# UTILIZATION OF A MODEL VACCINATION SYSTEM TO EVALUATE THE IMMUNOSTIMULATORY CHARACTERISTICS OF LICKM, A NOVEL CARRIER MOLECULE

by

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## ABSTRACT

The key objective of vaccination is the induction of an effective pathogen-specific immune response that leads to protection against infection and/or disease caused by that pathogen. New vaccine technologies have resulted in second-generation recombinant vaccines containing highly purified antigens with improved tolerability and safety profiles. Unfortunately, the immune responses they induce are suboptimal without the help of adjuvants. This project investigates the bacterial carrier molecule from *Clostridium thermocellum* a modified Lichenase (LicKM), to determine if it posses adjuvant-like capabilities. This thermostable enzyme contains a catalytic domain loop structure separating it into two regions; an N-terminal and C-terminal region (Musiychuk, 2007). Target protein sequences are expressed as either N or C terminal fusions; LicKM may contain a single or double fusions (Musiychuk, 2007).

We hypothesize, based upon preliminary results that this molecule serves a dual purpose, as a carrier of dominant epitopes for presentation to antigen presenting cells during vaccination and also as an adjuvant enhancing immunity. Here, we investigated expression of common dendritic cell (DC) markers in the presence of LicKM. Next, the ability of LicKM to suppress the immune response in the presence of a potent activator was assessed. To start to elucidate the potential mechanism of action of LicKM, the interaction of host immune cells with the carrier protein was analyzed. Finally, the humoral immune response following vaccination with LicKM fused target antigens was analyzed. The ultimate goal of this proposal is to

demonstrate if LicKM possesses novel adjuvant properties responsible for the development of enhanced cell-mediated responses.

# Chapter 1

# INTRODUCTION

## 1.1 Vaccine Basics

Vaccines are an important advancement in healthcare, as they serve to prime the immune system in healthy individuals to develop an immune response against a particular disease or infection (Makela, 2000). By allowing the immune system to develop this response when not under direct threat from the pathogen, the individual's ability to fight off the pathogen when directly exposed is enhanced. There are several vaccines that every child in developing nations receive over a lifetime which have served to reduce the risk of death and other complications. Vaccines have significantly reduced the risk of getting diphtheria, measles, mumps, rubella, and many others. Additionally, vaccination with small pox is no longer required due to high vaccination rates, which has lead to the eradication of small pox, indicating the effective nature of vaccines against common pathogens.

In order for a vaccine to be effective it must stimulate the proper response to the immunogenic dominant epitope. There are two different pathways of stimulation leading to either a humoral, antibody response, or cell-mediated, T-cell response. For example, a vaccine against an extracellular pathogen needs to generate a strong humoral response so that when the individual is exposed to the pathogen the body will be able to properly clear the infection with the developed antibodies. Alternatively, if a cell-mediated response was primarily generated by this vaccine, it may not be as effective in protecting the individual against the disease. Therefore, ensuring the vaccine generates the appropriate protective immune response against the target pathogen is important in vaccine design and development. Additional considerations

during vaccine development include the rigorous efficacy and safety testing before clinical trials can begin.

### 1.1.1 Adaptive Immune System

In order for a vaccine to generate effective protection the adaptive immune system must be activated. Within the adaptive immune system there are two different branches. One is a humoral immunity and the other is cell-mediated immunity. Within humoral immunity, T helper type 2, $CD4^+$ T-cells are important for fighting extracellular pathogens as memory B-cell and target-specific antibodies are generated (Abbas, 2012). These antibodies serve to protect the individual by coating the pathogen leading to opsonization, neutralization, and phagocytosis (Abbas, 2012).

Cell-mediated immunity, specifically T-helper type 1, $CD4^+$ T-cells is important against intracellular pathogens. These helper T-cells further differentiate into cells with specific functions essential to clear the specific types of infection (Abbas, 2012). More specifically, cytotoxic T-cells or killer T-cells can lyse infected cells using granzymes and perforin (Abbas, 2012).

### 1.1.2 Vaccine Development

Before vaccines are licensed for use for the general population they undergo strict testing to determine safety and efficacy. During this testing there are multiple phases they must pass before licensing is approved. In early vaccine development, vaccines were comprised of pathogens that were live-attenuated or killed with heat or formalin. However these vaccines had the ability to maintain virulence and potentially revert back to full virulence. As a result, they are not frequently found today. Due to such safety concerns, new vaccine approaches have focused on recombinant proteins, highly purified antigens. However, typically a protein by itself is not immunogenic and is unable to generate an adequate protective immune response. For these reasons, recombinant protein-based vaccines typically contain an adjuvant in the formulation. An adjuvant functions to stimulate an immune response by signaling through various

surface receptors that there is "danger present" (Glenny, 1921). This danger may be perceived as either a bacterial, viral, or fungal agent. Successful vaccine development must select the best immunogenic dominant epitope target, adjuvant, and administration strategy to be successful (Riese, 2013).

### 1.2 Adjuvants

Adjuvants function to stimulate an immune response by signaling to the immune system there is "danger present" (Glenny, 1921). In order for an adjuvant to be incorporated in a licensed vaccine it must also undergo strict safety and efficacy testing. An adjuvant should be able to be used with any antigen and predictably stimulate a specific type of response, either humoral or cell-mediated. Furthermore, the adjuvant employed must work with the intended route of vaccination developing the intended protective response. Most vaccines are administered intramuscularly and stimulate a strong systemic response suitable for fighting many pathogens such as fungal or parasitic infections (Belyakov, 2009). Some pathogens, whose primary site of infection is the mucosal region, would require an effective vaccine to stimulate a mucosal specific immune response as possibly produced by an oral vaccine (Harandi, 2003). Therefore, the adjuvant selection is as important as antigen selection for a successful protective vaccine formulation.

Adjuvants are beneficial on several different levels. First, adding an adjuvant can reduce the dose of antigen needed to elicit a robust immune response, which could be important if the vaccine is needed in a pandemic situation (Reed, 2013). Second, an adjuvant reduces the number of doses needed to elicit an effective immune response inducing protection quicker (Reed, 2013). Third, adjuvant inclusion can enhance antibody titer generation and antibody epitope recognition, helping combat antigenic shift and drift of pathogens (Reed, 2013). Fourth, an adjuvant could induce a larger proportion of functional antibodies (Reed, 2013). In addition, new more robust adjuvants could help to provide a better vaccine responses for the elderly who have undergone immunosenescence or help in the generation of a better therapeutic

vaccines for cancer, HPV, and others (Reed, 2013). Additionally, there is a significant need for adjuvants that could induce T-cell responses opening up a whole new area of vaccine development (Reed, 2013).

Currently, adjuvants are classified based on their properties and mechanism of action. There are adjuvants that work by stimulating the Toll-Like receptor (TLR) pathway, part of the innate immune system signaling through common motifs found on bacterial, fungal, or viral external surfaces. Additionally, there are adjuvants which signal through TLR independent pathways (Gregorio, 2013). An adjuvant can serve as either a stimulant to the immune system or it can provide better antigen presentation of the target antigen to the immune system (Reed, 2013). However, an adjuvant, which can both present the antigen and stimulate the immune system, would be most advantageous. At this time, only a select few adjuvants have been approved for human use.

### 1.2.1 Licensed Adjuvants

Although, adjuvant research has been conducted for a long time, only a few adjuvants have made it to the market in the United States and/or Europe. Currently licensed products include: aluminum salts (most well understood), oil-in-water emulsion (2 types), virosomes and AS04 (Reed, 2013). Each of these adjuvants works in a slightly different manner but overall they help to stimulate a protective and efficacious response in those vaccines for which they have been included.

#### 1.2.1.1 Aluminum Salts

Aluminum salts, one of the most common vaccine adjuvants, generate a strong humoral immune response (antibody). Current vaccines using aluminum salts include: diphtheria, tetanus, pneumococcus, and many others (De Gregorio, 2013). It is important to note that there are different formulations of aluminum salts including: aluminum potassium sulphate, aluminum hydroxide, and aluminum phosphate (Marrack, 2009). All formulations of aluminum salts have slightly different functions

due to different chemical structures, leading to some confusion as to how exactly this product enhances the immune system.

In 1926, Glenny and Pope found that precipitating the diphtheria toxoid onto aluminum salts increased the antibody response in guinea pigs (Glenny and Pope, 1926). By 1932, aluminum salts were being used in human vaccines (Marrack, 2009), interestingly this was done before the mechanism of action was fully understood. The depot theory was purposed by Glenny to explain how aluminum salts worked; keeping the antigen at the injection site providing a longer exposure time (Marrack, 2009). However, after further research the depot theory has not provided the full explanation. Holt (1950) found when the lymph node most closely located to the antigen-aluminum salts injection site was removed 14 days after immunization; a decrease in antibody titer did not occur suggesting that the adjuvant does not enhance B-cells or antibody titer generation. While another researcher, White and colleagues (1955) discovered that B cells were found in the lymph node 7 days after rabbits were immunized with aluminum salts plus antigen and at the injection site by day 14. However, by three weeks after immunization, negligible B-cells were found in the local lymph node (White, 1955). Additionally, Sharp (2009) detected pro-inflammatory mediators a few hours after injection with aluminum salts and antigen and Kool (2008) found innate immune cells were present at the site of injection within one day of immunization. Data from Holt and White provide strong evidence that a depot effect is not the proper explanation for the adjuvant activity of aluminum salts.

Since the depot effect does not explain the adjuvant activity of aluminum salts, other hypotheses have come to light. One being that aluminum salts are signaling through a TLR pathway (Marrack, 2009). MyD88 is an important signaling molecule for almost all TLR pathway signals (Schnare, 2001). Schnare (2001) showed mice deficient in MyD88 still had normal amounts of IgG1 in response to vaccination as compared to wild type animals. Gavin (2006) used mice that were deficient in both MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF), important signaling molecule for all TLR signaling, effectively inactivating all TLR signaling,

and found antibody titers and isotype profiles to be comparable to wild type animals. Taken together this provides evidence that the TLR pathway is not necessary for aluminum salt stimulated responses.

An additional theory for the adjuvant activity of aluminum salts is NLRP3 inflammasome activation (Marrack, 2009). There are two possible hypotheses: activation occurs through either a direct or indirect signaling. For the direct activation model, cells whose primary function is phagocytosis directly interacting with, and phagocytizing the aluminum salt leading to lysosomal damage, and ultimately activating the intracellular danger pathways (Hornug, 2008). The indirect activation model suggests that aluminum salt causes cytotoxicity, causing damage-associated molecular pattern molecules (DAMPs), such as uric acid, to be released causing NLRP3 activation (Kool, 2008). Kool (2008) provided strong evidence by showing that after vaccination with aluminum salts that the local concentration of uric acid was significantly increased.

The function of IL-4 was also investigated. Absence of IL-4 has been shown to reduce production of Th2 response upon vaccination (Brewer, 1996). Mice that were deficient in some part of the IL-4 signaling pathway were also deficient in IgE antibody production, and IgG1 production was at lower levels than wild type (Brewer, 1996). Additionally, these mice produced high IgG2a titers, which is indicative of a Th1-type response (Brewer, 1996). Upon establishing the importance of IL-4, studies looked to determine the location of IL-4 production. Jordan (2004) demonstrated that vaccination with aluminum salts caused a population of IL-4 producing $GR1^+$ cells (mainly eosinophils) to accumulate in the spleen within 6 days of infection/exposure. A follow up study by McKee (2008) showed this cell population accumulated in the spleen one-day post exposure along with an increase in B-cell proliferation and production of IgM antibody isotype. Additionally, McKee (2008) found that depletion of the $GR1^+$ cell population, followed by vaccination reduced levels of B-cell proliferation and IgM production, strongly suggesting a role for $GR1^+$ eosinophil-like cells.

Current research on the mechanism of action for aluminum salts can be summarized as affecting antigen uptake, inducing danger signals (pattern associated molecular patterns, PAMPs, patterns recognized by the innate immune system found on bacteria, fungi, or viruses) and various immune cell recruitment (Reed, 2013). Aluminum salts are known to generate a strong humoral response with IgG1 and IgE and a Th2 T-cell response due to IL-4 production (Marrack, 2009). Additionally, it has been well established in the literature that Th-2 T-cell responses inhibit Th1 T-cell responses (Grun, 1989).

#### 1.2.1.2 Oil-in-Water Emulsion

Emulsions are a two-part system that require a surfactant for stabilization of the antigen (Riese, 2013). The first oil-in-water emulsions, Incomplete Freund's adjuvants (IFA) and complete Freud's adjuvant (CFA), were developed in the 1940's (Brito, 2014). CFA has mycobacteria cell wall parts included and is used for initial vaccinations, where as IFA has no mycobacteria and is used for booster vaccinations (Brito, 2014). Both IFA and CFA enhanced cell mediated responses as well as humoral responses. These adjuvants were efficient but were not well tolerated, as they are potent stimulators of immune responses generating significant ulcerative side-effects, preventing their widespread use (Reed, 2013).

Currently, two oil-in-water emulsions are approved for human use: MF59 and AS03 (Reed, 2013). Both emulsions are used in influenza vaccines (De Gregorio, 2013). MF59 was first licensed in 1997 for use in influenza vaccines for the elderly, providing enhanced immunogenicity in this patient population (Riese, 2013). MF59 is a squalene nano-emulsion thought to work by creating an immunostimulating environment (Seubert, 2008). MF59 has also been shown to increase antigen uptake by dendritic cells (Calabro, 2011). This adjuvant induces a more proportional distribution of IgG1 and IgG2a titer responses (Reed, 2009). The wide scale use of MF59 in influenza vaccines has shown it is safe, effective, reduces the amount of antigen required, and induces a strong broad immune response (Durando, 2010). A

study to determine the mechanism of action for MF59 showed it is unable to stimulate dendritic cells but instead activates monocytes, macrophages, and granulocytes (Dupuis, 2001). This lead to the hypothesis that MF59 does not directly act on dendritic cells but acts to recruit dendritic cells to the area of injection (Dupuis, 2001). MF59 functions by creating a conducive environment for an immune response, increasing antigen uptake, resulting in an even distribution of antibody isotype response, and most importantly is safe for human use.

Lastly, AS03 is a squalene emulsion which is larger in emulsion size compared to MF59 and causes an immune response not only at the site of injection but in the whole muscle and draining lymph node (Garcon, 2012).

#### 1.2.1.3 Virosomes

Virosomes function by using common viral proteins to stimulate the immune system, protect the antigen at the site of injection. Virosomes are currently used in vaccines for hepatitis A and influenza in Europe (Reed, 2013). Virosomes are made *in vitro* and incorporate viral proteins on the surface membrane (Riese, 2013). The antigen is encapsulated into the virosomes thereby stabilizing the antigen and providing protection from degradation (Almeida, 1975). By combining the viral proteins and antigen the virosomes serves as a carrier system capable of stimulating the immune response to the target antigen (Gluck, 2005). One virosomes example is the hepatitis A vaccine, Epaxal, in which the hepatitis A virus is grown in human cells and inactivated with formaldehyde (Bovier, 2008). Virosomes based on influenza hemagglutinin (HA) have the ability to re-stimulate memory cells to HA allowing for a rapid response as compared to a naïve response. By using a previous immune response, the generated response will be stronger than naïve response. Epaxal is well tolerated and has a strong humoral response within 2 weeks of first vaccination (Bovier, 2008). Virosomes stimulate a strong humoral response to the antigen (Pevion, 2010). Recent studies have shown the possibility to use virosomes to present tumor antigens to dendritic cells for use in cancer treatment (Schumacher, 2005).

#### 1.2.1.4 AS04

AS04 is an improvement to aluminum salts, in which monophosphoryl lipid A (MPLA) is used in conjunction with aluminum salts (Reed, 2013). MPL is a non-toxic derivative based on lipopolysaccharide (LPS) from *Salmonella minnesota* which is a potent stimulator of the TLR4 pathway (De Gregorio, 2013). Mueller (2004) prepared single compounds and aggregates of LPS, then tested the preparations on human mononuclear cells for induction of cytokine production. It was found the LPS must be delivered in aggregates in order to function, as only the aggregates are biologically active (Mueller, 2004). MPL is the first adjuvant that has been licensed that can induce a sufficient T-cell response (Ismaili, 2002). AS04 is administered with aluminum salts, creating an antibody response along with a Th1 T-cell response (Didierlaurent, 2009). AS04 is currently found in Cervarix® the vaccine used for protection against a number of strains of HPV (Reed, 2013). Pre-clinical research has also demonstrated that AS04 may have application in allergy (Puggioni, 2005; Pfaar, 2011) and cancer vaccines (Cluff, 2010).

#### 1.2.1.5 Cholera Toxin B (CTB)

Cholera toxin contains two different subunits. Cholera toxin subunit A (CTA) is responsible for the excessive secretion of electrolytes and potentially leading to life-threatening dehydration whereas cholera toxin subunit B (CTB) is responsible for binding to the M cells of the intestinal Peyer's patches allowing CTA to enter (Holmgren, 1993). When used as an adjuvant, CTB alone mimics the toxin's ability to enter host cells. CTB binds to the GM-1 ganglioside receptor, which is present on the surface of the majority of mammalian cells. Binding then allows for the antigen to be presented to the dendritic cells (Riese, 2013). CTB is non-toxic as it only serves to bind to the receptor and cannot induce toxic effects. CTB was used as an oral adjuvant as in the vaccine Dukoral® for cholera (Svennerholm, 2011). However, after further testing, Dukoral® has been shown to have a short half-life and only partial protection

against cholera (Hill, 2006). Of greater concern is the temporary facial paralysis after the administration of the vaccine (Mutsch, 2004). Whole CTB requires additional safety testing before use in the United States. In order to eliminate safety concerns, recombinant CTB (rCTB) has been developed and used in vaccine trials (Svennerholm, 2011). Svennerholm (2011) found rCTB provided very high short-term protection over controls. Indicating a potential for rCTB to be used as an adjuvant in an oral vaccine against Cholera.

#### 1.2.1.6 Saponins

Quil-A is a partially purified saponin (sugar) from *Quillaja saponaria*, which is also a soapbark tree used as a veterinary adjuvant (Kensil, 1996). Quil-A elicits a strong Th1 and Th2 response and a modest $CD8^+$ T-cell response (Dalsgaard, 1974). Quil-A is inexpensive but not licensed for use in humans due to the strong Th1 and Th2 response it elicits (Cox, 1997).

QS-21 is a partially purified saponin (sugar) from *Quillaja saponaria*, which is also a soapbark tree (Kensil, 1996). QS-21 is currently under investigation for its adjuvant properties in a Phase 3 clinical trial against malaria epitopes (Reed, 2013). QS-21 has the ability to generate a humoral and cell-mediated response with $CD8^+$ T-cells being generated (Reed, 2013).

### 1.2.2 Adjuvant Development

Although, we have discussed multiple adjuvants currently in use or in stages of development at this time there is a significant need to develop new products especially those that can be employed to stimulate cell-mediated immune responses. In the future, a major area of focus will be adjuvants that induce a strong T-cell response or mucosal immunity (Riese, 2013).

During adjuvant development several constraints must be considered: quality production of the product, storage, stability, cost, safety, and immunization route are only a few constraints (Reed, 2013). The adjuvant must also be tailored to the type of

response required for optimal vaccine protection (Reed, 2013). At this time many potential adjuvants are in the pipeline for development. Again, they can be classified as TLR dependent or independent.

#### 1.2.2.1 TLR Dependent

Since the development and success of Monophosphoryl Lipid A (MPL) based on TLR4 binding and activation, there has been an interest in developing more adjuvants that mimic TLR agonists (Riese, 2013). An additional derivative was generated against TLR4, glucopyranosyl lipid A (GLA-SE) that is delivered in a squalene oil emulsion (Riese, 2013). GLA-SE generates a Th1 response similar to MPL (Coler, 2011). And has been evaluated in Phase I clinical trial vaccines for influenza and tuberculosis (Clinicaltrials.gov).

Macrophage-activating lipopeptide-2 (MALP-2) from *Mycoplasma fermentans* activates TLR2/6. (Rharaoui, 2002). MALP-2 is able to activate antigen-presenting cells (APC) via MHC I and generate a humoral and cellular immune response (Borsutzky, 2006). Additionally, MALP-2 has the potential to be used for mucosal immunity seen during the prime-boost against HIV-1 Tat protein with MALP-2 in BALB/c mice (Borsutzky, 2006; Rharaoui, 2002).

TLR5 agonist, bacterial flagellin has been shown to activate DC leading to protection against lethal pneumonic plague infection (Honko, 2006). Flagellin can also be found as a part of an influenza vaccine under evaluation in a Phase II clinical trial (Talbot, 2005).

Next, TLR7 agonist imiquimod, used for the treatment of genital warts, and TLR7/8 agonist resiquimod, that has antiviral and antitumor activity, have been shown to generate Th1 and CD8 T-cell responses in vaccinated mice (Thomsen, 2004). Natural TLR7/8 agonists are single-stranded RNA (Abbas, 2012). Lastly, TLR9 agonist thymosin α-1 (Tα-1) is a peptide naturally found in the thymus and is able to generate murine humoral and cellular immunity (Riese, 2013). Tα-1 promotes murine T cell maturation and differentiation (Romani, 2004), therefore, increasing murine

dendritic cells priming leading to better stimulation of the immune response and clearing of infection (Romani, 2006; Bozza, 2007).

#### 1.2.2.2 TLR Independent

Chitosan is a polysaccharide derived from chitin, a glucose derivative (Andrade, 2011). Jabbal-Gill (1998) immunized mice with filamentous hemagglutinin from *Bordetella pertussis* along with chitosan and found an increase in antibody responses. Intranasal vaccination with a mutated diphtheria toxin and chitosan in mice (McNeela, 2000) and humans (McNeela, 2004) has shown similar results. Chitosan works by slowing degradation of the antigen due to the encapsulation for longer exposure and greater immune response (Vila, 2004). Nishimura (1984) found chitosan activates macrophages for humoral cytokine production and increases antigen uptake at mucosal barriers.

Alpha galactosylceramide (αGalCer) a marine sponge *Agelas mauritianus* extract binds to CD1d receptor, a MHC I-like molecule on antigen presenting cells (APC) (Kronenberg, 2002). Bai (2012) found αGalCer interacts with natural killer T-cells (NKT cells), which in turn stimulates dendritic cells resulting in a Th1 and Th2 response. Additionally, αGalCer administered with antigen stimulates CD4 and CD8 T-cell production (Fujii, 2003). In 2009, αGalCer and hepatitis B vaccine entered a human phase II clinical trial of participants infected with hepatitis B (Woltman, 2009). Unfortunately, it showed little protection and has multiple safety issues (Woltman, 2009). These safety issues were thought to be due to the αGalCer structure, therefore it was structurally modified and became αGalCerMPEG and was shown to maintain affinity for the CD1d receptor (Ebensen, 2007). αGalCerMPEG appears to have improved humoral and cell mediated responses when compared to αGalCer and generates an IgA antibody response leading to the potential use as a mucosal adjuvant (Ebensen, 2007).

Two other natural compounds being evaluated, as potential adjuvants are cyclic di-nucleotides, either guanine (c-di-GMP) or adenine (c-di-AMP) bound to

ribose and phosphate (Libanova, 2012). Ebensen (2007) found these two compounds are able to produce a strong humoral and cell mediated immune responses. Cyclic di-nucleotides are able to activate DC and increase antigen specific humoral and cellular immune responses when administered either intramuscularly or mucosally (Karaolis, 2007; Karaolis, 2007). A recent study by Libanova (2010) showed the cyclic di-nucleotides were able to induce antibodies and a mixed Th1 and Th2 response.

#### 1.2.2.3 Combination of Adjuvants

One area of particular interest is combining adjuvants to provide better, longer lasting, protection. However, the need for additional safety and efficacy testing for combinations of adjuvants may arise (Riese, 2013).

One combination under investigation is resiquimod with a TLR3 or TLR4 agonist, as this has been shown to promote a Th1 response in human neonatal APC (Krumbiegel, 2007). Additionally, using MF59 in influenza vaccines with Tα-1 showed stronger immune responses than MF59 alone (Carraro, 2012). When aluminum salts, MPL and muramyl dipeptide (MDP) are used in combination a stronger additive immune response is produced (Giannini, 2006). Vaccination with CpG (DNA region where a cytosine is next to a guanine), CTB, along with antigen epitopes from *Chlamydia trachomatis* results in increases in the immune response without generating toxic effects (Cheng, 2009). AS02 oil-in-water emulsion with MPL and QS-21 (part of Quil-A) is able to generate a strong humoral and Th1 response and is being evaluated in several animal models of disease (Reed, 2009). Aide (2011) administered a malaria vaccine with AS02 to young children finding protection was generated for at least 18 months in the absence of side effects. AS01 is very similar to AS02 however, it consists of MPL, QS-21, and liposomes and its use in a malaria vaccine administered to two different populations of children demonstrated a greater level of protection when compared to AS02 (Lell, 2009; Ansong, 2011). Similar results were seen by Leach (2011) with a malaria vaccine in children and by Leroux-Roels (2012) with a tuberculosis vaccine in adults.

Murine research also points to CpG motifs as a potential TLR9 agonist, however in humans an additional stimulus is needed to generate protective responses (Riese, 2013). When CpG motifs are added to the Engerix B vaccine for Hepatitis B, increased protection in immune-compromised individuals is detected (Cooper, 2005). The combination of multiple adjuvants provides the possibility for very specific and long lasting protection.

### 1.3 Possible New Adjuvant, LicKM

LicKM, a lichenase from *Clostridium thermocellum,* (Musiychuk, 2007) has enzymatic activity towards lichenan and beta-glucan (Ahmed, 2009). LicKM was first investigated for its thermostable and carrier protein properties. When proteins were fused to LicKM the proteins acquired the thermostable properties of the enzyme (Ahmed, 2009). LicKM is a 25KDa protein made by Fraunhofer USA Center for Molecular Biotechnology, FhCMB) in a plant-based system. By producing LicKM in a plant system, safety concerns over bacterial LPS contamination are eliminated (Chichester, 2007). This plant platform provides a cost-effective and scalable approach to generating large-scale production of vaccine components (Chichester, 2007).

Vaccination with immunogenic epitopes fused to LicKM provided protection in several vaccination models. Immunization of monkeys with LicKM and *Y. pestis* epitope fusions, LicKM-LcrV and LicKM-F1, produced a strong antibody response and provided protection against lethal challenge with *Y. pestis* (Mett, 2007; Chichester, 2009). Immunization of mice with the same LicKM fusions demonstrated protection against *Y. pestis* and generated antibody and T-cell responses characterized by $CD4^+$ IL-2 production (Guth, 2012).

LicKM has also been used in the design of vaccines against the human papillomavirus (HPV) (E7 and E7GGG), *Bacillus anthracis* (PAD4 and LFD1) and *Plasmodium falciparum* (Pfs25). Human papillomavirus (HPV) contains several oncoproteins and E7 is of particular interest for vaccine development. E7 and E7GGG

(containing amino acid substitutions) fused to LicKM (Massa, 2007) generated high levels of IgG and IFN-γ production, demonstrating both humoral and cell-mediated responses to vaccination (Massa, 2007). Interestingly, prevention of tumor growth was observed in mice immunized with LicKM and E7 alone, no adjuvant (Massa, 2007). Immunization of mice with LicKM fusions and *B. anthracis* epitopes generated high IgG1 antibody titers capable of inhibiting anthrax lethal toxin *in vitro* (Chichester, 2007). Together this research indicates that LicKM may be serving as more than just a carrier molecule and possess immunomodulatory properties.

### 1.3.1 LicKM Development

The native lichenase protein was modified by replacing the signal sequence and by removing the docking domain and Pro-Thr-rich box therefore this modified protein is referred to as LicKM (Musiychuk, 2007). FhCMB has developed its plant-based rapid-response transient expression system for the engineering and production of a wide range of recombinant proteins, and demonstrated its utility for vaccine development in several research projects, progressing into clinical trials. FhCMB's system is applicable to a broad range of monomeric and multimeric proteins, and is based on transient expression vectors, vacuum infiltration delivery of the vectors into plant biomass, and the use of a multi-layered system for hydroponic generation of plant biomass (Musiychuk, 2007).

## 1.4 Infection with *Yersinia pestis* and Current Vaccine Development

*Yersinia pestis* is a Gram-negative, non-motile, non-spore forming, facultative intracellular bacterium with an optimal growth temperature of 28-30°C (Perry, 1997). *Y. pestis* has a typical Gram-negative cell wall and no true capsule but does have a carbohydrate protein envelope (F1) when grown at ≥33°C (Perry, 1997). *Y. pestis* is maintained in the wild rodent and flea populations in known foci around the world on all continents except Australia (MMWR, 1996).

### 1.4.1 Infection of *Y. pestis*

Usual modes of transmission occur most commonly in the United States from an infected flea or direct contact with an infected animal most commonly an infected cat (MMWR, 1996). Infection due to a fleabite results in bubonic plague leading to the development of buboes, which are painful swelling of the lymph nodes (Butler, 1983). Rapid antibiotic treatment is necessary to prevent development of sepsis and death (Butler, 1983). Pneumonic plague is infection occurring from inhalation of respiratory droplets from an infected animal or human, and is associated with a much higher mortality rate than the bubonic plague (Lien-The, 1926). Symptoms of pneumonic plague include severe headache, nausea, discomfort, fever, cough, and difficulty breathing (Smiley, 2008). As the infection progresses, the cough will generate infectious bloody mucous and infectious respiratory droplets (Smiley, 2008). Pneumonic plague is fatal unless the patient is treated during the first 24 hours after exposure (Butler, 1983).

Current antimicrobials treatment options are tetracycline, doxycycline, and trimethroprim-sulfamethoxazole all having different dosing recommendations for adults and children (White, 1980). Samples are routinely collected from infected patients to determine when the patient is no longer at risk of infecting others (Perry, 1997).

### 1.4.2 Immunogenic Epitopes of *Y. pestis*

Two proteins from *Y. pestis* have been under investigation for *Yersinia* vaccine development. The first protein, fraction-1 (F1), is a capsular protein that develops only when grown at 37°C, the normal human body temperature (Perry, 1997), indicating a role in its virulence in humans. The second common, target low-calcium release V antigen (LcrV), is a protein needed for proper function of the Type III secretion system, serving as a mode of entry for the bacteria into the host cell (Perry, 1997). LcrV was selected as the target antigen for the novel work presented in this thesis.

### 1.4.3 Need for Development of a *Y. pestis* Vaccine

After many years of vaccine research there is still no safe, effective and licensed vaccine against *Y. pestis.* The first vaccine was a fully virulent heat killed bacterial strain, which caused fever and severe adverse reactions and provided only partial protection (Haffkin, 1897). Next, small amounts of live-attenuated bacterium were used for a vaccine (Kolle, 1904). Many people in Indonesia, Madagascar, and Vietnam received this vaccine (Girard, 1963), however this vaccine is highly unstable and still has many virulence factors (Welkos, 2002). Because of its many adverse reactions this vaccine is no longer used (Meyer, 1974). The third vaccine, Plague Vaccine, USP, was a formalin-killed vaccine delivered in saline (Meyer, 1974). Plague Vaccine, USP was licensed and distributed for use in soldiers during the Vietnam War (Meyer, 1970). It was later removed from the market due to severe adverse reactions that became worse with each booster dose (Butler, 1983). This vaccine did not provide protection against pneumonic infection (Butler, 1983).

*Y. pestis* is considered a facultative intracellular organism, and therefore a humoral (Th2) response alone is not enough to protect the individual. Importantly, the proper antigenic epitopes must be used to generate a strong cell mediated response (Th1) with cytotoxic T-cells as the main method of clearing the infection. Additionally, the vaccine needs to produce a strong humoral (Th2) response to neutralize the bacteria before infection. The vaccine must be able to combat the ability of *Y. pestis'* to inhibit phagocytosis (Meyer, 1950). Additionally, fully virulent F1 negative strains of *Y. pestis* have been found. Therefore, a multi-faceted vaccine will be required to be effective against all modes of transmission and strains.

The need for a vaccine against *Y. pestis* would not only prevent future outbreaks of plague in current problematic countries such as the Democratic Republic of Congo, but also serve as a mechanism to protect the population from *Y. pestis* as a biological weapon (Inglesby, 2000). Due to the lack of an available vaccine, the utilization of an aerosolized delivery method combined with the high fatality rate and

relatively easy transfer from human-to-human, the effects of a bioterrorism attack with this bacterium would be devastating to the population (Inglesby, 2000).

## 1.5 Infection with *Plasmodium falciparum* and Current Vaccine Development

*Plasmodium falciparum* is tropical parasite causing Malaria. Malaria is both preventable and curable given the correct medication, artemisinin-based combination therapy (ACT) and precautionary measures such as mosquito nets and spraying with insecticide to kill mosquito breeding grounds (WHO, 2014). The transmission of *P. falciparum* is by *Anopheles* mosquitoes, the parasite is transmitted when the mosquito bites and collects a blood meal (Aly, 2009). Repetitive exposure to *P. falciparum* generates partial immunity, reducing the risk of severe disease (WHO, 2014). However children lack immunity and infection in this population is often fatal (WHO, 2014). In 2013, WHO estimated 584,000 deaths were caused by Malaria, the majority being children in Africa under the age of five. Current regions with high transmission rates include: Sub-Saharan Africa, Asia, Latin American, Middle East, and parts of Europe (WHO, 2014).

### 1.5.1 Infection of *P. falciparum* in Humans

As previously mentioned, infection with *P. falciparum* is due to the bite of an infected female *Anopheles* mosquito. During the up-take of a blood meal, sporozoites are released into the bitten individual (Aly, 2009). The sporozoites travel through the bloodstream into the liver where infection of hepatocytes occurs (Good, 1998). Sporozoites will grow and divide; with one sporozoite generating 30,000-40,000 merozoites, which are then released for infection of the red blood cells (RBC) (Good, 1998). Merozoites that infect RBC will divide until the RBC bursts releasing more merozoites to continue the cycle of infection. At this stage the individual will feel common symptoms including: fever, chills, headache, and vomiting (Good, 1998). Without treatment severe malaria can occur, consisting of: severe anemia, respiratory distress, or cerebral malaria (Good, 1998). Severe anemia is due to the rapid loss of

RBC as they become infected and burst, causing respiratory distress due to the loss of RBC for oxygen transfer.

In order for *P. falciparum* to propagate some merozoites will mature into gametocytes, which can then be taken up during a blood meal by the bite of an *Anopheles* mosquito (Good, 1998). The parasite then fertilizes itself inside the mosquito to develop a zygote, multiplying to ookinetes, which travel to the midgut of the infected mosquito and enter the salivary glands preparing for transfer during the next meal (Good, 1998).

### 1.5.2 Pfs25, Potential Candidate for a Transmission-Blocking Vaccine

Due to the complex life cycle of *P. falciparum* a protective vaccine has substantial challenges (Aly, 2009). One challenge is to break the parasite's life cycle with a vaccine that generates immunity. A particular area of interest is blocking the transmission of the parasite from the mosquito to another individual (Aly, 2009). A vaccine targeting transmission, works by inducing antibodies against the gametocytes, gametes, zygotes and/or ookinetes which in turn block the development of oocysts in the midgut of the mosquito (Good, 1998). During the blood meal the mosquito would receive the gametocytes but also the developed antibodies, stopping the transmission of the parasite. Seven potential antigens have been identified for use in a transmission blocking vaccine (TBV), with Pfs25 the most promising (Good, 1998). Pfs25 has been shown to protect lab animals against infection (Kaslow, 1997).

Kaslow and colleges were able to determine the sequence of Pfs25 and that it is expressed during the sexual stages of *P. falciparum,* occurring in the mosquito (Kaslow, 1988). Previously the sequence of Pfs25, the shared surface marker for zygotes, ookinetes, and gametocytes was unknown due to complications of collecting a large sample and due to the importance of the disulfide bonds for forming the potential antibody binding domains (conformational epitopes) (Kaslow, 1988). Pfs25 has a short hydrophobic anchor with no hydrophilic region at the C-terminal end

suggesting the presence of an anchor protein (Kaslow, 1988). Pfs25 has four tandemly repeated epidermal growth like factor domains (EGF-like domains) (Kaslow, 1988).

Vaccination of laboratory animals with Pfs25 provides transmission-blocking immunity (Barr, 1991). Additionally, when Pfs25 is produced in *Saccharomyces cerevisiae* and adsorbed to alum it is able to block transmission (Kaslow, 1994). Mice and monkeys were vaccinated showing transmission-blocking immunity in both species (Kaslow, 1994). Scientists believe that providing evidence for use of Pfs25 in a different vaccine platform would provide a safe and effective vaccine. Pfs25 (Pfs25-FhCMB) can be produced and purified in the plant-based system and mice and rabbits vaccinated with of Pfs25-FhCMB plus Alhydrogel produced a strong humoral response, transmission blocking capabilities, and had at least 6 months of protection (Jones, 2015).

### 1.5.3 *P. falciparum* Vaccine Development Needs

*P. falciparum* is responsible for causing Malaria, which continues to devastate the population of children in Sub-Saharan Africa. As was seen with previous drugs such as chloroquine, there are pockets of resistance appearing against the current treatment of ACT (Enserink, 2010). Historically, data shows that once chloroquine resistance begins it will spread to all other affected regions. Resistance to current control methods without new treatments would undermine control efforts (mosquito nets, pesticide spraying, and chloroquine) and could reverse the gains in child survival rates (WHO, 2014). Due to the devastation of the population of children in Sub-Saharan Africa (endemic region) and the inability of current drug treatments to provide relief, a vaccine is needed to combat the parasite as drug resistance to new treatments will continue.

## 1.6 Utilization of a Model Vaccination System to Evaluate the Immunostimulatory Characteristics of LicKM, a Novel Carrier Molecule

A variety of previous research has shown LicKM can act as a carrier molecule while also demonstrating adjuvant-like properties (Mett, 2007; Chichester, 2007; Chichester, 2009; Guth, 2012; Santi, 2006; Alvarez, 2006; Massa, 2007; Jones, 2015). The immunostimulatory effect of LicKM must be well understood before late stage clinical testing and licensing could move forward. Research to date has focused on the type of humoral response generated in response to vaccination with LicKM-target fusions. However, to begin to determine the mechanism of action of LicKM *in vitro* work needs to be completed. Additionally, to provide a full understanding of the responses generated by LicKM, the type of T-cell responses it stimulates must also be evaluated (Massa, 2007). In order to continue to establish the role of LicKM as a potential adjuvant, we investigated what host immune cells are interacting with LicKM. Additionally, due to preliminary evidence of LicKM stimulating an enhanced T-cell response (Massa, 2007), dendritic cells (DC), which are the only antigen presenting cells (APC) able to present to naïve T-cells, were investigated for activation. Any potential adjuvant must demonstrate stimulatory effects in the absence of inhibitory properties therefore; activation levels of common DC markers were assessed. Finally, vaccination of mice with various LicKM fusion products (LicKM-LcrV and LicKM-Pfs25) will help elucidate the T-cell response generated. Together this data will allow for a better understanding of how LicKM functions. With a prospective mechanism of action, this potential adjuvant would be able to undergo more safety and efficacy testing, which could lead to the licensing of a LicKM-containing product on the market. Any adjuvant licensed that is able to generate a humoral and cell mediated response will open up a whole new area for vaccine development.

# Chapter 2

# MATERIALS AND METHODS

## 2.1 Murine Models

Female BALB/c mice (Harlan Laboratories, Indianapolis, IN) at 5 to 10 weeks of age were used for generation of all bone marrow derived dendritic cells, splenocyte collection, and two vaccine trials. The Institutional Animal Care and Use Committee (IACUC) at the University of Delaware (Newark, DE) approved all animal protocols.

## 2.2 Determining LicKM's Interaction With Host Immune Cells

### 2.2.1 Splenocyte Collection and Stimulation

Spleens were dissected from female BALB/c mice (Harlan Laboratories, Indianapolis, IN) and put in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, Corning, NY) with 10% GemCell Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 2% L-glutamine (Corning, Manassas, VA), 1% non-essential amino acids (Corning, Manassas, VA), 1% penicillin/streptomycin (MP Biomedicals, LLC, Solon, OH), 0.1% 2-mercatptocethanol (Invitrogen, Grand Island, NY), 1% sodium pyruvate (Corning, Manassas, VA), and 1% HEPES (Corning, Manassas, VA) (cDMEM) on ice. Spleens were disrupted by crushing, pipetted into a conical tube and spun down for ten minutes at 2,000 rpm at 4°C, supernatants were removed. For each spleen dissected 500 µl of RBC lysing buffer (Sigma, St. Louis, MO) was added to re-suspend the pellet. Samples were put into a 37°C water bath for three minutes, after which 10 ml of cDMEM media was added to neutralize the RBC lysing buffer. Samples were spun for ten minutes at 2,000rpm, supernatants removed, and the pellet was re-suspended in 10 ml cDMEM media on ice. Splenocytes were seeded in a 96 well flat bottom plate (Corning, Corning, NY) at 2 X $10^7$ cells/mL. Each well received 50 µl cells, 100 µl cDMEM media, and 50 µl of protein (Ovalbumin (OVA), LicKM, LcrV, or LicKM-LcrV) at 10 µg/well. Plates were incubated for 1 or 3 hours at 37°C + 5%$CO_2$.

### 2.2.2 Splenocyte Staining for Flow Cytometry

Stimulated splenocytes were collected for flow cytometery analysis. Fcγ receptors on cells were blocked with 1μg MAb 2.4G2 (BioXCell, West Lebanon, NH) added in a final volume of 40 μl, for 15 minutes at room temperature. Antibodies specific for host immune cell surface markers were used at 1:100 dilution of 0.5mg/mL for FITC or a 1:200 dilution of 0.2mg/mL for PE, PE-Cy7, and APC in FACS buffer (1X PBS (Thermo Scientific, Waltham, MA) and 2% GemCell Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA)) Surface markers assessed were: CD3, CD4, CD8, CD11b, CD11c, CD19, B220, GR-1, NK1.1, and F4/80. All surface markers were purchased from Ebiosciene (San Diego, CA) or Tonbo (San Diego, CA). Cells were then fixed using 30 μl 4% formaldehyde (Fisher Scientific, Waltham, MA) and 25 μl FACS buffer for 20 minutes at room temperature in the dark. For intracellular staining assays samples were incubated with 180 μl permeabilization buffer (FACS wash and 0.5% saponin (MP Biomedicals LLC, Solon, OH)) for 10 minutes in the dark at room temperature. Intracellular staining was done using a primary antibody against LicKM (rabbit polyclonal anti-LicKM (Fraunhofer USA CMB, Newark, DE), Anti-LcrV (Abcam, Cambridge, MA) or OVA (BioLegend, San Diego, CA), followed by the appropriate secondary: LicKM (Anti-Rabbit IgG APC (Molecular Probes, Grand Island, NY), LcrV (Anti-mouse IgG1 APC (Tonbo, San Diego, CA)), or OVA (Anti-mouse IgG2a APC (Tonbo, San Diego, CA)). Samples were read by an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc, Ann Arbor, MI) using C-Flow Plus software (Accuri Cytometers, Inc, Ann Arbor, MI).

## 2.3 Determining LicKM's Effect on Dendritic Cells (DC)

### 2.3.1 Generation and Stimulation of Bone Marrow Derived Dendritic Cells (BMDC)

Femur and tibia were removed from mice and placed in RPMI 1640 (Thermo scientific, Waltham, MA) with 5% GemCell Fetal Bovine Serum (Gemini Bio-Products, West Sacramento, CA), 1% penicillin/streptomycin (MP Biomedicals, LLC, Solon, OH), 2% L-glutamine (Corning, Manassas, VA), 0.1% 2-mercaptoethanol (Invitrogen, Grand Island, NY), and 10mM HEPES (Corning, Manassas, VA) (cRPMI) on ice. Bones were flushed with cRPMI and bone marrow collected. The sample was pelleted and cells lysed using 500 µl RBC lysis buffer (Sigma, St. Louis, MO). Samples were put in a 37°C water bath for four minutes followed by the addition of 15 mL of cRPMI to neutralize the lysis buffer. Samples were spun down and the cells were pelleted followed by re-suspended in 10 mL cRPMI and run through a sterile 70µM filter (Fisherbrand, Waltham, MA).

BMDC were re-suspended and 1.6 X $10^7$ cells/mL were placed into each T-75 (Corning, Corning, NY), with a final concentration of 20 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ) and incubated at 37°C. BMDC were fed on day 3 and 5 with fresh cRPMI and 20 ng/mL GM-CSF (PeptroTech, Rocky Hill, NJ). Immature BMDC were collected on day 6 for experiments.

BMDC were counted and plated at 5 X $10^6$ cells/mLin a 96 well flat bottom plate (Corning, Corning, NY). Each well contained 50 µl cells, 50 µl protein (LicKM, LcrV, or LicKM & LcrV) at final concentrations of 10 µg/mL or 100 µg/mL, and 100 µl cRPMI media (Thermo scientific, Waltham, MA). Plates were incubated at 37°C + 5%$CO_2$ for 3, 6, 12, or 24 hours before analysis for cell surface activation markers.

### 2.3.2 BMDC Staining for Flow Cytometry

Stimulated BMDC were collected for flow cytometry analysis. Non-specific binding was blocked as previously described. Antibodies staining for surface markers

of interest were used at a 1:100 dilution of 0.5 mg/mL for FITC, or 1:200 dilution of 0.2 mg/mL for PE, PE-Cy7, and APC in FACS buffer for 10 minutes at 4°C. The activation markers used for BMDC were: CD11c, CD11b, CD40, CD80, CD86, MHC I and MHC II (Sun, 2002; Sokolovska, 2007). All markers were purchased from Ebioscience (San Diego, CA) or Tonbo (San Diego, CA). In addition, 2 µl of 1.0 mg/mL propidium iodine (PI) (Invitrogen, Carlsbad, CA) solution was added right before analysis to differentiate live versus dead cells. Samples were read by an Accuri C6 Flow Cytometer (Accuri Cytometers, Inc, Ann Arbor, MI) using C-Flow Plus software (Accuri Cytometers, Inc, Ann Arbor, MI).

### 2.3.3 Assessment of Potential LicKM Inhibitory Effects on BMDC

Two methods of activation were used for testing the possibility that LicKM could inhibit cell activation. The first method used BMDC plated at $2X10^7$ cells/mL or $5X10^6$ cells/mL in a flat bottom plate (Corning, Corning, NY) with 0.2, 0.1 or 0.05 µg/mL LPS (Innaxon, San Diego, CA) (Gagliardi, 2000; Gagliardi, 2002; Agrawal, 2003) for 16 hours. The cells were then restimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV.

The second method used BMDC plated at 5 X $10^6$ cells/mL in a 96 well flat bottom plate (Corning, Corning, NY). Each well contained cells and 0.1 µg/ml LPS (Innaxon, San Diego, CA), 100 µg/mL Monophosphoryl lipid A (InvivoGen,San Diego, CA) (MPLA) (Ismaili, 2002) or 1 µg/mL cholera toxin (Sigma, St. Louis, MO) (CT) (Bagley, 2005; Arce, 2006; Lapteva, 2007). BMDC were also stimulated with 10 µg/mL of LicKM, LcrV, or LicKM-LcrV.

Plates were incubated at 37°C for 3, 6, or 12 hours. BMDC were analyzed by flow cytometry for surface activation markers as described above. All markers were purchased from Ebioscience (San Diego, CA) or Tonbo (San Diego, CA). Markers used were: CD11c, CD40, CD80, CD86, CD184, CD197, and MHC II. Live/dead staining with propidium iodine (PI) (Invitrogen, Carlsbad, CA) was also performed.

## 2.4 Characterization of Vaccine Generated Response

### 2.4.1 Priming and Boosting BALB/c Mice Using LicKM and LicKM-LcrV Adsorbed onto Alhydrogel

Vaccines contained *Y.*pestis protein LcrV were engineered as previously described (Musiychuk, 2007). LcrV was fused onto the carrier molecule LicKM (Fraunhofer USA CMB, Newark, DE), a thermostable enzyme from *Clostridium* thermocellum and expressed in *Nicotiana benthamiana* (Musiychuk, 2007). Alhydrogel was used at 0.3% (Brenntag-Biosector). BALB/c mice were primed intramuscularly on Day 0 and boosted on Day 21. Vaccine groups were: Alhydrogel alone, PBS + Alhydrogel, LicKM at a 0.4 μg dose + Alhydrogel, LcrV at a 0.6 μg dose+ Alhydrogel, LcrV at a 0.6 μg dose + LicKM at a 0.4 μg dose + Alhydrogel, and LcrV-LicKM fusion at a 1 μg dose + Alhydrogel. LicKM + LcrV indicates the two proteins were mixed in the same vaccine. LicKM-LcrV indicates the two proteins were fused. LcrV was produced in *E. coli* (BEI Resources) instead of the plant-based system. Serum was collected for IgG titer analysis by enzyme-linked immunosorbent assay (ELISA) before priming at day 0, before boosting at day 21, and on day 42.

### 2.4.2 Priming and Boosting BALB/c Mice Using Pfs25 Adsorbed onto Alhydrogel

Vaccines contained *Plasmodium falciprium* protein Pfs25 were engineered as previously described (Musiychuk, 2007). Pfs25 was fused onto the carrier molecule LicKM (Fraunhofer USA CMB, Newark, DE), a thermostable enzyme from *Clostridium thermocellum* and expressed in *Nicotiana benthamiana* (Musiychuk, 2007). Alhydrogel was used at 0.3% (Brenntag-Biosector). Mice were primed intramuscularly on Day 0 and boosted on Day 21. Vaccine groups were: saline alone, Saline + Alhydrogel, LicKM at a 2.5 μg dose + Alhydrogel, Pfs25 at a 2.5 μg dose + Alhydrogel, Pfs25 at a 2.5 μg dose + LicKM at a 2.5 μg dose + Alhydrogel, and Pfs25-LicKM fusion at a 5 μg dose + Alhydrogel. LicKM + Pfs25 indicates the two

proteins were mixed in the same vaccine. LicKM-Pfs25 indicates the two proteins were fused.Serum was collected for IgG titer analysis by ELISA before priming at day 0, before boosting at day 21, and at day 42.

### 2.4.3 Serum IgG ELISA and Antibody Isotyping

Plates were coated with the appropriate recombinant protein: LcrV (*E.Coli* produced from BEI Resources), Pfs25 (plant produced by Fraunhofer USA CMB, Newark DE) or LicKM (plant produced by Fraunhofer USA CMB, Newark, DE) at 1 μg/mL in PBS. Antigen-specific IgG was detected using Goat anti-mouse IgG (Jackson Immunochemicals) or anti-mouse IgG isotype secondary antibodies (Southern Biotechnology Associates Inc., Birmingham, AL). Titers were determined as described in Mett et al (2007).

**Table 1- Surface Markers and the role in the Immune Response.** Surface markers assessed during this study. DC are dendritic cells, NK are natural killer cells, macs are macrophages. Adapted from Abbas, 2012.

| Surface Marker | Expressed By | Role in Immune Response |
|---|---|---|
| CD3 | T-cells | Part of the T-cell signaling complex |
| CD4 | Helper T-cells | Co-stimulator for helper T-cell activation |
| CD8 | Cytotoxic T-cells | Co-stimulator for cytotoxic T-cell activation |
| CD11b | Monocytes, DC, NK | Cell adhesion and apoptosis |
| CD11c | DC, NK | Cell adhesion |
| CD19 | B-cells | B-cell co-receptor signaling |
| CD40 | B-cells, DC, Macs | Co-stimulate B-cell for differentiation and isotype switching |
| CD80 | B-cells, T-cells, Macs, DC | Co-stimulate T-cell activation and proliferation |
| CD86 | Macs, DC, B-cells, T-cells | Co-stimulate T-cell activation and proliferation |
| CD184 | T-cells, B-cells, DC, Monocytes | Cell migration |
| CD197 | T-cells, B-cells, DC | T-cell adhesion and migration |
| B220 (CD45R) | B-cells | Regulates T-cell and B-cell antigen receptor signaling |
| F4/80 | Macs | Transmembrane protein for mac population |
| GR-1 (Ly-6G) | Granulocytes | Transmembrane protein for granulocyte population |
| MHC I | Macs, DC, B-cells | Antigen presentation to CD8 T-cells |
| MHC II | Macs, DC, B-cells | Antigen presentation to CD4 T-cells |
| NK1.1 (CD49) | NK | Adhesion and signaling |

# Chapter 3
# RESULTS

## 3.1 LicKM Trends Towards Up-Regulation of Activation Markers and Causes No Inhibition in the Presence of Various Stimuli

### 3.1.1 LicKM Stimulation of Dendritic Cells (BMDC) Results in Activation Marker Changes

Naïve dendritic cells from bone marrow of female BALB/c mice were stimulated with 100 μg/mL or 10 μg/mL OVA, LicKM, LcrV, or LicKM-LcrV for 6, 12, or 24 hours. Cells were then analyzed for changes in common BMDC activation markers using flow cytometry. To ensure only live BMDC cells were analyzed the following gating strategy was used for all experiments involving BMDC. Cells were first gated on size to ensure only whole cells were selected (Figure 1A). Next, only those live cells from that population which were propidium iodine (PI) negative were selected (Figure 1B). Next, from that population of cells only those which were CD11c positive were selected (Figure 1C). Lastly, the various cell surface markers were assessed (Figure 1D). In summary only the BMDC, which were the appropriate size, PI negative, and CD11c positive, were analyzed (Figure 1).

Incubation of BMDC for 24 hours with 100 μg/mL stimulation of vaccine antigen showed minimal changes in expression level of cell activation markers, suggesting the initial activation and up-regulation may possibly happen much earlier than 24 hours (Figure 2A). Several common activation markers showed the same result, CD86, CD80, MHC I, and CD40.

Next, BMDC were then incubated for 12 hours with 100 μg/mL stimulation of vaccine antigen. After stimulation, the BMDC showed minimal marker up-regulation for MHC II (Figure 2B), while CD86 and CD40 (Figure 2B) showed no difference in activation level between naïve and antigen stimulated cells.

Due to previous vaccine research conducted by Guth (2012) in which a smaller dose of LicKM-LcrV was still able to provide protection against a lethal dose of *Y. pestis*, BMDC were then stimulated with a lower dose of 10 μg/mL of vaccine antigen for 12 hours (Figure 2C). Stimulation with 10 μg/mL vaccine antigen provided the greatest differences in cell surface marker expression when stimulated and naïve cells were compared. The levels of CD86, MHC II, and CD40 (Figure 2C) demonstrated two distinct cell populations after 12 hours of incubation. Additionally, the shift in MHC II expression appears to be due to stimulation with LicKM carrier molecule rather than stimulation with the plague LcrV protein. Comparing the two plots of LicKM vs LicKM-LcrV to LcrV vs LicKM-LcrV, the peak shape of LicKM is more closely related to the shape generated by LicKM-LcrV stimulation than the peak from LcrV stimulation alone (Figure 2C), suggesting that LicKM by itself has the ability to activate BMDC.

In order to determine if the up-regulation happens earlier than 12 hours after antigen stimulation, BMDC were incubated for 6 hours with the different protein targets. However, little difference was detected in the expression level of the activation markers CD86, CD40, and MHC II after 6 hours (Figure 2D). This indicates that activation of BMDC by LicKM takes 12 hours of incubation before differences in activation levels can be determined using the methodology employed here.

### 3.1.2 Activation of BMDC With LicKM Does Not Result in Suppression of Cell Activation

Two different methods were used to investigate the ability of LicKM to inhibit immune activation. First, naïve BMDC generated from bone marrow of female BALB/c mice were stimulated with lipopolysaccharide (LPS) for 16 hours and then re-stimulated for 6 or 12 hours with LicKM, LcrV, or LicKM-LcrV and analyzed by flow cytometry. The second method was stimulating the BMDC the non-specific activators lipopolysaccharide (LPS), cholera toxin (CT), or monophosporyl lipid A (MPLA), concurrently with LicKM, LcrV, or LicKM-LcrV. BMDC were analyzed

using flow cytometry after 3, 6, and 12 hours of incubation for BMDC activation markers. All gating was performed as mentioned earlier according to Figure 1.

### 3.1.3 LicKM Does Not Inhibit the Immune Response When BMDC are Activated With Lipopolysaccharide (LPS)

BMDC were stimulated with 0.2, 0.1, or 0.05 μg/mL LPS for 16 hours then re-stimulated with vaccine proteins and controls for an additional 6 hours (Figure 3A). Surface marker expression of CD80, MHC II, CD86, CD197, CD40, and CD184 were assessed by FACS. No difference in activation levels were detected for all markers tested when naïve and LPS stimulated cells were compared (Figure 3A). The same experiment was conducted with 0.1 μg/mL and 0.05 μg/mL LPS stimulation showing similar results (Figure 3B and 3C). In order to assess cell viability after 0.2 μg/mL LPS stimulation, the use of Live/Dead staining with propidium iodine (PI) was employed. BMDC after 16 hours of stimulation with LPS showed ~75% cell death (Figure 4A). BMDC were then left un-stimulated, or stimulated with LicKM, LcrV, or LicKM-LcrV for an additional 6 hours to determine if the vaccine protein was up-regulating BMDC activation markers. Following the additional incubation, only ~10% of the naïve BMDC were alive, with ~8% viability in cells stimulated with LPS alone, and ~6% viability in cells stimulated with LPS followed by LicKM, LcrV or LicKM-LcrV (Figure 4A). Due to the fact that too few cells were alive at the end of the experiment prevented the formation of accurate conclusions.

Additionally, 0.1 μg/mL and 0.05 μg/mL concentrations of LPS were tested to determine if the LPS concentration was the cause of the cell death. However, both concentrations of LPS showed similar results of cell death after 16 hours stimulation then after an additional 6 hours of stimulation with vaccine protein, total cell viability was ~2-3% (Figure 4B). Incubation of the BMDC for 22 hours, killed the BMDC, therefore cell viability was assessed after 16 hours incubation (Figure 4C). In this experiment, prior to LPS stimulation ~84% of the BMDC were alive, however after 16 hours of incubation with 0.2 μg/mL or 0.1 μg/mL the cell viability was less than 1%

cell viability (Figure 4C). Due to the effects on cell viability seen after 16 hours incubation, and similar results were seen in a similar experiment run with BMDC incubated for an additional 12 hours (Figure 4D). However, viability of the cells in the culture was still low. Taken together, these results confirm that this activation procedure was unable to determine if LicKM caused any inhibition of the LPS stimulation due to the majority of BMDC being dead before stimulation with vaccine protein.

In order to determine if the plating concentration of the BMDC was too high for a 96 well plate re-stimulation contributing to the low viability results, cell concentrations were decreased to 5 X $10^6$ cells/mL (Figure 4E). After incubation for 16 hours the lower cell concentration of 5 X $10^6$ cells/mL demonstrated ~40% cell viability, showing little loss of viability after stimulation, whereas the higher cell concentration of 2 X $10^7$ cells/mL had ~22% viability after 16 hours incubation (Figure 4E). As a result, future experiments will use 5 X $10^6$ cells/mL as a plating concentration for the culture and activation of BMDC. In addition, a new procedure was used to evaluate LicKM inhibition of BMDC stimulation.

In this new procedure, BMDC were stimulated with both LPS and vaccine protein at the same time. Then surface marker expression levels were determined after 3, 6, and 12 hours by FACS. After 3 hours of stimulation CD80, CD197, CD40, and CD184 (Figure 5A) showed no difference in expression when comparing naïve to stimulated cell populations. However, CD86 levels demonstrated an additional peak upon stimulation when comparing naïve to LPS stimulated cells. This added peak is seen in all stimulation conditions. In contrast, MHC II has lower levels of expression after 3 hours of stimulation when compared to naïve cells.

After 6 hours of cell stimulation with vaccine protein and LPS, CD80, CD197, and CD184 still showed no difference in expression levels (Figure 5B). However, MHC II had a noticeable additional population in the LPS stimulated groups, which remains present when stimulated with the vaccine proteins. In addition, CD86 levels show an additional population present that can be seen across all stimulation

conditions (Figure 5B). Finally, CD40 showed up regulation upon stimulation with LPS, which remained present when vaccine proteins were added.

After 12 hours of incubation, all markers except CD197 and CD184 showed up regulation in the presence of LPS stimulation compared to naïve cells (Figure 5C). Additionally, when stimulated with LPS alone or LPS plus vaccine protein all samples showed no reduction in the expression levels of these markers. Finally, stimulation with LPS and vaccine protein induced a higher level of expression compared to stimulation with vaccine protein alone (Figure 5C). BMDC stimulated with LicKM in the presence of LPS does not cause suppression of activation.

### 3.1.4 LicKM Does Not Inhibit the Immune Response When BMDC are Activated With Cholera Toxin (CT)

BMDC were incubated with CT and vaccine protein then analyzed after 3, 6, and 12 hours of stimulation. After 3 and 6 hours there is little increase in expression increase between naïve and CT stimulated cells (Figure 6A and 6B). Again, all surface markers showed the same activation levels when they were stimulated with CT alone or with CT plus vaccine protein is present (Figure 6A, B). Finally, after 12 hours of incubation there remained little difference in expression levels between naïve and CT stimulated cells. However, all markers showed activation levels equivalent to cells stimulated with CT stimulated alone (Figure 6C). BMDC stimulated with LicKM in the presence of CT does not cause suppression of activation.

### 3.1.5 LicKM Does Not Inhibit the Immune Response When BMDC are Activated With Monophosporyl Lipid A (MPLA)

BMDC were incubated with MPLA and vaccine proteins for 3, 6, and 12 hours and then analyzed for changes in cell surface marker expression levels (Figure 7). After 3 hours of incubation all markers showed identical levels of expression except MHC II (Figure 7A). When cells were stimulated MPLA and vaccine proteins a new population of cells with an increased expression of MHC II became evident (Figure

7A), indicating a potential role for the vaccine proteins in MHC II. After 6 hours of incubation: CD80, CD197, CD40 and CD184 showed identical levels of expression in all test samples (Figure 7B). CD86 had slightly increased expression over naïve cells, which was seen across the conditions. Again, all conditions showed equal levels of expression when stimulated with MPLA alone or with vaccine products. By 12 hours of incubation, CD80, CD197, CD40 and CD184 were expressed at the same level as those detected after 6 hours of incubation (Figure 7C). MHC II showed multiple populations with different expression levels, indicating a less uniform population. However, no inhibition was demonstrated of surface expression markers by LicKM stimulation with MPLA. Finally, CD86 showed an additional population of cells when vaccine protein alone was present with MPLA (Figure 7C) indicating an additive advantage of vaccine protein for CD86 expression. BMDC stimulated with LicKM in the presence of MPLA does not cause suppression of activation.

### 3.2 LicKM Interacts With Some Host Immune Cells

Naïve splenocytes from female BALB/c mice were stimulated with LicKM. Cells were stained for LicKM and host immune cell surface markers then analyzed using flow cytometry. Prior to conducting the full experiment, a titration experiment for rabbit polyclonal LicKM antiserum, as previously described by Musychuk et. al (2007) was conducted (Figure 8A). The optimal dilution for the antibody was found to be 1:250. Cells were incubated for 1 hour with 10 μg/mL LicKM. After 1 hour of incubation double positive staining for LicKM and $CD3^+$ and $CD4^+$ staining of ~1% (Figure 8B). The ability of LcrV to bind to splenocytes was assessed and results showed that there was no direct interaction between LcrV and splenocytes (data not shown). OVA controls were also negative, indicating the lack of non-specific protein binding to the cells (data not shown). The interaction of LicKM-LcrV was conducted, however antibodies did not bind, failing to detect any cell populations (data not shown). After 3 hours of incubation, double positive staining was detected with CD3, CD19, B220, CD80, CD8, and CD4 cell surface markers and intracellular staining

with LicKM (Figure 8C, Table 1). Given the data, LicKM appears to be interacting with T-cells, B-cells, macrophages, and DCs of the immune system at low levels of detection.

### 3.3 Vaccination

Two vaccine studies were conducted following the same prime/boost strategy. BALB/c mice were immunized intramuscularly on day 0 and boosted on day 21. Serum was collected before day 0, before boost, and at the end of the study day 42. Each study consisted of six groups: saline or PBS alone, saline or PBS plus known adjuvant, LicKM plus adjuvant, LcrV or Pfs25 plus adjuvant, LicKM + LcrV or LicKM + Pfs25 plus adjuvant, and LicKM-LcrV or LicKM-Pfs25 plus adjuvant.

#### 3.3.1 Antibody Titer and Isotype Generated By Vaccination

##### 3.3.1.1 IgG1 is the Dominant Isotype Generated by BALB/c Mice Vaccinated With LcrV, LicKM, and Alum

BALB/c mice were vaccinated with PBS, PBS + alum, LcrV + alum, LicKM + alum, LicKM-LcrV + alum, or LicKM + LcrV + alum. Serum collected on day 42 of the study was used to determine the total IgG antibody titer specific to LicKM and LcrV. Groups of mice that received LcrV or LicKM individually generated high levels of antigen-specific IgG (Figure 9A). The dominant IgG isotype for LcrV + alum vaccination was IgG1, vaccination with LicKM + LcrV + alum generated a dominant IgG1 isotype against both LicKM and LcrV (Figure 9B). Vaccination with LicKM-LcrV + alum generated a dominant IgG1 isotype against LicKM and LcrV; vaccination with LicKM + alum generated a IgG1 dominant titer against LicKM (Figure 9B). Finally, vaccination with PBS alone or PBS + alum generated no detectable antibody titers against LicKM or LcrV (Figure 9B). Antibody titers generated against vaccination have an equal response across vaccinated conditions

(Figure 9B). Vaccination of BALB/c mice with LicKM, LcrV, and alum generates an increased humoral response dominated by IgG1 isotype.

#### 3.3.1.2 IgG1 is the Dominant Isotype Generated by BALB/c Mice Vaccinated With Pfs25, LicKM, and Alum

BALB/c mice were vaccinated with saline, saline + alum, Pfs25 + alum, LicKM + alum, LicKM-Pfs25 + alum, or LicKM + Pfs25 + alum. Serum collected on day 42 of the study was used to determine total IgG antibody titer to LicKM and Pfs25. All groups who received Pfs25 or LicKM responded appropriately, with increased antibody titers (Figure 10A). Vaccination with Pfs25 + alum generated a dominant IgG1 response against Pfs25, vaccination with LicKM + Pfs25 + alum generated a dominant IgG1 response against LicKM and Pfs25; additionally an IgG2a response against LicKM was also generated (Figure 10B). Vaccination with LicKM-Pfs25 + alum generated a dominant IgG1 response against LicKM and Pfs25 additionally vaccination generated an IgG2a response against LicKM and Pfs25 (Figure 10B). Vaccination with LicKM + alum generated a IgG1 dominant response but also a IgG2a response against LicKM (Figure 10B). Additionally, vaccination with LicKM + Pfs25 or LicKM-Pfs25 generated different antibody titer levels. Vaccination with LicKM + Pfs25 generates similar Ig titer responses as when proteins are given alone in vaccination, however, vaccination with LicKM-Pfs25 generates a stronger response than when proteins are given alone (Figure 10B). Vaccination of BALB/c mice with LicKM, Pfs25, and alum generates an increased humoral response dominated by IgG1.

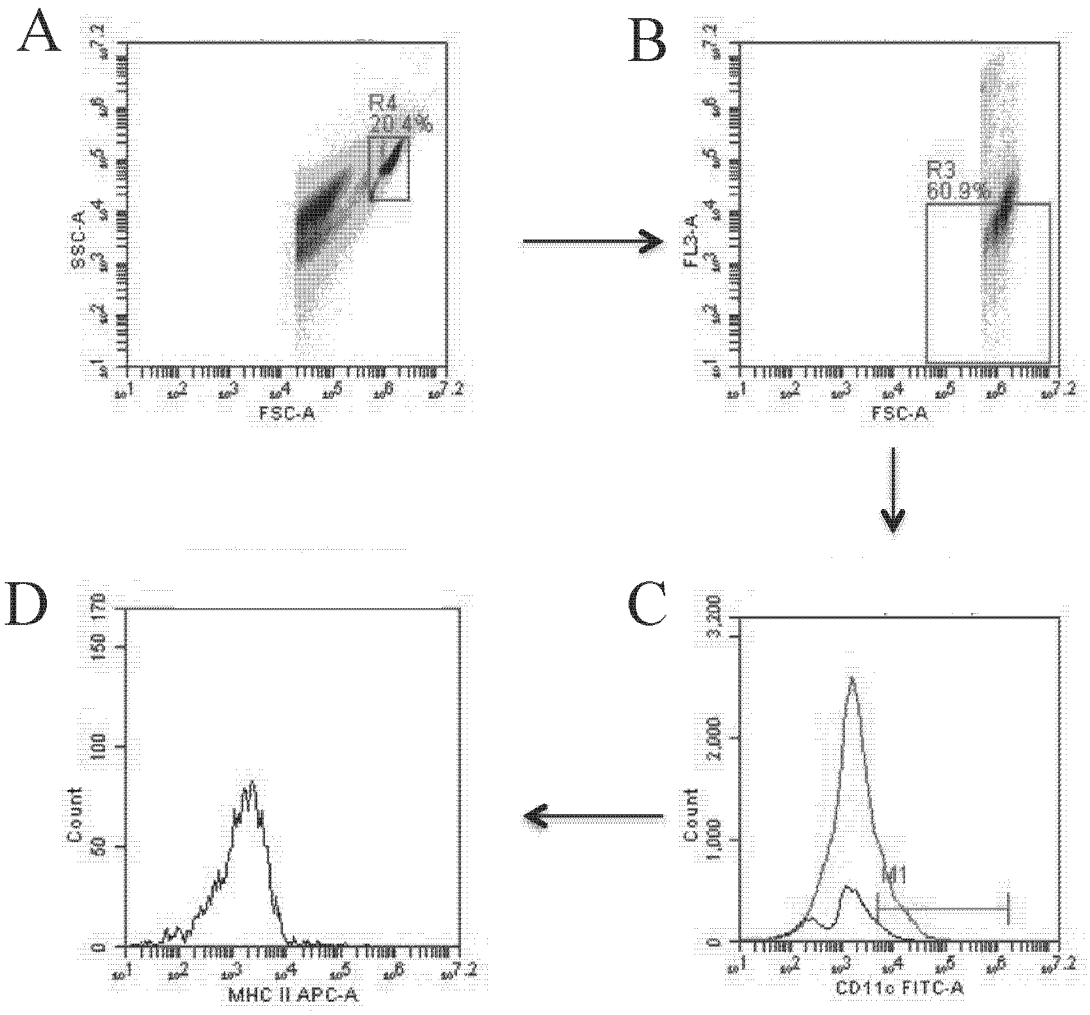


**Figure 1: Flow cytometric analysis.** BMDCs from naïve BALB/C mice were first visualized using forward and side scatter (A). Cells were then gated based on normal WBC size (A). Further gating using propidium iodide (P.I.) was performed to select viable cells (B). $CD11c^{+}$ cells were then selected to isolate the DC cell population (D). MHC II expression on live $CD11c^{+}$ BMDC.

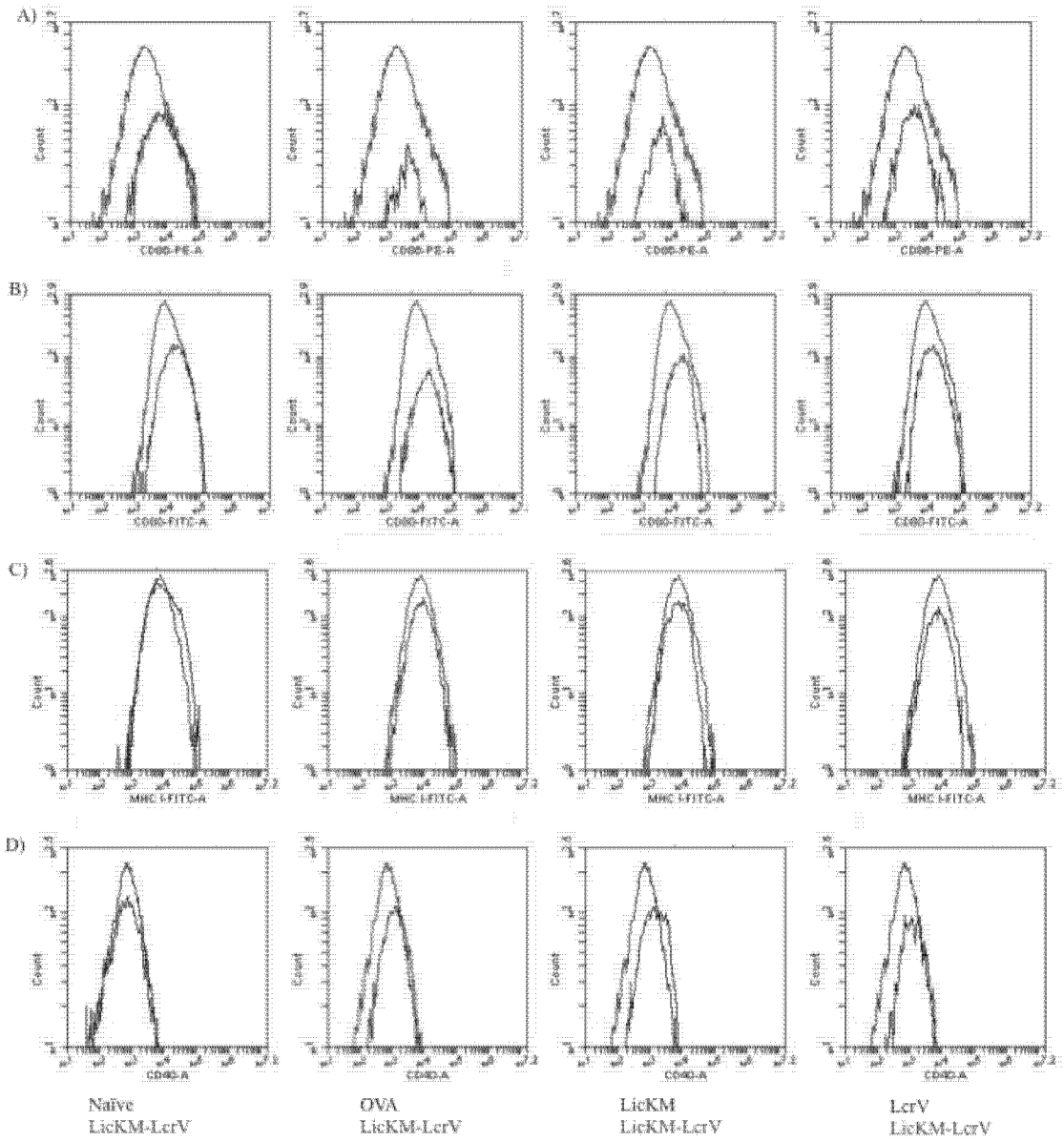


**Figure 2A:LicKM activation of BMDC happens before 24 hours of incubation.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 100 μg/mL OVA, LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 24 hours stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD86 B)CD80 C)MHC I D)CD40. *N*=1

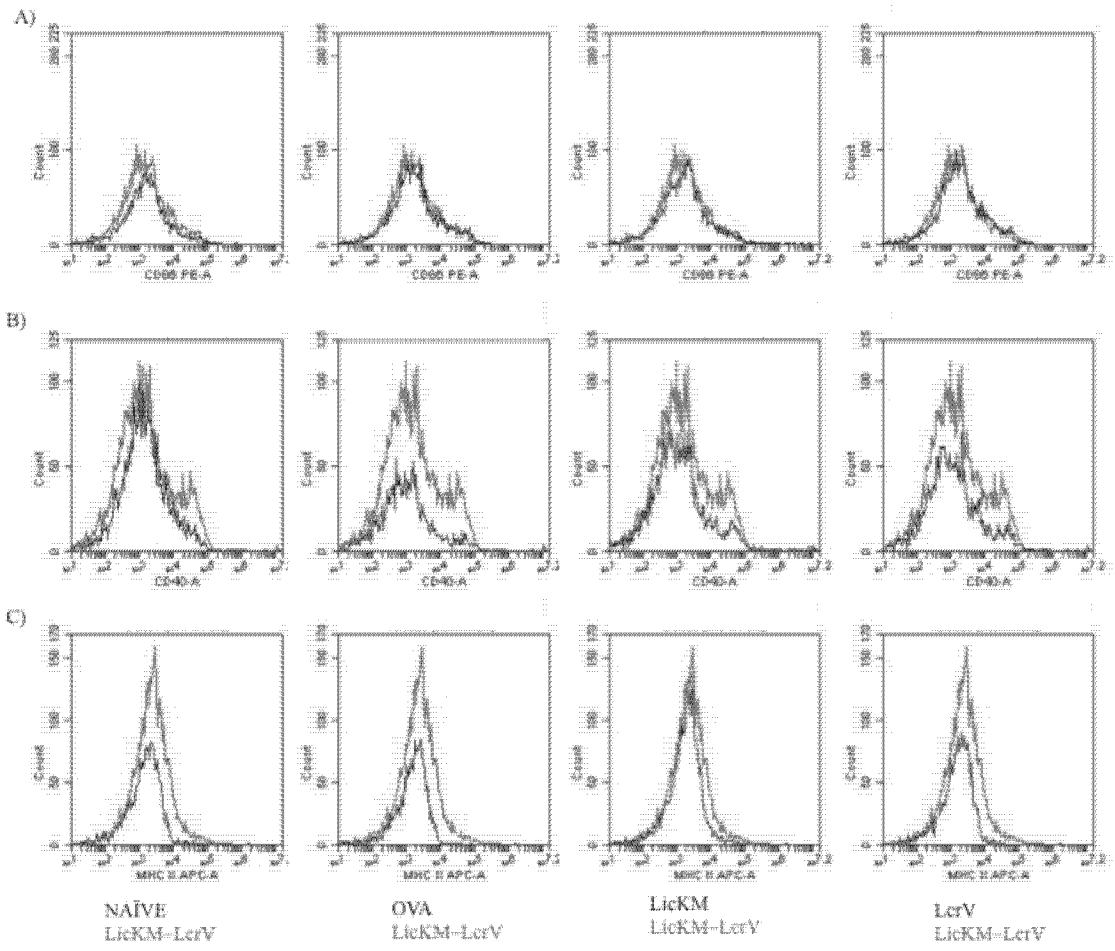


**Figure 2B: BMDC stimulated with 100 µg LicKM show changes in activation levels after 12 hours incubation.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 100 µg/mL OVA, LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 12 hours stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD86 B)CD40 C)MHC II. *N*=1

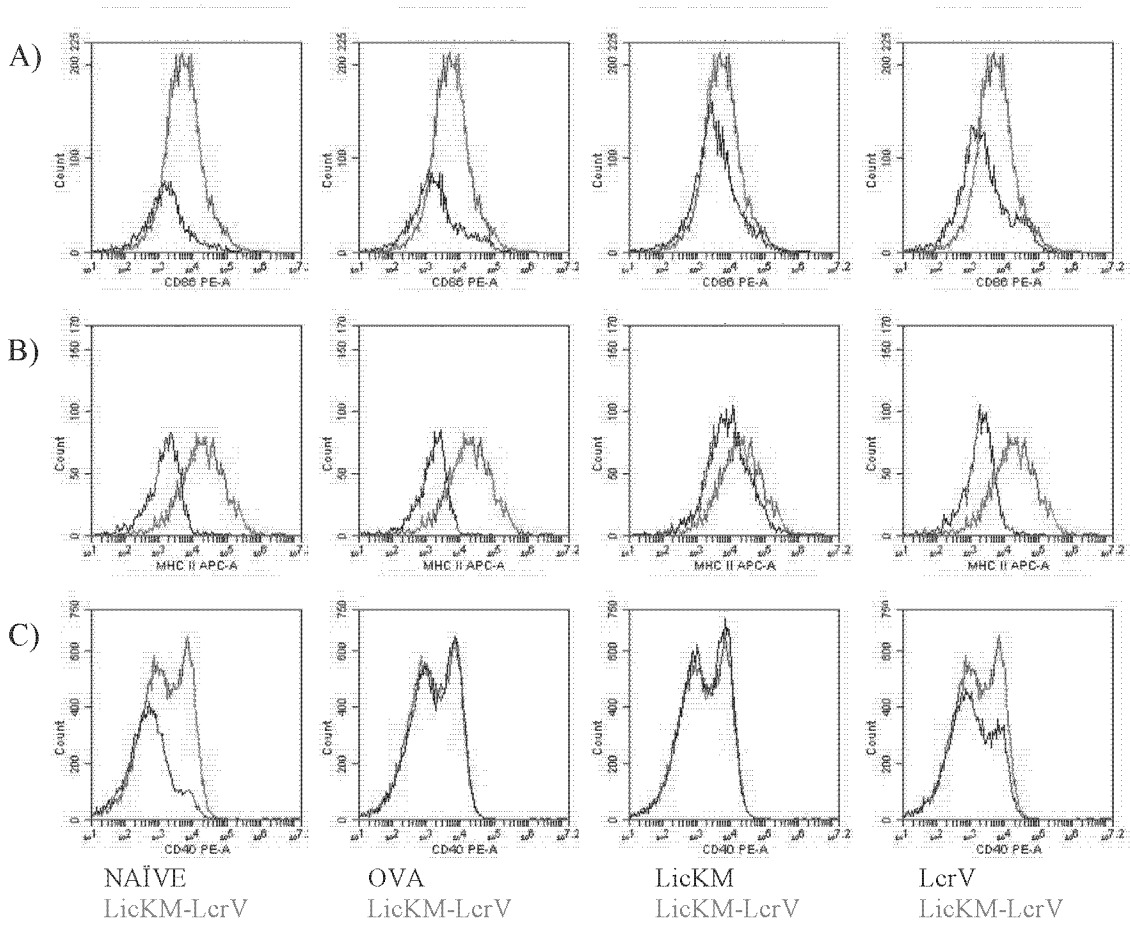


**Figure 2C: LicKM trends towards up regulation of BMDC activation markers after 12 hours stimulation.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 10 μg/mL OVA, LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 12 hours of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD86 B)MHC II C)CD40. *N*=4

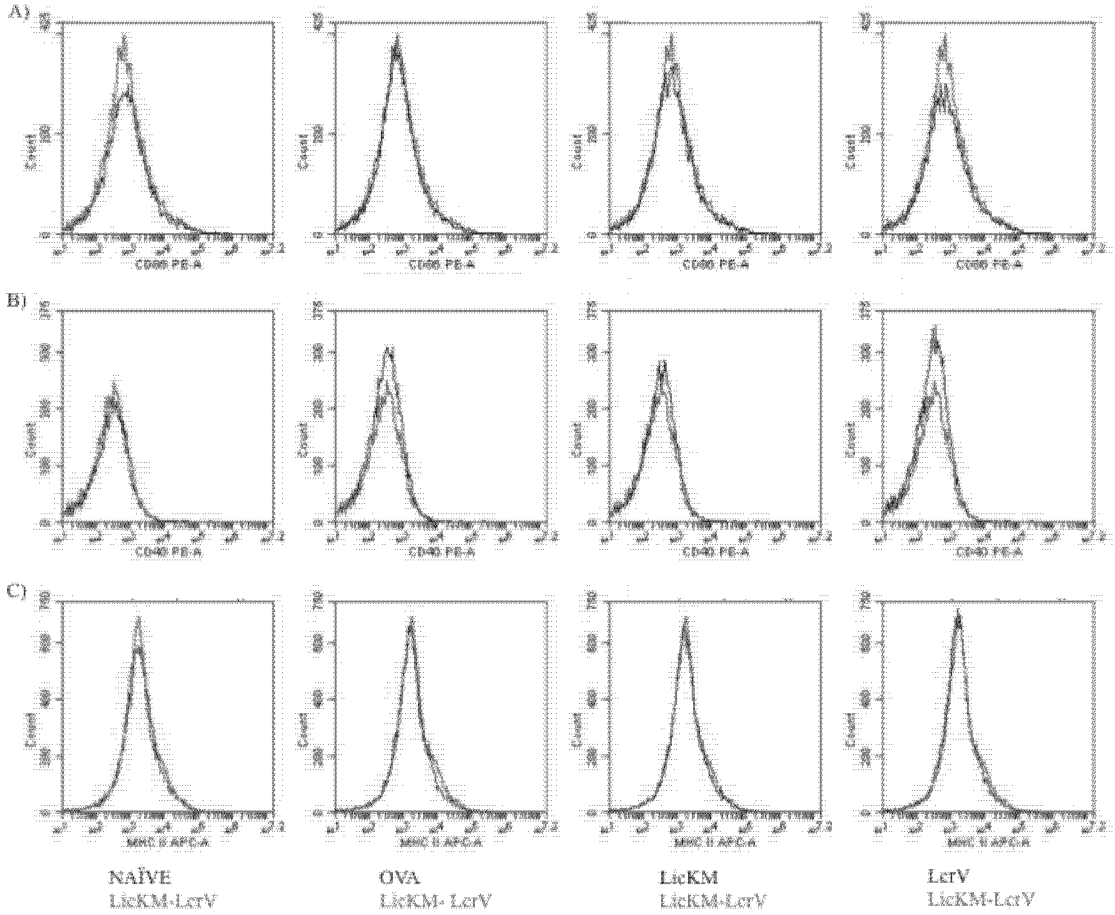


**Figure 2D: LicKM trends towards up regulation of dendritic cell activation markers after 6 hours of stimulation.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 10 µg/mL OVA, LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hours stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD86 B)CD40 C)MHC II. *N*=4

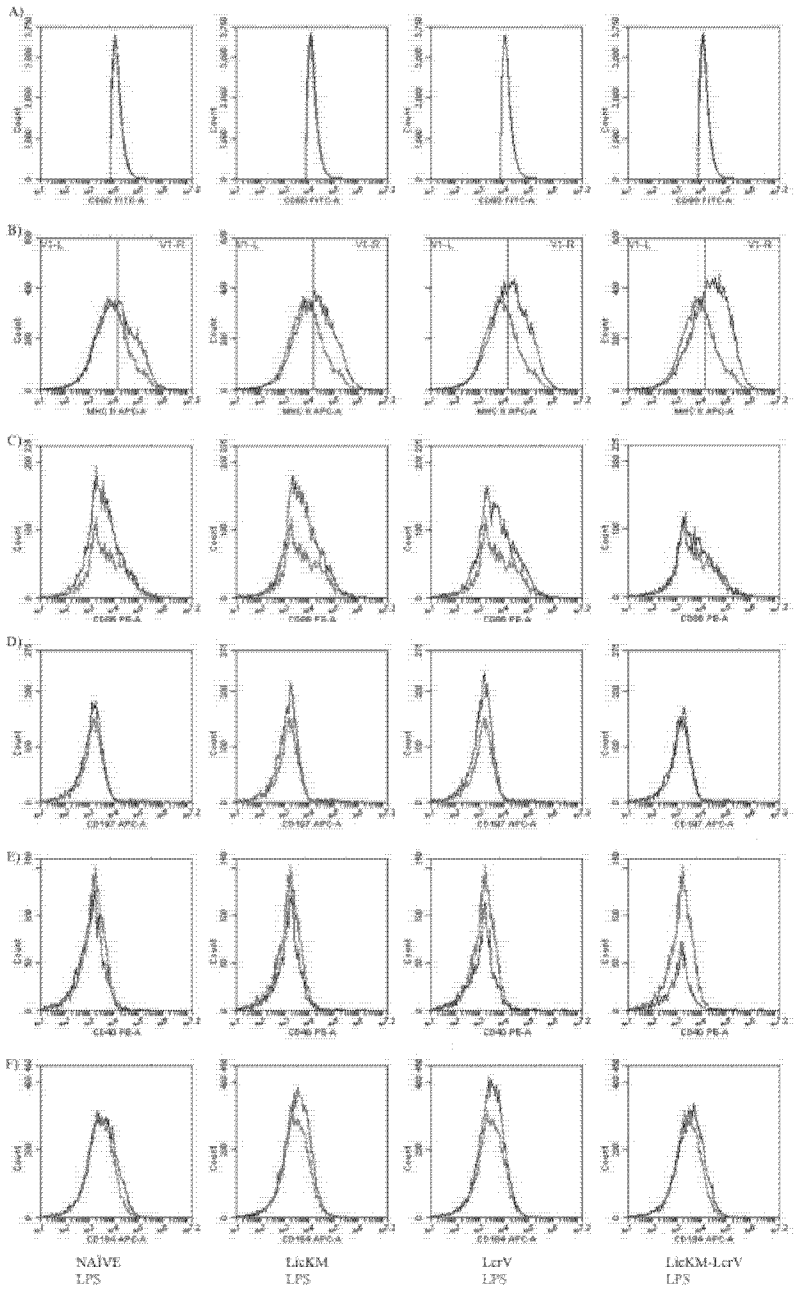


**Figure 3A: LicKM does not inhibit the generation of immune responses when in the presence of 0.2 µg/mL LPS.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.2 µg/mL LPS for 16 hours after which time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=4

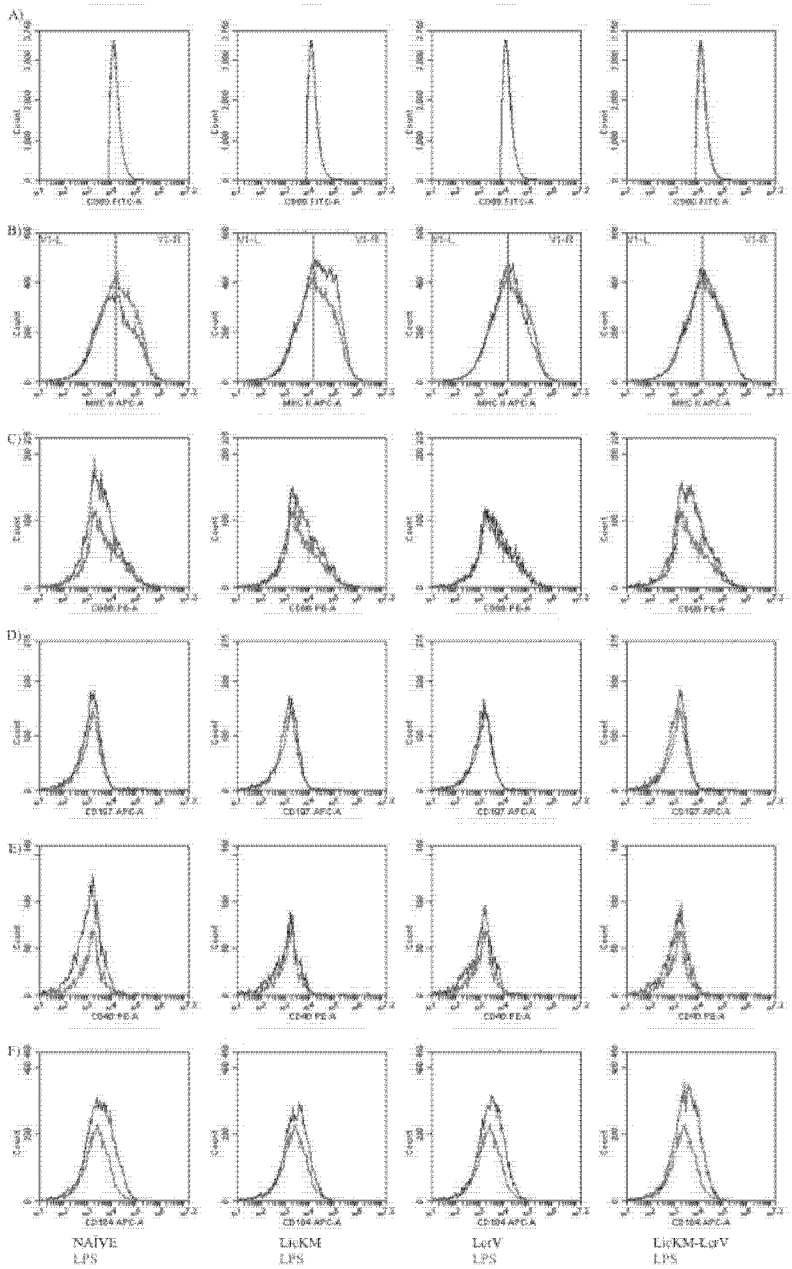


**Figure 3B: LicKM does not inhibit the generation of immune responses when in the presence of 0.1 µg/mL LPS.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 µg/mL LPS for 16 hours after which time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=4

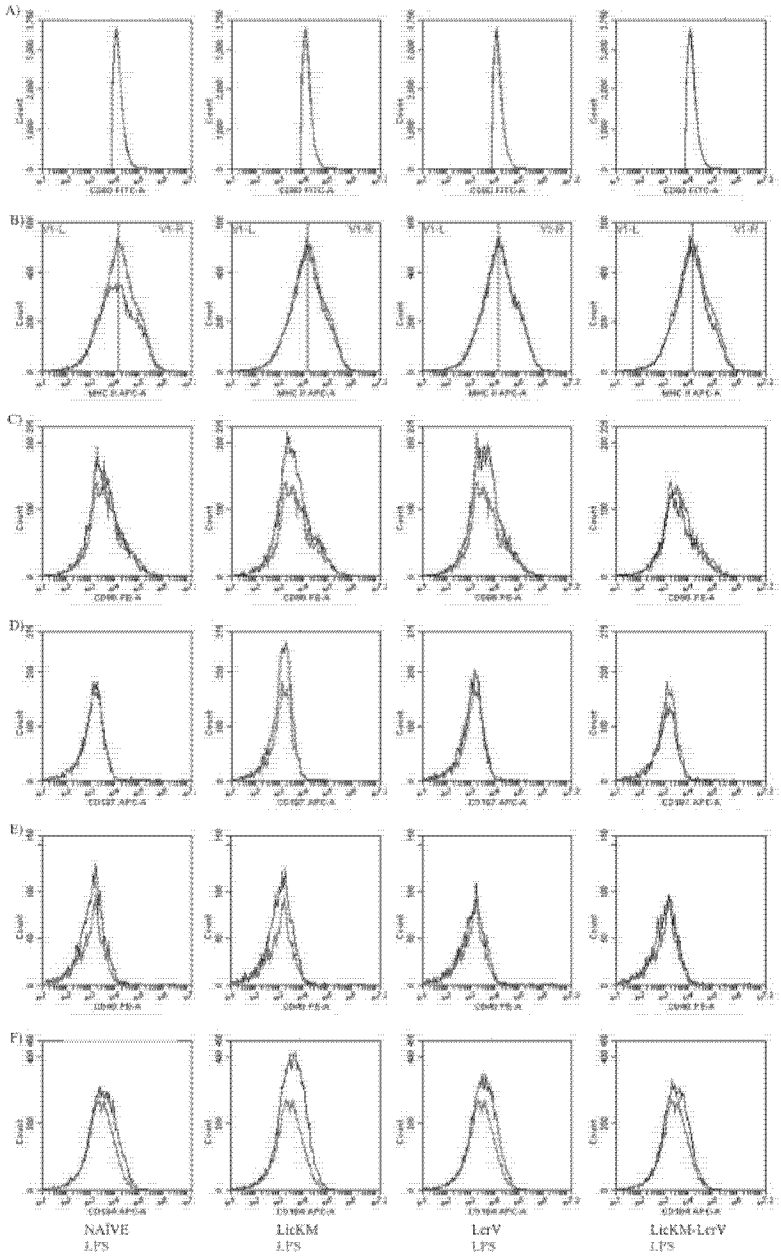


**Figure 3C: LicKM does not inhibit the generation of immune responses when in the presence of 0.05 µg/mL LPS.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.05 µg/mL LPS for 16 hours after which time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=4

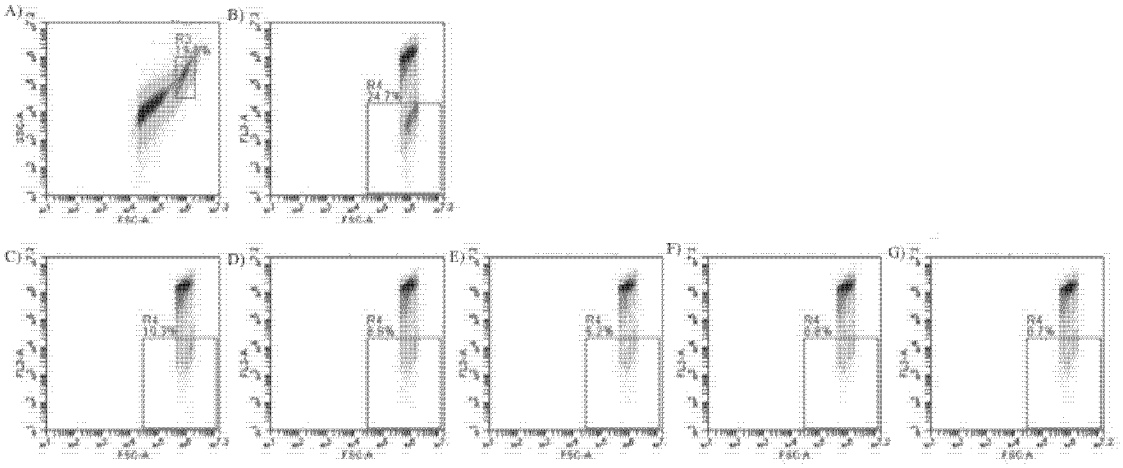

**Figure 4A: LPS stimulation for 16 hours kills BMDC as determined by propidium iodine staining.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 μg/mL LPS for 16 hours A) Lymphocyte population gate B) BMDC stimulated with LPS after 16 hours. After 16 hours time cells were stimulated with medium alone (D) or 10μg/mL LicKM (E), LcrV (F), LicKM-LcrV (G). Naïve cells after 22 hours incubation (C). Cells were stained for surface marker activation after 6 hrs stimulation with vaccine protein. Live/Dead staining using propidium iodine was conducted. Cells were analyzed using flow cytometry and Accuri 6 software. *N*=2

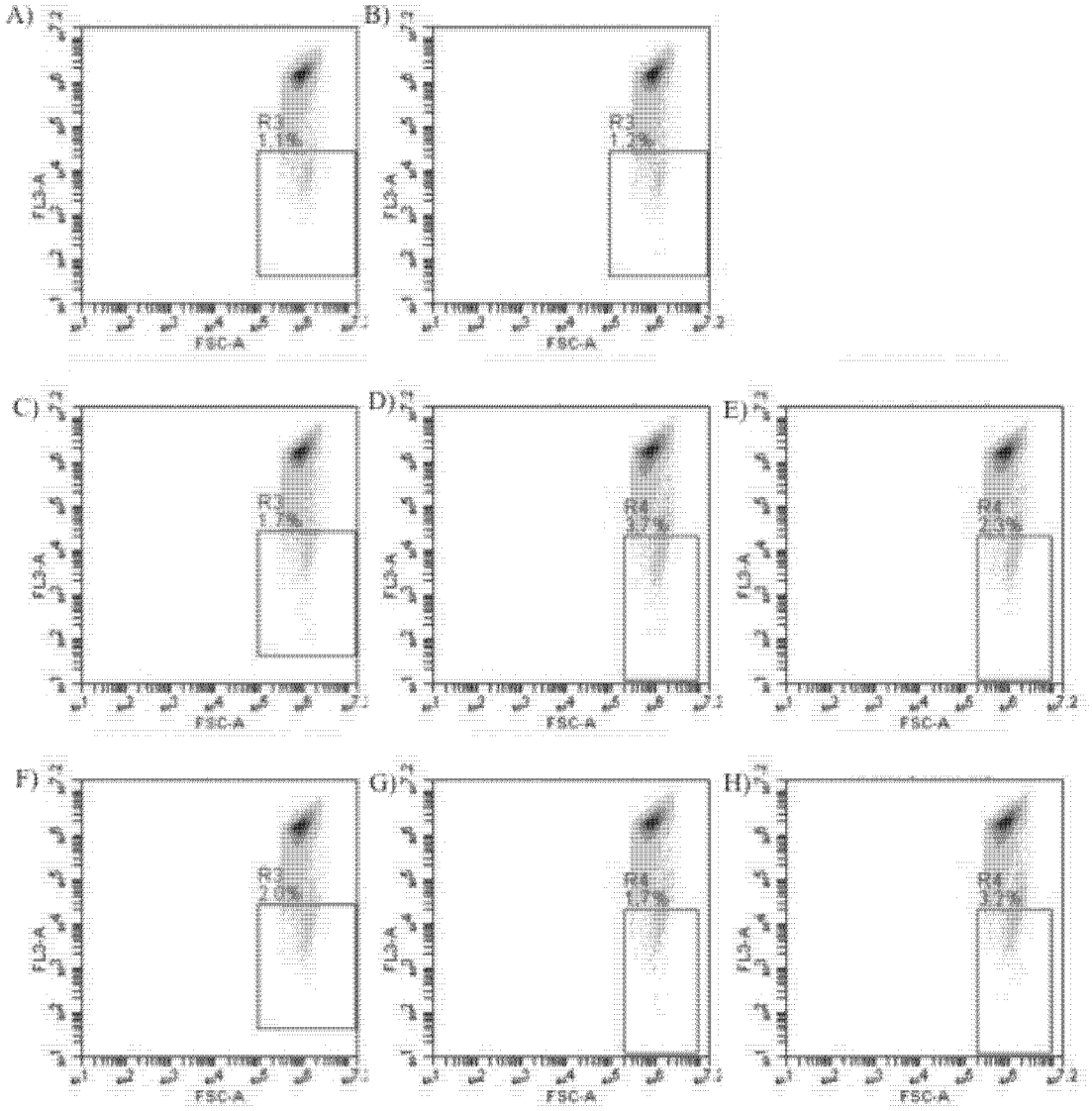


**Figure 4B: Incubation of BMDC for 22 hours kills the cells.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with medium alone (A), 0.2 (B), 0.1 (C), or 0.05 (F) μg/mL LPS for 16 hours. After which time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV for 6 hours. A) Naïve D) 0.1 μg/mL LPS plus LicKM E) 0.1 μg/mL LPS plus LcrV G) 0.05 μg/mL LPS plus LicKM H) 0.05 μg/mL LPS plus LcrV. Live/Dead staining using propidium iodine was conducted. Cells were analyzed using flow cytometry and Accuri 6 software. *N*=2

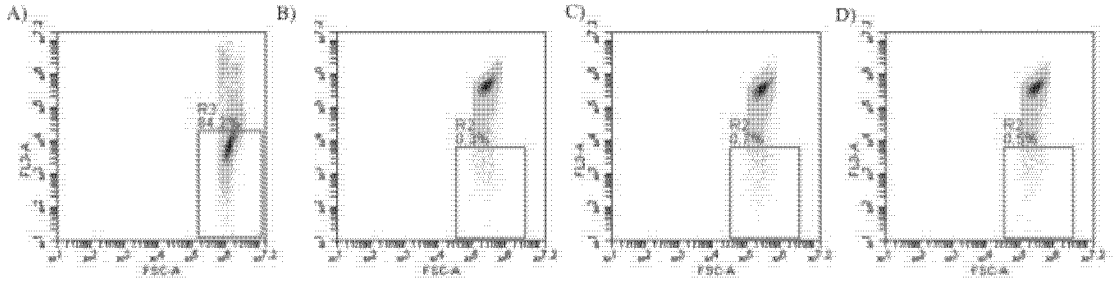


**Figure 4C: BMDC viability after 16 hours incubation with LPS is diminished.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.2 (C) or 0.1 (D) μg/mL LPS for 16 hours. BMDC before LPS stimulation (A). BMDC incubated for 16 hours with LPS (B). Live/Dead staining using propidium iodine was conducted. Cells were analyzed using flow cytometry and Accuri 6 software. *N*=2

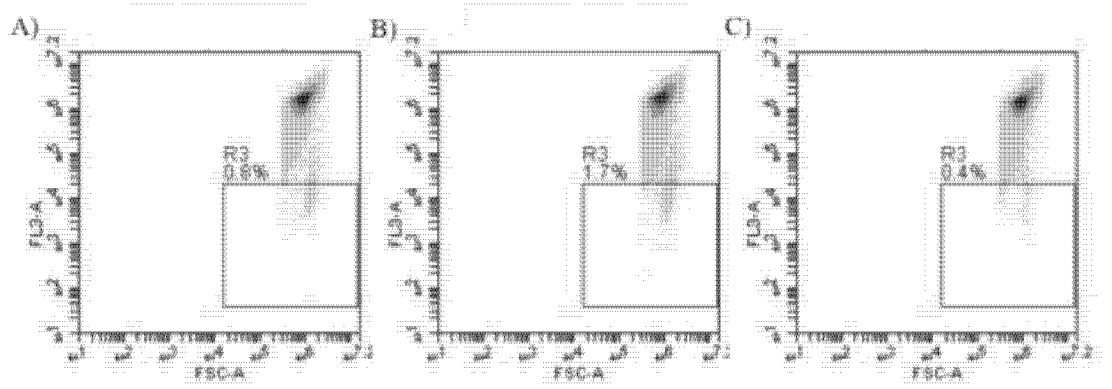


**Figure 4D: BMDC viability after 16 hours incubation with LPS followed by 12 hours incubation with vaccine protein is diminished.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 μg/mL (B) or 0.05 μg/mL (C) LPS for 16 hours. BMDC before LPS stimulation (A). Live/Dead staining using propidium iodine was conducted. Cells were analyzed using flow cytometry and Accuri 6 software. *N*=2

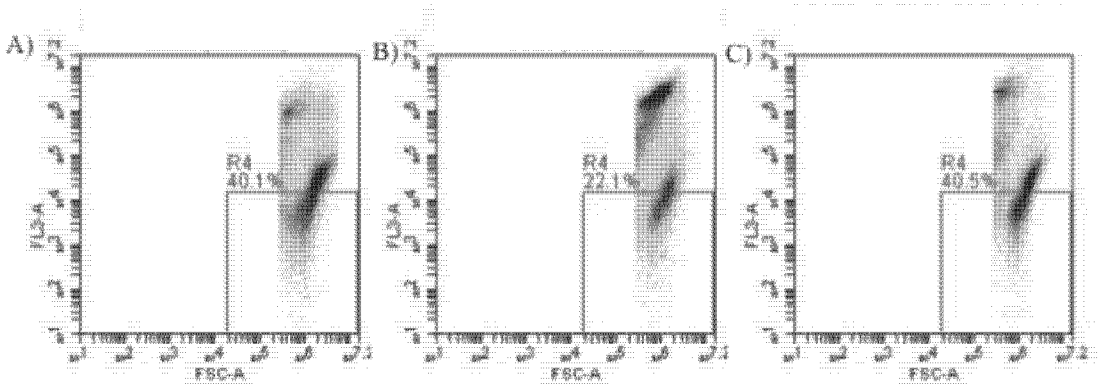


**Figure 4E: BMDC cell concentration of 5X10$^6$ cells/mL increases viability after 16 hours incubation with LPS.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and seeded at 2X10$^7$ cells/mL (B) or 5X10$^6$ cells/mL (C) then stimulated with 0.1µg/mL LPS for 16 hours. BMDC before LPS stimulation (A). Live/Dead staining using propidium iodine was conducted. Cells were analyzed using flow cytometry and Accuri 6 software. *N*=2

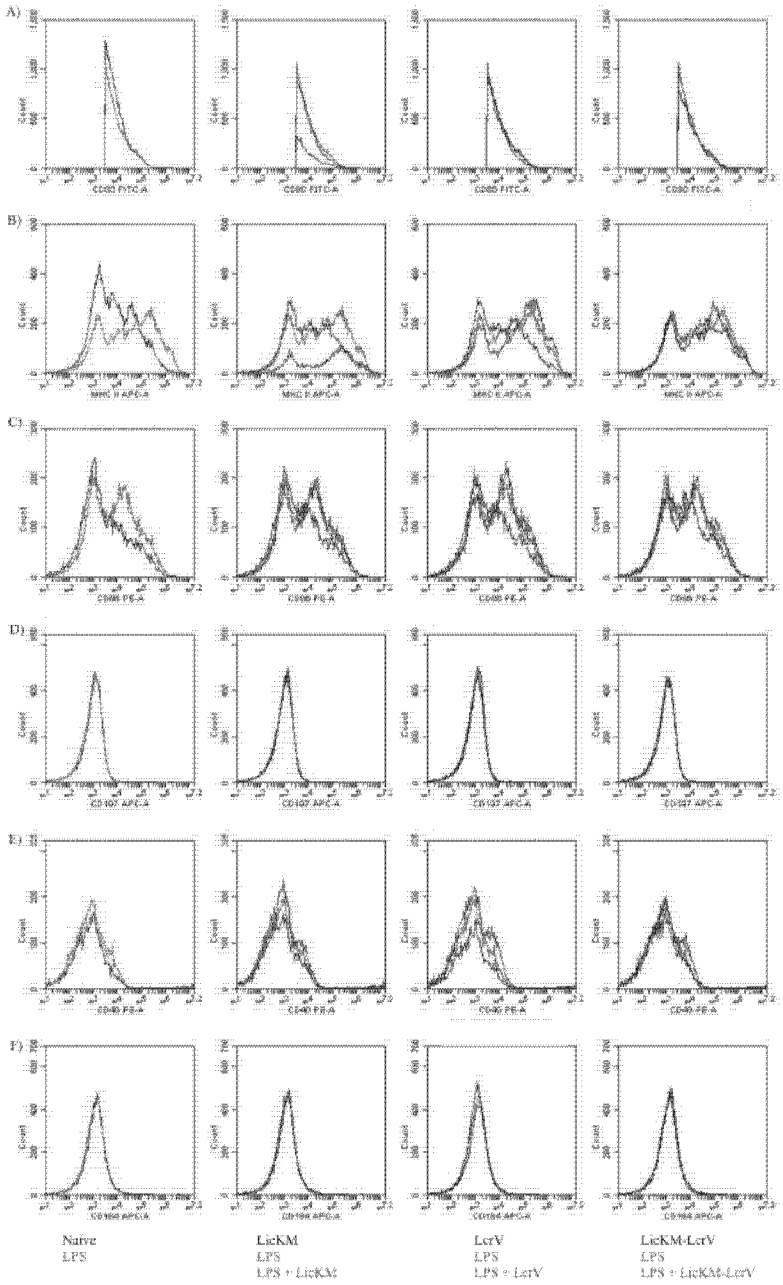


**Figure 5A: LicKM does not inhibit the generation of immune responses when in the presence of 0.1 µg/mL LPS for 3 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 µg/mL LPS. At the same time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 3 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=6

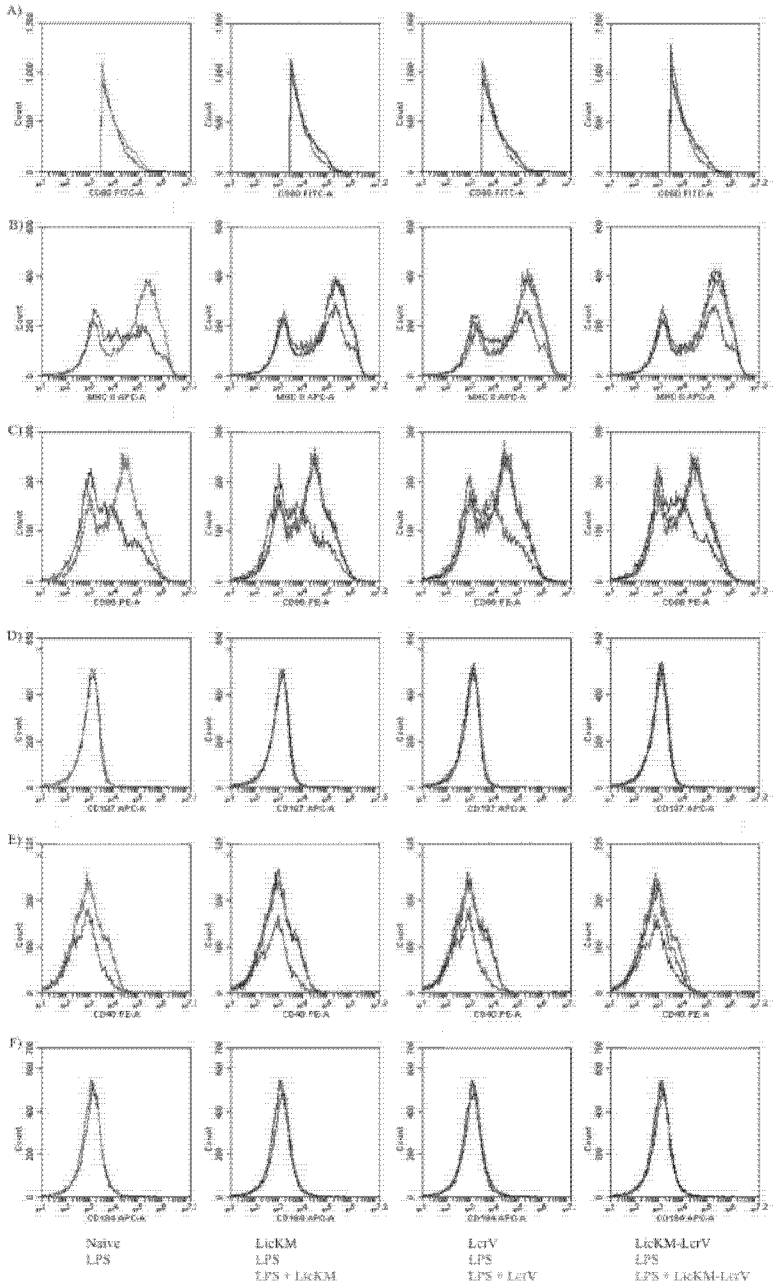


**Figure 5B: LicKM does not inhibit the generation of immune responses when in the presence of 0.1 µg/mL LPS for 6 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 µg/mL LPS. At the same time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=6

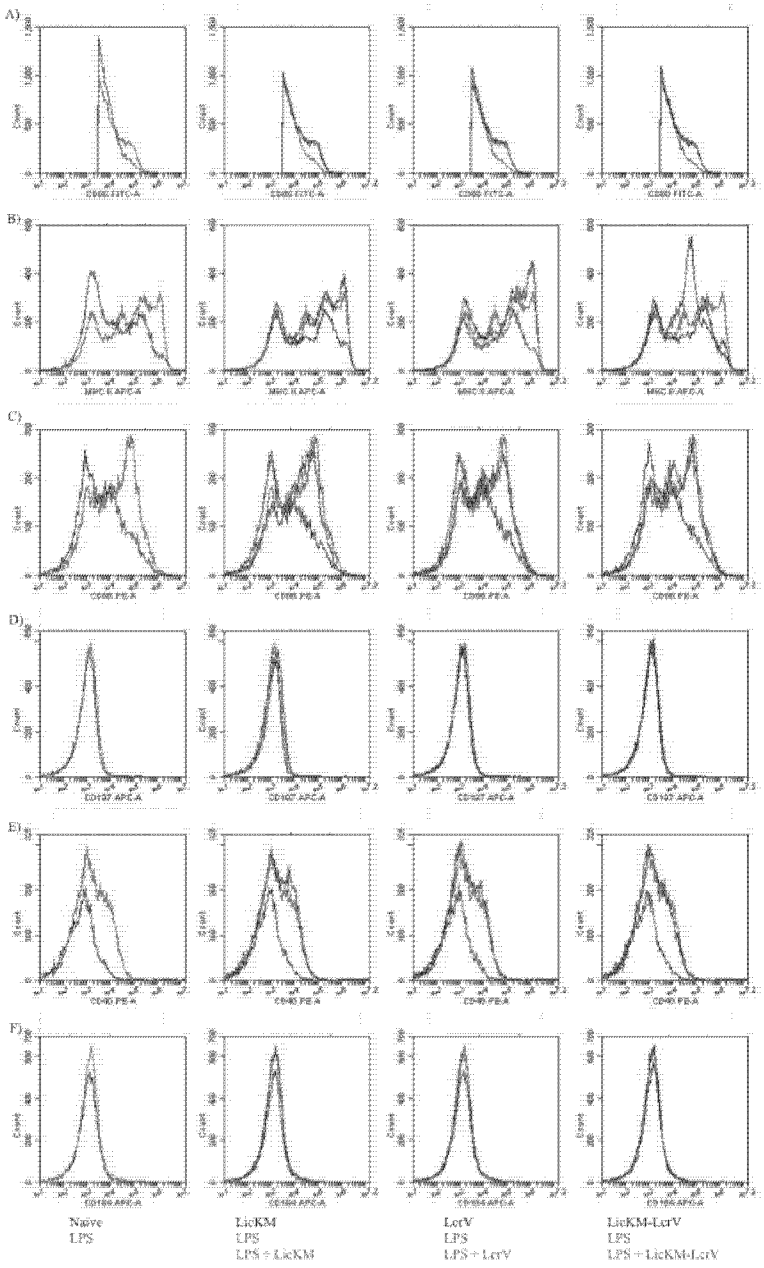


**Figure 5C: LicKM does not inhibit the generation of immune responses when in the presence of 0.1 µg/mL LPS for 12 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 0.1 µg/mL LPS. At the same time cells were stimulated with 10 µg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 12 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=6

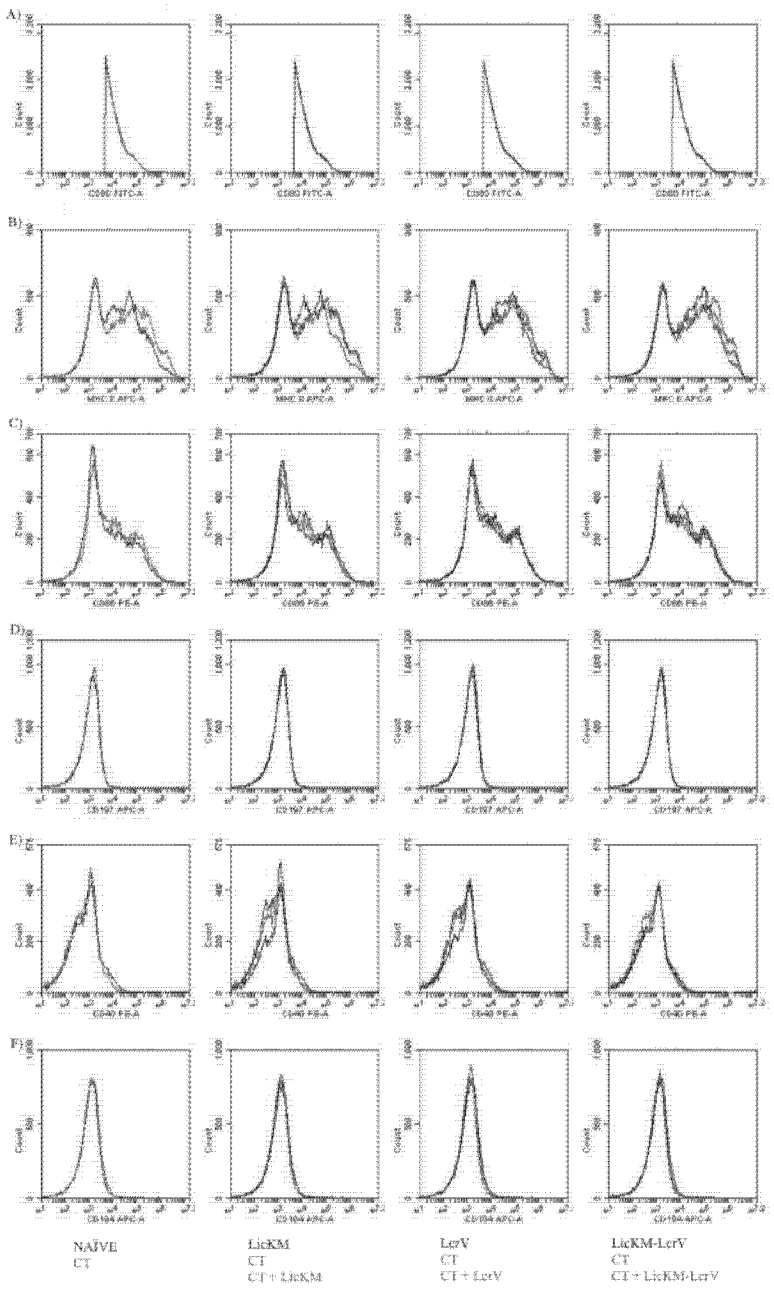


**Figure 6A: LicKM does not inhibit the generation of immune responses when in the presence of 1 μg/mL Cholera Toxin (CT) for 3 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 1 μg/mL CT. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 3 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD40 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=3

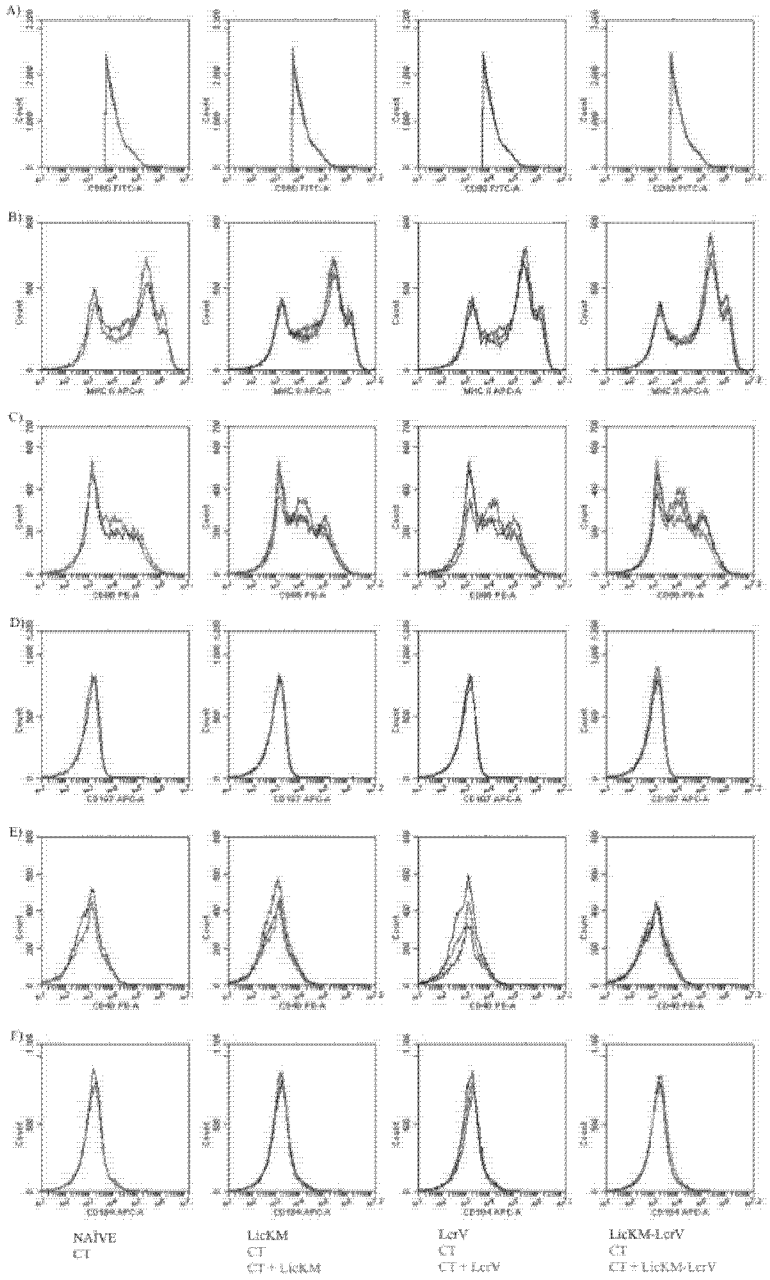


**Figure 6B: LicKM does not inhibit the generation of immune responses when in the presence of 1 μg/mL Cholera Toxin (CT) for 6 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 1 μg/mL CT. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD40 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=3

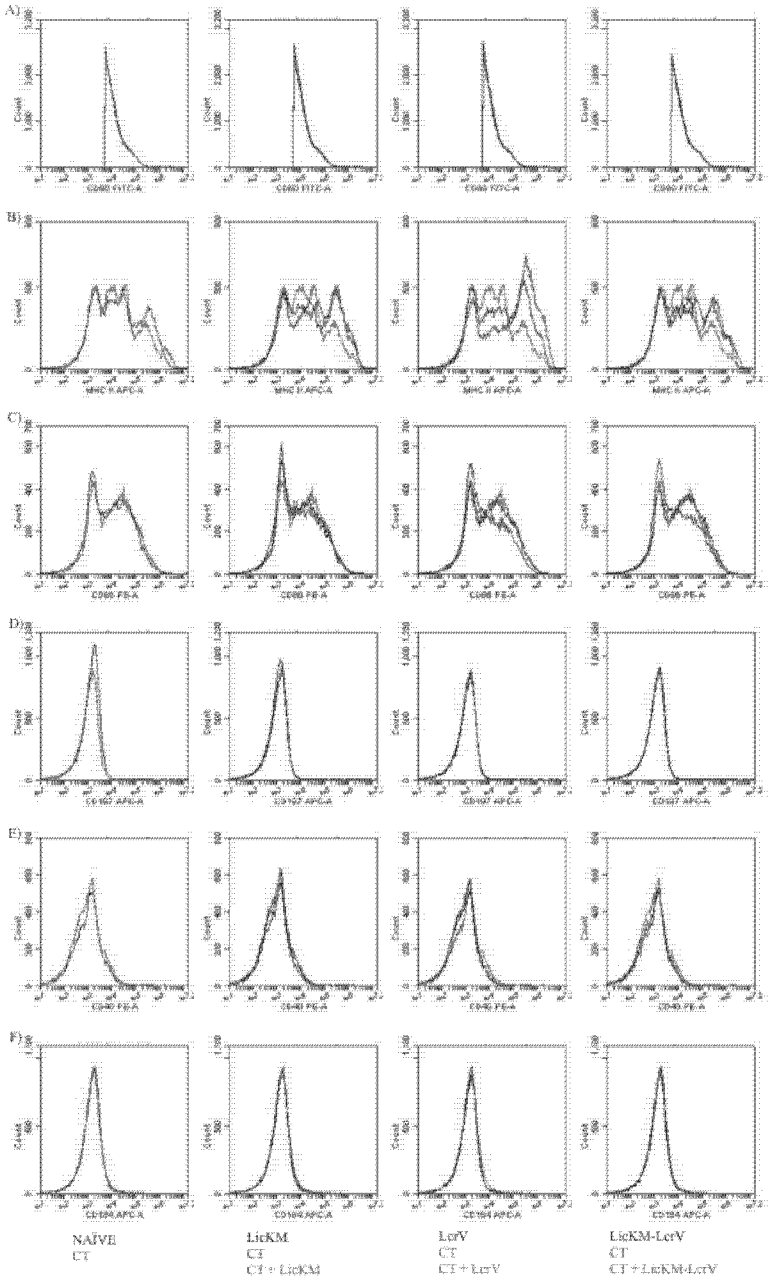


**Figure 6C: LicKM does not inhibit the generation of immune responses when in the presence of 1 μg/mL CT for 12 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 1 μg/mL CT. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 12 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD40 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=3

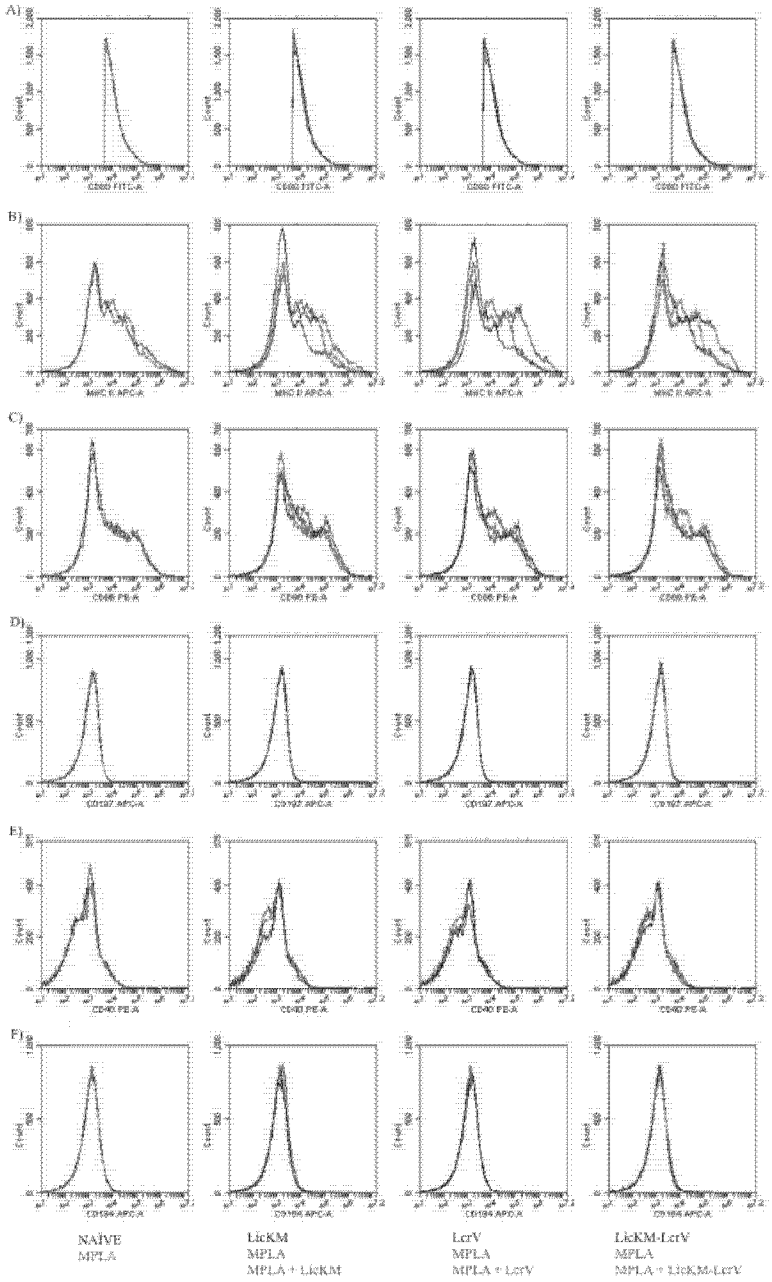


**Figure 7A: LicKM does not inhibit the generation of immune responses when in the presence of 100 μg/mL Monophosporyl Lipid A (MPLA) for 3 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 100 μg/mL MPLA. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 3 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=2

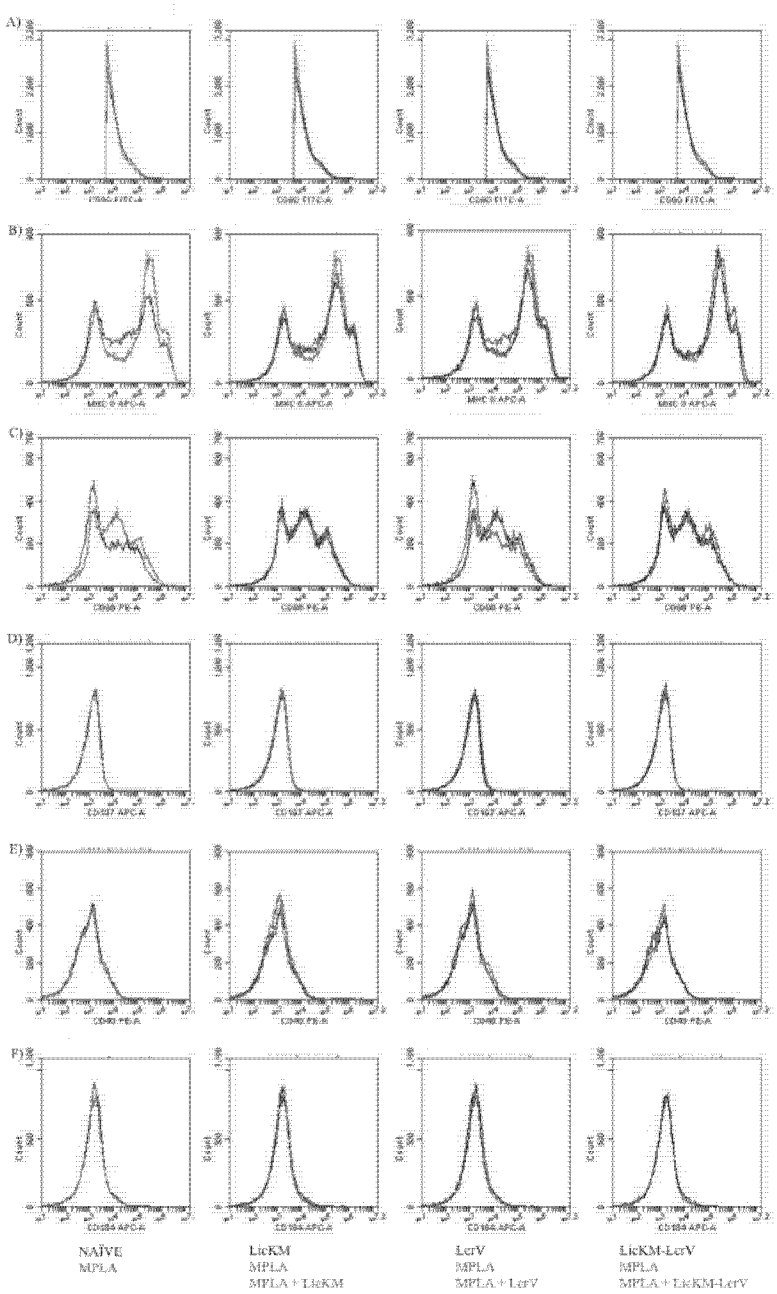


**Figure 7B: LicKM does not inhibit the generation of immune responses when in the presence of 100 μg/mL Monophosporyl Lipid A (MPLA) for 6 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 100 μg/mL MPLA. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 6 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=2

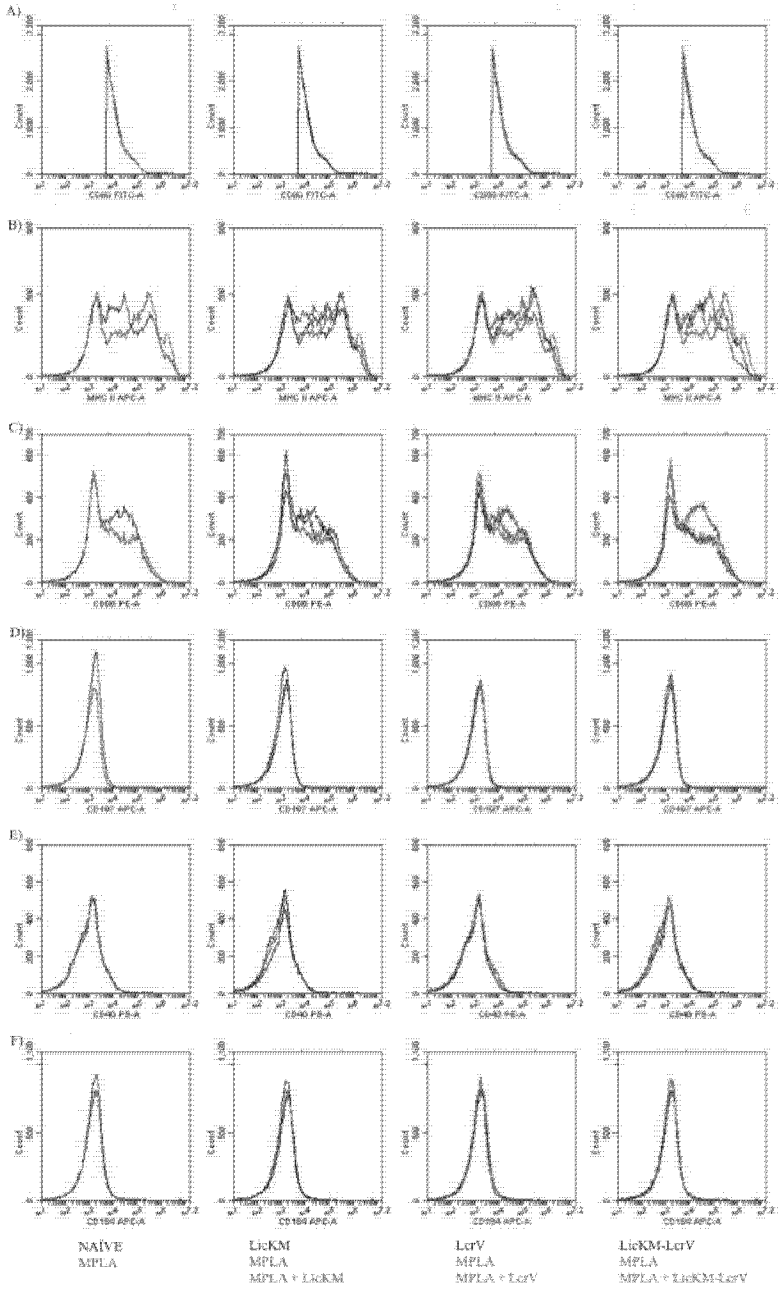


**Figure 7C: LicKM does not inhibit the generation of immune responses when in the presence of 100 μg/mL Monophosporyl Lipid A (MPLA) for 12 hours.** Bone marrow derived dendritic cells (BMDC) were propagated from BALB/c mice using GM-CSF. BMDC were collected and stimulated with 100 μg/mL MPLA. At the same time cells were stimulated with 10 μg/mL LicKM, LcrV, or LicKM-LcrV. Cells were stained for surface marker activation after 12 hrs of stimulation. Cells were analyzed using flow cytometry and Accuri 6 software. A)CD80 B)MHC II C)CD86 D)CD197 E)CD40 F)CD184. *N*=2

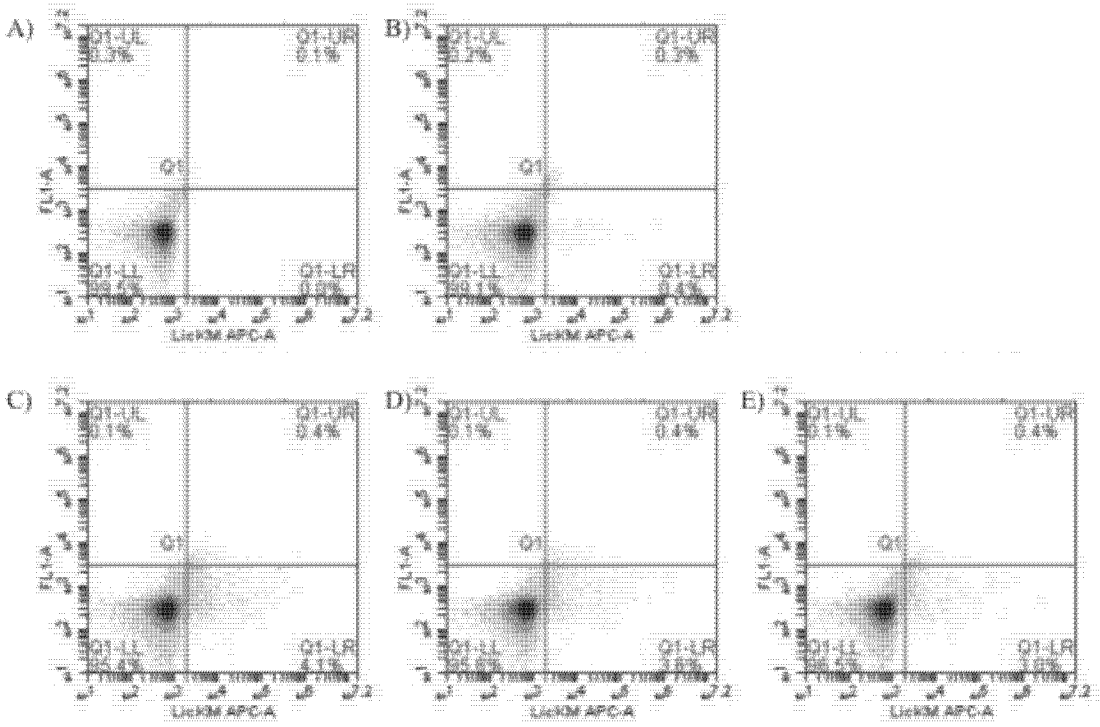


**Figure 8A: Detection of LicKM using a rabbit polyclonal antibody.** Splenocytes were collected from a naïve BALB/c mouse and cultured with 10 µg/mL LicKM for 3 hours. Spenocytes were stained for LicKM using different dilutions of a rabbit polyclonal anti-LicKM antiserum. Samples were analyzed using flow cytometry and Accuri 6 software. A) Unstained B) No primary antibody C) 1:250 dilution D) 1:500 dilution E) 1:1000 dilution $n$=1

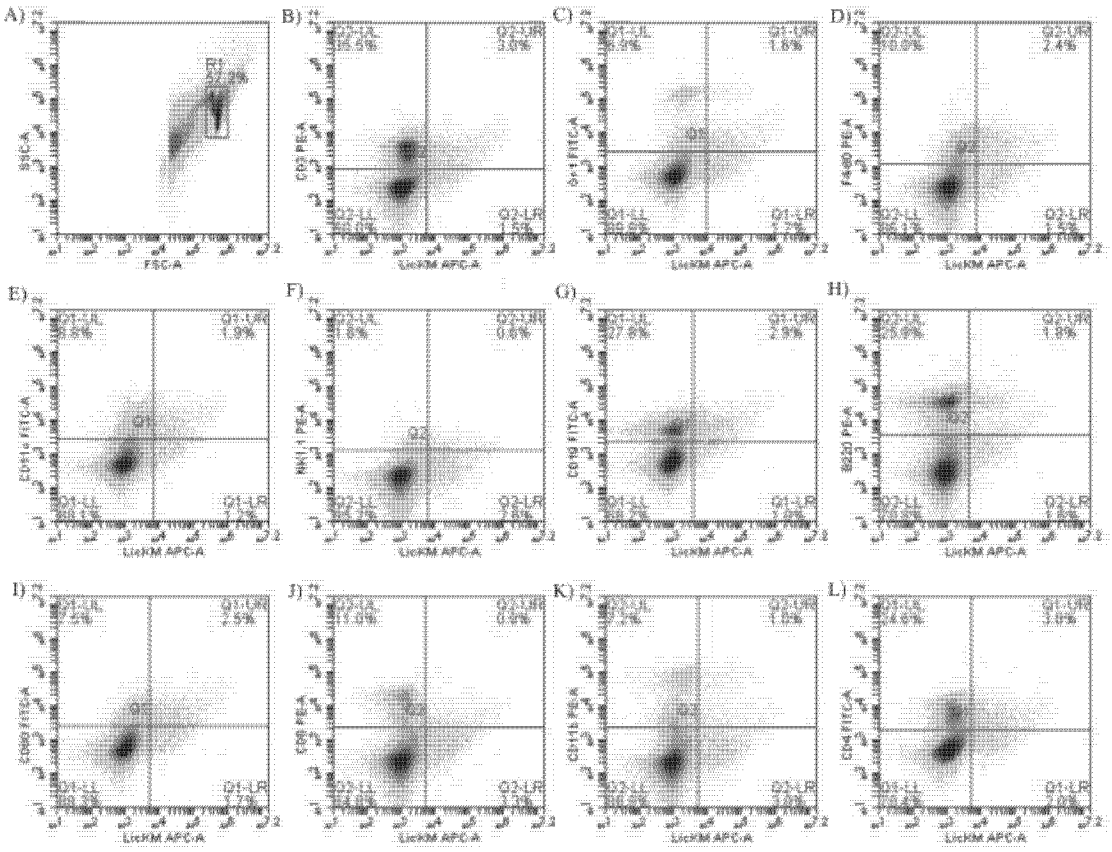

**Figure 8B: LicKM interacts with CD3 and CD4 T-cells after 1 hour of incubation.** Splenocytes were collected from a naïve BALB/c mouse and cultured with 10 µg/mL OVA, LicKM, LcrV, or LicKM-LcrV for 1 hour. Splenocytes were stained using surface markers for immune cells and intracellular staining for LicKM using a rabbit polyclonal anti-LicKM antiserum. Samples were analyzed using flow cytometry and Accuri 6 software. All samples were gated based on size of lymphocytes (A). Non-specific binding ~1%. B)CD3 C)Gr-1 D)F4/80 E)CD11c F)NK1.1 G)CD19 H)B220 I)CD80 J)CD8 K)CD11b L)CD4. *n*=1

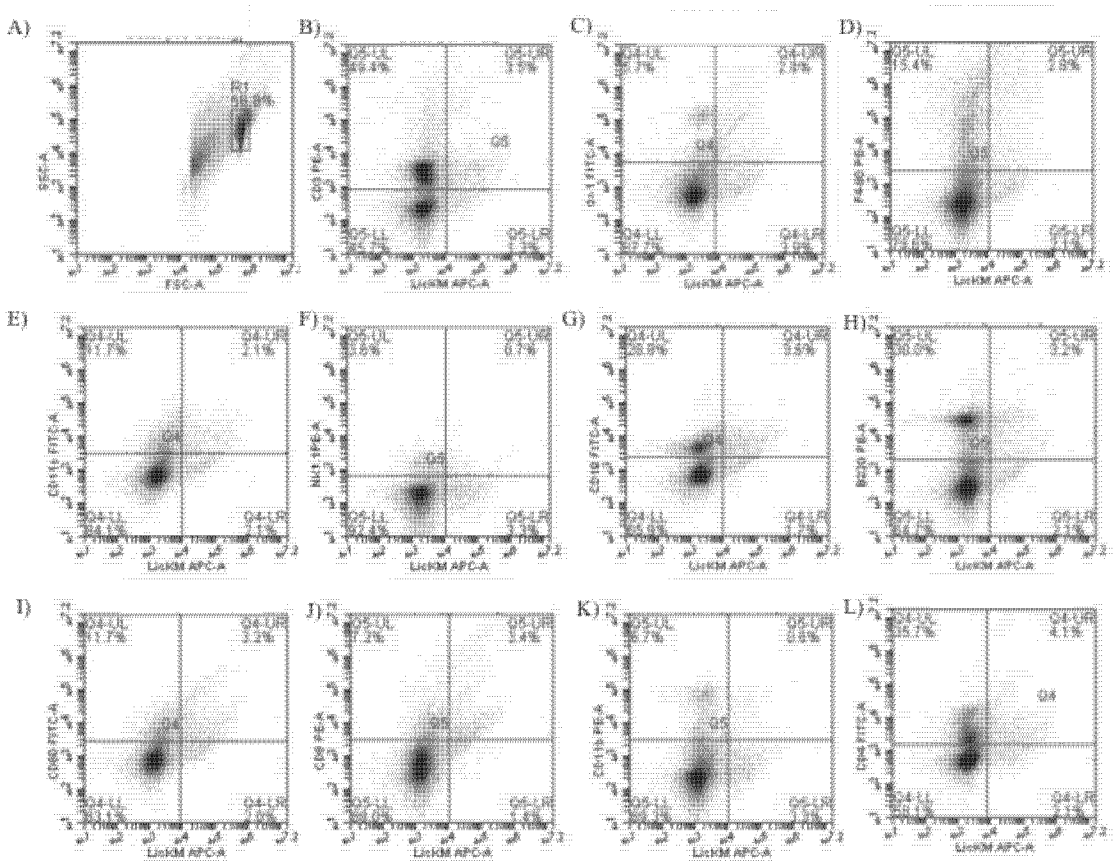

**Figure 8C: LicKM interacts with CD3, CD19, B220, CD80, CD8 and CD4 surface markers after 3 hours of incubation.** Splenocytes were collected from a naïve BALB/c mouse and cultured with 10 µg/mL OVA, LicKM, LcrV, or LicKM-LcrV for 3 hours. Splenocytes were stained using surface markers for immune cells and intracellular staining for LicKM using a rabbit polyclonal anti-LicKM antiserum. Samples were analyzed using flow cytometry and Accuri 6 software. All samples were gated based on size of lymphocytes (A). Non-specific binding ~1%. B)CD3 C)Gr-1 D)F4/80 E)CD11c F)NK1.1 G)CD19 H)B220 I)CD80 J)CD8 K)CD11b L)CD4. *n*=2

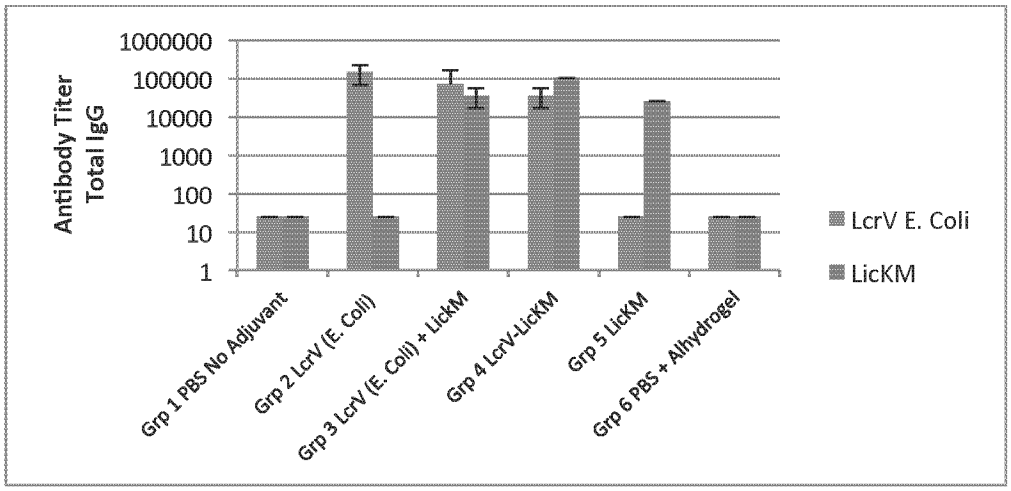


**Figure 9A: IgG titers in BALB/c mice vaccinated with LicKM and LcrV vaccine candidates.** BALB/c mice were primed and boosted intramuscularly with PBS, LcrV, LcrV + LicKM, LcrV-LicKM, LicKM or PBS + adjuvant. All groups except the PBS only group (group 1) contained 0.3% Alhydrogel. On day 42, post 2nd dose, serum was collected and an ELISA was performed to determine the total IgG titer against LcrV (blue bars) and LicKM (red bars) vaccine components. *N*=1

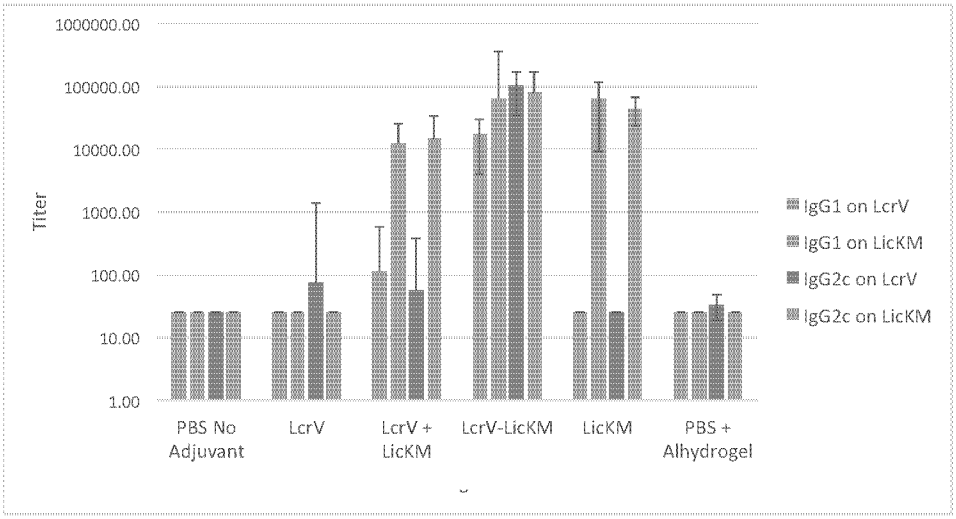


**Figure 9B: IgG1 versus IgG2a responses in BALB/c mice vaccinated with LicKM and LcrV vaccine candidates.** BALB/c mice were primed and boosted intramuscularly with PBS, LcrV, LcrV + LicKM, LcrV-LicKM, LicKM or PBS + adjuvant. All groups except the PBS only group (group 1) contained 0.4% Alhydrogel. On day 42, post 2nd dose, serum was collected and an ELISA was performed to determine the titer of IgG1 versus IgG2a generated against LcrV (red and purple bars, respectively) and LicKM (blue and green bars, respectively) vaccine components. *N*=1

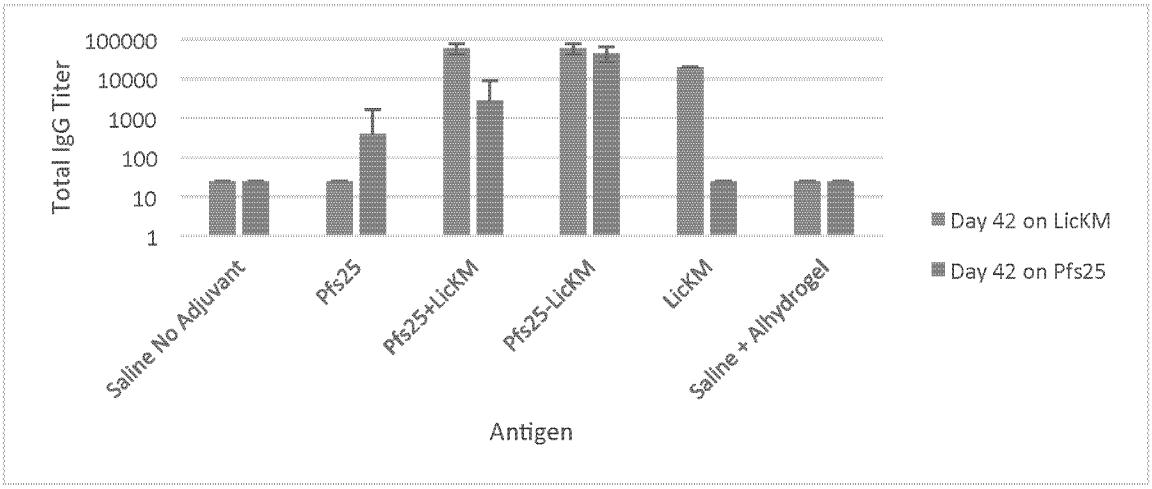


**Figure 10A: IgG titers in BALB/c mice vaccinated with LicKM and Pfs25 vaccine candidates.** BALB/c mice were primed and boosted intramuscularly with saline, Pfs25, Pfs25 + LicKM, Pfs25-LicKM, LicKM or saline + adjuvant. All groups except the saline only group (group 1) contained 0.3% Alhydrogel. On day 42, post 2$^{nd}$ dose, serum was collected and an ELISA was performed to determine the total IgG titer against Pfs25 (red bars) and LicKM (blue bars) vaccine components. *N*=1

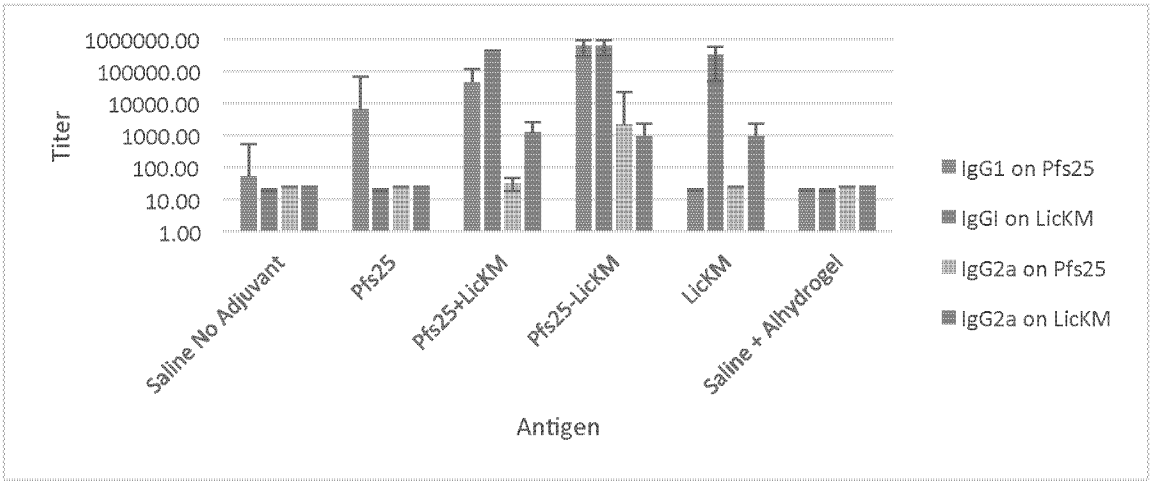


**Figure 10B: IgG1 versus IgG2a responses in BALB/c mice vaccinated with LicKM and Pfs25 vaccine candidates.** BALB/c mice were primed and boosted intramuscularly with saline, Pfs25, Pfs25 + LicKM, Pfs25-LicKM, LicKM or saline + adjuvant. All groups except the saline only group (group 1) contained 0.3% Alhydrogel. On day 42, post 2$^{nd}$ dose, serum was collected and an ELISA was performed to determine the titer of IgG1 versus IgG2 generated against Pfs25 (blue and green bars, respectively) and LicKM (red and purple bars, respectively) vaccine components. *N*=1

# Chapter 4
# DISCUSSION

## 4.1 Vaccines Serve to Stimulate the Immune System

The development of vaccines serve as an important advancement in healthcare. Vaccines are able to stimulate the immune system to develop a protective response to a particular disease or infection (Makela, 2000). By stimulating the immune system before coming in contact with the pathogen, the individual will be protected when directly exposed. The majority of vaccines received during childhood protect against diseases that often claimed young children before vaccine development.

Effective vaccines stimulate the adaptive immune response generating both humoral and cell-mediated responses. Antibodies developed through vaccination protect the individual through opsonization, neutralization, and/or phagocytosis (Abbas, 2012). A cell-mediated response following vaccination includes the generation of helper $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells. The generated T-cell responses protect the individual against intracellular pathogens, such as viruses.

## 4.2 Current Adjuvants are Lacking in the Ability to Generate a Cell-Mediated Response

Currently, there is a lack of adjuvants to stimulate a cell-mediated response. In order for subunit vaccines to pass safety testing, the immunostimulatory capabilities usually have to removed. This leads to the need for an adjuvant to be added to the vaccine to stimulate the immune system by triggering a "danger signal" (Glenny, 1921). Adding an adjuvant has several other benefits including: reducing the dose of antigen needed to generate a protective response, reducing the number of vaccinations necessary to generate a protective response, and increasing the amount of functional antibody produced (Reed, 2013). In order for the correct adjuvant to be selected during vaccine development, the type of immune response needed for protection and the intended mode of delivery for the vaccine must be determined.

The current list of licensed adjuvants used in vaccine products includes: aluminum salt, oil-in-water emulsion, virosomes, and AS04 (Reed, 2013). Aluminum salts generate a strong humoral response and are the most widely used adjuvant in vaccines. Oil-in-water emulsions generate enhanced cell-mediated and humoral responses over aluminum salts, however, in the general population this adjuvant is not well tolerated (Reed, 2013). Virosomes stimulate a strong humoral response and protect the antigen from degradation (Pevion, 2010). The virosome protects the antigens from degradation by forming a protective coating around the antigen. It is important to protect the antigen from being degraded before an immune response can be generated. AS04 generates a humoral and cell mediated response but must be delivered in aggregates to function (Ismaili, 2002).

Of the current licensed adjuvants, all but two generate a dominant antibody response and little to no a cell-mediated response. Of the two adjuvants, oil-in-water is not well tolerated and AS04 must be delivered in aggregates. Both of these adjuvants have limits that make them an unsuitable choice for future vaccines leading to the need for new and better adjuvants that are able to target and enhance cell-mediated responses. Additionally, a new adjuvant that is able to generate both a strong cell-mediated response and a strong humoral response will provide the best protection to the individual. The work described here evaluates the potential of a molecule to stimulate both branches of adaptive immunity.

### 4.3 LicKM Stimulates Humoral and Cell-Mediated Immune Response

LicKM is a 25KDa thermostable lichenase from *Clostridum thermocellum* (Musiychuk, 2007). Previous research was done using *Yersina pestis* protein, LcrV, fused to LicKM. Monkeys immunized with LcrV + F1 vaccine produced a strong antibody response and were protected from a lethal dose of *Y. pesits* (Mett, 2007; Chichester, 2009). Mice immunized with LicKM, F1, and LcrV produced an antibody and a T-cell response (Guth, 2012). Vaccination of mice with human papillomavirus (HPV) fusions with LicKM showed a humoral (IgG) and cell-mediated response

against HPV (Massa, 2007). Additionally, when no other adjuvant is present in the vaccine, tumor growth was suppressed (Massa, 2007). Vaccination with *Bacillus anthracis* antigen domain 4 of protective antigen (PAD4) and domain 1 of lethal factor (LFD1) and LicKM generates a high antibody titer of IgG1, however T-cell response were not tested (Chichester, 2007). Given the previous research, there is a strong indication that LicKM is able to generate not only a humoral response but also a cell-mediated response. The goal of this work was to further characterize the immune response stimulated by LicKM.

### 4.4 LicKM Trends Towards Up-Regulation of DC Activation Markers *In Vivo*

Previous research has focused on evaluating LicKM *in vivo* using *Y. pestis* as a model (Mett, 2007; Chichester, 2009; Guth, 2012), therefore this work evaluated LicKM *in vitro* using *Y.pestis* as a vaccine candidate. In order to look at the *in vitro* effects of LicKM, dendritic cells (DC) from BALB/c mice were stimulated with vaccine proteins. Guth's work (2012) investigating IL-2 levels after mice vaccination with LicKM, F1, and LcrV showed a memory T-cell response to LicKM. Due to the ability of LicKM to generate a memory T-cell response, DC cells were investigated because DC are the only APC able to stimulate naïve T-cells. After some investigation to determine the stimulation concentration and incubation time to detect stimulation, 10 μg/mL of vaccine protein, incubated for 12 hours shows up regulation of common DC expression markers. Cells stimulated with 100 μg/mL vaccine protein for 24 hours, showed expression levels that were consistent across conditions, leading to the conclusion that up-regulation happens before 24 hours. Cells stimulated with 100 μg/mL vaccine protein for 12 hours, showed differences in expression levels, however, the changes are not clearly distinguishable from each condition. By reducing vaccine protein to 10 μg/mL and incubating for 12 hours, the changes in activation levels can be clearly documented. We know from other vaccine studies that a lower concentration is better at stimulating the immune system than larger concentrations.

The shift in the population for MHC class II expression present when comparing naïve to LicKM-LcrV stimulated cells can be attributed more to the LicKM vaccine component than the LcrV protein. As determined when comparing LicKM to LicKM-LcrV and LcrV to LicKM-LcrV, a smaller shift in the population is seen with LicKM stimulation alone than with LcrV stimulation alone. Finally, an earlier time point was investigated for earlier expression changes. After 6 hours of incubation, there was no difference in expression levels, indicating LicKM requires more than 6 hours for activation of DC surface markers. Therefore, murine BMDC incubated with 10 μg/mL LicKM for 12 hours causes increased expression of common DC activation markers. The expression changes are caused by LicKM and not LcrV, indicating the potential immunostimulatory ability of LicKM.

### 4.5 LicKM Does Not Inhibit the Immune Response When BMDC are Stimulated With LPS, CT, or MPLA

In order for a new vaccine adjuvant to enter clinical trials, researchers must prove that this potential adjuvant does not cause adverse events, such as immune suppression. Therefore BMDC from BALB/c mice were stimulated with LPS for 16 hours and then re-stimulated with vaccine proteins for 6 or 12 hours. Suppression of DC activation markers was not detected after 6 hours of incubation with vaccine proteins. However, after titration of LPS it was determined the majority of the BMDC were dead before being re-stimulated with vaccine protein. To confirm these findings BMDC were evaluated after 22 hours or 28 hours of stimulation and also found to be mainly dead. Therefore, this method of stimulating for 16 hours with LPS then re-stimulating with vaccine protein for 6 or 12 hours was found to be ineffective to determine if LicKM was causing suppression of activation.

Therefore a different procedure was used in which BMDC from BALB/c mice were incubated with various potent activators and vaccine protein to determine expression levels. The viability of cells was greatly increased when the concentration of cells plated was reduced. Reduction of cells plated, could have allowed for less

competition between cells for nutrients. For each activator used, no difference in expression was seen when comparing stimulated with potent activator and those stimulated with activator and vaccine protein. All common DC expression markers analyzed showed no suppression at any time points. Potent activators used were LPS, CT, and MPLA. Therefore, *in vitro* data concludes that the presence of LicKM during LPS, CT or MPLA stimulation does not affect the activation profile of murine BMDCs.

### 4.6 LicKM Interacts at Low Levels with Cells of the Immune Response

In order to determine if LicKM interacts with host immune cells, splenocytes were collected from BALB/c mice and incubated with vaccine protein. LicKM antiserum was first titrated to find the optimal dose for staining. Naïve spleens from BALB/c mice were collected and stimulated with vaccine protein. After staining with LicKM antiserum and known immune cell surface markers after 1 and 3 hours of incubation double positive staining was found with several markers. CD3 and CD4 were positive for interaction with LicKM after 1 hour of incubation. CD3, CD4, CD8, CD19, B220, and CD80 were positive after 3 hours of incubation. CD4 is found on helper T-cells. CD8 is found on cytotoxic T-cells. CD3 is found on both CD4 and CD8 T-cells. CD19 and B220 are found on B-cells. CD80 is associated with macrophages, dendritic cells, and activated B-cells. T-cells and B-cells are the immune cells responsible for generating acquired immunity. T-cells are responsible for generating immune regulation and for a cell-mediated response primarily against intracellular pathogens. B-cells are responsible for generating humoral immunity responsible for clearing extracellular pathogens. LicKM appears to be interacting with APCs indicating a role in activating acquired immunity.

## 4.7 Vaccination With LicKM Stimulates Both a Humoral and Cell-Mediated Response

### 4.7.1 LicKM Elicits a Strong IgG1 Antibody Response

In order to determine the antibody response against LicKM upon vaccination of BALB/c mice, serum samples were collected and analyzed by ELISA. Groups immunized with LicKM showed an increase in total IgG against LicKM when compared to those without LicKM. The antibody response against LcrV is unchanged when in the presence or absence of LicKM. To determine the primary isotype, an additional ELISA was performed for IgG1 and IgG2a. LicKM and LcrV both generated a strong IgG1 response as compared to IgG2a data. LicKM does appear to generate a small response of IgG2a when compared to controls, however IgG1 is the dominant response.

In another vaccine trial with BALB/c mice and Pfs25 in which serum samples were collected and analyzed by ELISA. Total IgG titers were increased upon vaccination as expected, when LicKM + Pfs25 + alum was used to vaccinate a lower IgG response was generated against Pfs25 as compared to vaccination with a LicKM-Pfs25 fusion and alum. Additionally, when IgG antibody titers are investigated, the fusion product generates a strong IgG1 response and IgG2a response that the non-fused product is unable to do. Indicating an additive benefit of the fusion product over the two proteins in the same vaccine. This data taken together indicates the potential of LicKM to generate a strong IgG1 antibody response and depending on the protein fused an IgG2a response in BALB/c mice.

## 4.8 Utilization of a Model Vaccination System to Evaluate the Immunostimulatory Characteristics of LicKM, a Novel Carrier Molecule

In conclusion, this work showed that LicKM can affect the humoral and cell-mediated immune responses against a vaccine antigen it is fused to. This work was focused on starting to elucidate the mechanism of action of LicKM. LicKM is able to

stimulate naïve DC through up regulation of surface activation markers. In addition, no suppression of activation was seen when DC were stimulated with LicKM plus LPS, CT, or MPLA. LicKM appears to interact at low levels with B-cells, T-cells, macrophages, and DCs all of which are important for stimulating the immune system. Vaccination in the presence of LicKM generates a strong IgG1 humoral response in BALB/c mice. A stronger antibody response is generated when immunogenic epitopes are fused with LicKM. Further investigation into the mechanism of action of LicKM could provide a new adjuvant capable of stimulating a humoral and cell-mediated response.

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WHO Malaria Fact Sheet, Updated Regularly: http://www.who.int/mediacentre/factsheets/fs094/en/index.html. Assessed on April 9, 2015.

# Appendix

## INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE FORM



**University of Delaware**
**Institutional Animal Care and Use Committee**
**Request to Amend an Animal Use Protocol**

| | |
|---|---|
| **Title of Protocol:** Yersinia infection of mice | |
| **AUP Number:** 1166-2015-A | ← (4 digits only) |
| **Principal Investigator:** Michelle A. Parent | |

**Requested Changes**

**I am requesting a change to:** *(Check all that apply)*

- ☐ Animal Species *(Complete Section 1)*
- ☒ Animal Numbers *(Complete Section 2)*
- ☒ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* [illegible]

*(Complete Section 7)*

**Changes MUST NOT be initiated until IACUC approval is granted**

**Official Use Only**

IACUC Approval Signature: Glen Tabin, DVM

Date of Approval: 1/14/15