuringiensis* spores that were incapable of germination, the cytokine responses observed were quite similar to those observed in the studies using *B. anthracis* spores described above. Spore-stimulated murine bone marrow-derived dendritic cells produced TNF-α, IL-6, IL-12p70, and interleukin-10 (IL-10). Additionally, BMDCs also produced nitric oxide in response to UV-inactivated spore exposure. The production of nitric oxide indicates the presence of active inducible nitric oxide synthase (iNOS). The expression of iNOS by murine BMDCs requires the presence of IFN-γ, therefore these data suggest that UV-inactivated spores are also inducing the expression of IFN-γ cytokine as no

exogenous IFN-γ was provided [245]. Further experiments are currently underway to directly compare the cytokine responses of dendritic cells to spores from *B. thuringiensis* to those elicited by *B. anthracis* and *B. subtilis.*

The most notable difference between the germination-competent *B. anthracis* and the studies conducted here with UV-inactivated *B. thuringiensis* was that IL-1β was absent in the spore-stimulated BMDC samples assessed in this study. However, the potential for murine BMDCs to produce IL-1β was confirmed by stimulation with lipopolysaccharide. The primary function of IL-1β is to initiate the release of inflammatory cytokines such as IL-6 and TNF-α as well the acute phase response. As discussed earlier, IL-1β is produced as a pro-protein in response to cell stimulation with pathogen-associated molecular patterns. Although the IL-1β pro-protein is expressed in response to microbial exposure, the release mature of IL-1β from the cell is controlled by additional cell signaling pathways. The IL-1β pro-protein must first be cleaved by active caspase-1 enzyme. Caspase-1 is a member of the prototypical NOD-like receptor inflammasome, a multiprotein complex that responds to extracellular stimuli such as flagellin, LPS, silica and alum. In the absence of these secondary signals, caspase-1 is inactive and unable to cleave the IL-1β pro-protein [246]. Although, the exact mechanism of IL-1β secretion is not yet elucidated, in the presence of these additional signals, caspase-1 becomes activated and able to cleave the IL-1β pro-protein for release. The current hypothesis from this work is that exposure to UV-irradiated spores is sufficient to induce the expression of IL-1β pro-protein, but does not provide the additional stimuli to activate the

inflammasome which is responsible for the cleavage and release of mature IL-1β protein. Further studies are currently being conducted to elucidate the mechanisms and requirements necessary for murine BMDCs to produce mature IL-1β following exposure to UV-irradiated spores.

D. Implications of the innate immune responses on spore-based vaccine design

One of the reasons for conducting the studies in Chapter IV was to elucidate the innate immune responses induced by *B. thuringiensis* spores, so that these responses may be further modulated using additional heterologous adjuvants. The results of these experiments indicate that UV-irradiated *B. thuringiensis* spores induce strong inflammatory responses that may be beneficial in promoting vaccine-specific immune responses. They also suggest that the UV-irradiated spores induce the production of cytokines involved in the development of both antigen-specific B and T-cell responses.

The data demonstrating that the spores induce the upregulation of MHCII, CD80, and CD86 along with the effect of spore exposure on phagocytosis indicate that the spores efficiently induce the maturation of BMDCs. In addition, the data imply that spores prolong the life with BMDCs in culture. This maturation and prolonged survival of dendritic cells following spore exposure suggest that the presence of UV-inactivated *B. thuringiensis* spores may enhance the presentation of antigenic peptides to T-cells. This phenomenon has been observed in other studies using *B. subtilis*-based vaccine platforms [116]. Further experiments need to be conducted using *B. thuringiensis* spore-

stimulated BMDCs to determine if spore exposure is able to improve antigen presentation to CD4 T-cells. Also, to determine if spores are able to enhance cross presentation of heterologous antigens to CD8 T-cells the cell surface expression of major histocompatibility class I (MHC I) molecules should be investigated to determine if spore exposure results in increased levels of MHC I. Furthermore, cross presentation assays with spore-exposed BMDCs should also be performed with a well characterized antigen such as chicken ovalbumin (OVA) to determine if spore-exposure results in enhanced presentation of antigenic peptides to CD8 T-cells.

In addition to the maturation of murine BMDCs, exposure to UV-irradiated *B. thuringiensis* spores also induced the upregulation of proinflammatory cytokine expression and iNOS. The expression of the pro-inflammatory cytokines TNF-α and IL-6 suggests that the spores are capable of initiating strong innate immune responses, which will consequently lead to the recruitment of additional inflammatory and antigen presenting cells [247]. This type of environment is well suited to vaccine administration due to the presence of multiple cell populations that are capable of potentiating immune responses to heterologous antigens. For example, IL-6 is well characterized as a potent B-cell stimulator, which in part, explains the high titer antibody responses to the spore-displayed antigens β-galactosidase and LcrV [248].

The presence of IL-12 and nitric oxide in spore-stimulated BMDC cultures suggests that the spores are also capable of generating T-helper 1 (TH1)-type CD4 T-cell responses to the displayed antigens. IL-12 has been well

characterized in the establishment of TH1 CD4 T-cell responses and as a growth factor for cytotoxic CD8 T-cells. Indications of a mixed TH1/TH2 type of response were observed in both the LcrV and β-galactosidase-specific IgG1 and IgG2a titers. Although CD4 T-cell responses were not examined in this study, the ratio of IgG1 to IgG2a has been used previously as an indication of the type of helper T-cell responses [249]. High antigen-specific IgG1 titers typically indicate a TH2-biased response, while high IgG2a titers suggest stronger TH1 responses. When the ratios of IgG1 to IgG2a were assessed in mice immunized with spore-displayed LcrV or spore-displayed β-galactosidase, higher serum levels of IgG2a were observed in responses to spore displayed antigens when compared to soluble antigens in the absence of spores, suggesting a shift towards a balanced TH1/TH2-type CD4 T-cell response.

The anti-inflammatory cytokine IL-10 was also observed in spore-stimulated BMDC cultures. IL-10 has been demonstrated to be a negative regulator of T-cell responses and has been shown to reduce the antigen presentation capacity of dendritic cells [250-252]. The presence of IL-10 in these spore-stimulated cultures may be due to the expression of TNF-α following toll-like receptor activation as demonstrated in other innate immune studies [253]. The expression of IL-10 may have some negative impacts on the efficacy of antiviral vaccines due to the ability of IL-10 to suppress CD8 T-cell responses [254]. Further studies should be carried out to first elucidate the effect of IL-10 on vaccine-specific responses as well as determine if the IL-10 response can be modulated

with the addition of heterologous adjuvants such as polyinosinic:polycytidylic acid (polyI:C) which has been shown to diminish IL-10 responses [255].

E. Antigen-specific immune responses to spore-displayed antigens

The ultimate goal of this dissertation was to develop a self-adjuvanted, spore-based microparticle vaccine platform that was able to elicit high-titer antibody responses specific to heterologous antigens displayed on the spore surface. Previous experiments with recombinant *B. subtilis* spores have demonstrated that antigens expressed on the spore surfaces are immunogenic [124, 256]. Collectively, the data presented in this document suggest that high titer antibody responses can be raised against heterologous spore-displayed antigens following either subcutaneous or intranasal immunization.

Initial “proof of concept” experiments were conducted using streptavidin-linked β-galactosidase displayed on the surface of biotinylated, UV-inactivated *B. thuringiensis* spores. In these experiments, the titers of β-galactosidase-specific serum IgG were quantified in mice that were immunized subcutaneously with soluble, streptavidin-linked β-galactosidase in PBS or the protein displayed on the surface of biotinylated spores. As an additional control, mice were also immunized with the soluble protein mixed with non-biotinylated spores, as the presence of *B. subtilis* spores have been demonstrated to enhance immune responses to co-administered antigens [116]. The results of these experiments demonstrate that immunization with spore-displayed β-galactosidase resulted in significantly higher titers than immunization with protein alone or the protein simply mixed with spores (Figure 8). These data suggest that the spore-

displayed antigen platform was the most efficient method examined for exposing antigens to the immune system and eliciting antibody responses. In addition, they confirm what has been observed by others which demonstrate that high titer antibody responses can be generated by displaying antigens on the surface of Bacillus spores [104]. However, this is the first use of *B. thuringiensis* spores for this application as all of the previous attempts at spore-based antigen display systems have used recombinant *B. subtilis or B. anthracis* spores as microparticles [103-105].

To further examine the immunogenicity of spore-displayed antigens using a pathogen-derived antigen, a series of experiments were conducted using recombinant low calcium response V antigen (LcrV), a well characterized antigen derived from *Yersinia pestis* [201, 203, 206]. A series of preliminary immunogenicity experiments were conducted to ensure that detectable levels of LcrV-specific IgG were detectable following one or two subcutaneous immunizations of spore-displayed LcrV. The results of these initial studies indicated that spore-displayed LcrV induced high titer LcrV-specific serum IgGs that were boosted 10-fold with a second immunization two weeks later (Figure 19). Furthermore, in pilot experiments, two intranasal immunizations with spore-displayed LcrV elicited LcrV-specific IgG responses in the serum of vaccinated mice (Figure 23). These results were expected as LcrV has been shown previously to be a highly immunogenic protein [201, 203, 206]. Furthermore, the results from the experiments with β-galactosidase suggested that the spore-

display of heterologous antigens significantly increases antigen-specific IgG responses.

Once it had been established that spore-displayed LcrV was immunogenic, further analyses were carried out to compare the titers of mice intranasally immunized with spore-displayed LcrV to those of mice immunized intranasally with soluble recombinant purified LcrV. In addition, both vaccines were formulated with the adjuvant Alhydrogel® which has previously been used in LcrV vaccine studies. Interestingly, after three intranasal doses of vaccine, there were no significant differences between any of the groups which had received spore-displayed LcrV and soluble LcrV protein, with or without Alhydrogel® ($p=0.83$, one-way ANOVA). These data indicate that spore-displayed LcrV was as efficient as more conventional vaccine formulations in the induction of LcrV-specific antibody responses.

The results described above were not expected. As demonstrated in Chapter III, the display of β-galactosidase on the surface of biotinylated spores resulted in a significant increase in serum IgG titers compared to protein alone. In addition, the formulation of soluble LcrV protein with Alhydrogel® has previously been shown to increase LcrV-specific serum IgG titers compared to immunization with soluble protein alone [257]. LcrV has been characterized as a highly immunogenic protein, and many of the studies utilizing LcrV as a vaccine antigen have used very small quantities of soluble protein [257]. One possible explanation of the results observed in these experiments is that the dosage of antigen administered in these experiments resulted in maximal IgG responses.

This hypothesis is reinforced by the results obtained from mice immunized with soluble LcrV formulated with Alhydrogel®. The mean IgG response in the serum of these mice was no different than mice which had received LcrV protein alone. The addition of Alhydrogel® has been shown to boost the antibody responses to LcrV as well as other antigens [13, 257]. Alhydrogel® did not increase immune responses to the dose of LcrV used in this study which suggests that no further increases in serum IgG were possible. Further studies need to be conducted in order to first, identify the optimal dose which will allow for the observation of the adjuvant effect of both Alhydrogel® as well as the spores and second, to further characterize the effect of spore display on the immunogenicity of LcrV at this optimal dosage.

To extend the knowledge of immune responses against spore-displayed antigens, the serum IgG1 and IgG2a isotype responses specific for both β-galactosidase and LcrV were also characterized. In both cases the immune responses were dominated by IgG1 antibodies. With the LcrV antigen, this response was observed following either subcutaneous or intranasal administration. IgG1 responses are typically observed with non-replicating vaccines. However, mice which had received spore-displayed antigens displayed significantly higher IgG2a titers compared to mice who received soluble antigens, which indicates an increased TH1 type CD4 T-cell response (Figure 8 and Figure 24). This increase in antigen-specific IgG2a has been described previously with other spore-based vaccines [107, 116]. The increase in IgG2a has been attributed to an increase in a TH1-type CD4 responses which

may result from the activation of pathogen recognition receptors (PRR) on dendritic cell subsets that can affect TH1 or TH2 CD4 T-cell development [116]. PRR activation has also been alluded to in this dissertation in which murine BMDC's that were coincubated with spores produced IL-12p70 and iNOS which suggests the presence of IFN-γ (Figure 14). The presence of these cytokines suggests that spore-stimulated dendritic cells may be able to instruct TH1 CD4 responses

As described above, the linkage system used to display molecules on the surface of spores also allows for additional molecules to be linked to spores in order to further modulate immune responses to spore-displayed antigens. Preliminary studies were conducted here to assess the ability of CpG-containing oligodeoxynucleotides (CpG-ODN) to affect immune responses to spore-displayed LcrV. In these experiments, the co-administration of CpG-ODN, significantly increased the levels of LcrV-specific serum IgG responses following either subcutaneous or intranasal immunizations. However, the addition of CpG-ODN did not affect the IgG1 to IgG2a ratio. This result was not expected as the addition of this CpG-ODN (1826) has been demonstrated previously to enhance IgG2a responses when used as a molecular adjuvant to hepatitis B surface antigen in Balb/c mice [258-259]. Moreover, this CpG-ODN has also been demonstrated to increase IFN-γ production when co-administered to C57BL/6 mice with tetanus toxin-containing microspheres, both data suffest the capacity of the CpG-ODN to induce a TH1 shift [64]. There are several possible explanations for why this phenomenon was not observed in the current study.

First, the spores potentially contain a number of different PRR ligands that are able to potently instruct a mixed TH1/TH2 CD4 T-cell response. These signals may have outcompeted the CpG-ODN signals which would have induced a TH1 biased CD4 T-cell response. The second potential explanation for this lack of IgG2a shift is that the dose of antigen was too high. Previous studies by Ruedl *et al.* have demonstrated that high doses of antigen favor TH2 responses [260].

Additional experiments should be conducted to further understand the effects of this and other CpG-ODN; such as CpG-ODN 1980 which has also been demonstrated to induce TH1 responses, on the immunogenicity of spore-displayed antigens. Another CpG-ODN (CpG-ODN 2006) has been used previously with plague vaccines. This CpG not only increased the LcrV and F1-specific IgG responses, it was also shown to favor the IgG2a isotype, indicating a TH1 shift in CD4 responses [19].

The experiments described here did not investigate the consequences of linking the CpG-ODN molecules to the spore surface; instead they only assessed the effects when they were co-immunized. Therefore, additional experiments should also be conducted to determine the outcome of physically linking the adjuvant to the spore.

Although this dissertation thoroughly characterized the innate immune responses to spores and the antibody responses to the spores and spore-displayed antigens, further studies need to be carried out to fully realize the capacity of this system to induce antigen-specific immune responses. This study did not assess the CD4 T-cell responses to either the spores, or spore-displayed

antigens. These studies can and should be conducted using antigens with well described MHC II epitopes such as chicken ovalbumin (OVA). In addition, the co-administration of *B. subtilis* spores has been suggested to enhance the cross priming of CD8 T-cells to soluble antigens [116]. This data combined with the mixed TH1/TH2 CD4 T-cell responses suggest that spore display is a possible solution to the inability of non-replicating vaccines to induce strong CD8 cytotoxic T-lymphocyte (CTL) responses. Here again, the well characterized T-cell antigen, OVA may be used to assess the ability of the spore-displayed antigen system to generate antigen specific CTL responses.

F. Protective efficacy of spore-displayed antigens

In this dissertation, intranasal immunizations with spore-displayed LcrV were used in challenge studies to determine the protective efficacy of the spore-displayed antigen platform. Pneumonic infection with *Yersinia pestis* has previously been demonstrated to be lethal to C57BL/6 mice [261]. Some monoclonal and polyclonal LcrV-specific antibodies have also been demonstrated to protect against lethal intranasal plague infections [132]. Furthermore, *in* vitro analyses in this dissertation suggested that sera from mice immunized with spore-displayed LcrV were able to at least partially neutralize *Yersinia pestis* bacteria (Figure 21). Therefore, it was anticipated that immunizations with spore-displayed LcrV would protect the recipients from an intranasal plague challenge.

The challenge data shown in Figure 25 and Figure 26 indicate that following the intranasal challenge with either 30 or 250 LD_{50} of *Yersinia pestis,* mice that

were immunized with spore-displayed LcrV were protected when compared to sham immunized mice or control mice immunized with spores alone (p=0.04 and p=0.03 respectively, Mantel-Cox). These results suggest that vaccination with spore-displayed antigens does result in immune responses that can protect the recipient. However, there were no significant differences in the protection of mice vaccinated with spore-displayed LcrV compared to the previously described vaccine preparations of soluble LcrV with Alhydrogel® or soluble LcrV alone [206]. This result may be explained by the similar serum levels of LcrV-specific IgG antibodies demonstrated in each group of mice two weeks after the third intranasal immunization.

Surprising results were obtained from mice that were challenged with spore-displayed LcrV formulated with 1% Alhydrogel®. When challenged with 250 LD_{50} of *Yersinia pestis,* there were no significant differences between this group of mice and the other groups immunized with spore-displayed LcrV, LcrV alone, or LcrV formulated with Alhydrogel®, suggesting that the vaccine did elicit protective immunity (Figure 26). However, when this group of mice was challenged with 30 LD_{50} of bacteria, none of the mice from this group survived. One potential explanation for these data, as discussed earlier, is the unreliability of intranasal administration of this extremely viscous vaccine formulation. One possible solution to this issue would be to utilize a soluble adjuvant such as CpG-ODN 1826 which has been demonstrated in this dissertation to improve total IgG responses to spore-displayed antigens following intranasal administration.

Another surprising result was a lack of correlation between IgG and IgG1 titers and survival. Several previous studies using LcrV and LcrV-F1 fusion vaccines to protect against plague challenge have related the levels of these antibodies to the survival of mice following subcutaneous or intranasal *Yersinia pestis* challenge [33, 201, 206]. It has also been shown however, that not all antibodies specific for LcrV are neutralizing [132]. It may be possible that the methods of immunization used in this study did not generate sufficient titers of neutralizing epitope-specific antibodies to protect against the stringent challenge doses used in these experiments. Furthermore, other qualities of the immune response may have a role in the response to intranasal plague challenge, such as CD4 T-cell responses or mucosal IgA antibodies. LcrV contains several MHC II epitopes that have been recently characterized [37]. It is possible that these epitopes may be used to further investigate the quality of the CD4 response generated by spore-displayed LcrV compared to protein alone or formulated with Alhydrogel®. In addition, the IgG and IgG1 antibody responses to LcrV may also be further characterized using peptides corresponding to neutralizing and non-neutralizing epitopes of the LcrV protein [262].

G. Future applications of the spore-displayed antigen system

Although this dissertation describes the use of the spore-displayed antigen system as a monovalent vaccine platform, the potential to develop multivalent vaccines was alluded to in Chapter III. These data demonstrated that two small molecules can be displayed on the surface of a single spore, or that two different populations of individually labeled spores can be mixed, creating a divalent

vaccine. Furthermore, it was also demonstrated by enzymatic assays that a single population of spores can display two different, biologically active antigens. PLGA microparticles have been previously used as multivalent vaccines against tetanus and diphtheria with some success [263]. Further experiments do need to be conducted in order to determine the immunogenicity of co-displayed antigens. Because some antigens are more immunogenic than others, further experiments need to be conducted to determine if immunodominance is a potential problem. In the event it does affect the outcome of immunizations with spores displaying multiple antigens, experiments should be conducted to optimize the ratios of the two antigens on the spore surface to ensure that the response to the dominant antigen does not overwhelm the response to the subdominant antigen.

The nature of the spore-displayed antigen systems have also been shown to allow for the display of small molecules linked to streptavidin. Microparticles have previously shown to efficiently present haptens to the immune system [264]. Therefore, there is potential utility in the spore-displayed antigen system for the delivery of haptens as vaccines. This hypothesis could be effectively tested by using commercially available streptavidin-linked FITC. In addition, other linkage chemistries that do not involve the large molecular weight streptavidin such as those described earlier should also be explored.

Another vaccine related application that should be investigated further is the ability of the spore-displayed antigen system to induce virus-specific immune responses. *B. subtilis* spores have been demonstrated previously to enhance the cross presentation of a displayed antigen such as ovalbumin. Furthermore,

the ability of *B. thuringiensis* spores to induce the production of IL-12 and nitric oxide, which are indicative of TH1-type CD4 T-cells responses, was demonstrated in this study. To support this type of CD4 T-cell responses, spore-displayed antigens were repeatedly shown to induce a mixed IgG1/IgG2a antibody response. Collectively, these data suggest that the spore-displayed antigen system may be able to induce cytotoxic, CD8+ T-cell responses to displayed antigens. Further experiments should be conducted to determine if spore-displayed antigens are able to efficiently induce CTL responses following immunization.

As suggested in Figure 1, the modular nature of the spore-display system also allows for the display of antibodies on the spore surface. It is possible that these antibodies may be utilized to target specific cell subsets that have been previously demonstrated to be involved in priming of potentiating immune responses. Antibody targeting has been used in multiple studies and has resulted in the increased presentation of MHCI and MHCII peptides, which then led to increased CD8 and CD4 T-cell responses, respectively [37-38]. Additionally, antibody-mediated targeting has also been demonstrated to increase antigen-specific antibody responses [265].

One example of this antibody-mediated targeting is the use of DEC-205-specific antibodies which have been used to target antigens for enhanced cross presentation [37-38]. DEC-205 is a receptor expressed at high levels on the surface of plasmacytoid and CD8+ myeloid dendritic cells. Both of these subsets of dendritic cells have been shown to efficiently cross prime CD8+ T-cells. The

chemical conjugation of OVA to a DEC-205-specific antibody has been demonstrated to significantly increase OVA-specific CD8 T-cell responses in mice [38, 266]. Additionally the DEC-205 antibody has also been shown to enhance CD8 T-cell responses to microparticle-displayed OVA [267]. Together these data suggest that spores displaying a CTL antigen and a DEC-205-specific antibody may be more effective at generating antigen-specific CD8 CTL.

The stability of the spore under harsh chemical conditions suggests that they may provide excellent oral vaccine delivery vehicles. Many of the early experiments with recombinant *B. subtilis* spores were designed to develop oral vaccines [105, 134, 161]. Although only subcutaneous and intranasal routes of immunization were investigated during the course of this study, the spore-displayed antigen system does have the potential to deliver antigens to the gut. Several considerations must be taken into account. First, the ability of the linkage chemistries used to display the antigens on the spore surface must be able to withstand the low pH conditions of the gut. Second, the dosage requirement for orally administered vaccines has been shown to be much higher than other routes of administration. Therefore, dose titrations should be conducted to determine the optimal dosage.

The focus of this study was the development of a vaccine delivery platform, however the spore-display system may be used for additional applications, such as the delivery of biologically active enzymes. Recently, a study successfully used recombinant spores to display phytase, an enzyme that assists in the breakdown of undigestible phytic acid [268]. Microparticles have also been used

to deliver the enzyme prolidase to the cytoplasm of patient fibroblasts in an effort to treat prolidase dysfunction [269]. This dissertation also presents data that enzymatically active proteins can be displayed on the spore surface which indicates that the spores may be utilized to deliver similar enzymes to certain cell types (Figure 6). However, stability and *in vivo* activity assays need to be conducted to evaluate the ability of spores to deliver these molecules effectively.

Additionally, biodegradable microparticles have been investigated as delivery vehicles for small molecule pharmaceuticals [270-272]. This document also describes experiments in which the small molecule FITC was displayed at high densities on the surface of biotinylated spores, which suggests that these biotinylated spores may also be efficient carries of small molecule pharmaceuticals. Further studies should be carried out to determine the efficiency of spore-displayed enzyme or molecule uptake as well as the stability and activity of the small molecules displayed.

Chapter VII: Literature Cited

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VITA

Curtis Pritzl was born March 31st, 1979 in Northern Wisconsin. He was raised in the small town of Park Falls, Wisconsin by his loving parents Lawrence and Sheila Pritzl. During high school he was excessively active in student organizations such as the concert, marching, and jazz bands; as well as drama and the FBLA. After high school, he moved as far away from Northern Wisconsin as he could and attended Salem International University where he completed his undergraduate degree. He then continued his education there in the field of molecular immunology under the supervision of Dr. Patrick Lai. Once he finished his Master's degree, Curtis then moved to Columbia, Missouri to begin work on his Ph.D. under the guidance of Dr. Dan Hassett. During this period, he not only managed to accomplish this dissertation, but also to accumulate a small hobby farm that consisted of two dogs, two goats, two horses and two ponies and six chickens. Since completing his doctoral degree, Curtis has continued his research in immunology, mentored by Dr. Bumsuk Hahm.