

DISSERTATION

MODULATION OF IMMUNE RESPONSES ON MUCOSAL SURFACES THROUGH
VACCINATION AND DIETARY INTERVENTION

Submitted by

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ABSTRACT

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Numerous pathogenic organisms enter the body at the mucosal surfaces and therefore the mucosal immune response must function as the first line of defense. The ability of the body to induce protective immune responses on the mucosal surfaces is a powerful strategy for the prevention of disease. Therefore, understanding the mechanism of induction associated with protection is critical if there is to be improvement in current treatments. In these studies, the use of vaccination and diet were investigated as potential strategies for the induction of potent immune responses on the mucosal surfaces.

The principle of vaccination has been used successfully for centuries. However, there is still a great need for the development of vaccines against mucosal pathogens such as HIV, TB, and newly emerging pathogens. The primary way to improve mucosal vaccination is through the use of a potent vaccine adjuvant. The first part of this project focuses on the use of cationic-liposome plasmid DNA complexes (CLDC) as a mucosal vaccine adjuvant for enhancing the immune response to both particulate and soluble antigens. In these studies, intranasal vaccination using CLDC resulted in a balanced humoral and cellular immune response capable of protecting against a lethal pulmonary

bacterial challenge. We found that mucosal immunization with CLDC adjuvant resulted in the increase in the pro-inflammatory cytokines IL-6 and IFN- γ . Also, cellular immune responses were shown to be dependent on MyD88 signaling. Finally, resident airway myeloid dendritic cells (DC) efficiently phagocytosed the CLDC adjuvant and efficiently trafficked the associated antigen to the draining lymph node. Therefore the effectiveness of CLDC as a mucosal vaccine adjuvant appears to depend on strong cytokine induction and efficient antigen presenting cell activation and migration.

In a similar manner, dietary modulation has been shown to significantly impact the intestinal immune environment and has only recently begun to be investigated. It represents a novel approach for enhancing protective responses against pathogens and inflammatory diseases. The focus of the second part of this study is the ability of dietary rice bran to modulate the mucosal immune response as a potential mechanism to prevent disease. We found that a diet containing 10% rice bran resulted in an increase in local IgA concentrations and surface expression of IgA on mucosal B cells. Also, dietary rice bran induced a significant increase in myeloid dendritic cells residing in the lamina propria and mesenteric lymph nodes, and increased the colonization of native *Lactobacillus*, a beneficial gut microorganism known for its ability to positively influence the mucosal immune system.

This work has increased our knowledge of the impact of vaccination and dietary modulation for the protection of the mucosal surfaces. More specifically, these findings have revealed that CLDC is a potent vaccine adjuvant and that incorporating rice bran in a balanced diet can augment the mucosal immune environment.

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LIST OF PUBLICATIONS
(Related to Dissertation Work)

Henderson, A.J., Kumar, A., Barnett, B., Dow, S.W., and Ryan, E.P. Consumption of Rice Bran Increases Mucosal IgA Concentrations and Numbers of Intestinal *Lactobacilli*. *Journal of Medicinal Food*. 2011 [Accepted with revisions].

Henderson, A., Propst, K., Kedl, R. and Dow, S. Mucosal immunization with liposome-nucleic acid adjuvants generates effective humoral and cellular immunity. *Vaccine* **29**, 5304-5312 (2011).

Whitlock, G.C., **Duffy, A.J.**, *et al.* Protective response to subunit vaccination against intranasal *Burkholderia mallei* and *B. pseudomallei* challenge. *Procedia in Vaccinology* **2**, 73-77 (2010).

Jones, A., **Duffy, A.**, *et al.* Protection against pneumonic plague following oral immunization with a non-replicating vaccine. *Vaccine* **28**, 5924-5929 (2010).

Kumar, A., **Henderson, A.**, Forester, G.M., Goodyear, A.W., Weir, T.L., Leach, J.E., Dow, S.W., and Ryan, E.P. Dietary Rice Bran Promotes Resistance to *Salmonella enterica* serovar Typhimurium Colonization in mice. *BMC Microbiology* 2011 [Submitted].

CHAPTER 1

LITERATURE REVIEW

1.1 Mucosal Immune System

1.1 (1) Unique Characteristics of the Mucosal Immune System

The mucosal surfaces comprise an enormous area that the immune system must protect. From air exchange to nutrient absorption, these surfaces play very important physiological roles. However, they are typically thin and permeable which makes them vulnerable to invasion by a large number of infectious agents [1]. As a result, unique immunological mechanisms have evolved to protect these surfaces from invasion.

The first mechanism involves the general lay-out and structure of the mucosa-associated lymphoid tissue (MALT). Diffuse collections of lymphoid tissue are present at various portals of entry throughout the body. These collections are made up of structures such as the Peyer's patches in the gut-associated lymphoid tissue (GALT) and the tonsils in the bronchus-associated lymphoid tissue (BALT). These lymphoid structures are essential for the quick initiation of appropriate immune responses against pathogenic or innocuous antigens [1]. The mucosal surfaces are further protected by numerous effector lymphocytes, macrophages, and dendritic cells (DCs) which are scattered throughout the connective tissue underlying the mucosa.

Another distinctive feature of the mucosal immune system is the complex network of effector cells circulating between the various mucosal compartments. Mucosally activated lymphocytes are conditioned to return and function on the mucosal surfaces following systemic circulation. This occurs as a result of mucosal DCs inducing the expression of $\alpha_4\beta_7$ on their surfaces during antigenic priming [2]. The $\alpha_4\beta_7$ integrin binds to MAdCAM-1, a mucosal homing molecule found on the vasculature of all mucosal tissues. This binding results in the extravasation of the mucosally-activated lymphocytes into the tissues [2-3]. For instance, a lymphocyte primed in the Peyer's patches is capable of binding to the MAdCAM-1 present in the airway vasculature and entering the mucosal tissue of the lungs. This interconnected network is referred to as the common mucosal immune system and represents clear advantages for the development of protective immunity that spans multiple mucosal sites [4].

One unique challenge to the defense of the mucosa is the constant exposure to the outside environment. In comparison to most of the other immune organs which function in a relatively sterile environment, the mucosal surfaces interface with the environment and must distinguish pathogens from harmless environmental antigens. Pathogens contain numerous virulence factors and structural patterns which alert and activate a mucosal immune response. On the other hand, a soluble non-replicating antigen would not promote a strong mucosal response, but instead induce a state of antigen-specific hyporesponsiveness [5]. The mechanisms for the induction of tolerance to non-pathogenic antigens on the mucosal surfaces involve clonal deletion, anergy, and suppression by T regulatory cells (T_{reg}). T_{reg} cells function by secreting anti-inflammatory cytokines such as TGF- β and IL-10 [6]. Also, DCs have been shown to induce hyporesponsiveness to airway antigens through the secretion of IL-10 [5]. B_{10} cells, a newly emerging immunoregulatory cell, also dampen inflammation through the production of IL-10 and may play a part in mucosal tolerance [7]. Therefore, the mucosal immune system must maintain a delicate balance in order to adequately protect against pathogenic organisms and disregard non-pathogenic antigens.

1.1 (2) Mucosal Innate Immune Responses

The innate immune system is critical for the immediate removal of infectious agents and the shaping of the adaptive immune response. The primary responsibility of the mucosal innate immune response is to maintain a balance between defending the mucosa from infection and limiting an inflammatory response which can be detrimental to the integrity of the mucosal surfaces. Some of the essential innate immune components are pattern recognition receptors (PRRs), epithelial cells, and antigen presenting cells (APCs) [8].

The innate responses are relatively non-specific in comparison to the adaptive immune responses, although pathogens can still be distinguished from non-pathogens. This is done primarily through a series of receptors called pattern recognition receptors (PRRs) commonly

expressed on epithelial cells and APCs [9-10]. The primary PRRs expressed by the mucosal surfaces are the Toll-like receptor (TLR) family, the Nod-like receptors (NLRs) and C-type lectins [11]. Pathogens contain unique motifs referred to as pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs by PRRs results in the production of pro-inflammatory cytokines and other immune mediators [9-10]. On the mucosal surfaces, PRRs assist in distinguishing pathogenic organisms from commensal bacteria or innocuous material through the spatial regulation of the receptors. [12]. For instance, TLR2, TLR4, and TLR5 have been shown to be expressed mostly on the baso-lateral side of mucosal epithelial cells [12-14]. This finding supports the hypothesis that highly inflammatory TLR responses are only induced following a breach of the epithelium as a result of infection by a pathogenic organism [12].

The epithelial cells can be viewed as central modulators of the mucosal immune response. The barrier set up by epithelial cells represents not only a physical defense, but a means for the initial assessment of foreign antigen. Epithelial cells interact with antigens through a diverse array of PRRs [15]. As a result, epithelial cells can modulate the adaptive immune response through the secretion of cytokines, chemokines, and other mediators to help amplify as well as polarize the immune response [15-16]. For example, airway epithelial cells are known producers of thymic stromal lymphopoietin (TSLP), a cytokine responsible for activating DCs to induce a T_H2 response in the airways of asthmatics [17]. Also, through the release of granulocyte-macrophage colony-stimulatory factor (GM-CSF) mucosal epithelial cells can prolong the survival of neutrophils and monocytes as well as further amplify an inflammatory response [18].

One of the most important cell types of the innate immune response is the antigen presenting cell (APC). DCs, macrophages, and B cells are all classified as APCs; the DCs being the most potent at priming an adaptive immune response. DCs use PRRs to recognize and internalize pathogens prior to carrying the antigens to the draining lymph nodes. The phagocytosis of non-pathogenic antigens that do not induce PRR signaling can result in semi-

mature DCs. These DCs are able to upregulate MHC and co-stimulatory molecules, but fail to produce the necessary cytokines to induce protective immunity [19]. The primary APCs in the airways are alveolar macrophages (AVMs) and resident myeloid DCs. During steady state conditions AVMs constitute 90% of the cells present in the airways [15]. The main role of AVMs is phagocytosis and the sequestration of antigen from DCs in order to block overt immune responses [15]. Also, AVMs actively suppress the function of DCs, evidenced by the enhanced DC function following AVM depletion [15, 20-21]. Similar regulatory mechanisms are in place on the mucosal surfaces of the intestinal tract in order to minimize inflammatory responses. For example, most of the intestinal macrophages lack CD14, the co-receptor for TLR4 signaling. This results in impaired TLR4 signaling and subsequent lack of pro-inflammatory cytokine production [22-23]. Also, DCs found in the Peyer's patches produce high levels of IL-10 in comparison to splenic DCs [22].

1.1 (3) Mucosal Adaptive Immune Responses

If the innate immune system is unable to control a pathogenic infection, the development of a potent adaptive immune response is critical. Unlike the innate responses, the adaptive responses are antigen specific which makes them highly efficient at eliminating infectious agents. The mucosal adaptive immune responses are distinctively tailored to protect the unique environment of the mucosal surfaces. Key mechanisms that are involved include the induction of high titers of antigen-specific IgA antibodies and the positioning of large numbers of effector cells throughout the mucosal tissues.

The dominant antibody isotype of the mucosal immune system is IgA [24]. Activated mucosal B cells undergo IgA antibody class switching under the influence of TGF- β , IL-10, and IL-6 [24-27]. Class switching occurs prior to systemic circulation in the lymphoid structures of the mucosal tissues, such as in the nasal associated lymphoid tissue (NALT) and in the Peyer's patches [28]. A majority of the IgA⁺ B cells and IgA-secreting plasma cells return to function

within the mucosal tissues [29]. The locally produced IgA antibodies are an essential part of the antigen-specific defense against mucosal pathogens. The effectiveness of IgA is attributed to its ability to achieve both immune protection and pathogen exclusion in a non-inflammatory manner [25]. Immune exclusion involves blocking the access of pathogenic organism to the mucosa in order to minimize inflammatory immune responses. A secondary source of mucosal IgA production comes from B-1 cells located in the lamina propria [30]. B-1 cells differ in phenotype and function from conventional B-2 cells [31]. For instance, B-1 cells produce IgA independent of T cell help. This results in an antibody that has not undergone somatic hypermutation and binds antigen with a lower affinity [31]. Intestinal B-1 cells require the presence of commensal bacteria in order to produce IgA. As a result, it is hypothesized that a majority of the intestinal IgA produced by B-1 cells are specific for the current commensal bacterial population [32-33].

Although IgA is the chief antibody responsible for humoral protection on the mucosal surfaces, secretory IgM and serum-derived IgG induce additional protective responses [34]. In the case of pulmonary viral infections, IgA antibodies have been shown to locally control viral replication and reduce pathology in the upper airways [35]. In comparison, systemically-derived IgG responses have demonstrated the ability to neutralize replicating virus and prevent further spread of the infection [36]. Furthermore, protective IgG1 responses have been observed in the lamina propria in the absence of secretory IgA antibodies [37-38].

Once effector cells are positioned within the mucosal tissues, they can be found in two main locations which are within the epithelium and throughout the lamina propria. The majority of the cells found embedded between the epithelial cells are cytotoxic CD8 T cells and DCs [39]. The lamina propria exhibits a wider collection of cell types, such as CD4 T cells, CD8 T cells, plasma cells, DCs, macrophages, and mast cells [39]. In the lungs, effector cells can also be found in the airway spaces. Regardless of the composition, the mucosal tissues contain an increased amount of effector cells in comparison to most other parts of the body. Tissues in other

compartments of the body are patrolled by innate immune cells such as DCs. Only during a chronic infection are there numerous effector cells present in order to fight off infection. The nature of the immune response on mucosal surfaces is different due to the continual bombardment of antigenic stimulants from the external environment. As a result, numerous effector cells permanently reside in the mucosal tissues in order to quickly respond to foreign antigens. It is important to note that despite the heightened potential for immunological effector responses, the mucosal surfaces are not in a constant state of inflammation. Powerful regulatory mechanisms including T_{reg} cells elicit the maintenance of mucosal homeostasis through the use of mediator such as TGF- β , IL-10, and nitric oxide [15, 40].

1.2 Mucosal Vaccine Development

1.2 (1) Vaccine Basics

Vaccines are considered by many to be one of the most successful medical interventions for disease prevention in the 20th century [41-42]. However, there is still great need for further investigation into the development of new vaccines, the improvement of current substandard vaccines, and the rapid construction of vaccines against emerging pathogens [43-45]. The infectious agents, HIV, malaria, and *Mycobacterium tuberculosis* are responsible for the deaths of millions of people worldwide [46]. In spite of numerous attempts, successful vaccines for these agents have not yet been developed. The ultimate goal of vaccination is to produce long term immunological protection by inducing a memory response capable of protecting against “re-exposure” to a pathogen [47]. Also, successful vaccination depends on multiple factors including the choice of the antigen, the route of administration, the immunostimulatory nature of the antigen and/or adjuvant, and the status of the host immune system. All vaccines can be classified into the following three main categories based on the state of the antigen: modified live,

killed/inactivated, or subunit vaccines [44]. Each category has distinct advantages and disadvantages that are described below.

Modified live vaccines induce the most potent and longest lasting immune responses of the three vaccine categories [44]. The ability of live organisms to replicate in the host results in constant antigen exposure and reduces the number of necessary vaccine doses. These organisms retain the natural conformation of immunogenic epitopes and secreted molecules resulting in a more specific and stronger adaptive immune response. They are typically attenuated through genetic modification in order to reduce virulence as is demonstrated in the Sabin oral polio vaccine and Flu-mist [48-49]. The biggest disadvantages for the use of modified live vaccines are the potential for the organism to revert back to virulence and the possibility of individuals remaining persistently infected. A healthy individual is capable of safely responding to modified live vaccines; but the elderly, children, and immune compromised individuals are at more risk for complications due to their under-developed or weakened immune systems.

Killed or inactivated vaccines do not carry the same risks as modified live vaccines. These vaccines are developed by treating whole organisms with heat or chemicals in a way that renders them unable to replicate, but keeps the surface epitopes intact allowing for the induction of potent immune responses [44]. Based on the inability to replicate, killed vaccines typically need multiple doses and potentially a vaccine adjuvant to enhance immunity. One safety concern associated with killed vaccines involves intact surface TLR agonists like LPS that induce inflammatory responses such as fever and pain [44]. A few examples of current vaccines containing killed/inactivated organisms include Salk polio vaccine, whole cell *B. pertussis*, Hepatitis A, and *Yersina pestis* [44, 48].

In light of the adverse affects of modified live and killed vaccines, subunit vaccines have recently gained in popularity due to advantages in safety [50-51]. The antigenic components of subunit vaccines are pathogen-specific proteins, peptides, and carbohydrate motifs that are capable of inducing an adaptive immune response. The ability to specifically design subunit

vaccines to target and stimulate key immune responses is a clear advantage over whole organism based vaccines. However, subunit vaccines tend to lack molecules such as PAMPs, which are necessary to initiate an immune response. As a result, subunit vaccines are poorly immunogenic typically requiring multiple doses and a vaccine adjuvant. The best example of an approved subunit vaccine is the Hepatitis B vaccine which contains one of the viral envelop proteins, HBsAg, mixed with the Alum adjuvant [48].

1.2 (1a) Vaccine Adjuvants

Vaccine adjuvants can be functionally defined as components added to vaccine formulations that enhance the immunogenicity of an antigen *in vivo* [42]. Potent adjuvants improve the efficacy of vaccination in the following ways: acceleration of the initiation of an immune response, extension in the duration of the protective response, improvement in the avidity of the antibody responses, and by inducing the activation of a cytotoxic response [52-53]. Also, vaccine adjuvants tend to reduce the cost of vaccination by reducing the concentration of antigen needed to induce a successful immune response. Vaccine adjuvants can be described based on their mechanism of action and are typically categorized as either “delivery-based” or as immune potentiators [42].

The characteristics associated with delivery-based vaccine adjuvants are variable. For instance, some carrier molecules such as immune stimulating complexes (ISCOMs) are stimulatory where as others such as microparticles are naturally inert [54]. Regardless of the immunomodulatory characteristics, vaccine adjuvants classified as delivery-based adjuvants excel at antigen protection, display, and targeting to APCs. A few examples of the commonly used delivery system adjuvants include aluminium salts (alum), liposomes, virosomes, emulsions, and ISCOMs [53]. The recent use of immune potentiators as vaccine adjuvants is a result of an increased understanding of the molecules involved in stimulating the innate immune system. These adjuvants tend to contain common PAMPs that induce cytokine production and activation

of APCs. Examples of immunostimulatory adjuvants include but are not limited to MPL, CpG oligonucleotides, and double stranded RNA (poly I:C) [53].

Recent attention has been focused on the development of combination adjuvants that contain both a delivery system and an immunostimulatory component [54-55]. These adjuvants are being investigated for the various synergistic immune responses [56]. An example is the herpes subunit vaccine using AS02, a combination adjuvant containing alum and MPL that has shown partial protection against HSV-1 and HSV-2 [54]. The AS02 adjuvant and others are listed in **Table 1.1** with a description of their potential mechanisms of action and their current status in clinical trials.

Table 1.1 Characteristics of Current and Promising Vaccine Adjuvants. This table was adapted from [42, 56].

Adjuvant	Description (Mechanism)	Most Advanced Clinical Phase
Mineral salts Aluminum Salts Calcium Salts	Bacterial and viral antigens are adsorbed onto the alum and Ca salts	Licensed (Alum)
Oil emulsions and surfactant-based MF59 QS-21 AS02 Montanide ISA-51 Montanide ISA-720	Micro-fluidized detergent-stabilized emulsions MF59: oil-in-water QS-21: purified saponin AS02: Oil-in-water emulsion + MPL + QS-21 ISA-51: water-in-oil ISA-720: water-in-oil	Licensed Phase II (cancer) Phase IIb Phase II (HIV) Phase II (cancer, HIV)
Particulate delivery vehicles Virosomes PLG ISCOMS Liposomes	Antigens and adjuvants can be trapped inside or coated onto the surface of particles	Licensed (Flu) Phase I Phase I Phase I (<i>H. pylori</i>)
Microbial derivatives MPL CpG ODN CT/LT OmpI	Bacterial products or synthetic mimics are potent stimulators of the innate immune system (Most of these agents signal through TLRs)	Licensed (melanoma) Phase I Phase II Preclinical
Cells and cytokines Dendritic cells IL-12 and GM-CSF	Antigen-loaded DCs can be potent activators of the immune response Cytokines stimulate cells of the immune system	Phase II (cancer) Phase II

Despite intense research, the only vaccine adjuvant approved for use in the United States is alum [45, 57]. The alum adjuvant elicits a strong humoral response but fails to induce a cell-mediated response. The failure to successfully vaccinate against intracellular pathogens such as HIV, *M. tuberculosis*, and malaria highlights the current need for the development of a vaccine adjuvant capable of eliciting a cytotoxic CD8 T cell response as well as a strong humoral response [48]. There are two main strategies that have shown promise for the induction of CD8 T cells. One strategy involves the use of live bacterial vectors and virus like particles (VLPs) capable of inducing antigen uptake into APCs [51]. The other strategy involves the use of lipid-based delivery mechanisms [58-59]. Both of these strategies are capable of inducing the presentation of exogenous antigen within the MHC class I molecule on DCs, a phenomenon referred to as cross-presentation [60]. Mechanisms involved in the induction of cross-presentation are still largely unknown and further research is required before it is implemented in vaccine development.

The main reason that alum is the only approved vaccine adjuvant is because of its solid safety record. Regardless of the ability of experimental adjuvants to induce increased vaccine efficacy, an adjuvant must demonstrate stability and safety in order to progress beyond clinical trials [54]. The progression of most adjuvants is halted or slowed as a result of induced toxicities [57]. Therefore, further work is needed to understand the innate characteristics and mechanisms of action elicited by promising vaccine adjuvants prior to entering clinical trials.

1.2 (2) Advantages and Challenges in Mucosal Vaccination

The majority of infections occur at, or emanate from mucosal surfaces [61] which provides a unique opportunity for inducing local immunity at sites where pathogens typically infect. Mucosal immune responses have been shown to be most effective when vaccines are administered directly onto the mucosal surfaces [45]. Comparatively, injectable vaccines are

typically poor inducers of mucosal immunity since they are administered away from mucosal surfaces [45, 62]. Regardless of the potential advantages of mucosal immunization, the majority of vaccines today are still administered parenterally [45].

A great number of studies have been performed in order to verify the advantages associated with mucosal vaccination in the hopes of increasing future implementation. For instance, a variety of mucosal vaccines have been tested that investigate the following routes: oral, ocular, pulmonary, nasal, vaginal, and rectal [63]. The nasal route of vaccination has shown the most promise due to patient acceptability, increased tendency towards the induction of a balanced immune response, and the ability to use a lower concentration of antigen [63-64]. One clear advantage for the use of mucosal vaccination is the elimination of the use of needles and specialists required for administering the vaccinations [63]. Mucosal immunizations without needles could greatly improve vaccine safety and compliance issues. Moreover, another advantage to priming an immune response on the mucosal surfaces results from activating antigen-specific lymphocytes. These cells are seeded at distant mucosal sites along with the vaccinated mucosa in order to further amplify the protective response to future pathogen exposure [24].

Despite numerous vaccination attempts and compositions, only a few mucosal vaccines have been approved for human use. These vaccines include the poliovirus, influenza, rotavirus, *Salmonella typhi*, and *Vibrio cholera* vaccines [4, 65]. All of these approved mucosal vaccines are composed of live, attenuated organisms [66-67]. There have been many attempts to develop non-living mucosal vaccines, but with limited success due to the nature of the mucosal surfaces. The mucosal secretions tend to dilute the vaccine which causes difficulty in calculating the antigenic dose resulting in the use of high concentrations of antigen [45]. In addition, although the immunosuppressive nature of the mucosal surface is beneficial for dampening responses to commensal bacterial and particulate matter, it presents difficulties for vaccine development [45]. Mucosal vaccines are exposed to the same host defenses that pathogens experience including

dilution in mucosal secretions, capture in mucus gels, attack by proteases and nucleases, and exclusion from the epithelial barrier [45, 57]. Therefore, in order to successfully design mucosal vaccines, especially non-living vaccines; the use of a vaccine adjuvant is essential.

1.2 (2a) Mucosal Vaccine Adjuvants

The advancement of mucosal vaccination requires adequate vaccine adjuvants capable of enhancing a potent protective response in spite of the immunosuppressive mucosal environment. At present, several experimental adjuvants have been used with non-replicating mucosal vaccines, including mutated cholera toxin and *E. coli* labile toxins, synthetic TLR agonists, cytokine adjuvants, and liposomal-based adjuvants. For many years the “gold standards” of mucosal vaccine adjuvants have been cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) due to their potent enhancement of mucosal immunity [53]. CT adjuvants elicit strong humoral immunity following mucosal administration. However, the risk of systemic toxicity and especially neurotoxicity renders current CT adjuvants generally unsuitable for vaccines intended for human use. One option to reduce systemic toxicity is to use the modified cholera toxin subunit B (CTB) adjuvant which has been shown to be fairly effective as a mucosal adjuvant through its retained ability to induce IgA production. Another option is the use of site directed mutagenesis in order to keep the cholera toxin subunit A (CTA) but render it non-toxic. Such mutations reduce the adjuvant capabilities, but researchers have found that conjugating targeting motifs to the modified CTA returns adjuvanticity [4, 68-69].

CpG oligonucleotides (ODN) and Monophosphoryl lipid A (MPL) have been widely used as both parenteral vaccine adjuvants and as mucosal vaccine adjuvants [4, 70-78]. Studies have shown the CpG and MPL adjuvants potently activate immune responses by stimulating innate immune signaling via TLR9 and TLR4, respectively [79-81]. CpG ODNs have been shown to be effective mucosal vaccine adjuvants [4, 70-78] through the production of T_H1-type cytokines and the induction of the maturation of antigen presenting cells [82-83]. CpG ODN-

based mucosal adjuvants may be used alone or in conjunction with existing adjuvants such as aluminum hydroxide. MPL has been shown to induce activation of APCs and production of pro-inflammatory cytokines such as TNF- α and IL-12 [70, 84-87] without causing toxic effects [88]. MPL, in combination with alum, has been shown to be a safe and effective component in the licensed vaccines for hepatitis B and papilloma viruses [89].

Cytokines including GM-CSF, IL-12, and type I interferons (IFN) have also been used as mucosal vaccine adjuvants [51, 78, 90-91]. These adjuvants are often used to skew the mucosal immune response towards increased production of IgA [92] or to the induction of specific subsets of T cell responses [93]. Cytokine adjuvants are typically administered as either genetic elements (plasmid DNA vaccines) or as recombinant cytokines. Cytokine adjuvants can be incorporated into mucosal vaccines, but may be less effective than other adjuvants because they do not physically associate with antigens unless chemically coupled.

Some of the most promising “delivery-based” vaccine adjuvants involve the use of lipid-based structures for the entrapment of the vaccine antigen [4]. Liposome-based mucosal adjuvants have been thoroughly investigated by using a variety of different antigens [94-99]. The impact of mode of antigen association with the liposome (encapsulation, conjugation, and absorption) and the physiochemical properties of the liposome (size, charge, lipid composition) on immune responses have also been studied [100]. At present, cationic liposomes are particularly advantageous as mucosal adjuvants due their ability to enhance the uptake of the vaccine by antigen presenting cells (APC) and to induce APC activation [101-103]. Indeed, numerous studies have shown that liposomes are essential to achieve improved immune responses [99, 104-105]. Many liposome-based adjuvants can induce mucosal production of IgA, systemic IgG production, and some have even shown the ability to induce effective CD8⁺ T cell responses.

1.2 (2b) CLDC Induced Mucosal Immune Modulation

Cationic liposome-DNA complexes (CLDC) were first developed to be used as non-viral gene delivery vehicles, but have since been shown to be an effective immunotherapeutic in the areas of cancer, infectious disease, and vaccine development [106]. Recent studies in our laboratory have also revealed that intranasal administration of CLDC as an immune therapeutic generates rapid, non-specific, innate immune protection against inhalational challenge with rapidly lethal bacterial pathogens including *Burkholderia* and *Francisella* [107-108]. More importantly, we reported the use of CLDC as a vaccine adjuvant to elicit balanced cellular and humoral immunity following parenteral administration [109]. We believe the majority of the success of the CLDC adjuvanted parenteral vaccines can be attributed to the combination of the liposome (carrier) and the plasmid DNA (immunostimulant).

The cationic liposome component of CLDC aids in the binding of proteins or peptide antigens directly onto the surface of the liposome through charge-charge interactions. The presence of the liposomal component targets the vaccine antigen to mucosal APCs. Following uptake by APCs, the plasmid DNA component of CLDC is able to interact with TLR9 found in the endosome and potentially the DAI (DNA-dependent activator of IFN-regulatory factors) receptor found in the cytoplasm. DNA binding triggers a strong local activation of the innate immune system and can induce the production of TNF- α , IL-12, IFN- γ , and IFN- α [108, 110-112]. Also, CLDC is able to elicit increased cellular activation, such as the increased cytotoxicity of NK cells and the upregulation of co-stimulatory molecules on DCs and macrophages [106, 113].

In light of the effectiveness of CLDC following intranasal administration and the success of CLDC parenteral vaccinations, we believe the investigation of CLDC as a mucosal vaccine adjuvant holds great promise for future non-living subunit vaccine development.

1.3 Dietary Modulation of the Gastrointestinal Tract

1.3 (1) Implications of diet on overall health

The impact of food on health has been acknowledged for thousands of years [114], but the study of complex mechanisms for how food components function and are available to aid in disease prevention is an emerging area of research. Current, ongoing research is focused on identifying functional or health-promoting foods with either beneficial effects on overall health or reduction in disease. A variety of functional foods such as whole grains, vegetables, and fatty acids have gained positive attention due to increasing evidence of dietary involvement in the treatment of diseases such as diabetes, heart disease, and cancer [115-116]. A list of some promising functional foods and their associated benefits are displayed in **Table 1.2**. However, recent studies hypothesize that the complex interactions of diet, the microbiota, and the mucosal immune response may also play a role in etiology of inflammatory bowel diseases [117-118]. There is a clear need for enhanced understanding of the effects of diet on overall health, specifically on the gut microbiota and the mucosal immune system.

Table 1.2 Partial list of functional foods and their associated effects. This table was adapted from [119].

Food	Physiologic effect
Apple, barley, blackberry, blueberry, carrot, eggplant, oats, garlic, ginger, ginseng, mushroom, onion, rice, soybean, tea	Lipid lowering
Lemon, apple, cranberry, garlic, beet, cucumber, squash, soybean, cabbage, Brussels sprouts, cauliflower, kale, broccoli, spinach	Enhanced drug detoxification
Ginseng, licorice, oats, parsley, green tea [120]	Anti-inflammatory
Cranberry, garlic, onion, green tea	Anti-microbial
Anise, fennel, soybean, cabbage	Anti-estrogenic
Orange, green tea, garlic	Anti-proliferative

1.3 (1a) Effect of diet on the gut microbiota

The composition of the gut microbiota is unique to each individual. The initial colonization is imparted by the mother during childbirth [118]. Following birth, the microbiota is influenced by factors such as diet, antibiotic use, host genetics, and other environmental factors [118]. Recently diet has been shown to play a dominant role in shaping the gut microbiota, superseding even host genetics [121]. A study performed by Turnbaugh et al. showed the dramatic effect of diet on the human gut microflora present in gnotobiotic C57BL/6J mice [122]. The mouse diet was switched from a low fat/high plant polysaccharide diet to a diet high in fat and sugar and low in plant polysaccharides. Within 24 hours a shift was observed in the microbial composition resulting in an enrichment of bacteria in the phylum Firmicutes and a decrease in bacteria in the Bacteroidetes phylum, a ratio which is also shown to predominate in obesity [123]. This finding was reinforced by a human study showing a similar Firmicutes:Bacteroidetes ratio in the gut microbial populations of European children that consumed a western diet high in fat and sugar when compared with a high fiber diet consumed by children in rural Africa [123].

A primary mechanism through which diet may affect the composition of the gut microbial population is the presence of dietary components, mostly insoluble fibers that avoid digestion and reach the bacteria in the colon [124-125]. These compounds, often referred to as prebiotic components, shape the microbiota by acting as substrates for the beneficial bacteria known as probiotics. The resulting increase in probiotic concentration induced by prebiotics has been shown to modulate the mucosal immune responses as well as antagonize pathogens through competition for resources and antimicrobial production [126-127]. Also, the fermentation of these prebiotic components can affect the intestinal environment through the release of byproducts such as short-chain fatty acids (SCFA). SCFA have been shown to enhance the health

of colonic enterocytes as well as interact with mucosal immune cells [124, 128-129]. An example of promising function foods containing high concentration of prebiotic components are cereal grains. Grains such as rice, wheat, barley, and oats contain significant amounts of dietary fiber and have been shown to be fermented by probiotic bacteria [130]. Therefore, the ability of dietary food components to modulate the beneficial microbial populations is an important criterion to consider when investigating the benefits of various functional foods.

1.3 (1b) Effect of diet on the immune system

The concept of dietary immune modulation was first observed in breast-fed infants that had a lower incidence of diarrheal diseases [131]. Breast milk oligosaccharides were shown to be partially responsible for helping stimulate the development of the mucosal immune system and to provide subsequent protection from enteric pathogens [131]. Some of the primary effects of dietary derived prebiotic components on the mucosal immune system include: 1) the modulation of bacterial numbers and subsequent byproducts that induce immune activation and cytokine production, 2) the production of SCFA and their interactions with immune cells, and 3) the modulation of mucin production [132].

It is well known that prebiotics increase the numbers of beneficial bacteria. Bacteria from the genera *Lactobacilli*, *Bifidobacteria*, and *Bacteroides* [125, 133] have been shown to be capable of producing molecules with the potential to influence the immune system. For example, polysaccharide A produced by *Bacteroides fragilis* induces CD4⁺ T cells to produce IL-10, which has been shown to protect against inflammatory bowel disease [134]. Also, *Lactobacillus* has been shown to influence the innate immune system through increased macrophage recruitment, phagocytic activity [135-137], and the induction of pro-inflammatory cytokine production [138]. The influence of *Lactobacillus* on the mucosal immune system may also be dependent on antigenic particles crossing the epithelium as well as the viability of the microorganism [139].

The SCFAs produced by the fermentation of prebiotic components by beneficial bacteria have been shown to act as ligands for receptors found on immune cells. The three major SCFA are butyrate, acetate, and propionate; and they regulate inflammation through binding the G-protein coupled receptor, GPR43 [140]. The expression of GPR43 is found on neutrophils, eosinophiles, and activated macrophages. The interaction of the SCFA with the immune cells results in the production of reactive oxygen species (ROS), enhanced phagocytosis, and neutrophil recruitment [140]. Therefore, diet and dietary induced microbial changes result in the modulation of the mucosal immune system and have great potential to aid in disease protection.

1.3 (2) Rice bran

Rice (*Oryza sativa*) is staple food for over half the world's population. Due to stability and consumer acceptance, rice is typically polished to produce white rice and the nutrient-rich rice bran is discarded or used for animal feed. Based on the more than 70 million tons of rice bran produced worldwide each year from milling; dietary rice bran has the potential to provide a cheap, safe, and non-toxic food source to aid in prevention and/or alleviation of numerous diseases [141]. Whole dietary rice bran contains a balanced nutritional profile of proteins, carbohydrates, fats, and minerals. It also contains dietary fibers (6-14.4 g/100g) in the form of hemicellulose, β -glucan, pectin, and gum, some of which have been shown to be reservoirs for components active against cancer [141-142]. When compared to other cereal crops, rice bran contains a handful of unique bioactive components, such as γ -oryzanol, β -sitosterol, and tocotrienols-tocopherols [141]. Dietary rice bran can be easily stabilized using a high heat treatment in order to inactivate the lipase enzyme responsible for spoiling [143]. Therefore, rice bran offers a novel and promising staple food byproduct with bioactive components capable of modulating the gastrointestinal tract.

Some of the bioactive components found in rice bran include but are not limited to γ -oryzanol, tocopherols, tocotrienols, polyphenols, phytosterols, and carotenoids [141]. An

overview of the biologic activities associated with the above-mentioned compounds is outlined in **Table 1.3**. Rice bran also contains numerous essential amino acids and micronutrients associated with beneficial health effects, such as tryptophan, histidin, methionine, cysteine, and arginine as well as magnesium, calcium, phosphorous, manganese, and 9 B-vitamins [144-145].

Not only does rice bran differ from other cereal food, there are significant differences between the varieties of rice grown worldwide. The variability in characteristics and components found in rice bran have been investigated for agricultural purposes [146]. However, the importance of these difference in improving health and preventing disease have yet to be identified. Further study is required to identify the varieties with the most beneficial characteristics.

Table 1.3 Bioactive components of Rice bran associated with disease prevention.

Rice Bran Compound	Examples	Biologic Activity	References
γ-Oryzanol (Rice Bran Oil)		- Antibacterial - Antioxidant - Reduces cholesterol absorption	[147-152]
Tocopherols & Tocotrienols		- Anti-tumor - Antioxidant - Antibacterial	[147, 150-154]
Polyphenols	Ferulic acid α -lipoic acid	- Antioxidant - Anti-proliferative effect on cancer - Antibacterial - Anti-inflammatory	[155-157]
Phytosterols	β -sitosterol Campesterol Stigmasterol	- Reduces cholesterol absorption - Anti-inflammatory - Antioxidant - Stimulates lymphocyte proliferation	[158-161]
Carotenoids	α -carotene β -carotene Lycopene Lutein Zeaxanthin	- Antioxidant - Reduces the risk of cancer	[150, 153, 162-163]

1.3 (3) Benefits of dietary rice bran consumption

Rice bran has demonstrated unique chronic disease fighting properties in the areas of cancer prevention [147, 156], coronary artery disease [142, 164], diabetes [165], and inflammatory bowel disease [166]. The abilities of rice bran to modulate the gastrointestinal tract has been investigated using various formulations, including stabilized full-fat and defatted rice bran, rice bran oil, methanol extracted rice bran components, and fermented rice bran components. To date, the consumption of dietary rice bran has been shown to lower cholesterol, block the proliferation of cancer cells, and enhance innate immune responses.

Full-fat rice bran (not de-fatted rice bran) has been shown to reduce serum cholesterol in humans and hamsters through what is hypothesized to be the binding and sequestering of bile salts in the intestine. Binding results in serum cholesterol being shunted into bile salt production thus lowering serum cholesterol concentrations [142, 154, 164]. The cholesterol lowering capability of dietary rice bran may also be due to the known phytosterol components found in rice bran, which act as cholesterol absorption inhibitors. Rice bran components elicited by methanol extraction induce antioxidant activity through free-radical scavenging and iron-chelating activity [167] [147]. In support of this finding, methanol-extracted rice bran components such as phenols have been shown to inhibit the proliferation of human breast and colon cancer cells [156].

1.3 (3a) Immunological responses to dietary rice bran

Minimal attention has been given to understanding the effect of dietary rice bran and its components on the mucosal immune response. To date, studies have been conducted only on fermented rice bran and methanol extracted rice bran components. The patented food supplement, Biobran MGN-3, is produced by fermenting rice bran with a Shitake mushroom enzyme [168]. Biobran MGN-3 has been shown to enhance percentages and phagocytic activities of macrophages as well as induce the maturation of dendritic cells [168-169]. Also, Biobran MGN-3 induces IL-6, TNF- α , IL-10, and NO production [168, 170]. The effect of methanol treated dietary rice bran on the complement system was recently investigated using black rice

bran. The studies showed rice bran components were able to inhibit the complement system in vitro, which may help reduce pathogenesis in a variety of inflammatory diseases [171]. Finally, the immunomodulatory abilities of rice bran oil were investigated and were shown to enhance the proliferation of B-lymphocytes [172]. These studies reveal beneficial effects of dietary rice bran on the mucosal immune system.

1.4 References

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CHAPTER 2

MUCOSAL IMMUNIZATION WITH LIPOSOME- NUCLEIC ACID ADJUVANTS GENERATES EFFECTIVE HUMORAL AND CELLULAR IMMUNITY

I acknowledge the contribution of Dr. Ross Kedl for the production of the H-2K^b MHC class I tetramers, Dr. Scott Hafeman for the production of the liposomes, Dr. Katie Propst-Graham in the *Burkholderia* challenges and production of survival curves (Figures 2.5 and 2.6), and Dr. Mark Estes for providing the *Burkholderia* recombinant proteins.

2.1 Research Rationale

There is a significant need for the development of vaccine adjuvants that can induce potent mucosal protection. Many mucosa-targeting pathogens such as HIV and *Mycobacterium tuberculosis* do not have adequate vaccines resulting in high morbidity and mortality rates worldwide. Although minimal induction of mucosal immunity following an injection-based vaccines has been demonstrated, the complexity of modulating the mucosal surfaces has resulted in slow progress for the development of mucosal adjuvants. In the last few decades, there have been many discoveries that have produced a better understanding of the innate immune responses and the role of pattern recognition receptors. We believe there is great potential for the ability to modulate the mucosal immune responses using vaccine adjuvants that target these immune receptors. The first two aims of this dissertation (Chapters 2 and 3) investigate mucosal immune modulation elicited by cationic-liposome plasmid DNA complexes (CLDC) in association with soluble and particulate proteins. This chapter is focused on both investigating the adaptive immune responses to intranasal immunization with a CLDC adjuvant, and the ability of the induced responses to protect against a lethal pulmonary challenge. Chapter 3 will discuss the mechanisms utilized by CLDC to enhance the immune response.

2.2 Summary

Development of effective mucosal vaccine adjuvants is becoming a priority given the increase in emerging viral and bacterial pathogens. We previously reported that cationic liposomes complexed with non-coding plasmid DNA (CLDC) were effective parenteral vaccine adjuvants. However, little is known regarding the ability of liposome-nucleic acid complexes to function as mucosal vaccine adjuvants. To address this question, antibody and T cell responses were assessed in mice following intranasal immunization with CLDC-adjuvanted vaccines, and these responses were compared to leading mucosal vaccine adjuvants. Also, the ability of CLDC adjuvant to protect against pulmonary bacterial challenge was investigated. We found that mucosal immunization with CLDC-adjuvanted vaccines effectively generated potent mucosal IgA antibody responses, as well as systemic IgG responses. Notably, mucosal immunization with CLDC adjuvant was very effective in generating strong and sustained antigen-specific CD8⁺ and CD4⁺ T cell responses in the airways of mice. Finally, CLDC-adjuvanted vaccines induced significant protection from lethal pulmonary challenge with *Burkholderia pseudomallei*. These findings suggest that liposome-nucleic acid adjuvants represent a promising new class of mucosal adjuvants for non-replicating vaccines, with notable efficiency at eliciting both humoral and cellular immune responses following intranasal administration.

2.3 Introduction

Many pathogens attach to or invade mucosal surfaces and mucosal immunity is often the key to controlling the initial infection. Mucosal immune responses are generated most efficiently when vaccines are administered mucosally, though the majority of vaccines available today are administered parenterally [1-4]. Indeed, only a few mucosal vaccines have been approved for human use, including poliovirus, influenza, rotavirus, *Salmonella typhi*, and *Vibrio cholera* vaccines [1, 5]. Currently, most mucosal vaccines are prepared using live, attenuated organisms [6-7]. Though effective, such vaccines are costly to prepare, require careful attention to storage conditions, and pose some potential risk to immunosuppressed individuals. Therefore, there is continued interest in the development of effective, non-replicating mucosal vaccines. However, most mucosal antigens are poorly immunogenic and require the use of potent vaccine adjuvants.

At present, several adjuvants have been used with non-replicating mucosal vaccines, including mutated cholera toxin and *E. coli* labile toxins, as well as synthetic TLR agonist, such as CpG oligodeoxynucleotides (ODN). [4-5, 8-11]. Cholera toxin (CT) adjuvants elicit strong humoral immunity following mucosal administration, though the risk of systemic toxicity and especially neurotoxicity renders current CT adjuvants generally unsuitable for use in human vaccines. A modified cholera toxin subunit B (CTB) adjuvant is relatively effective as a mucosal adjuvant and eliminates the risk of systemic toxicity. CpG ODN have been widely used as parenteral vaccine adjuvants and as effective mucosal vaccine adjuvants [5, 12-20]. Studies have shown that CpG ODN adjuvants potently activate innate immune responses by stimulating signaling via TLR9 [21-23]. While each of these adjuvants has certain desirable properties, there are also some characteristics about CTB and CpG that raise efficacy and safety concerns [24-28]. Therefore, there remains a need for more potent, more quickly acting, and safer mucosal adjuvants.

Liposome-based mucosal adjuvants have been extensively investigated, using a variety of different antigens [29-34]. The impact of mode of antigen association with the liposome (encapsulation, conjugation, and absorptions) and the physiochemical properties of the liposome (size, charge, lipid composition) on immune responses have also been studied [35]. At present, cationic liposomes are particularly advantageous as mucosal adjuvants due to their ability to enhance the uptake of the vaccine by antigen presenting cells (APC) and to induce APC activation [36-38]. Indeed, numerous studies have shown that liposomes are essential to achieve efficient immune responses [34, 39-40]. Many liposome-based adjuvants can induce mucosal production of IgA, and some also induce systemic IgG production, but few have been shown to induce effective CD8⁺ T cell responses. Therefore, there is still a need for broadly effective mucosal vaccine adjuvants, capable of eliciting both humoral and cellular immune responses.

We previously reported that a vaccine adjuvant consisting of cationic liposome-DNA complexes (CLDC) effectively elicited balanced cellular and humoral immunity following parenteral administration [41]. We attribute a majority of the success of the CLDC adjuvanted parenteral vaccines to the combination of the liposome (carrier) and the plasmid DNA (immunostimulant). Combination vaccine adjuvants have recently become an area of interest due to the synergistic effects of combining antigen delivery with potent stimulation of the innate immune system [42-43]. CLDC can be classified as a combination adjuvant, and the need for physical association of all three of the components of the CLDC-based vaccines has recently been shown in our laboratory. Mice immunized with Ova plus liposome alone or Ova plus plasmid DNA alone failed to generate significant immune responses [41]. The efficacy of CLDC-based vaccines for immunization against a variety of different antigens in several different species has also been reported, including studies in guinea pigs, woodchucks, non-human primates, and more recently in normal human volunteers [44-49]. Moreover, recent studies in our laboratory have also revealed that intranasal administration of CLDC as an immune therapeutic could generate

rapid, non-specific, innate immune protection against inhalational challenge with rapidly lethal bacterial pathogens including *Burkholderia* and *Francisella* [50-51].

Therefore, we tested the **hypothesis that the use of CLDC as a mucosal vaccine adjuvant would elicit a potent immune response in the lungs and airways following intranasal vaccination**. To address this question, we investigated the mucosal adjuvant properties of CLDC combined with soluble protein antigens, delivered by the intranasal (i.n.) route. The ability of the CLDC adjuvant to elicit humoral and cellular immune responses was investigated. Also, the ability of CLDC-adjuvanted vaccines to elicit protective immunity using heat-killed bacteria and recombinant protein antigens was assessed in a model of lethal pulmonary *Burkholderia pseudomallei* challenge. In the course of these studies, we identified properties shared by CLDC adjuvants and other mucosal adjuvants, as well as properties unique to CLDC-based mucosal adjuvants.

2.4 Materials and Methods

2.4 (1) Mice

Specific pathogen-free 6-8-week-old female C57BL/6 and BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All protocols involving animal experiments described in this study were approved by Institutional Animal Care and Use Committee at Colorado State University.

2.4 (2) Reagents and biochemicals

Ovalbumin was purchased from Sigma-Aldrich (St Louis, MO) and was prepared as a 1 mg/ml solution in diH₂O. All cell preparations were resuspended in complete RPMI (Invitrogen, Carlsbad, CA) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Invitrogen), 1X non-essential amino acids (Invitrogen), 0.075% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

2.4 (3) Preparation of cationic liposomes-DNA complexes and vaccines

Liposomes were prepared by combining cationic liposome DOTIM octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolium chloride and cholesterol in equimolar concentrations as described previously [52]. Cationic liposome-DNA complexes (CLDC) were freshly prepared at room temperature and administered within 30 min. Non-coding plasmid DNA (0.2 mg/ml, Juvaris Biotherapeutics) was diluted in sterile Tris-buffered 5% dextrose water. The cationic liposomes were then added with gentle pipetting at a concentration of 100 µl of liposomes per 1 ml of solution, resulting in the spontaneous formation of CLDC. To formulate the CLDC-adjuvanted vaccines, the protein antigen was added to the diluted plasmid DNA solution prior to the addition of the cationic liposomes.

2.4 (4) Intranasal immunizations

Prior to immunization, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100mg/kg) with xylazine (10mg/kg). Each mouse was immunized with a total of 20 μ l vaccine, which was administered by an equal amount in each nares and allowing the mice to inhale the vaccine. Control mice were not vaccinated. For most experiments, mice were immunized with a total of 2 μ g ovalbumin (Ova). Mice were immunized once and boosted 10 days later. Serum was collected 5 - 7 days after the boost for analysis of cellular and humoral immune responses. Saliva was collected following i.p. injection of 10 μ g pilocarpine (Sigma-Aldrich) in PBS.

2.4 (5) Antibody response in serum, saliva, and BAL fluid

Antibody responses to Ova were assessed as described previously [53-54]. Briefly, ELISA plates were coated with Ova, blocked to reduce non-specific binding, then incubated with serial dilutions of samples from vaccinated and control mice. Antibody titers were determined using endpoint dilution assay and were expressed as the log reciprocal of the highest dilution of a sample with an OD reading of 0.1 above background.

2.4 (6) Cell collection

Bronchoalveolar lavage (BAL) cells were obtained by airway lavage, as previously described [55]. Cells from the 3-4 washes per mouse were pooled, centrifuged at 1,200 rpm for 5 min at 4°C. The cells were further purified by NH₄Cl lysis of the RBC. Lung cells were prepared by first mincing the tissues, then digesting in a solution of 5 mg/ml collagenase (type 1A, Sigma-Aldrich) plus DNAase (50 U/ml) and soybean trypsin inhibitor (10 mg/ml) for 20 min at 37°C, as described previously [55]. The cells were then mechanically disrupted through an 18-gauge needle as previously described [56] and further purified by NH₄Cl lysis. Cells from each organ

source were counted and resuspended in complete medium on ice prior to immunostaining and analysis.

2.4 (7) Antibodies and flow cytometric analysis

Directly conjugated antibodies used for these analyses were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). The following antibodies were used: anti-CD8b (FITC; clone H35-17.2) and anti-I-A/I-E (MHC class II, FITC; clone NIMR-4). Immunostaining was done as described previously [55]. In most cases, cells were fixed in 1% paraformaldehyde for 20 min and stored in FACS buffer at 4°C for 1-2 days prior to analysis. Analysis was carried out with a Cyan ADP flow cytometer (Beckman Coulter, Fort Collins, CO). Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

2.4 (8) Ova stimulation assay

Airway lavage, lung, and mediastinal lymph node cells were collected and processed as described above. Cells from the lavage fluid and lungs were further purified by positive selection of the CD4⁺ T cells using CD4 (L3T4) Microbeads on a magnetic cell separation column (MACS Miltenyi Biotech, Auburn, CA). Sample preparation was done according to the manufacturer's instructions. Single cell suspensions were added to triplicate wells of 96-well plates at a concentration of 1×10^6 cells in 100 μ l of complete RPMI media. Naïve spleen cells (4×10^5 cells) were added to airway lavage and lung cells as a source of antigen presenting cells. Cells were stimulated with 50 μ g/ml Ova for 84 hours at 37°C in 5% CO₂ in 96-well round bottom plates. After stimulation, the cells were centrifuged, and the supernatants were harvested and kept at -20°C until assayed for cytokine release.

2.4 (9) MHC-peptide tetramers

Soluble H-2K^b MHC class I tetramers containing the ova8 peptide, SIINFEKL, were produced as described previously [57]. The CD8⁺ T cell response in mice vaccinated against ovalbumin was assessed in C57BL/6 mice. Single cell suspensions (typically 5x10⁵ to 1x10⁶ cells suspended in 100 µl of complete media) from the lung, peripheral bone marrow, and mediastinal lymph node were incubated with tetramer at 37°C for 90 min. Splenocytes from OT-1 mice (Ova8-specific TCR transgenic mice, provided by T. Potter, National Jewish Medical and Research Center, Denver, CO) were used as positive controls for tetramer staining. Staining and analysis of tetramer-labeled cells was done as described previously [41].

2.4 (10) Vaccinations for protection from *Burkholderia* pulmonary challenge

Heat killing of *Burkholderia pseudomallei* was performed as described previously [58]. Briefly, bacteria were washed and resuspended in PBS, then heated to 80°C for 1 hour. Complete bacterial killing was confirmed by agar plating on LB agar plates. To assess the ability of CLDC adjuvanted vaccines to elicit protection from a lethal infectious challenge, BALB/c mice were vaccinated i.n. with CLDC adjuvant alone, 1 x 10⁵ heat-killed *Burkholderia pseudomallei* organisms alone, or heat-killed bacteria mixed with 10 µl CLDC in a total volume of 20 µl. Mice were boosted in the same manner 10 days later, and then subjected to lethal i.n. challenge with 7,500 CFU live *B. pseudomallei* 1026b (8 x LD₅₀) 14 days after the boost, using a bacterial challenge protocol described previously [50]. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

The ability of CLDC to elicit protection in combination with recombinant protein antigens was also assessed. The proteins being evaluated for potential use as vaccine antigens were BimA, BopA, and LolC (provided by Dr. Mark Estes, University of Texas Medical Branch, Galveston, TX) [59]. BimA, BopA, and LolC are secreted and surface proteins from *B. pseudomallei* and *B. mallei* (refer to Appendix 1). BALB/c mice were vaccinated i.n. with D5W

(sham), BimA alone (no adjuvant), BimA with CLDC, BopA with CLDC, or LolC with CLDC. Each vaccine consisted of 2 µg of protein antigen in a total volume of 20 µl. Mice were boosted 1-2 more times in the same manner 10-14 days following the primary vaccination. Mice were then subjected to lethal i.n. challenge with 3×10^3 CFU live *B. pseudomallei* 1026b ($3 \times LD_{50}$) 14 days after the final boost. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

2.4 (11) Statistical analyses

Statistical analysis was performed using Prism 5.0 software (Graph Pad, La Jolla, CA). For comparison of more than two groups, a non-parametric ANOVA (Kruskal-Wallis test) was done, followed by Dunn's multiple means comparison test. Survival times were determined using Kaplan-Meier curves, followed by the log-rank test. The Bonferroni correction was applied for comparison of more than 2 survival curves. For all comparisons, differences were considered statistically significant for $p < 0.05$.

2.5 Results

2.5 (1) Mucosal immunization with CLDC adjuvant elicits systemic and local antibody responses.

To assess the mucosal adjuvant properties of CLDC, we first investigated the ability of vaccines delivered intranasally (i.n.) with CLDC to generate systemic humoral immune responses, using the model antigen ovalbumin (Ova). Mice were typically immunized twice, 10 days apart. Mice immunized i.n. with a CLDC/Ova vaccine developed significant increases in total serum ova-specific IgG titers, compared to mice vaccinated with Ova alone (**Figure 2.1A**). CLDC adjuvanted vaccines also elicited significant increases in serum ova-specific IgG₁ titers (**Figure 2.1B and 2.1C**).

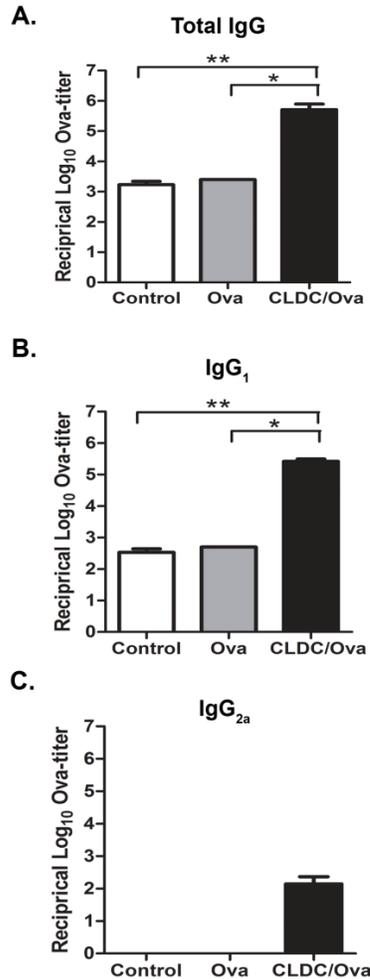


Figure 2.1. Mucosal immunization with CLDC adjuvant elicits systemic IgG. (A-C) C57BL/6 mice ($n = 5$ per group) were intranasally vaccinated twice with 2 μ g ovalbumin protein alone or in conjunction with a CLDC adjuvant as described in Materials and Methods. At three weeks post-vaccination serum was collected. An ELISA for ova-specific antibodies was performed on serial dilutions using secondary antibodies to (A) total IgG, (B) IgG₁, and (C) IgG_{2a}. The antibody titers are expressed as the reciprocal of the highest dilution of serum with an OD reading of 0.1 above background. Similar results were seen in one additional experiment. Significant differences (* $p < 0.05$, ** $p < 0.01$) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

The ability of CLDC-adjuvanted vaccines to induce local IgA responses was assessed next. Intranasal immunization resulted in a significant increase in ova-specific IgA titers in saliva of CLDC/Ova vaccinated mice, compared to mice vaccinated with Ova alone (**Figure 2.2A**). CLDC/Ova also induced significant ova-specific IgA titers in the airways of mice, as assessed in the BAL fluid (**Figure 2.2B**). Thus, it was apparent that the mucosal administration of CLDC

adjuvanted vaccines was capable of eliciting significant mucosal IgA responses, as well as significant systemic IgG responses.

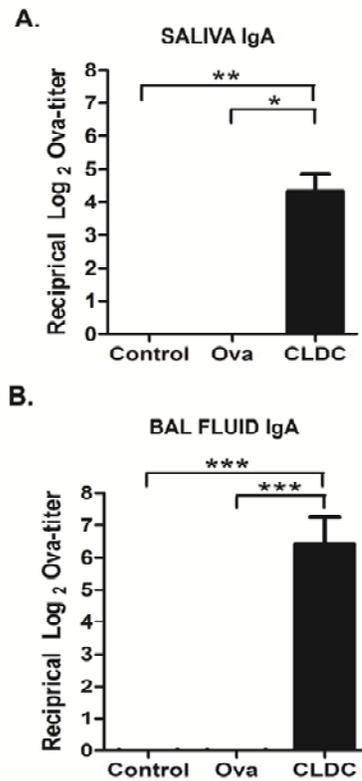


Figure 2.2. Mucosal immunization with CLDC adjuvant elicits local IgA responses. C57BL/6 mice ($n = 10$ per group) were intranasally vaccinated twice with 2 μg ovalbumin protein alone or in conjunction with a CLDC adjuvant as described in Materials and Methods. At three weeks post-vaccination saliva and BAL fluid were collected. Results were pooled from two independent experiments. An IgA ELISA for ova-specific antibodies was performed on serial dilutions. The antibody titers are expressed as the reciprocal of the highest dilution of a sample with an OD reading of 0.1 above background. Significant differences ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

2.5 (2) Mucosal immunization with CLDC adjuvant induces antigen-specific T cell responses.

We reported previously that CLDC-adjuvanted vaccines administered parenterally produced strong T cell responses and were particularly effective in stimulating cross-priming and generating antigen-specific CD4⁺ T cell and CD8⁺ T cell responses against protein antigens [41].

Therefore, we assessed T cell responses to Ova following immunization with CLDC-adjuvanted vaccines administered mucosally. First, CD4⁺ T cell responses were evaluated using cells recovered from the airways, lung tissues, and mediastinal lymph nodes (MLN) of immunized mice. For assay of T cell responses in the lungs and BAL, CD4⁺ T cells were enriched using magnetic bead separation, whereas unseparated T cells were assayed in the MLN samples. Cells were incubated with 50 µg/ml Ova protein and the release of IFN-γ into the supernatants was assessed using an IFN-γ ELISA. We found that following Ova re-stimulation, BAL CD4⁺ T cells recovered from CLDC/ova vaccinated mice produced significant IFN-γ concentrations in comparison to mice vaccinated with Ova alone (**Figure 2.3**). CD4⁺ T cells from the MLN and lung tissues failed to produce IFN-γ when re-stimulated in vitro (data not shown). These data suggested that mucosal immunization with CLDC results in activated antigen-specific CD4⁺ T being targeted to the airways.

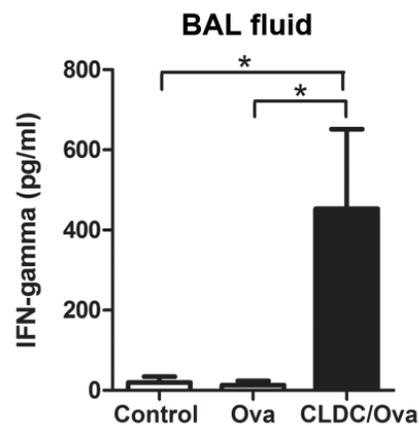


Figure 2.3. Mucosal immunization with CLDC-based vaccines results in IFN-γ producing CD4⁺ T cells in the BAL fluid. C57BL/6 mice (*n* = 5 per group) were intranasally vaccinated twice with 2 µg ovalbumin protein in a CLDC adjuvant, a CTB adjuvant, or a CpG adjuvant, as described in Materials and Methods. Two weeks after the second immunization, CD4⁺ T cell responses were measured using IFN-γ production following ovalbumin stimulation of enriched CD4⁺ T cells from the BAL fluid. Results were pooled from 2 independent experiments, * denotes significant differences (*p*<0.05) determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

We next investigated CD8⁺ T cell responses to CLDC-adjuvanted vaccines. For these experiments, numbers of Ova-specific CD8⁺ T cells in blood, BAL fluid, and lung tissues were enumerated using H-2K^b-ova8 tetramers and flow cytometry, as previously described [41]. Following i.n. immunizations, a significant increase in numbers of Ova-specific CD8⁺ T cells was noted in all three sites evaluated (blood, lung parenchyma, and airways) (**Figure 2.4B**). The expansion of Ova-specific CD8⁺ T cells was particularly dramatic in the airways of vaccinated mice, with 34.7% of all airway CD8⁺ T cells being Ova-specific. It is clear therefore that CLDC-adjuvanted vaccines are quite effective in generating CD8⁺ T cell responses in pulmonary mucosal tissues of vaccinated animals. The presence of antigen specific CD8⁺ T cells in the airways could be very beneficial for inducing protection against inhaled viral and bacterial pathogens.

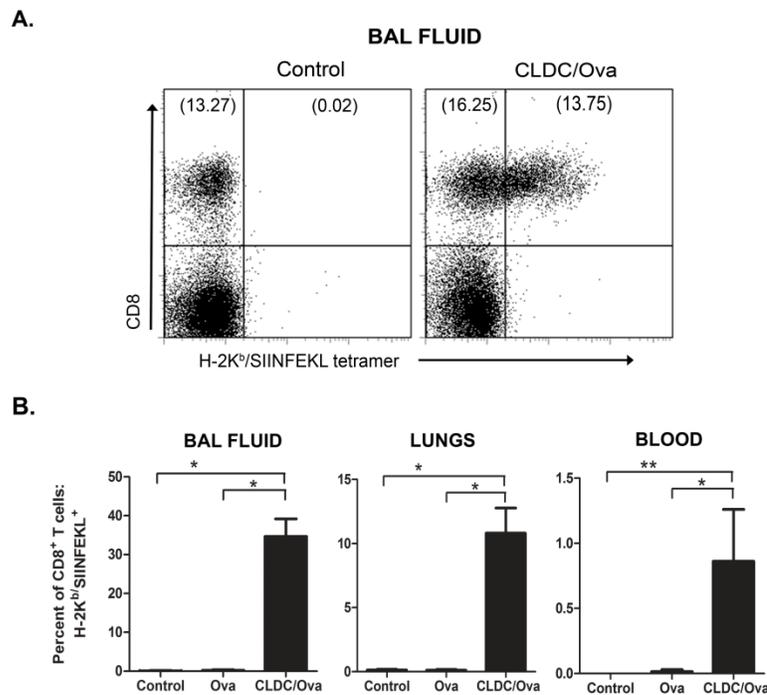


Figure 2.4. Mucosal immunization with CLDC-based vaccines results in the cross-priming of CD8⁺ T cells. C57BL/6 mice ($n = 5$ per group) were intranasally vaccinated twice with 2 μ g ovalbumin protein alone or in conjunction with a CLDC adjuvant as described in Materials and Methods. One week after the second immunization, CD8⁺ T cell responses were measured using H-2K^b/SIINFEKL tetramers as described in Materials and Methods. (A) Representative FACS plot of SIINFEKL-specific CD8⁺ T cells elicited by vaccination with ova peptide in CLDC adjuvant in the BAL fluid. (B) Total CD8⁺ T cells were gated for analysis (after excluding MHC class II⁺ cells), and the percentage of the total CD8⁺ T cells that

were H-2K^b/SIINFEKL⁺ was plotted for the BAL fluid, lungs, and peripheral blood mononuclear cells. Similar results were seen in one additional experiment, significant differences (*p<0.05, **p<0.01) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

2.5 (3) Mucosal immunization with heat killed bacteria and CLDC adjuvant generates effective protective immunity against lethal pulmonary challenge with *Burkholderia pseudomallei*.

Experiments were conducted next to assess the potential for CLDC-adjuvanted mucosal vaccines to generate protective immunity against an inhaled pathogen. For these experiments, we used a mouse model of lethal *Burkholderia pseudomallei* pneumonia, based on recent studies conducted by our laboratory [50, 60]. BALB/c mice were vaccinated and boosted i.n. with CLDC adjuvant alone, 1×10^5 heat-killed *Burkholderia pseudomallei* organisms alone, or heat-killed bacteria mixed with 10 μ l CLDC in a total volume of 20 μ l. Control mice were not vaccinated. All mice were then subjected to i.n. challenge with 8 x LD₅₀ (7.5×10^3) CFU *B. pseudomallei* 2 weeks after the last immunization and survival times were determined by the Animal Care and Use Committee at Colorado State University.

All unvaccinated control mice reached end-point prior to day 3 after challenge, and the CLDC alone mice succumbed to disease by day 4. In contrast, 4 of the 9 mice vaccinated with heat-killed bacteria alone survived for > 40 days (**Figure 2.5**). However, it is important to note that all of the surviving mice vaccinated with heat-killed *B. pseudomallei* only eventually succumbed to chronic disease by day 60 post-challenge (data not shown). In contrast, 100% of mice vaccinated with heat-killed bacteria plus CLDC survived bacterial challenge for > 40 days (Fig. 6). Five of these 9 mice survived past day 60 post-challenge and were considered long-term survivors. Long-term survival tends to correlate with clearing of the organism, but cultures were not performed to confirm this fact. These results indicate that mucosal vaccination using a

CLDC-adjuvanted vaccine elicited significant protective local and systemic immunity against a lethal challenge with a very virulent bacterial pathogen.

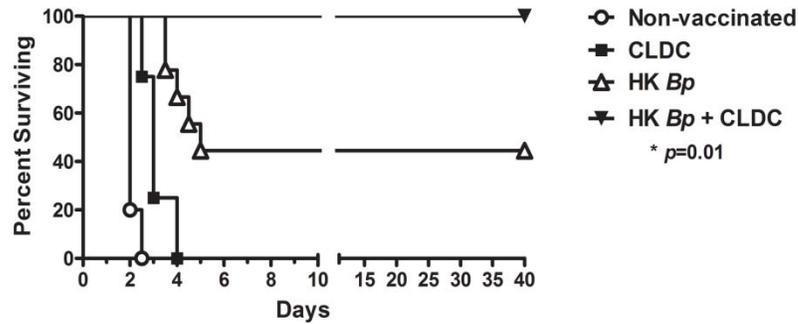


Figure 2.5. Mucosal immunization with heat killed bacteria and CLDC adjuvant generates effective protective immunity against lethal pulmonary challenge with *Burkholderia pseudomallei*. BALB/c mice ($n = 4-5$ mice per non-vaccinated control and CLDC groups, and 9 mice per HK *Bp* and HK *Bp* + CLDC groups) were primed intranasally with 1×10^5 CFU heat-killed *B. pseudomallei* 1026b suspended in D5W buffer or with heat-killed bacteria complexed to the CLDC adjuvant. Mice were boosted in the same manner 10 days later. Mice in the CLDC alone group were primed and boosted with this adjuvant alone. All animals were then challenged intranasally with 7500 CFU live *B. pseudomallei* 1026b 14 days following the boost, and survival was monitored. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test. The Bonferroni corrected threshold was applied and comparisons with $p < 0.013$ were considered significant. ($*p = 0.01$ for mice vaccinated with heat-killed bacteria alone vs. those vaccinated with heat-killed bacteria complexed to CLDC). Data shown are representative of 2 combined independent experiments.

2.5 (4) Mucosal immunization with recombinant protein antigens and CLDC adjuvant generates short-term protection against acute pneumonic *Burkholderia* infection.

Based on the promising results following CLDC-adjuvanted immunization with heat killed bacteria (**Figure 2.5**), we were interested in investigating the ability of CLDC to enhance the immune response to recombinant protein antigens. These experiments utilized the lethal *Burkholderia pseudomallei* pneumonia mouse model and the *Burkholderia* purified proteins BopA, BimA, and LolC. Please refer to Appendix 1 for the complete study on these recombinant vaccine antigens. BALB/c mice were vaccinated and boosted i.n. with D5W (sham), BopA alone (no adjuvant), BopA mixed with CLDC, BimA mixed with CLDC, and LolC mixed with CLDC.

Mice were vaccinated with 2 µg recombinant protein in a total volume of 20µl. All mice were then subjected to i.n. challenge with 3 x LD₅₀ (3 x 10³) CFU *B. pseudomallei* 2 weeks after the last immunization. All unvaccinated animals as well as those animals vaccinated with BopA alone succumbed to disease by days 4 and 3.5 respectively. In contrast, 40-60% of animals vaccinated with a recombinant protein in conjunction with CLDC adjuvant survived acute disease until around day 20 (Figure 2.6). However, the vaccination did not protect the mice from chronic disease, as few mice survived until day 60. Therefore, the ability of CLDC adjuvant to elicit protective immunity when combined with recombinant protein antigens shows promise but is less potent in comparison to vaccination with heat killed bacteria.

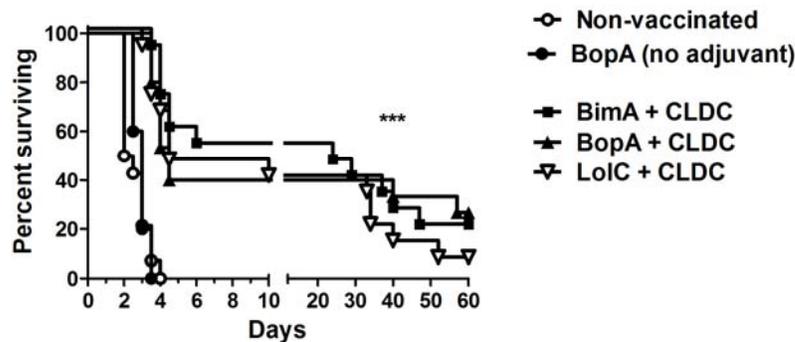


Figure 2.6. Mucosal vaccination with *Burkholderia* protein subunits in conjunction with CLDC elicits short-term protection from pneumonic *B. pseudomallei*. BALB/c mice ($n = 5$ per group) were vaccinated with the BopA subunit alone, and other groups ($n = 15$ mice per group) were vaccinated with BimA, BopA, or LolC, all in conjunction with the CLDC adjuvant. Control mice ($n = 14$) were left unvaccinated. Vaccinated mice were primed and boosted 1-2 times, and challenged with 3×10^3 CFU live *B. pseudomallei* 1026b ($3 \times \text{LD}_{50}$) two weeks after the final boost. Survival was monitored for 60 d post-challenge, and mice were euthanized according to pre-determined humane end-points. Approximately 40-60% of the mice were protected until day 20 from lethal *B. pseudomallei* challenge. All antigens tested conferred increased survival and time to death compared to non-vaccinated control animals (***, $p < 0.0001$ for BimA, BopA, and LolC vaccinated groups vs. non-vaccinated controls). Data shown are representative of 3 combined independent experiments.

2.5 (5) Potency of CLDC adjuvant equivalent or superior to that of conventional mucosal vaccine adjuvants.

Lastly, to place the potency of CLDC mucosal adjuvant properties in context, CLDC-elicited vaccine responses were compared to those generated by the conventional mucosal adjuvants cholera toxin B (CTB) and CpG oligonucleotides (ODN) [9-10, 12]. Mice were therefore vaccinated i.n. with 2 ug Ova admixed with CLDC, CTB (5 μ g), or CpG (10 μ g) [19, 61-64]. Intranasal immunization using each of the three different adjuvants elicited significant increases in ova-specific IgA titers in the BAL fluid of vaccinated mice (**Figure 2.7A**). Of the three adjuvants only the CLDC adjuvant generated significant increases in ova-specific IgG titers in the BAL fluid (**Figure 2.7B**). However, it should also be noted that only the CpG ODN adjuvant elicited significant increases in ova-specific IgG_{2a} titers (data not shown). Mucosal adjuvants were also compared for their ability to generate CD8 T cell responses in the lungs. Intranasal immunization with CLDC adjuvant appeared particularly effective in generating antigen-specific CD8 T cell responses, especially in the airways of vaccinated mice (**Figure 2.7C**). Overall, these results suggested that at least for soluble protein antigens, CLDC based adjuvants are likely as effective as current mucosal vaccine adjuvants.

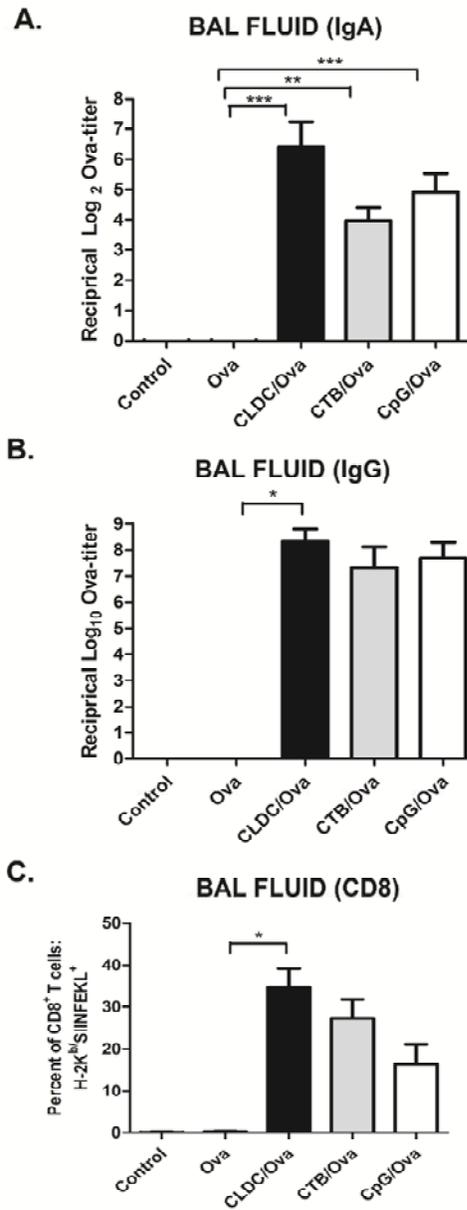


Figure 2.7. Mucosal immunization with CLDC adjuvant elicits potent immune responses equivalent to leading mucosal vaccine adjuvants. (A-C) C57BL/6 mice ($n = 5$ per group) were intranasally vaccinated twice with 2 μ g ovalbumin protein alone or in conjunction with a CLDC adjuvant, a CTB adjuvant (5 μ g), or a CpG adjuvant (10 μ g), as described in Materials and Methods. One week after the second immunization the BAL fluid was collected. (A,B) An ELISA for ova-specific antibodies was performed on serial dilutions of the BAL fluid using secondary antibodies to (A) IgA and (B) Total IgG. The antibody titers are expressed as the reciprocal of the highest dilution of serum with an OD reading of 0.1 above background. (C) Total CD8⁺ T cells were gated for analysis (after excluding MHC class II⁺ cells), and the percentage of the total CD8⁺ T cells that were H-2K^b/SIINFEKL⁺ was plotted for BAL fluid. Similar results were seen in one additional experiment. Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

2.6 Discussion

After assessing both humoral and cellular immune responses to soluble antigens (Ova) delivered intranasally using the CLDC adjuvant, we concluded that CLDC was indeed an effective mucosal vaccine adjuvant. CLDC adjuvanted vaccines were found to be particularly effective at generating mucosal IgA responses, as well as intrapulmonary T cell responses. The ability of the CLDC adjuvant to increase the immunogenicity of a complex particulate antigen (ie, heat-killed bacteria) as well as recombinant protein antigens was also demonstrated.

A variety of immunological properties that have been attributed to cationic liposomes are likely to have contributed to the effectiveness of CLDC as a mucosal vaccine adjuvant. For one, positively charged liposomes rapidly adhere to negatively charged surfaces of cells such as APCs and epithelial cells, increasing their uptake [38, 65]. In addition, cationic liposomes have been shown to directly activate APCs such as DC [38, 66-67]. Finally, the size of the CLDC particles used in this study (approximately 250 nm diameter) is ideal for uptake by DC, including pulmonary DC [66, 68]. Therefore, the mucosal adjuvant properties of CLDC are likely dependent on the unique characteristics of the cationic liposomes. Although the uptake and activation of DC following CLDC administration still remains unclear, it represents a potential angle to investigate.

The adjuvant activity of cationic liposome-nucleic acid-based adjuvants is also influenced by the nucleic acid component of the vaccine [69]. It has been shown that the non-coding plasmid DNA used in the preparation of CLDC contains many CpG motifs and these motifs are known to activate innate immunity most likely through TLR9 signaling [69-70]. Indeed, previous studies in our laboratory have shown that cellular immune responses to vaccination with CLDC adjuvanted vaccines were almost completely abolished in MyD88^{-/-} mice when the vaccine was administered parenterally [41]. We believe MyD88 signaling will play an equally important role in mucosal immunization with CLDC adjuvant and warrants further investigation. While it is currently not known how well other TLR agonists might function as mucosal adjuvants when

complexed to cationic liposomes, it is known that TLR3 agonists, such as polyI:C, are effective at stimulating immune responses with cationic liposomes [41].

The unique ability of CLDC to elicit the cross-priming of CD8 T cells to protein antigens has been explored previously in the context of parenteral vaccines [41] as well in a therapeutic vaccine used to suppress hyperresponsiveness in the airways [71]. In the present study we found that mucosal vaccination using CLDC as an adjuvant was also capable of rapidly generating pulmonary CD8⁺ T cell responses. While the mechanism of CLDC mediated cross-priming is not fully understood, it is believed that the cationic liposome component of CLDC results in the slight instability of the endosome resulting in the leakage of endosomal contents into the cytoplasm, leading to the processing and presentation of peptide fragments via MHC class I [36]. The ability of CLDC to elicit activated antigen-specific CD4⁺ T cells in the airways highlights a potential mechanism for enhancing both the humoral and CD8⁺ T cell immune responses.

The capacity of CLDC-adjuvanted vaccines to induce protective responses to both complex particulate antigens as well as recombinant proteins speaks to the CLDC's versatility and potential use in multiple types of vaccines. The ability of CLDC to elicit a better protective response to the heat-killed bacteria may be due to the exposure of multiple epitopes to the immune system in comparison to the more limited epitopes available on recombinant proteins. Therefore, CLDC has been proven to be a vaccine adjuvant capable of eliciting protective immunity with a variety of antigens.

The ability of the CLDC adjuvant to induce mucosal immune responses is not confined to the respiratory tract. For example, we have recently reported that oral administration of CLDC-adjuvanted vaccines is also capable of generating substantial protective immunity against pulmonary challenge with *Yersinia pestis*[72]. Thus, induction of efficient mucosal immunity, particularly at pulmonary surfaces, appears to be a general property of CLDC-based adjuvants. Moreover, we also found here that CLDC adjuvants performed well when compared to other conventional adjuvants in terms of potency of both humoral and cellular immunity. In summary,

these findings suggest that liposome-nucleic acid based adjuvants are an important new category of mucosal vaccine adjuvant that generates considerable activity when combined with protein antigens.

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CHAPTER 3

MUCOSALLY ADMINISTERED LIPOSOME-NUCLEIC ACID ADJUVANT INDUCES INCREASED ANTIGEN UPTAKE AND TRAFFICKING BY AIRWAY DENDRITIC CELLS

I acknowledge the contribution of Dr. Ross Kedl for the production of the H-2K^b MHC class I tetramers, Dr. Laurel Lenz for providing the MyD88^{-/-} mice, and Dr. Scott Hafeman for the production of the liposomes.

3.1 Research Rationale

The development of safer, less expensive, shelf-tolerant mucosal vaccines is necessary and could greatly impact the health of people worldwide. The ability to administer these vaccines through methods such as intranasal spray or sublingual tablet instead of a needle offers many advantages, especially in third world countries [1]. As a result, there is great interest in the development of vaccines and vaccine adjuvants administered directly onto the mucosal surfaces. The strategic choice of a mucosal vaccine adjuvant has many benefits including antigen protection and targeting or polarizing of the immune response. Understanding the mechanism of action elicited by newly discovered mucosal vaccine adjuvants allows for their optimal use in developing vaccines. Recent investigation has revealed the use of cationic liposome-DNA complexes (CLDC) as a potent mucosal vaccine adjuvant (Chapter 2). However, the mechanisms of immune modulation need to be elucidated in order to understand the full potential of liposome-nucleic acid based adjuvants, an important category of mucosal vaccine adjuvants. This chapter investigates the effects of CLDC on innate immune signaling as well as the activation and mobilization of antigen presenting cells in the airways.

3.2 Summary

The development of novel vaccine adjuvants can no longer be undertaken empirically, but requires in-depth investigation of cellular and molecular events responsible for adjuvant activity. Cationic liposome-DNA complexes (CLDC), when administered mucosally, have been shown to be effective vaccine adjuvants through the induction of a balanced humoral and cellular immune response. However, little is known regarding the mechanism of action elicited by CLDC adjuvant when utilized in mucosal vaccination. Therefore, we conducted studies to assess the ability of CLDC to modulate the innate immune system in order to better understand the initiation of the adaptive response. Following the intranasal (i.n.) administration of CLDC, relevant cytokines and cellular population were enumerated in the airways of mice. MyD88 signaling as well as antigen uptake and trafficking to the draining lymph nodes were also investigated as potential modes of immune modulation elicited by CLDC. We found that mucosal immunization with CLDC adjuvant resulted in the increase in the pro-inflammatory cytokines IL-6 and IFN- γ and cellular immune responses were dependent on MyD88 signaling. MCP-1 (CCL2) concentration and inflammatory monocyte populations were increased in the airways following CLDC treatment. However, the CLDC-adjuvanted adaptive responses appeared to be independent of monocyte recruitment into the airways. Finally, resident airway myeloid dendritic cells (DC) efficiently phagocytosed the CLDC adjuvant and trafficked the associated antigen to the draining lymph node. These findings suggest that the mechanism of action elicited by the mucosally administered CLDC adjuvant involves modulating the airway environment through cytokine induction and antigen presenting cell activation.

3.3 Introduction

In recent years the trends in vaccine development have shifted to safer, non-replicating subunit vaccines requiring adjuvants in order to enhance poorly immunogenic antigens [2-4]. Vaccine adjuvants typically modulate the immune system in two ways: by increasing the magnitude of the adaptive response, and by polarizing the immune response via signaling through innate immune receptors [5-6]. Vaccine adjuvants are composed of a variety of compounds such as microbial compounds, mineral salts, microspheres, liposomes, and emulsions; whose functions are diverse and mechanisms of action mostly unknown [5, 7]. The handful of licensed vaccine adjuvants have mainly been discovered empirically without any clear understanding of the mechanism of action. Until a recent study showing potential signaling through NALP3, aluminum compounds have been used in vaccines in humans for almost 70 years with limited knowledge as to their cellular and molecular mechanisms [8].

The development of new vaccine adjuvant candidates has been on the rise during the last 20 years due to breakthroughs in the understanding of the innate immune system [6]. The innate immune response, orchestrated mainly through pattern recognition receptors (PRRs), induces the production of key molecules involved in priming, expansion, and polarizing the adaptive immune responses [5]. More specifically, PRR signaling results in increased pro-inflammatory cytokine and chemokines production, and the up-regulation of MHC class I and II surface expression. For example, MF59, an oil-in-water emulsion approved in the UK for use with the influenza vaccines in the elderly, modulates the immune system by binding TLR4 and recruiting dendritic cells [9]. Other adjuvants in the late stages of clinical development have been shown to bind PRRs such as: Poly-IC (TLR3, MDA5), MPL (TLR4), Flagellin (TLR5), Imiquimods (TLR7/8), and CpG Oligonucleotides (TLR9) [5].

In addition to the ability of vaccine adjuvants to induce signaling through PRRs, many adjuvants are also potent stimulators of cellular migration to draining lymph nodes. The particulate nature of adjuvants such as ISCOMs and liposomes enables non-specific targeting and

activation of APCs [10]. In the airways, pulmonary dendritic cells (DCs) constitutively migrate to the lymph nodes; but following activation, the migration of DCs dramatically increases through the binding of CCR7 and CCR8 [11]. Therefore, the ability of various vaccine adjuvants, such as MF59 and HSP70L1, to induce the up-regulation of CCR7 expression on DCs highlights a potent mechanism of action induced by these vaccine adjuvants [12-13].

We previously reported that a vaccine adjuvant consisting of cationic liposome-DNA complexes (CLDC) effectively elicited balanced cellular and humoral immunity following mucosal vaccination [14]. CLDC-adjuvanted vaccines were also shown to protect against lethal bacterial infections with particulate and soluble antigens [14-15]. Understanding the mechanism of action utilized by CLDC will aid in its consideration as a potent mucosal vaccine adjuvant capable of eliciting protection in various infectious disease models. We attribute a majority of the success of these CLDC adjuvanted vaccines to the combination of the liposome (carrier) and the plasmid DNA (immunostimulant). One of the hypothesized mechanisms of action attributed to CLDC is the ability of the positively charged liposome to rapidly adhere to negatively charged surfaces of cells such as APCs and epithelial cells resulting in cellular uptake [16-17] and activation [17-19]. The other proposed mechanism involves the nucleic acid component of the vaccine adjuvant [20]. The non-coding plasmid DNA used in the preparation of CLDC contains many CpG motifs. It is known to activate innate immunity via TLR9 signaling inducing the secretion of pro-inflammatory cytokines such as IFN- γ and TNF- α as well as promote the maturation of APCs and B cells [21].

Therefore, **we tested the hypothesis that CLDC generates effective adaptive immune responses as a result of enhanced antigen uptake and trafficking to draining lymph nodes.** We investigated the ability of CLDC to modulate the cytokine environment and performed experiments to confirm the importance of MyD88 signaling. Also, experiments were conducted to identify mucosal antigen presenting cells (APCs) responsible for antigen uptake and trafficking to regional lymph nodes. In the course of these studies, we identified several mechanisms

through which the CLDC adjuvant elicits strong mucosal immune responses following intranasal vaccination.

3.4 Materials and Methods

3.4 (1) Mice

Specific pathogen-free 6-8-week-old female C57BL/6, and ICR mice were purchased from the Jackson Laboratories (Bar Harbor, ME) or Harlan Laboratories (Indianapolis, IN). MyD88^{-/-} mice bred on the C57Bl/6 background were obtained from Dr. Laurel Lenz (National Jewish Medical, Denver, CO). CCR2^{-/-} mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred in the laboratory animal facilities at Colorado State University. All protocols involving the animal experiments described in this study were approved by Institutional Animal Care and Use Committee at Colorado State University.

3.4 (2) Reagents and biochemicals

Ovalbumin was purchased from Sigma-Aldrich (St Louis, MO) and was prepared as a 1 mg/ml solution in diH₂O. Fluorescent Alexa Fluor 647 ovalbumin was purchased from Invitrogen (Carlsbad, CA) and was resuspended in PBS at a concentration of 1mg/ml prior to use. All cell preparations were resuspended in complete RPMI (Invitrogen) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Invitrogen), 1X non-essential amino acids (Invitrogen), 0.075% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen).

3.4 (3) Preparation of cationic liposomes-DNA complexes and vaccines

Liposomes were prepared by combining cationic liposome DOTIM octadecenoyloxy(ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolium chloride and cholesterol in equimolar concentrations as described previously [22]. Cationic liposome-DNA complexes (CLDC) were freshly prepared at room temperature and administered within 30 min. Non-coding plasmid DNA (0.2 mg/ml, Juvaris Biotherapeutics) was diluted in sterile Tris-buffered 5% dextrose water. The cationic liposomes were then added with gentle pipetting at a concentration

of 100 μ l of liposomes per 1 ml of solution, resulting in the spontaneous formation of CLDC. To formulate the CLDC-adjuvanted vaccines, the protein antigen was added to the diluted plasmid DNA solution prior to the addition of the cationic liposomes.

3.4 (4) Intranasal immunizations

Prior to immunization, mice were anesthetized by intraperitoneal (i.p.) injection of ketamine (100mg/kg) with xylazine (10mg/kg). Each mouse was immunized with a total of 20 μ l vaccine, which was administered by an equal amount in each nares and allowing the mice to inhale the vaccine. Control mice were not vaccinated. For most experiments, mice were immunized with a total of 2 μ g ovalbumin (Ova). Mice were immunized once and boosted 10 days later. Serum was collected 5 - 7 days after the boost for analysis of cellular and humoral immune responses. Saliva was collected following i.p. injection of 10 μ g pilocarpine (Sigma-Aldrich) in PBS.

3.4 (5) Antibody response in serum

Antibody responses to Ova were assessed as described previously [23-24]. Briefly, ELISA plates were coated with Ova, blocked to reduce non-specific binding, then incubated with serial dilutions of samples from vaccinated and control mice. Antibody titers were determined using endpoint dilution assay and were expressed as the log reciprocal of the highest dilution of a sample with an OD reading of 0.1 above background.

3.4 (6) Cell collection

Bronchoalveolar lavage (BAL) cells were obtained by airway lavage, as previously described [25]. Cells from the 3-4 washes per mouse were pooled, centrifuged at 1,200 rpm for 5 min at 4°C. The cells were further purified by NH_4Cl lysis

of the RBC. Lymph node cells were prepared by mechanical disruption and screening through a 70- μ m nylon mesh screen (BD Biosciences), followed by NH_4Cl lysis. Lung cells were prepared by first mincing the tissues, then digesting in a solution of 5 mg/ml collagenase (type 1A, Sigma-Aldrich) plus DNAase (50 U/ml) and soybean trypsin inhibitor (10 mg/ml) for 20 min at 37°C, as described previously [25]. The cells were then mechanically disrupted through an 18-gauge needle as previously described [26] and further purified by NH_4Cl lysis. Cells from each organ source were counted and resuspended in complete medium on ice prior to immunostaining and analysis.

3.4 (7) Antibodies and flow cytometric analysis

Directly conjugated antibodies used for these analyses were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). The following antibodies were used in various combinations: anti-CD8b (APC, FITC; clone H35-17.2), anti-I-A/I-E (MHC class II, FITC; clone NIMR-4), anti-CD11c (PE-Cy7; clone N418), anti-CD11b (Pacific Blue, biotin; clone M1/70), anti-Ly6G (PE; clone 1A8), anti-Ly6C (Biotin, FITC; clone AL-21), anti-CD45 (Pacific Blue; clone 30-F11). Immunostaining was done as described previously [25]. In most cases, cells were fixed in 1% paraformaldehyde for 20 min and stored in FACS buffer at 4°C for 1-2 days prior to analysis. Analysis was carried out with a Cyan ADP flow cytometer (Beckman Coulter, Fort Collins, CO). Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

3.4 (8) MHC-peptide tetramers

Soluble H-2K^b MHC class I tetramers containing the ova8 peptide, SIINFEKL, were produced as described previously [27]. The CD8⁺ T cell response in mice vaccinated against ovalbumin was assessed in C57BL/6 mice. Single cell suspensions (typically 5x10⁵ to 1x10⁶ cells suspended in 100 μ l of complete media) from the lung and peripheral bone marrow were

incubated with tetramer at 37°C for 90 min. Splenocytes from OT-1 mice (Ova8-specific TCR transgenic mice, provided by T. Potter, National Jewish Medical and Research Center, Denver, CO) were used as positive controls for tetramer staining. Staining and analysis of tetramer-labeled cells was done as described previously [15].

3.4 (9) Cytokine analysis

Cytokine production in lung and BAL samples were assessed using a cytometric bead array (CBA; Becton Dickinson). Lung homogenates were prepped as described previously [28] and the lavage was performed using 1.5 ml of a PBS with EDTA (1mM) solution and processed as previously described [29]. The assay was performed according to the manufacturer's instructions. Analysis was carried out using a Cyan ADP flow cytometer and data was analyzed using Flowjo software. The limit of detection for this assay for each cytokine was reported by the manufacturer to be 5 pg/ml.

3.4 (10) Distribution of labeled CLDC in the airways

The uptake and distribution of CLDC by APCs in the airways and draining lymph nodes was assessed using liposomes labeled with the fluorochrome BODIPY, as described previously [15]. The labeled CLDC were administered i.n. and at various time points (3 hr, 6 hr, 12 hr, and 24 hr) after administration the BAL fluid was collected. Single-cell suspensions were prepared, immunostained with surface antibodies, and analyzed by flow cytometry.

3.4 (11) Uptake of labeled Ovalbumin in the draining lymph node

Uptake and trafficking of Ova by cells in the airways and distribution to the draining lymph node was assessed using Alexa647-labeled Ova (Invitrogen). Alexa647-ova alone, or Alexa647-ova complexed to CLDC, were administered intranasally to mice. Six hours after

administration, the mediastinal lymph node was collected for immunostaining and analysis by flow cytometry.

3.4 (12) Statistical analyses

Statistical analysis was performed using Prism 5.0 software (Graph Pad, La Jolla, CA). For comparisons between two groups, two-tailed non-parametric (Mann-Whitney) t-tests were performed. For comparison of more than two groups, a non-parametric ANOVA (Kruskal-Wallis test) was done, followed by Dunn's multiple means comparison test. For all comparisons, differences were considered statistically significant for $p < 0.05$.

3.5 Results

3.5 (1) Mucosal immunization with CLDC adjuvanted vaccine induces production of IFN- γ and IL-6 in the airways and in the lung tissues.

Experiments were conducted first to assess the effects of CLDC on local induction of cytokine responses in the airways and in lung tissues. In prior studies from our lab it was reported that intranasal (i.n.) administration of CLDC stimulated pulmonary production of pro-inflammatory cytokines, including IL-12, TNF- α , and IFN- α [28, 30-32]. Here we were interested in studying levels of IFN- γ in response to i.n. CLDC to better understand the ability of CLDC to polarize the adaptive immune response. We found a significant increase in IFN- γ levels in the airways and the lung tissue following i.n. CLDC treatment (**Figure 3.1**) indicating the tendency of CLDC-adjuvanted vaccines to induce a T_H1 type immunity. Also, based on the ability of mucosally administered CLDC-adjuvanted vaccines to elicit increased antigen-specific IgA [14], we were interested in examining CLDC induction of cytokines known to be involved in IgA antibody class switching, including IL-6, IL-10, and TGF- β [33-36]. While i.n. administration of CLDC did not induce significant increases in IL-10 or TGF- β in the lungs (data not shown), we found that administration of CLDC induced significant increases in IL-6 in both the airways and lung tissues (**Figure 3.1**). Thus, the ability of CLDC to elicit high levels of local IFN- γ and IL-6 may account in part for the ability of the CLDC adjuvant to modulate the adaptive immunity following mucosal administration.

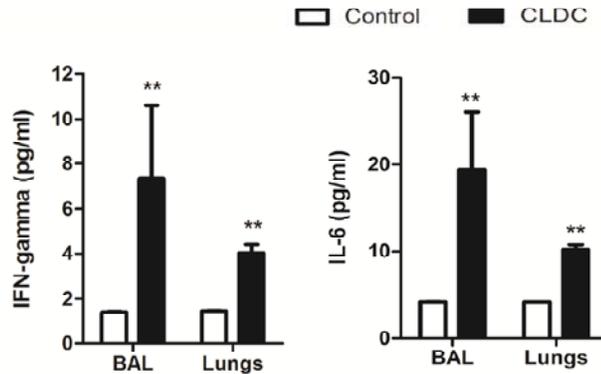


Figure 3.1. Mucosal immunization with CLDC adjuvanted vaccines induces the production of IFN- γ and IL-6 in the airways and lung tissues. C57BL/6 mice ($n = 5$ per group) were given intranasal CLDC 24 hours prior to collecting the BAL fluid and lung tissue. The lung supernatant was collected after tissue homogenization, as described in the Materials and Methods. The mouse inflammatory cytometric bead array was used to determine the concentration of IFN- γ and IL-6 produced following stimulation with CLDC, as described in the Materials and Methods. Similar results were seen in one additional experiment, the asterisks denote significant differences (** $p < 0.01$) determined by non-parametric Mann-Whitney t Test.

3.5 (2) Effective mucosal immunization by CLDC adjuvant is partially MyD88-dependent.

Given the potency of immune responses induced by CLDC-adjuvanted vaccines[14-15], and the increased production of IFN- γ ; the ability of the CLDC plasmid DNA component to modulate the mucosal immune response through binding of TLR9 following mucosal immunization was next to be investigated. While it is known that immune activation by bacterial DNA (eg, plasmid DNA) is mediated via MyD88 and TLR9 dependent pathways, the role of this pathway in mucosal immune activation by liposome-nucleic acid adjuvants has not been previously explored. Therefore, MyD88^{-/-} mice and wild type mice were immunized twice i.n. with CLDC/Ova vaccines and the humoral and cellular immune responses were assessed. We found that Ova-specific CD8⁺ T cell immune responses were significantly reduced in MyD88^{-/-} mice vaccinated with CLDC/Ova, compared to wild type, vaccinated mice (**Figure 3.2A**). For example, the percentage of Ova-specific CD8⁺ T cells in the blood was reduced by 60% in the vaccinated MyD88^{-/-} animals compared to wild type animals.

We also examined the requirement of MyD88 signaling for generating antibody responses by CLDC adjuvants. Multiple studies have shown that following parenteral

immunization using alum, FCA, FIA, MPL, and α -GalCer adjuvants, that MyD88 is not required for generation of antibody responses [37-40]. We found that serum IgG titers in MyD88^{-/-} mice vaccinated i.n. with CLDC adjuvant were equivalent to those of vaccinated wild type mice (**Figure 3.2B**). Thus, these studies indicate that as is the case with other adjuvants, the generation of antibody responses following mucosal immunization with CLDC adjuvant is also MyD88-independent.

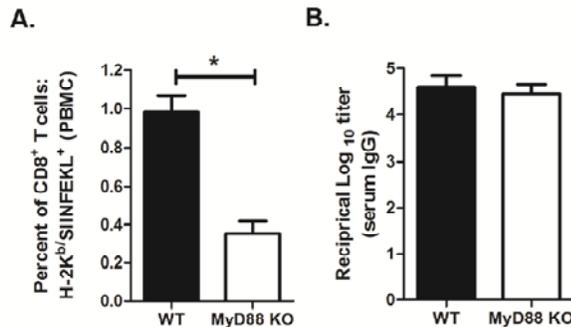


Figure 3.2. Cellular and humoral immune responses to CLDC/Ova vaccination are partially dependent on MyD88 signaling. Wild type C57BL/6 and MyD88^{-/-} mice ($n = 5$ per group) were immunized i.n. twice with 2 μ g ovalbumin protein in CLDC adjuvant, as described in Materials and Methods. (A) Three weeks after immunization, numbers of ova-specific CD8⁺ T cells in blood of wild type and MyD88^{-/-} mice were determined using tetramers and flow cytometry, as described in the Methods. (B) Antibody responses were determined in wild type and MyD88^{-/-} mice. At three weeks post-vaccination serum was collected. An ELISA for ova-specific antibodies was performed on serial dilutions using a secondary antibody to total IgG. The antibody titers are expressed as the reciprocal of the highest dilution of serum with an OD reading of 0.1 above background. Statistical comparisons of groups were made using the non-parametric Mann-Whitney t test, and significant differences between groups were noted (* $p < 0.05$). Similar results were obtained in one additional experiment.

3.5 (3) CLDC are taken up efficiently in the airways of immunized mice.

In order to investigate the initiation of adaptive immune responses following CLDC-adjuvanted i.n. vaccination, we next investigated the ability of CLDC to mobilize antigen presenting cells (APC). Within the respiratory tract, the major APC include resident airway dendritic cells (DC), alveolar macrophages (AM), and inflammatory monocytes recruited to the airways. We therefore assessed cellular immune responses in the airways over the first 24 hours following vaccination with CLDC (**Figure 3.3**). At 3 hours after CLDC administration, there was

a significant increase in inflammatory monocytes within the lungs and the airways. The monocyte population remained elevated for 12 hours after CLDC administration. In contrast, the population of AM in the airways was significantly reduced for at least 12 hours following CLDC immunization. The resident population of CD11b⁺CD11c⁺ DC in the airways also initially decreased, but then increased beginning at 24 hours after CLDC administration. These data show an effect of the CLDC adjuvant on the mobilization of airway APCs within 3 hours of administration, which is important for rapid response to inhaled antigen upon vaccination.

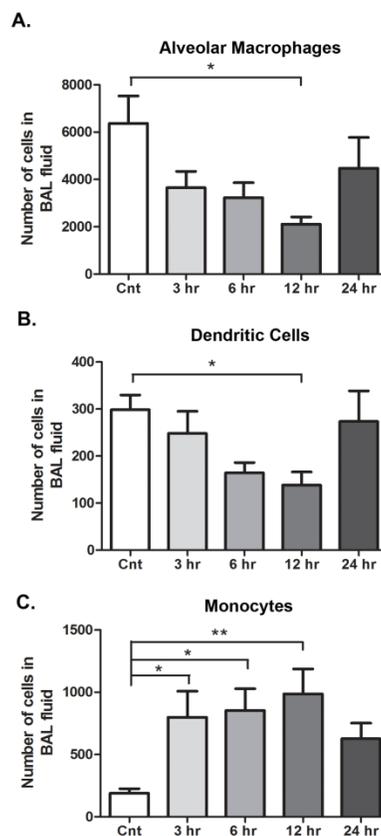


Figure 3.3. Airway cellular response to i.n. administration of CLDC. C57BL/6 mice ($n = 5$ per group) were administered intranasal CLDC at the indicated time points prior to collection of the BAL fluid for cell analysis by flow cytometry. Gating strategy for identification of relevant APC populations in the airways is similar to that displayed in Figure 3.4A. Cellular phenotypes are defined as A) CD11c⁺CD11b⁻ B) CD11c⁺CD11b⁺ C) CD11c⁺CD11b⁺Ly6C⁺Ly6G⁻. Data was pooled from two independent experiments. Significant differences (* $p < 0.05$, ** $p < 0.01$) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison.

Cationic liposomes have been shown to induce uptake and activation of epithelial cells and antigen presenting cells [16-19]. Therefore, we investigated the uptake and distribution of CLDC in the airways and lungs following i.n. immunization. For these experiments, fluorescently-labeled liposomes were used to track CLDC *in vivo* (**Figure 3.4**). Prior to analysis, all external fluorescence was quenched to ensure only internalized CLDC was investigated. At 6 hours following i.n. administration of labeled CLDC, 87% of all the inflammatory monocytes in the airways contained labeled CLDC. In contrast, 40% of DC and 53% of AM contained labeled liposomes (**Figure 3.4B**). The inflammatory monocytes not only took up the complexes more efficiently, but they also appeared to take up more CLDC complexes per cell, as the average mean fluorescence intensity of labeled monocytes was 1307, compared to an average MFI of 680 for AMs and 491 for the DC (data not shown). Thus, mucosally administered CLDC were efficiently taken up by APCs in the airways, most notably by monocytes.

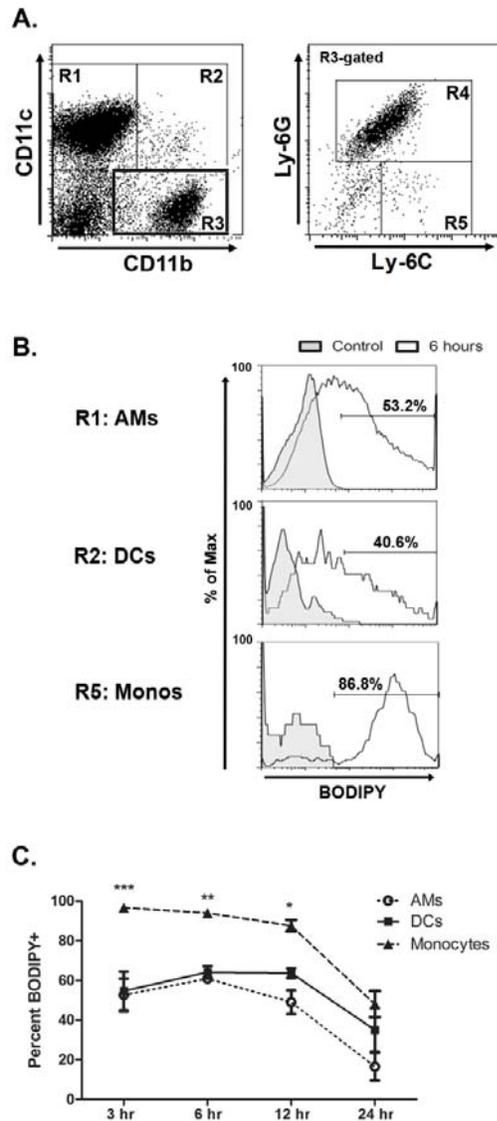


Figure 3.4. Uptake of labeled CLDC by APC in airways of mice. C57BL/6 mice ($n = 5$ per group) were administered BODIPY-labeled CLDC i.n. and airway cells were collected by BAL at the indicated time points after CLDC administration. (A) Gating strategy for identification of relevant APC populations in the airways; including alveolar macrophages (R1), conventional myeloid DCs (R2), and monocytes (R5). (B) BODIPY⁺ cells within the 3 cell populations 6 hours after administration of BODIPY-CLDC, in control and treated mice. (C) Graphical representation of percentage of BODIPY⁺ cells in the airways over time. Between group differences over time were analyzed statistically using 2-way ANOVA, with Bonferroni post-test. Statistically significant differences were noted as (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Similar results were obtained in one additional experiment.

3.5 (4) Effective immunization with CLDC adjuvant is CCR2 independent.

The ability of airway inflammatory monocytes to efficiently phagocytose labeled CLDC, and the fact that CLDC administration elicited MCP-1 (CCL2) production in the airways (**Figure 3.5A**), suggested that inflammatory monocytes may play a role in antigen presentation following CLDC vaccination. To address the role of recruited monocytes in immune responses to mucosal immunization with CLDC adjuvant, we assessed immune responses in CCR2^{-/-} mice, which previous studies have shown are severely impaired in their ability to mobilize inflammatory monocytes to the lungs [28, 41-43]. CCR2^{-/-} mice and wild type mice were immunized twice i.n. with CLDC/Ova vaccine and humoral and cellular immune responses were assessed.

We found that antigen-specific CD8⁺ T cell responses were not significantly different in CCR2^{-/-} mice versus wild type mice (**Figure 3.5B**). Moreover, the Ova specific IgG titers were not significantly different between vaccinated CCR2^{-/-} mice and vaccinated wild type animals (**Figure 3.5B**). These results suggested that antigen targeting and presentation generated by the CLDC adjuvant most likely did not rely on MCP-1-dependent recruitment of inflammatory monocytes into the airways. However, we could not rule out a role for antigen presentation by inflammatory monocytes, since the airways and lungs of CLDC-vaccinated CCR2^{-/-} mice contained only slightly diminished numbers of monocytes compared to wild type mice (**Figure 3.5C**). Therefore, these results suggested that i.n. administration of CLDC stimulated CCR2-independent recruitment of monocytes to the lungs, and that monocytes recruited by other mechanisms may still play an important role in CLDC-induced vaccine immunity [44-45].

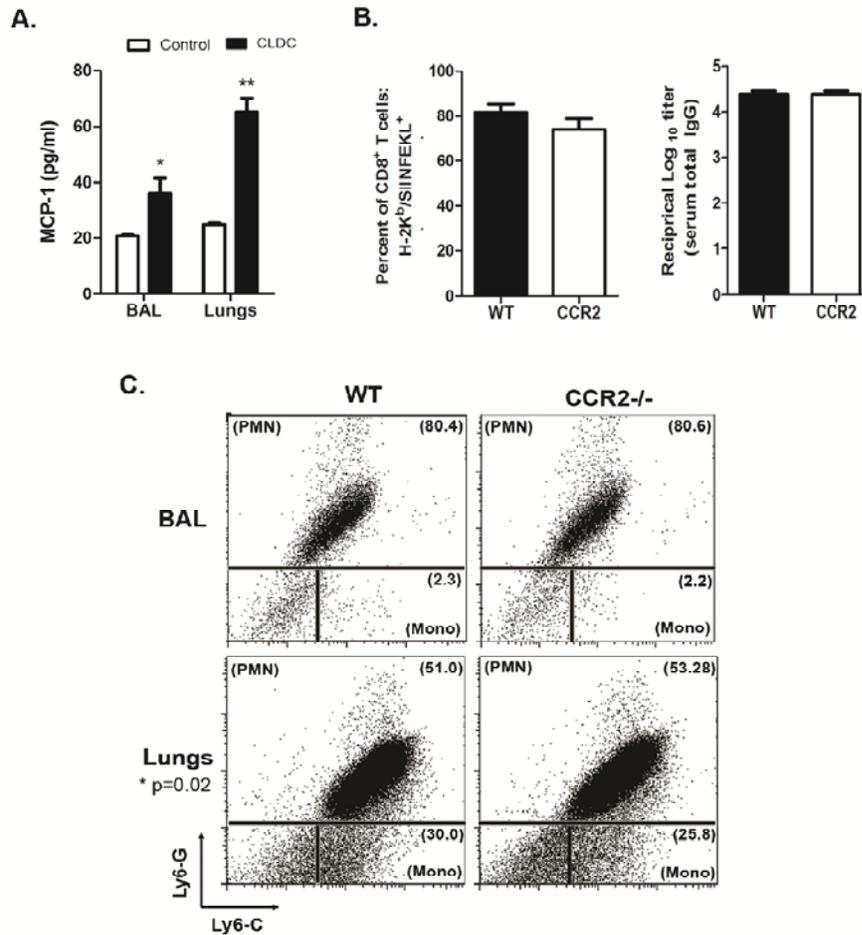


Figure 3.5. Cellular and humoral immune responses to CLDC/Ova vaccination are independent of signaling through the CCR2 receptor. (A) C57BL/6 mice ($n = 5$ per group) were given intranasal CLDC 24 hours prior to collecting the BAL fluid and lung tissue. The lung supernatant was collected after tissue homogenization, as described in the Materials and Methods. The mouse inflammatory cytometric bead array was used to determine the concentration of MCP-1 produced following stimulation with CLDC, as described in the Materials and Methods. (B) Wild type C57BL/6 and CCR2^{-/-} mice ($n = 5$ per group) were vaccinated twice with 2 μ g ovalbumin protein in CLDC adjuvant, as described in Materials and Methods. Antigen-specific CD8⁺ T cell responses in the airways and lung tissues were measured using tetramers and flow cytometry as described in Materials and Methods. Antibody responses were also quantified in vaccinated mice using an Ova ELISA. (C) Wild type C57BL/6 and CCR2^{-/-} mice on the C57BL/6 background ($n = 5$ per group) were administered i.n. CLDC and 6h later, BAL fluid and lung tissues were obtained for analysis of infiltrating monocyte and neutrophil populations. A significant difference was found in the percent of Ly6C⁺Ly6G⁻ lung monocytes in the wild type C57BL/6 and the CCR2^{-/-} mice. (* $p=0.02$). Group means were compared statistically using the non-parametric Mann-Whitney t test (* $p<0.05$, ** $p<0.01$). Similar results were obtained in one additional experiment.

3.4 (5) Antigens complexed to CLDC are delivered efficiently to the mediastinal lymph nodes.

The ability of airway APCs to efficiently take up CLDC suggests a potential role for these cells in priming the immune response in the lymph node. Experiments were conducted next to directly assess the ability of CLDC to enhance delivery of soluble antigens to draining lymph nodes. For these experiments Ova labeled with AlexaFluor 647 was used to facilitate uptake and trafficking studies. Mice were immunized i.n. with Alexa647-Ova alone or Alexa647-Ova complexed to CLDC. Six hours later, antigen uptake in the mediastinal lymph nodes (MLN) was assessed using flow cytometry. We found that administration of Alexa647-Ova complexed to CLDC resulted in significantly greater antigen delivery to the MLN, compared to administration of Alexa647-Ova alone (**Figure 3.6A and 3.6B**).

Next, the impact of CLDC on antigen uptake and delivery by airway APCs was further assessed. We found that the uptake of labeled Ova by CD11c⁺ DC in the MLN was significantly greater when the antigen was complexed to CLDC than when it was administered alone (**Figure 3.6C**). The effect of CLDC on uptake of Ova by other APCs in the lung was also investigated. We found that administering Ova complexed to CLDC did not enhance antigen uptake and trafficking to the MLN by B cells or macrophages (data not shown). Thus, these results show that CLDC are effective vaccine adjuvants in the lungs because they enhance antigen uptake and delivery by airway DC.

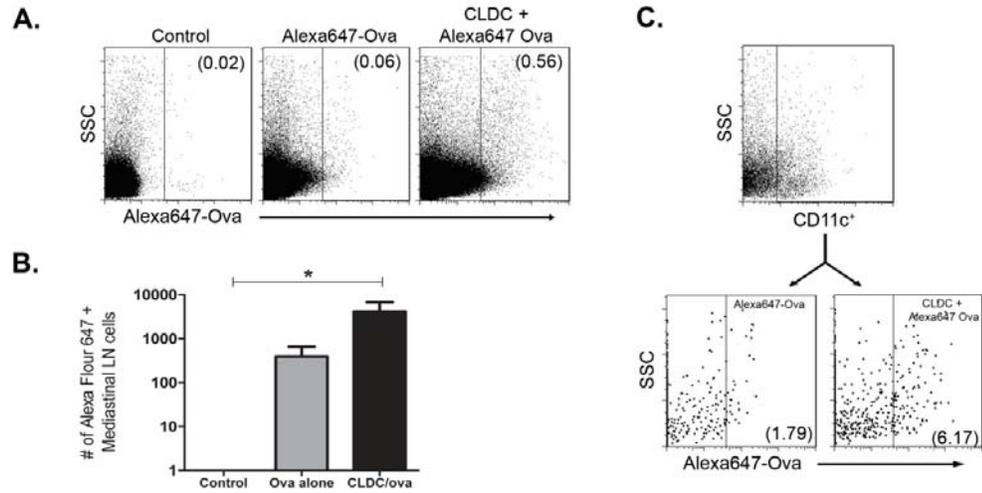


Figure 3.6. Antigens complexed to CLDC were delivered to the mediastinal lymph node following mucosal immunization and were taken up efficiently by dendritic cells. Mice ($n = 5$ per group) were given intranasal Alexa647 ovalbumin (5 ug) in association with CLDC 6 hours prior to the collection of the draining mediastinal lymph node. (A) Representative FACS plots of Alexa647⁺ cells found in the lymph node, the numbers represent the percent of Alexa647⁺ cells. (B) Quantification of Alexa647⁺ cells in the lymph node. Significant differences ($*p < 0.05$) were determined by non-parametric ANOVA followed by Dunn's multiple means comparison. (C) Representative FACS plot of CD11c⁺ dendritic cells found in the lymph node. Following the staining of CD11c, the cells were analyzed for the uptake of Alexa647 ovalbumin, and a significant difference was found between the Alexa647-ova alone group and the Alexa647-ova⁺ CLDC group ($**p = 0.007$). Similar results were seen in one additional experiment.

3.6 Discussion

After assessing the immune responses following intranasal administration of CLDC, we conclude that some of the mechanisms utilized by CLDC to enhance mucosal vaccination are the induction of signaling through innate immune receptors and the activation of airway APCs. CLDC was found to increase key T_H1 cytokine concentrations and induce the recruitment of monocytes in the airways. In addition, CLDC was shown to enhance antigen uptake and trafficking by airway dendritic cells to the draining lymph nodes.

The adjuvant activity of cationic liposome-nucleic acid-based adjuvants is clearly influenced by the nucleic acid component of the vaccine [20]. In the current study, the non-coding plasmid DNA used in the preparation of CLDC contains many CpG motifs known to interact with TLR9. The ability of the CpG motifs in CLDC to activate TLR9 signaling was believed to be highly probable following the detection of IFN- γ , an important pro-inflammatory cytokine induced by TLR9 signaling. The role of TLR9 signaling in the mechanism of action for CLDC was further confirmed using MyD88^{-/-} mice. Indeed, in our studies we found that cellular immune responses to vaccination with CLDC adjuvanted vaccines were nearly completely abolished in MyD88^{-/-} mice, indicating that TLR signaling in the lungs was critical to the activity of CLDC adjuvants.

The capacity of CLDC to modulate the airway cytokine environment is another critical feature to consider when assessing the influence of the adjuvant. Intranasal administration of CLDC has previously been shown to induce the production of IL-12, TNF- α , IFN- γ , IFN- α and IFN- β [28, 30-32]. In the current study we found that CLDC administration induced pulmonary expression of IL-6, a cytokine linked to the induction of IgA class-switching [36, 46-50]. IL-6 has also been shown to stimulate T cell proliferation [51-53], and to enhance generation of protective immunity following vaccination against respiratory pathogens [54-55].

The ability of CLDC to modulate the recruitment of cell populations in the airways following intranasal vaccination is an important function to study. Resident and in fluxing

inflammatory monocytes are important pulmonary cells involved in protection and early immune responses and are of interest when investigating adjuvant modulation of the airway environment. In this study we were able to show not only an increase in MCP-1 (CCL2) cytokine levels in the airways but also an increase in CD11b⁺Ly6C⁺Ly6G⁻ monocytes in the airways at 3 hours post CLDC treatment. In light of the apparent effect of CLDC on monocyte populations, CCR2^{-/-} mice were vaccinated with a CLDC adjuvant. The generation of antibody and cellular immune responses were not altered in CCR2^{-/-} mice, though it should be noted that these mice did not exhibit a defect in monocyte mobilization into the airways. Therefore, the role of inflammatory monocytes in antigen uptake and presentation following immunization with CLDC adjuvants remains unclear. There is the potential for inflammatory monocytes to be recruited through alternative cytokines or be differentiated into DC by the time they arrive in the draining lymph nodes [56-57], though prior experiments suggest that this is unlikely to occur in such a short time frame [11]. Another possibility is that MCP-1 recruited inflammatory monocytes may be playing an immunoregulatory role upon entry into the airways following CLDC vaccination. Previous studies have suggested that under certain circumstances monocytes can act to inhibit an active immune response [58-59].

The influence of the liposome component of the cationic liposome-nucleic acid-based adjuvants strongly enhances the delivery of the intranasally-administered antigens to the airway immune cells. We were able to show an increased uptake of fluorescently labeled CLDC in resident myeloid dendritic cells (DC), inflammatory monocytes, and alveolar macrophages (AVM) in the airways. Also, the numbers of DCs and AVMs following i.n. CLDC treatment decreased, suggesting cellular activation and potential trafficking to the draining lymph nodes. Based on previous studies indicating AVMs as inefficient cells for presenting antigen and stimulating naïve T cell responses [60-61], we do not believe the AVMs to be responsible for uptake and trafficking of vaccine antigens out of the airways. This was confirmed by the use of a labeled antigen (Ova), which enabled us to directly visualize the interaction of the antigen with

APCs, as well as assess how CLDC affected the movement of the antigen. We found that complexing the antigen to CLDC resulted in preferential targeting of antigen to resident airway DC. We believe that DCs are the key cells responsible for increased antigen presentation following antigen delivery with CLDC. We also noted the uptake of labeled antigen by B cells and macrophages in the MLN, but the addition of CLDC did not enhance antigen uptake by these cells. Therefore, we believe that the uptake of antigen by B cells and macrophages in the MLN may have resulted from passive transport of soluble or CLDC bound antigen directly through the lymphatics, without cell associated transport.

In summary, these findings suggest the mechanism of action utilized by liposome-nucleic acid based adjuvants involves the binding of TLR9 and subsequent production of innate immune molecules as well as antigenic targeting to relevant antigen presenting cells capable of trafficking to the drain lymph node and initiating the adaptive immune response. The synergistic effect of the delivery vehicle and immunostimulator components of the CLDC adjuvant highlights the potential use of CLDC with multiple vaccine antigens and makes it a good candidate for continued study.

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CHAPTER 4

CONSUMPTION OF RICE BRAN INCREASES MUCOSAL IgA CONCENTRATIONS AND NUMBERS OF INTESTINAL *LACTOBACILLI*

I acknowledge the contribution of Dr. Elizabeth Ryan and Ajay Kumar for the development of the 10% rice bran diet, and Brittany Barnett for the confirmation of the *Lactobacillus* spp. through real time PCR.

4.1 Research Rationale

The use of dietary intervention to improve overall health has been recognized for many years, but the mechanisms induced by food components to modulate the mucosal immune system require further study. A variety of food components have been useful in disease prevention against cancer, diabetes, and inflammatory diseases. An example is whole dietary rice bran. It contains many bioactive and prebiotic components. Studies have revealed the beneficial effects of methanol extracted rice bran [1] and fermented rice bran components [2-4] on the immune response. In light of the effectiveness of these formulations, the potential use of whole dietary rice bran warrants investigation. Whole dietary rice bran has great potential as a food component for disease prevention based on worldwide accessibility and a limited need for manipulation. Therefore, this chapter will focus on investigating the effects of whole dietary rice bran consumption on mucosal immune responses. The ability of rice bran to modulate beneficial bacterial populations will also be investigated. A mechanistic approach to understanding rice bran's potential to modulate the mucosal environment is essential for future studies investigating protection against enteric diseases.

4.2 Summary

Gut-associated lymphoid tissue (GALT) maintains mucosal homeostasis by combating pathogens and inducing a state of hypo-responsiveness to food antigens and commensal bacteria. Dietary modulation of the intestinal immune environment represents a novel approach for enhancing protective responses against pathogens and inflammatory diseases. Dietary rice bran consists of bioactive components with disease fighting properties. Therefore, we conducted a study to determine the effects of whole dietary rice bran intake on mucosal immune responses and beneficial gut microbes. Mice were fed a 10% rice bran diet for 28 days. Serum and fecal samples were collected throughout the study to assess total IgA concentrations. The Peyer's patches, lamina propria, and mesenteric lymph nodes were collected for cellular immune phenotype analysis and concentrations of *Lactobacillus* spp. were enumerated in the fecal samples. We found that dietary rice bran induced an increase in total IgA locally and systemically. In addition, B lymphocytes in the Peyer's patches of mice fed rice bran displayed increased surface IgA expression compared to control mice. Antigen presenting cells were also influenced by rice bran, with a significant increase in myeloid dendritic cells residing in the lamina propria and mesenteric lymph nodes. Increased colonization of native *Lactobacillus* was observed in rice bran fed mice compared to control mice. These findings suggest that rice bran induced microbial changes may have contributed to enhanced mucosal IgA response. Therefore, we conclude that increased rice bran consumption represents a promising dietary intervention to modulate mucosal immunity for protection against enteric infections and induction of beneficial gut bacteria.

4.3 Introduction

Rice is a staple food for a large portion of the world's population. Rice is typically polished to make white rice for reasons of stability and consumer acceptability. The removal of the bran results in the loss of many prebiotic components and beneficial nutrients including various polyphenols, essential fatty acids, and numerous antioxidants [5-7]. Dietary rice bran intake has been shown to aid in protection against gastrointestinal cancers [5, 8-9], improve lipid metabolism in diabetic rats [10], and significantly lower cholesterol levels in humans [11-12] and hamsters [13]. Also, rice bran derived prebiotics suppress inflammatory bowel disease [14-15] by decreasing ulceration and inducing beneficial effects on the intestinal mucosa through the production of anti-inflammatory cytokines.

Prebiotics are defined as non-digestible food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract [16]. Prebiotics promote the growth of beneficial bacteria, commonly referred to as probiotics [17-18]. In addition, the fermentation of prebiotic components via probiotic bacteria elicits the production of short-chain fatty acids. This results in the acidification of the colonic environment [19-21] which is detrimental to pathogenic bacteria and advantageous for colonic epithelial cells.

The microbiota of the gastrointestinal tract has received increased research attention due to its abundance, ranging from 10^3 - 10^7 CFU/gram, its role in inflammatory diseases, and its responsiveness to dietary patterns [22-24]. Prominent diet modifiable phylum such as *Bacteroidetes* and *Firmicutes* [23] are commonly referred to as resident commensal bacteria. Probiotic bacteria tend to be classified as non-colonizing members of the microbiota with the exception of *Lactobacillus* found in low concentration in the intestines [25]. The mechanisms by which probiotics benefit the host include strengthening of the mucosal surface [26], modulating the immune response, and antagonizing pathogens via competition or antimicrobial agents [27]. Changes in the gut microbiota have been shown to significantly affect the mucosal immune system [28] most notably through the increased production and secretion of IgA [29-30]. For

example, mice administered oral *L. acidophilus* were shown to have increased production of IgA by B cells in the Peyer's patches as a result of local DCs secreting TGF- β [31]. Also, probiotic bacteria have been associated with the enhancement of innate immunity through increased macrophage recruitment and phagocytic activity [17, 32-33] as well as pro-inflammatory cytokine production [34].

Emerging evidence supports the beneficial effects of single rice bran components [35], enzyme-treated rice bran [15], and fermented rice bran [7, 14] on the immune system. Although these studies have uncovered important information, whole dietary rice bran may be more practical for public health intervention in the developing and developed world when compared with extract preparations. Given the strong potential for dietary inclusion of whole rice bran as a functional food ingredient for chronic disease control and prevention [5, 11, 13, 36], studies focused on the effects of dietary rice bran intake on the gut mucosal immune response and the microbiota are warranted. Therefore, **we tested the hypothesis that the intake of whole dietary rice bran would enhance the mucosal immune response and alter the beneficial gut microorganisms.** To address this hypothesis, we investigated whether a 10% rice bran intake compared to a control diet would lead to changes in cellular and humoral immune responses as well as alterations in the native commensal probiotic *Lactobacilli*. The 10% rice bran dose was selected as relevant for these studies because this intake level demonstrated disease-fighting activities and is achievable for humans.

4.4 Materials and Methods

4.4 (1) Animals and feeding schedule.

Specific pathogen-free 4-6 week-old female ICR outbred mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were provided water ad libitum and fed a maintenance diet AIN-93M (Harlan Teklad, Madison, WI) for one week prior to randomization. Mice were randomized into two groups (AIN-93M Control Diet and 10% rice bran diet) and remained on the control diet and the 10% rice bran diet for up to 28 days. Mice were weighed daily with no significant difference in body weight observed between the experimental and control groups (data not shown). Institutional Animal Care and Use Committee at Colorado State University approved all protocols involving the animal experiments described in this study.

4.4 (2) Diet Composition.

Rice bran from the Neptune rice variety was provided as a gift from Dr. Anna McClung at USDA Rice Research Unit (Stuttgart, AK, USA). The Neptune rice variety was chosen based on its availability for rice grown in the U.S. Animal diets were formulated to match macronutrients (e.g. protein, carbohydrates) across diet groups. The differences in macronutrient composition were balanced using purified diet components. The percent of rice bran incorporated into the diet is expressed as g/100g of diet. Harlan Laboratories (Madison, WI) mixed and pelleted the 10% rice bran containing diet with AIN-93M control purified diet components. The composition of rice-bran-containing diet was calculated based on published reports [37-38] that demonstrated rice bran disease fighting activity and were stored at -20°C until fed to the mice. Diet formulations are shown in **Table 4.1**.

TABLE 4.1 Composition of Experimental Diets (g/kg)

Components	Control Diet *	10% Rice Bran Diet **
Casein	140.0	140.0
L-Cystine	1.8	1.8
Corn Starch	465.7	422.7
Maltodextrin	155.0	155.0
Sucrose	100.0	100.3
Corn Oil	40.0	19.0
Cellulose	50.0	29.0
Mineral Mix	35.0	35.0
Vitamin Mix	10.0	10.0
Choline Bitartrate	2.5	2.5
TBHQ, antioxidant	0.008	0.008
Rice Bran	-	100.0

* Control Diet produced by Harlan (AIN-93M).

** 10% Rice Bran Diet produced by Harlan using AIN-93M in addition to rice bran from the Neptune rice variety, adjusted to match total fat, protein, and carbohydrates.

4.4 (3) Sampling procedures.

Mice were fed the experimental rice bran diet for 28 days. Fecal and serum samples were collected from all mice on day 0, 4, 7, 14, 18, 21, 25, and 28 following commencement of experimental diets. Fecal extracts were made by using a modification of methods previously described [39]. Briefly, PBS containing 5% nonfat dry milk and protease inhibitors were added to the fresh fecal pellets at a volume of about 5 μ l/mg feces. Solid fecal matter was suspended by extensive vortexing and separated by centrifugation of 16,000 \times g for 10 min. The clear supernatants were transferred to a fresh tube and frozen at -20°C to be used for ELISAs as described below.

4.4 (4) Quantification of total IgA and *Lactobacillus*-specific IgA antibody. Total IgA antibody titers were assessed in mouse feces and serum by ELISA. Nunc-Immuno plates (Thermo Scientific) were coated overnight at 4°C with purified rat anti-mouse IgA (C10-3, BD,

San Diego, CA) at a concentration of 5 µg/ml in carbonate-bicarbonate buffer (pH 9.6). Non-specific protein binding sites were blocked with PBS containing 3% bovine serum albumin (BSA, Sigma). Dilutions (1% BSA) of the samples and standard were applied to the plate (50 µl/well) and incubated for 1.5 hours at room temperature (RT). Plates were incubated with biotin rat anti-mouse IgA (C10-1, BD) at a dilution of 1:1000 for 1 hour at RT. Next, plates were incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch) at a concentration of 1:1500 for 20 min. at RT. Plates were developed with 3,3',5,5' tetramethylbenzidine (TMB, Sigma). The reaction was stopped using 1N HCl and the optical densities were read at 450 nm. Purified Mouse IgA (eBioscience, San Diego, CA) was used to create the standard curve for the ELISA.

The *Lactobacillus* IgA ELISA used a similar protocol as the total IgA ELISA. The *Lactobacillus* spp. used to coat the plate were isolated from fecal samples collected on D18 following commencement of dietary rice bran. The *Lactobacilli* was expanded in MRS broth prior to undergoing heat-killing as described previously [40]. ELISA plates were coated overnight at 4°C with 10⁸ CFU/ml heat-killed *Lactobacilli* in carbonate-bicarbonate buffer. Non-specific binding sites were blocked with PBS containing 5% non-fat dried milk (NFDM). A positive control sample was obtained by vaccinating intraperitoneally an ICR mouse with the heat-killed *Lactobacilli* and collecting serum after 14 days.

4.4 (5) Tissue harvest and immune cell preparation.

After the 28 day feeding period, mice were killed following anesthetization by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) with xylazine (10 mg/kg). The mesenteric lymph nodes (MsLN) and Peyer's patches (PP) were collected from each mouse and stored in HBSS + 2% FBS on ice. Cell from the MsLN and PP were prepared by mechanical disruption and screening through a 70-µm nylon mesh screen (BD Biosciences), followed by NH₄Cl lysis. Next, a 10 cm section of the small intestine was collected (ileum and distal jejunum) and the intestinal lamina propria lymphocytes were prepared as previously described [41]. Briefly, the

intestinal section was cut longitudinally and then into smaller sections. The small sections were incubated with 5 mM DL-Dithiothreitol (DTT, Sigma) to remove the mucous, followed by 30 mM EDTA to remove epithelial cells. Next, the tissue sections were incubated at 37°C in RPMI media containing collagenase solution (40 Units/ml). The tissue was disrupted through a 70- μ m nylon mesh screen prior to being run through a density gradient (Optiprep density gradient media, Accurate Chemical & Scientific Corp). All cell preparations were re-suspended in complete RPMI (Invitrogen, Carlsbad, CA) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), 2mM L-glutamine (Invitrogen), 1X non-essential amino acids (Invitrogen), 0.075% sodium bicarbonate (Fisher Scientific, Pittsburgh, PA), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). Cells were kept on ice prior to immunostaining and analysis.

4.4 (6) Small Intestine homogenization and cytokine quantification.

A 3 cm section of the small intestine (ileum) was collected in ice-cold PBS with protease inhibitor. The tissue section was then homogenized for 10 seconds using a Tissue-Tearor prior to centrifugation at 16,000 x g for 10 min. The clear supernatants were transferred to a fresh tube and frozen at -80°C. Concentrations of IL-6 and TGF- β were measured by commercial ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

4.4 (7) Flow cytometric analysis.

Directly conjugated antibodies used for these analyses were purchased from eBioscience (San Diego, CA) or BD Pharmingen (San Diego, CA). The following antibodies were used in various combinations: anti-CD4 (PE; clone GK1.5), anti-CD8b (FITC; clone H35-17.2), anti-CD5 (FITC; clone 53-7.3), anti-CD27 (APC; clone LG.7F9), anti-CD11c (PE-Cy7; clone N418), anti-CD11b (APC-Cy7; clone M1/70), anti-CD45R (B220, APC-Cy7; clone RA3-6B2), and anti-mouse IgA (biotin; clone C10-1). Immunostaining was completed as described previously [42]. In most cases, cells were fixed in 1% paraformaldehyde for 20 min. and stored in FACS buffer at

4°C for 1-2 days prior to analysis. Analysis was performed with a Cyan ADP flow cytometer (Beckman Coulter, Fort Collins, CO) and data analyzed using FlowJo software (Tree Star, Ashland, OR).

4.4 (8) Fecal *Lactobacillus* levels.

Fecal samples were collected from all mice on day 0, 4, 7, 14, 18, 21, 25, and 28 following commencement of experimental diets. Approximately 5-6 fresh fecal pellets (0.1 grams) were collected per mouse and re-hydrated in 1 ml of PBS for 15 min. Samples were vigorously vortexed prior to performing a ten-fold dilution in sterile PBS. The diluted samples (100 µl per quad) were then plated on a MRS agar (enrichment agar for *Lactobacillus*) and placed in a 37°C incubator containing 5% CO₂ for 48 hours. All colonies grown on MRS agar were confirmed as *Lactobacillus* spp. using real time PCR as previously described [43].

4.4 (9) Statistical analyses.

Statistical analysis was performed using Prism 5.0 software (Graph Pad, La Jolla, CA). Two-tailed non-parametric (Mann-Whitney) t-tests were performed for comparisons between the two groups. In order to complete comparison of two groups over time, a repeated measures (mixed model) 2-way ANOVA with a Bonferroni post-test was performed. Differences were considered statistically significant for $p < 0.05$ for all comparisons.

4.5 Results

4.5 (1) Dietary rice bran intake induces local and systemic IgA production.

The effect of daily rice bran intake on the mucosal immune system was first investigated in mice for changes in serum, intestinal, and fecal IgA. Control and 10% rice bran diets (**Table 4.1**, Materials and Methods) were fed to mice for 28 days and the total IgA antibody levels were measured throughout this time period. The concentration of total IgA in the feces of the rice bran fed mice (**Figure 4.1A**) was significantly increased on day 18 and remained high through day 21 in comparison to mice on the control diet. A similar difference was detected systemically in the serum (**Figure 4.1B**) with the total IgA concentration peaking at day 18.

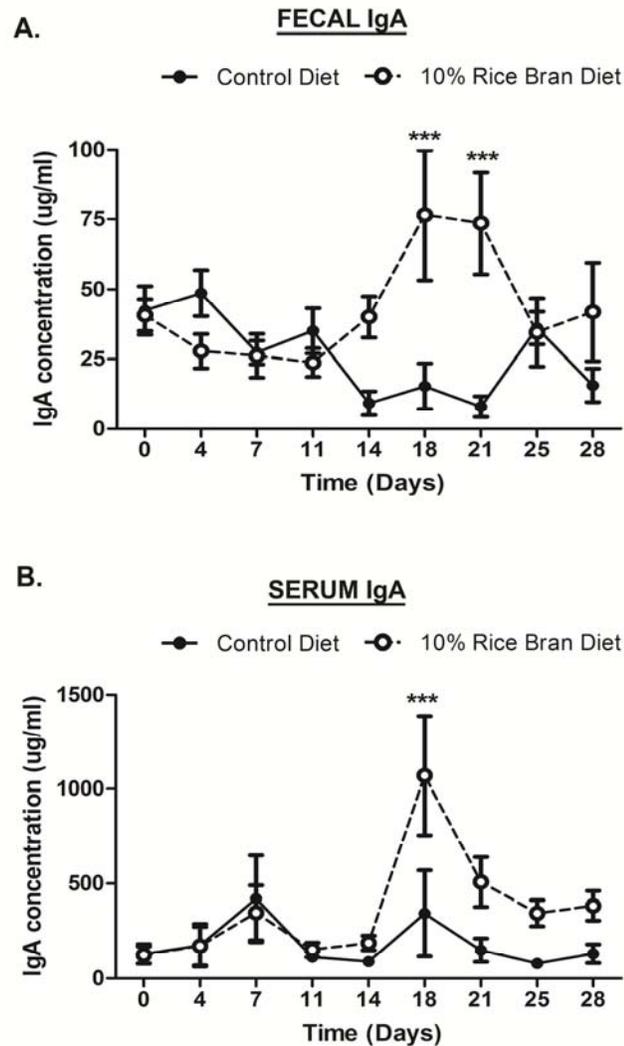


Figure 4.1 Effect of dietary rice bran on local and systemic total IgA responses. A total IgA ELISA was performed on serial dilutions of fecal (A) and serum (B) samples collected for 28 days. Antibody concentrations were determined through comparison to a standard control. Data are shown as mean \pm SEM ($n = 12$ mice/group). Results were pooled from 2 independent experiments. Significant differences (** $p < 0.001$) were determined by a repeated measures (mixed model) 2-way ANOVA followed by Bonferroni's post-tests.

Next, we assessed the expression of IgA on the surface of intestinal B cells to better understand the transient IgA response following dietary rice bran intake. Increased expression of surface IgA indicates activation of local B cells as well as an increased potential to respond to commensal and pathogenic bacteria in a T cell independent fashion [30]. Mice were euthanized after 7, 14, and 28 days of rice bran consumption and B220⁺ B cells were analyzed for surface

IgA expression (**Tables 4.2**). A significant difference was observed in the number of IgA molecules expressed on the B cell surface between control and rice bran fed mice, determined by mean fluorescence intensity (MFI). The MFI for IgA expression on the B220⁺ B cells in the Peyer's patches was 166.7 ± 19.7 in the 10% rice bran fed group and 98.7 ± 23.1 in the mice fed control diet (**Table 4.2**). These results are consistent with the idea that dietary rice bran induces mucosal immunity via systemic and local IgA production as well as increased activation of IgA⁺ B cells found in the Peyer's patches.

TABLE 4.2 Increased expression of surface IgA on B220⁺ B cells in the Peyers Patches following 14 days of rice bran diet consumption

Cell Populations	Lamina Propria		Peyers Patches		Mesenteric Lymph Nodes	
	Control	10% RB	Control	10% RB	Control	10% RB
B220 ⁺ IgA ⁺ cells (%)	1.4 ± 0.3	1.6 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	0.6 ± 0.1	0.7 ± 0.2
IgA (MFI) on B220 ⁺ B cells	193 ± 36	194 ± 51	98.7 ± 23.1	$166.7 \pm 19.7^*$	81.3 ± 24	88.6 ± 28

Data are represented as mean \pm SEM,. Data was pooled from two independent experiments, n=10. *p<0.05 as determined by comparing control diet vs 10% rice bran diet in each cell population using a non-parametric Mann-Whitney t test. RB, Rice Bran; MFI, mean fluorescence intensity

4.5 (2) Increased mucosal CD11c⁺CD11b⁺ dendritic cells induced by rice bran.

In order to determine whether dietary rice bran intake influences antigen presenting cells as a mechanism for IgA induction, we examined cellular immune phenotypes previously shown to be required for IgA class switching [44-45]. Following 7, 14, and 28 days of daily 10% rice bran intake, mice were sacrificed and the cellular responses were assessed in the lamina propria, Peyer's patches, and mesenteric lymph nodes. By day 14, the percent of the myeloid dendritic cells (CD11c⁺CD11b⁺) in both the lamina propria and the mesenteric lymph nodes were significantly increased in mice fed the 10% rice bran diet in comparison with the mice receiving control diet (**Table 4.3**). These data suggested that rice bran mediated induction of dendritic cell

recruitment into the mucosal tissues resulting in increased antigen presentation and subsequent IgA production. No significant differences were detected in T or B cell populations (**Table 4.3**). The increased presence of dendritic cells in the lamina propria also enhances the mucosal innate immune response at the site where pathogens invade, and reveals a potential protective mechanism induced by dietary rice bran.

TABLE 4.3 Influence of dietary rice bran on cellular populations in the intestinal immune compartments following 14 days of diet consumption

Cell Population (%)	Lamina Propria		Peyer's Patches		Mesenteric Lymph Nodes	
	Control	10% RB	Control	10% RB	Control	10% RB
CD4 ⁺ T cells	7.4 ± 0.8	9.6 ± 1.0	20.3 ± 1.5	19.7 ± 1.9	66.8 ± 1.7	67.6 ± 1.5
CD8 ⁺ T cells	16.5 ± 3.3	18.4 ± 3.3	6.3 ± 0.8	4.5 ± 0.4	17.1 ± 0.6	16.3 ± 0.9
B220 ⁺ B cells	33.7 ± 5.9	31.3 ± 3.1	66.1 ± 3.6	66.4 ± 4.1	10.8 ± 3.6	20.4 ± 0.9
B220 ⁺ CD5 ⁺ B cells	0.9 ± 0.1	1.3 ± 0.1	3.3 ± 0.7	3.8 ± 0.8	7.4 ± 1.5	8.7 ± 0.8
B220 ⁺ CD27 ⁺ B cells	0.8 ± 0.2	1.0 ± 0.3	0.2 ± 0.04	0.2 ± 0.09	0.3 ± 0.05	0.3 ± 0.05
CD11c ⁺ CD11b ⁺ DCs	6.8 ± 0.9	11.1 ± 0.9 **	1.1 ± 0.1	1.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.04 *

Data are represented as mean ± SEM. Data was pooled from two independent experiments, n=10. *p<0.05, **p<0.01 as determined by comparing control diet vs 10% rice bran diet in each cell population using a non-parametric Mann-Whitney t test. RB, Rice Bran

4.5 (3) Dietary rice bran failed to induce IL-6 or TGF-β production in the small intestine.

Experiments were conducted next to assess the effects of dietary rice bran on local induction of cytokine production in the mucosal tissues. We were most interested in examining cytokines known to be involved in IgA antibody class switching, including IL-6 and TGF-β [46-49]. Following 7 days of daily 10% rice bran intake, mice were sacrificed and small sections of the ileum were collected and homogenized as described in materials and methods. Concentrations of IL-6 and TGF-β were determined by ELISA. There was no significant

difference in the intestinal concentrations of either cytokine between the rice bran fed animals and the control animals (**Figure 4.2**). These negative results indicate that the rice bran induced mechanism of IgA class-switching does not involve IL-6 or TGF- β .

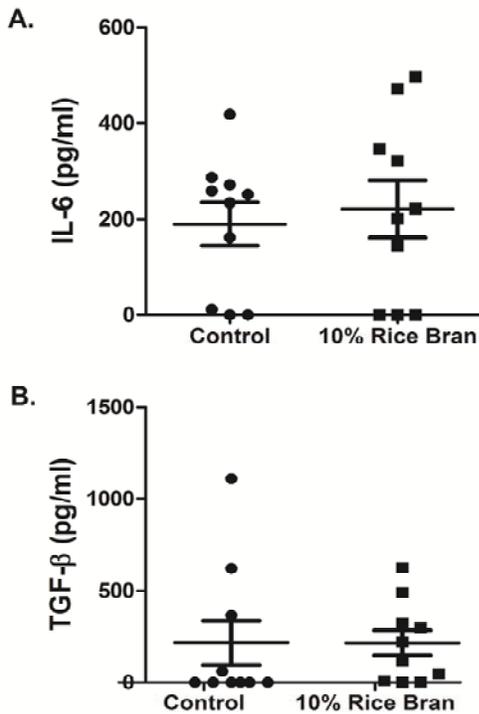


Figure 4.2 Dietary rice bran intake fails to induce the secretion of IgA class switch cytokines. (A) IL-6 and (B) TGF- β ELISAs were performed on small intestine homogenate samples collect on day 7 following the commencement of experimental diets as described in materials and methods. Data are shown as mean \pm SEM ($n = 10$ mice/group). Results were pooled from 2 independent experiments. Significant differences (IL-6 $p=0.91$, TGF- β $p=0.23$) were determined by non-parametric Mann-Whitney t Tests.

4.5 (4) Dietary rice bran increased *Lactobacillus* colonization.

Given the associations of probiotic bacteria with the modulation of the mucosal immune system [28], we next determined the titers of the probiotic, *Lactobacillus* spp., in the rice bran fed mice compared to control. *Lactobacillus* was chosen for its aerobic growth conditions as well as its ability to protect against a variety of infectious diseases [50]. For these experiments, we

processed and plated fresh fecal pellets for the presence of *Lactobacillus* spp. over a 28 day time period as described in the Materials and Methods. On days 11, 18, 21, and 25 the numbers of *Lactobacillus* spp. in the feces were significantly higher from the rice bran fed mice in comparison to a more cyclical pattern seen in the mice fed control diet (**Figure 4.3**). The nearly 500% increase in numbers of *Lactobacillus* spp. (CFU/gram feces) became apparent on day 11 and remained significantly high for 14 days. Both the control and rice bran diets did not contain any *Lactobacilli*. Next, we determined if the increased IgA titers were *Lactobacillus*-specific. A *Lactobacillus* IgA ELISA was performed and revealed low to undetectable *Lactobacillus*-specific IgA, expressed as reciprocal endpoint titers (data not shown). A positive control was in place to ensure the reliability and sensitivity of the *Lactobacillus* IgA ELISA as described in materials and methods. Therefore, these results indicate a potential role for dietary rice bran in modulating the intestinal microbiota through eliciting increased concentrations of beneficial bacteria.

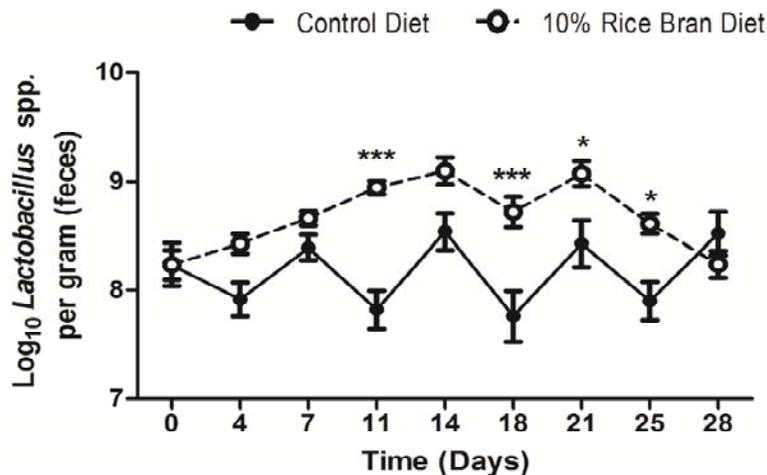


Figure 4.3 Effect of dietary rice bran on the fecal titers of *Lactobacillus*. Fresh fecal pellets were collected, processed, and plated on MRS agar every 3-4 days in order to determine the Log₁₀ *Lactobacillus* titer per gram feces. Data are shown as mean \pm SEM ($n = 10$ mice/group). Results were pooled from 2 independent experiments. Significant differences (* $p < 0.05$, *** $p < 0.001$) were determined by a repeated measures (mixed model) 2-way ANOVA followed by Bonferroni's post-tests.

4.6 Discussion

The unique health promoting properties and chemical composition of rice bran make it a promising candidate for dietary supplementation, nutritional therapy, and for prevention of chronic disease [2-6, 8-13, 35, 51]. In this study we found the effects of whole dietary rice bran intake on the mucosal immune system to be the induction of IgA (Figure 4.1) and the enhancement of the innate cellular immune response (Table 4.2 and 4.3). In addition, the ability of dietary rice bran to promote increased intestinal colonization of *Lactobacillus* spp. (Figure 4.3) highlights a novel role for rice bran prebiotic components to influence the intestinal microbiota [6, 52].

Understanding the effect of dietary rice bran on the innate immune system and antigen presentation is crucial to elucidating the mechanisms by which rice bran may induce protective responses at mucosal surfaces. The increased percentages of myeloid dendritic cells in both the lamina propria and the mesenteric lymph nodes following rice bran consumption speaks to the targeted affect of rice bran on the key cells involved in shaping an immune response. Intestinal dendritic cells have the unique ability to induce IgA production from B cells through secretion of retinoic acid, IL-5 and IL-6 [53]. Therefore, the enhanced dendritic cell population may help in the initiation of an adaptive immune response through the activation of B cells and subsequent IgA class-switching. Also, dendritic cells can assist in the surveillance of the intestinal compartments that are vulnerable to invasion by pathogenic bacteria, such as *Salmonella* and *Vibrio*.

Understanding the mechanisms behind the ability of dietary rice bran to induce IgA production is important for future studies aimed at protection against enteric disease. TGF- β , IL-6, IL-10, IL-5, IL-2, and IL-15 have all been associated with induction of IgA class switching [46-49, 54]. We found the intake of dietary rice bran had no effect on the levels of IL-6 or TGF- β in the small intestine (Figure 4.2). This lack of cytokine production could imply the IL-6 and TGF- β concentrations peaked at a different time following the initiation of the rice bran diet or

that other IgA class switch cytokines are responsible for the enhanced IgA response. For example, IL-15 derived from intestinal epithelial cells has been shown to induce B-1 peritoneal B cells to undergo IgA class switching [54]. Therefore, the potential for dietary rice bran to elicit IL-15 and other IgA class switch cytokines is of great interest and requires further investigation.

Evidence for the modulation of the mucosal IgA response associated with the consumption of dietary components has been limited to fructooligosaccharides (FOS) and pectin [55-56]. Similarly, this study demonstrated the ability of dietary rice bran to induce increased IgA concentrations systemically and locally. A variety of bioactive and prebiotic components other than FOS that are present in rice bran are hypothesized to be involved in the enhancement of immunity. These phytochemicals include, but are not limited to γ -oryzanol, polyphenols, fatty acids, as well as some essential amino acids and micronutrients [6]. Understanding the mechanism of IgA induction is of considerable importance when evaluating the dietary capacity of rice bran to elicit protection against enteric infections. Current evidence suggests that the majority of IgA molecules in the gut are induced by commensal bacteria, and that there is a role for this transient and stable population to induce a specific IgA response [30, 57-62]. A recent study performed by Hapfelmeier et al. confirms the role of commensal bacteria, but also describes the specific IgA response to be less robust and have a slower onset [59] consistent with our findings. Another relevant finding by Macpherson et al. showed induction of commensal-specific IgA antibodies to be mostly T cell independent but dependent largely on the B1 peritoneal B cells [30]; a potential angle that should be evaluated in rice bran fed mice.

Based on the emerging evidence for commensal involvement in shaping the mucosal IgA population, the effect of dietary rice bran on the native gut probiotic *Lactobacillus* spp. was evaluated as a potential mechanism of immune modulation (Figure 4.3). The antibody enhancing ability of *Lactobacillus* was described previously in a study showing increased production of rotavirus-specific antibodies when fermented milk was administered during the acute phase of a rotavirus infection [63]. The low to nonexistent *Lactobacillus*-specific IgA antibodies in our

model may speak to the unique ability of rice bran to enhance antibody responses specific for other commensal and/or pathogenic bacteria in the course of infection. The low titers may also reflect the fact that dietary rice bran contains growth substrates for other resident bacteria and that the specificity of the increased IgA is heterogeneous, thus making the *Lactobacillus* titer too low for detection by ELISA. We hypothesize that rice bran induced modulation of multiple commensal bacteria resulted in increased luminal IgA concentrations of the intestine. The next step will be to elucidate the effect of dietary rice bran on the entire intestinal microbiota through the use of 454 pyrosequencing or other high throughput microbiome analytical approaches.

The ability of dietary rice bran to promote the growth of the probiotic, *Lactobacillus*, and subsequent enhancement of mucosal immune cell populations offers numerous health promoting and disease fighting possibilities. For example, the potential use of rice bran as a dietary vaccine adjuvant is highlighted by the recent application of *Lactobacillus* in an intramuscular influenza vaccine [64]. Also, the beneficial effects of rice bran and probiotics on inflammatory diseases such as IBD [14-15, 65-66] and Type 1 diabetes [10, 67-69] emphasizes a unique research avenue to study the probiotic enhancing effects of dietary rice bran on these nutritionally relevant diseases. In summary, the ability of whole dietary rice bran to modulate the immune system as well as promote the growth of important probiotics holds great promise in protection against enteric pathogens and modulation of chronic inflammatory diseases.

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CHAPTER 5

CONCLUDING STATEMENTS

5.1 Introduction

The work presented in this dissertation is intended to further the development and understanding of potential strategies for enhancing protective immune responses on the mucosal surfaces. In a time where there are newly emerging pathogens, antibiotic resistant bacteria, and escalating incidences of inflammatory mucosal diseases, the ability to target and enhance the mucosal immune responses is crucial. We have shown that mucosal modulatory techniques such as vaccination and dietary intervention have the capacity to induce a protective environment capable of eliciting appropriate responses to foreign organisms or antigens.

5.2 Chapters 2 and 3

Vaccines are considered by many to be the most successful medical strategy for disease prevention in the 20th century [1-2]. The development of mucosally-administered vaccines is now of great interest due to the fact that many pathogens enter the body through mucosal surfaces. One of the most promising strategies used to improve mucosal vaccination is the addition of a potent vaccine adjuvant. Therefore, Chapter 2 investigated the potency of cationic-liposome plasmid DNA complexes (CLDC) as a mucosal vaccine adjuvant and Chapter 3 focused on understanding its mechanism of action.

According to the work presented, CLDC is a potent mucosal vaccine adjuvant. We demonstrated that CLDC-based vaccine adjuvants induced balanced humoral and cellular immune responses; but most importantly, these induced responses protected the mucosa from a lethal bacterial challenge. The combination of the liposomal and the CpG-rich plasmid DNA components of CLDC were critical for the modulation of the mucosal surfaces. In order to understand the success of CLDC-based intranasal vaccination, it is necessary to go step-by-step through the fate of the vaccine antigen and the development of the immune responses.

Components of the innate immune system were the first to respond to the intranasal administration of CLDC-based vaccination. Within hours after vaccination, we observed that the liposomal component of CLDC targeted the associated antigen to resident airway APCs (AVMs, DCs, and monocytes). Following phagocytosis of CLDC-antigen, the CpG-rich plasmid DNA component of CLDC interacted with the TLR9 receptor found in the endosome of the APCs evidenced by the increased secretion of airway IFN- γ and IL-6. We concluded that CLDC induced the activation of mucosal APCs through liposomal targeting and the initiation of TLR signaling. In addition to cytokine production, activated APC were also induced to migrate to the nearest lymph node for the purpose of antigen presentation. We showed evidence of APC migration by the observation of decreased airway APC numbers and increased numbers of CLDC-antigen containing DCs in the mediastinal lymph nodes.

The initiation of the adaptive immune response to intranasal CLDC-based vaccination did not begin until after the vaccine antigen had been transported to the draining lymph node by airway DCs. In general, antigen-loaded DCs are involved in activating naïve antigen-specific lymphocytes. These lymphocytes include antigen-specific CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, and B cells. CLDC-based vaccination resulted in the activation of CD4⁺ helper T cells, which we were able to demonstrate through the identification of activated and functional CD4 T cells located in the airways three weeks after vaccination. Next, in order to demonstrate the activation of antigen-specific B cells following vaccination, we measured the presence of antigen-specific antibody. We demonstrated an increase in both antigen-specific IgA titers in the BAL fluid and antigen-specific IgG titers in the serum three weeks after vaccination. We hypothesized that the IL-6 secretion induced by administration of i.n. CLDC was most likely produced by the airway DCs. Therefore, the presence of the IL-6 producing DCs in the lymph nodes resulted in the induction of IgA class switching by the B cells. Finally, we showed the ability of CLDC- adjuvanted vaccines to induce a potent cytotoxic T cell response. We observed large percentages of antigen-specific CD8 T cells residing in the airways and lung tissue three weeks post

vaccination. These findings not only revealed that the CLDC-adjuvanted vaccination caused a balanced immune response, but also that the mucosal surfaces were well protected by effector and memory cells following vaccination. We believe that these findings will help in future use of CLDC as a potent adjuvant to enhance mucosal vaccination.

A number of CLDC-adjuvanted immune responses stand out as potential avenues for future studies. The ability of the CLDC adjuvant to induce a potent antigen-specific cytotoxic CD8 T cell response holds significant promise for vaccine development against intracellular pathogens. Many mucosa-targeting pathogens do not have an adequate vaccine. These pathogens typically establish an intracellular infection, and in order to be eliminated require a strong cytotoxic CD8 T cell response. Therefore, the use of CLDC has the potential to result in the enhancement of vaccines against infectious organisms such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and herpes simplex virus (HSV). Another area of significant need in vaccine development is the identification of an adjuvant capable of enhancing vaccination in the elderly and in those who are poor seroconverters. Based on the combined effects of the liposomal and DNA components of CLDC, there is increasing interest in the potential use of the CLDC adjuvant in vaccine development for these populations. In conclusion, CLDC exhibits promising characteristics as an effective mucosal vaccine adjuvant and continued investigation using other disease platforms is warranted.

5.3 Chapter 4

The use of dietary intervention for the improvement of overall health is not a new concept; but recent research has revealed a number of foods with disease fighting properties. A variety of these functional foods have been useful in the areas of cancer prevention, diabetes, and inflammatory diseases. One example is whole dietary rice bran. It contains many bioactive and prebiotic components. Understanding the mechanisms of action elicited by dietary rice bran on

the mucosal surfaces would be of supreme interest for further disease prevention studies. Therefore, Chapter 4 investigated the ability of whole dietary rice bran to modulate the mucosal immune system and influence the composition of the microbiota.

Based on the work presented herein it can be concluded that rice bran consumption represents a promising dietary intervention to enhance mucosal immune responses for protection against enteric infections. We have discovered a few key mechanisms elicited by dietary rice bran to induce a protective mucosal environment; they include the enhancement of the innate immunity, the induction of increased IgA molecules present in the lumen of the intestine, and the promotion of increased colonization of native *Lactocillus* spp.

The first mechanism of modulation involved the innate immune system. We demonstrated an increase in myeloid DCs in the lamina propria and the mesenteric lymph nodes. These cells induce the protection of the mucosa through the phagocytosis of invading pathogens and through the ability to polarize an effective immune response. The second rice bran induced mechanism involved the most prominent mucosal antibody, IgA. Following dietary rice bran intake, we noted an increase in IgA titers in a pattern representative of a primary response. The more pronounced IgA responses were observed on the mucosal surfaces along with significant IgA titers that briefly appeared systemically. We were unable to identify the specificity of the IgA molecules; but we believe them to have been produced in response to shifting commensal bacterial populations as a result of rice bran consumption. The final mechanism induced by rice bran involved the intestinal microbiota. We demonstrated significant increases in *Lactobacillus* spp. concentrations in the lumen of the intestine following about 10 days of dietary rice bran consumption. The ability of rice bran to influence the intestinal microbiota through the enhancement of probiotic bacterial species represents a promising direction for modulating the mucosal environment. Based on previous studies into the effects of *Lactobacillus* on the mucosal immune system, we believe that the rice bran induced microbial changes may have contributed to the observed enhancement of mucosal IgA responses. Regardless of the direct or indirect effects

of rice bran on the immune system, these findings highlight the mechanisms responsible for the success of dietary rice bran in modulating the mucosal immune responses.

Our findings showing that dietary rice bran can modulate the mucosal immune system through IgA induction and microbial changes establishes a solid foundation for continued study into this very promising food. As a result of limited research into the effects of dietary rice bran on the mucosal environment, we realize the need for further investigation into certain unanswered questions. First, in order to understand the full effect of rice bran on the composition of the intestinal microbiota, other bacterial species affected by the dietary intervention must be identified. This can be elucidated through the use of 454 pyrosequencing or other high throughput microbiome analytical approaches. Next, the ability of dietary rice bran to modulate mucosal immunity and establish protective responses in the presence of infectious enteric organisms needs to be investigated. These studies would include readouts not only in survival and bacterial burden, but also in antibody specificity, immune cell activation, and changes in microbial populations. To extend the scope even further, the ability of dietary rice bran to induce protective responses in non-infectious diseases, such as IBD and Crohn's disease, represents another potential avenue of study. In conclusion, whole dietary rice bran exhibits many promising characteristics to warrant its inclusion into a balanced diet and further investigation into disease prevention.

5.4 References

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APPENDIX 1

3rd Global Vaccine Congress, Singapore 2009

Protective response to subunit vaccination against intranasal *Burkholderia mallei* and *B. pseudomallei* challenge

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Abstract

Burkholderia mallei and *B. pseudomallei* are Gram-negative pathogenic bacteria, responsible for the diseases glanders and melioidosis, respectively. Furthermore, there is currently no vaccine available against these *Burkholderia* species. In this study, we aimed to identify protective proteins against these pathogens. Immunization with recombinant *B. mallei* Hcp1 (type VI secreted/structural protein), BimA (autotransporter protein), BopA (type III secreted protein), and *B. pseudomallei* LolC (ABC transporter protein) generated significant protection against lethal inhaled *B. mallei* ATCC23344 and *B. pseudomallei* 1026b challenge. Immunization with BopA elicited the greatest protective activity, resulting in 100% and 60% survival against *B. mallei* and *B. pseudomallei* challenge, respectively. Moreover, sera from recovered mice demonstrated reactivity with the recombinant proteins. Dendritic cells stimulated with each of the different recombinant proteins showed distinct cytokine patterns. In addition, T cells from immunized mice produced IFN- γ following *in vitro* stimulation. These results indicated therefore that it was possible to elicit cross-protective immunity against both *B. mallei* and *B. pseudomallei* by vaccinating animals with one or more novel recombinant proteins identified in *B. mallei*.

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Key words: *Burkholderia*, *B. mallei*, *B. pseudomallei*, vaccine, subunit vaccination, intranasal infection

Introduction

Vaccines are the most efficacious and cost-effective means of protecting human and animal populations from infection. At present, there are no approved vaccines available for use in protecting animals or humans against the Gram-negative

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bacterial pathogens *Burkholderia mallei* and *B. pseudomallei*. Therefore, we sought to develop and evaluate vaccines that might be used to generate cross-protective immunity against both pathogens.

B. mallei is a non-motile bacterium responsible for glanders. This disease mainly affects horses, which are considered to be the natural reservoir for infection, although mules and donkeys are also susceptible (1). Humans are accidental hosts of *B. mallei* following prolonged and close contact with infected animals. *B. mallei* infects humans by entering through open wounds and surfaces of the eyes or nose. Symptoms of glanders are dependent on the route of infection (2). *B. pseudomallei* are motile bacteria causing melioidosis (3). Melioidosis is a life-threatening disease that is mainly acquired through skin inoculation or pulmonary contamination, although other routes have been documented. This saprophyte inhabitant of soil environments is mainly encountered in Southeast Asia and Northern Australia, but is sporadically isolated in subtropical and temperate countries (4).

Both *Burkholderia* species are highly pathogenic and are classified as such in list B by the Centers for Disease Control and Prevention (5). *Burkholderia* infections are difficult to treat with antibiotics and there are several reports that indicate it is feasible to protect against melioidosis, at least in animal models of disease, with non-living vaccines (6). There has also been some progress in identifying partially protective subunits. Passively administered antisera raised against flagellin, polysaccharide, or conjugates of polysaccharide and flagellin, protect diabetic rats against challenge with *B. pseudomallei* (7-9). However, *B. mallei* are not motile and do not produce flagella. Moreover, the ability of flagellin to induce protection against an aerosol, or intranasal challenge has not been reported. Therefore, we assessed flagellin as a potential candidate for inclusion in a *Burkholderia* vaccine and found it unsuitable (our unpublished data). In contrast, all of the current evidence indicates that other surface-expressed or secreted proteins are immunogenic and structural similarity exists between the proteins in *B. pseudomallei* and *B. mallei* (10-11). In this study, we aimed to identify *Burkholderia* protective proteins that could be administered in vaccines to generate cross-protective immunity against both *B. mallei* and *B. pseudomallei*. We hypothesize that cross-protection is possible based on the similarities in antigenic composition and mechanisms of protection between these organisms, and if this is true, development of a single vaccine which stimulates T-cell and antibody responses against melioidosis and glanders-producing bacterial agents is feasible. If cross-protective immunity is observed, then it may be possible to develop a single vaccine capable of generating protection against both melioidosis and glanders.

2. Recombinant protein expression and purification

Bioinformatics analysis of target sequences was used to indicate the presence (or absence) of an N-terminal secretion sequence, transmembrane domains and homology to published crystal structures. The programs used were SignalP v.3.0, TMHMM v.2.0 and PHYRE v.0.2, respectively (12-14). DNA sequences coding for *B. mallei* proteins BopA (BMA_A1521; AA 23 - 512), BimA (BMA_A0749; residues 19 - 265), Hcp1 (BMA_A0742; residues 1 - 169) and the *B. pseudomallei* protein LolC (BPSL2277; residues 44 - 266) (15) were cloned into the pET28a (+) expression vector (Novagen). Primers were designed to PCR-amplify and clone the selected sequences in frame with a C-terminal 6x Histidine tag, for all four targets. Expand high fidelity DNA polymerase (Roche) was used to amplify targets from *B. mallei* ATCC 23344 or *B. pseudomallei* K96243 genomic DNA. Once ligated into pET28a (+), plasmid DNA was electroporated into *Escherichia coli* DH5 α . Cloned sequences were verified by DNA sequencing, using T7 promoter / terminator oligonucleotide primers.

Target protein expression in *E. coli* (Δ DE3) Rosetta was induced by growth in Overnight Express instant TB medium (Novagen) for 18 – 20 h. Bacterial pellets were lysed using 10x CelLytic B (Sigma), and 6x His-tagged proteins were purified by Ni²⁺ affinity chromatography. Purified proteins were dialyzed against two changes of 10 mM Hepes / 150 mM NaCl, pH 7.4, aliquoted and stored at -80 °C. Protein concentrations were determined using the BCA kit (Pierce) using bovine serum albumin (BSA) as a standard, and sample purity assessed by SDS-PAGE.

3. Vaccination and challenge with *B. pseudomallei* or *B. mallei*

To evaluate the potential of *Burkholderia* surface expressed or secreted proteins to generate protective immunity, the purified recombinant proteins were used individually or in combination to vaccinate mice via the intranasal (i.n.) route. For immunization of mice against *B. mallei* challenge, 6-8 week old female BALB/c mice (n = 8 per group) were primed with 10 μ g of recombinant proteins mixed with adjuvant (12.5 μ g of CpG oligodeoxynucleotide (ODN) 2395 (Coley Pharmaceuticals) and mixed with 12.5 μ g immune-stimulating complex (ISCOM) AbISCO 100 (Isconova AB), followed by a 2 week boost of 5 μ g recombinant proteins with adjuvant. Four weeks post-boost, animals were infected by intranasal inoculation with 2 LD₅₀ of *B. mallei* ATCC 23344 administered i.n. to anesthetized mice. Control animals were vaccinated with non-specific protein (Bovine Serum Albumin, BSA) and adjuvant. Animals were observed closely following challenge and euthanized immediately when pre-determined endpoints were reached and these time points were used to calculate survival times. All animal studies were approved by the Institutional Animal Care and Use Committee at UTMB.

Following challenge with *B. mallei*, 12.5% of control animals survived for > 21 days (Figure 1A). In contrast, survival percentages were significantly increased to 100% up to 21 days post-infection in mice vaccinated with recombinant BimA or BopA. The surviving animals were euthanized at day 21 post-challenge, and the lungs and spleens were homogenized and bacterial counts determined. In all the surviving animals, *B. mallei* were not recovered from the lungs. However, *B. mallei* were recovered from the spleens of all surviving animals (data not shown).

To determine whether antibodies from infected mice reacted with the recombinant *Burkholderia* proteins, serum was obtained from surviving animals. These sera were then tested for recognition of the purified recombinant *Burkholderia* antigens Hcp1, BimA, BopA and LolC by Western blot. Serum from infected mice recognized each of the recombinant antigens tested except for the LolC protein (Figure 2A) and were recognized by both IgG1 and IgG2a isotypes (Figure 2B).

Experiments were also conducted to determine whether the *Burkholderia* recombinant antigens were also capable of generating protective immunity against *B. pseudomallei* challenge (Figure 1B). For these experiments BALB/c mice, (n = 5 per group) were primed by i.n. inoculation with two adjuvant systems and immunized with 2 µg of the purified recombinant BimA, BopA, or LolC proteins given with CLDC adjuvant (cationic liposome-DNA complex), and then boosted 2 weeks later and again 2 weeks after that. The adjuvant used for these studies consisted of cationic liposome-DNA complexes, as reported previously (16) for use in non-specific immunotherapy of *B. pseudomallei*. Controls consisted of mice administered CLDC adjuvant alone, diluent only or BopA antigen alone. Two weeks after the last immunization, mice were subjected to lethal i.n. challenge with 2 LD₅₀ of *B. pseudomallei* strain 1026b, as described previously (17). Survival times were determined as noted above, and all the *B. pseudomallei* animal challenge studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

Our data indicate that the vaccine candidates protected the animals from an initial, acute infection, but failed to confer sterilizing immunity. Our future studies are focusing on the identification of the immunogenic domains in the proteins coupled with optimization in the vaccination strategies to develop a fully protective vaccine.

We observed that immunization separately or as a combination of each of the four recombinant *Burkholderia* antigens conferred at least 75% protection against *B. mallei* infection at 21 days compared to control mice (Figures 1A and 1B). Notably, one of the vaccine antigens (BopA) also conferred significant 60% longer term (> 60 days) protection against *B. pseudomallei* challenge. Furthermore, when surviving BopA-vaccinated mice were sacrificed and lung, spleen, and liver tissues plated for detection of *B. pseudomallei*, 25% of the surviving animals were sterile, at least within the limits of detection of the organ culture (typically 50 CFU/organ).

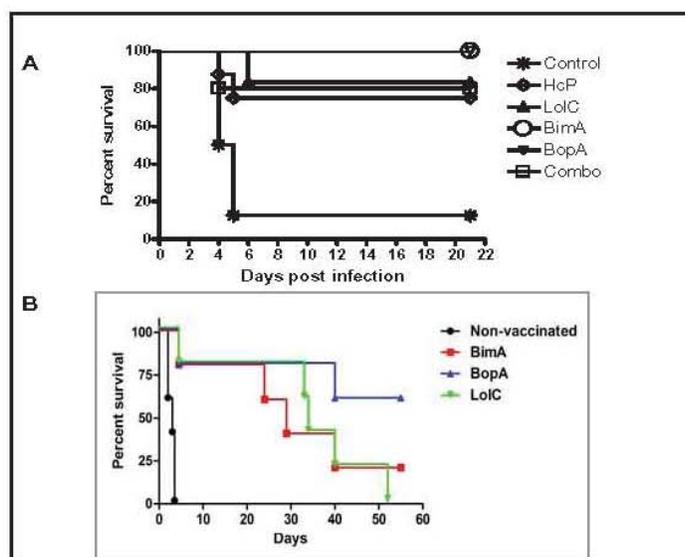


Fig. 1. Survival of BALB/c mice immunized with different recombinant proteins and challenged with *B. mallei* ATCC 23344 and *B. pseudomallei* 1026b.

BALB/c mice were challenged i.n. with 2 LD₅₀ *B. mallei* 4 weeks following intranasal vaccination with BimA (n=2), BopA (n=5), Combo (n=5), LolC (n=6), Hcp1 (n=8) or Control (n=8). BopA- and BimA-vaccinated animals resulted in 100% survival up to 21 days post-challenge. (B) BALB/c mice (n = 15, pooled data from 3 separate experiments) were immunized 3 times with the indicated antigens, then challenged i.n. with 2 LD₅₀ *B. pseudomallei* strain 1026b and survival times determined.

5. Discussion

Immunization with recombinant LolC, BimA, Hcp1HCP and BopA proteins provided significant protection against *B. mallei* ATCC 23344 and *B. pseudomallei* 1026b (Figures 1A and B), in which *B. mallei* BopA gave the best results. The combination of all subunits for protection from *B. mallei* may have some utility as a combined vaccine, although not remarkably better than BopA alone. The serological results suggest that optimal level of Th1 (IgG2a) and Th2 (IgG1) responses are important for protection in *B. mallei* infection. An interesting observation is that in control group mice, which received only CpG2395 and ISCOM, we have observed over 78% survival, whereas in a previous study, mice receiving only ISCOM had ~12% survival after infection with 2 LD₅₀ of *B. mallei* ATCC 23344, indicating that CpG2395 itself also offers protection (data not shown). There are several reports showing immune-enhancing activity of CpG (18-24). Consistent with this study, all future studies will be repeated using a more prolonged time to challenge at 4 weeks to reduce background protection offered by CpG2395, and a control group without CpG treatment will be included as a control as was done in these studies.

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