DEVELOPMENT OF VACCINES AGAINST CANCER AND INFECTIOUS DISEASES FOR ORAL, BUCCAL AND TRANSDERMAL DELIVERY

By

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A Dissertation Submitted to the Graduate Faculty of Mercer University College of Pharmacy in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATLANTA, GA
2017

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DEDICATED TO MY BELOVED PARENTS
ACKNOWLEDGEMENT

As I near the completion of my Ph.D., I look back at the contributions of many individuals without whose help and support the successful completion of this Ph.D. would have been extremely difficult if not impossible. I take this opportunity to express my thankfulness to each of them.

I would first and foremost thank God for giving me the opportunity to pursue a Ph.D., something that I myself did not believe I could do. He gave me the strength to overcome many an obstacle that I would not have been able to cross without His constant guidance and grace. This experience has made me a much stronger person than I was when I started off and I thank God for His constant presence through it.

I am grateful to my parents – Mr. Praful Gala and Mrs. Parul Gala and brother – Mr. Parshv Gala for their constant support and belief in me. Their prayers and blessings have given me hope and helped me realize my dreams.

I would like to express my heartfelt gratitude to Dr. Martin J. D'Souza for letting me be a part of his lab and for his valuable support through these years. The spirit of camaraderie and friendship that we share as a lab because of him is one of a kind and I believe it has helped me grow both personally and professionally. I truly look up to him and sincerely hope I can be as good a
Mentor as him in my future career.

I am grateful to my committee members Dr. Ajay Banga, Dr. Grady Strom and Dr. Ravi Palaniappan for their advice and guidance with my research and for their constant encouragement. I would like to thank Dr. Bernadette D’Souza for agreeing to be part of my Ph.D. dissertation committee and for her suggestions whenever I needed it.

I thank Dean Hewitt ‘Ted’ Matthews for his unstinting support to all graduate students. His speeches were a source of inspiration to us and helped keep our spirits high when times were tough.

I would like to take this opportunity to thank my collaborators and mentors, Dr. Susu M. Zughaier, Dr. Gregory Knipp, and Dr. Carmen Popsecu. They have been a constant source of guidance, motivation and a true inspiration during my time as a graduate student. It has been an honor working with them and learning from their experiences. I would like to once again thank my major advisor, Dr. Martin J. D’Souza for having faith in me and providing the opportunity to work on these collaborative projects.

I acknowledge and thank the staff at Mercer University who has helped me in ways I cannot count. I thank Cherilyn D’Souza for rubbing off her good cheer on me and being a huge help in lab. I thank Frank Morris, Vivienne Brown and Theresa Montague for their assistance at all times. A huge thanks to the Swilley Library, Atlanta and the Academic Resource Center (ARC) for all their help with the academic resources.
Last but definitely not the least; I thank all my friends at Mercer with whom I share some of the most wonderful memories of my life. I thoroughly enjoyed my time at Mercer because I believe we made it our home away from home. Thanks for being there for me every time I needed one of you. I hope our paths cross again and destiny gives us a chance to meet again.
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ABSTRACT

DEVELOPMENT OF VACCINES AGAINST CANCER AND INFECTIOUS DISEASES FOR ORAL, BUCCAL AND TRANSDERMAL DELIVERY

Rikhav Praful Gala
(Under the direction of Dr. Martin J. D’Souza)

Vaccination has been widely used as a mode of protection against various diseases by taking advantage of host’s immune system. Even though vaccination has provided a relief from many infectious diseases, it remains to be a challenge to prepare a vaccine against cancer. Some of the current challenges in vaccine development are its stability, delivery form and patient compliance. Our approach is to formulate vaccines in microparticles/nanoparticles composed of biodegradable and biocompatible polymers encapsulating the vaccine antigen. The particulate vaccine system can also utilized to incorporate vaccine adjuvants, which are act as catalysts to boost the immune response to the vaccine. These microparticulate vaccine systems offer several advantages to its solution form in terms of higher uptake by macrophages, sustained release of the antigen/adjuvant and its protective functions. Also, these microparticles can be incorporated into various conventional dosage forms such as capsules, suspensions and also in novel dosage forms such as microneedles, oral dissolving films etc.

In the ovarian cancer vaccine development, previously we had shown the efficacy of the whole cell lysate to generate a protective immune response. In this study, we have screened various adjuvants to potentiate the efficacy of the vaccine. We also tested the vaccine via two different route of administration – oral and transdermal. We found that certain adjuvants such as Alum and MF59 had a significant effect on the potentiation of
the ovarian cancer vaccine. Moreover, both the oral and the transdermal route with the aid of microneedles showed promising immunization strategies.

Secondly, in order to evaluate oral dissolving films (ODF) as potential buccal immunization strategy, we incorporated measles vaccine microparticles in the ODF. This ODF’s were formulated and characterized for their mechanical and tensile properties. From our in-vivo immunization studies in pigs, we found a significantly higher level of antibodies being produced by the ODF loaded measles vaccine. Thus, ODF via buccal delivery could be a potential route for immunizations.

In the last part of this project, we formulated a novel microparticulate vaccine against gonorrhea infections by using dead whole cell gonorrhea bacteria as the vaccine antigen. These microparticles were incorporated into maltose based dissolving microneedles for transdermal immunization. In our preliminary studies, we have reported generation of antibodies by the vaccine administered transdermally.

Overall, we have reported the potentiation of an immune response when the addition of an adjuvant to the formulation. Adjuvants such as Alum, MF59, MPL-A have shown promising candidates for vaccine development. Secondly, ODF for buccal delivery, microneedles for transdermal delivery are promising drug delivery and immunization strategies.
CHAPTER 1

INTRODUCTION

Vaccines are one the most effective modern medicines that help prevent infectious diseases and cancer. Edward Jenner was the first to use of a vaccine against smallpox in 1796; since then the use of vaccines has become imperative in disease prevention. In recent times, more than 70 vaccines have been licensed for use against approximately 30 microbes, protecting millions across the globe. The reason for the popularity of vaccines is the fact that they represent the least expensive and most effective way to protect against crippling epidemics. Economic benefits along with preserving the social well-being of society is a major consequence of vaccinations. Therefore, vaccines provide a very cost-effective means to maintain a high quality of life, save lives and preserve good health (Nabel, 2013).

Prophylactic and therapeutic interventions are the two main types of therapies available for vaccination. Prophylactic vaccines are primarily used for prevention of bacterial, viral or parasitic infectious diseases such as HIV, influenza, malaria, tuberculosis, pneumonia, polio and smallpox, which are mainly caused by antigens that are foreign to the body (“Nanoparticulate Vaccine Delivery Systems,” n.d.). Despite achieving significant strides in vaccine development, several infectious diseases and cancers still lead to significant loss of human life. The Global Alliance for Vaccines and Immunization (GAVI) estimates that every year at least 1.5 million children will die from vaccine-preventable diseases and vaccines for human immunodeficiency virus (HIV) infection, tuberculosis, and malaria, are yet to be developed. Currently, vaccines are administered through conventional routes such as intramuscular, subcutaneous and intravenous, which are less patient compliant and require medical supervision. Therefore, conventional vaccines are not only painful but also
expensive, which makes it harder to eradicate debilitating diseases in developing countries. Vaccine administration through non-conventional routes such as oral, transdermal, intranasal, buccal and pulmonary routes are under intense investigation (Akalkotkar, Chablani, Tawde, D’Souza, & D’Souza, 2015; Bhowmik et al., 2011a; Gala et al., 2017; Tawde, Chablani, Akalkotkar, & D’Souza, 2016). The oral route is particularly attractive primarily owing to its patient compliance and robust immune system activation. Oral administration of antigens have the potential to elicit mucosal and systemic immunity due to efficient antigen sampling by Microfold cells (M-cells) in the Peyer’s patches (Akalkotkar, Tawde, Chablani, & D’Souza, 2012; Bejugam, Uddin, Gayakwad, & D’Souza, 2008; Bhowmik et al., 2011b; Chablani, Tawde, Akalkotkar, et al., 2012a; Gala et al., 2016; Lai & D’Souza, 2007; Shastri, Kim, Quan, D’Souza, & Kang, 2012a; Ubale, D’Souza, Infield, McCarty, & Zughaier, 2013a; Uddin, Bejugam, Gayakwad, Akther, & D’Souza, 2009a). Although there are several advantages of oral administration, challenges such as degradation of antigen in harsh gastric and intestinal conditions is a significant concern. Along with identifying appropriate routes of vaccine administration, antigen selection and adjuvant therapy also determine vaccine efficacy. In case of cancer, vaccine antigen selection becomes extremely crucial because the patient’s immune system is primed to specifically recognize tumors and eliminate them. Cancer immunotherapy is, therefore, an attractive strategy to initiate anti-tumor activity as compared to monotherapy with chemotherapeutics and/or radiation therapy. Ovarian cancer is one such malignancy which has received significant attention in recent years as a target for immunotherapy. Every year more than 200,000 women are diagnosed with ovarian cancer in the US alone, making it one of the most prevalent forms of cancer among women in the US. Commercial
vaccines are always administered with adjuvants such as alum and MPL, which are approved by the FDA for use in humans. Adjuvants boost the innate and adaptive immune responses to vaccine antigens and hence improve vaccine efficacy. There are several important adjuvants under clinical investigation that have the potential to increase anti-tumor activity.

In this thesis, we have looked and development of microparticulate vaccines against ovarian cancer and other infectious diseases such as meningitis, measles, and gonorrhea. We have also developed a method to formulate novel dosage forms such as Oral dissolving films (ODF), dissolving microneedles (MN) containing the microparticulate vaccines for immunization via different routes of administration such as buccal, sublingual, oral, IM, IV and transdermal. We also looked at the potential of adjuvants in improving the immunogenicity of these vaccines. Here is the list of specific aims of each of these projects:

Specific Aim 1: The formulation and development of an oral and transdermal vaccine against ovarian cancer.

Specific Aim 2: Screening of adjuvants for potentiating the effects of the meningitis vaccine.

Specific Aim 3: Development of Oral dissolving films (ODF) for buccal delivery of measles vaccine.

Specific Aim 4: Development of a novel vaccine against Gonorrhea using the whole cell for transdermal delivery using microneedles.
CHAPTER 2
LITERATURE REVIEW

With the evolution of different challenging diseases, there is an urgent need for vaccine development against them to save lives of millions throughout the world. Moreover, in case of existing vaccines, there is still a need to address issues with respect to safety, effectiveness, ease of administration, time of preparation and most importantly, the cost. The use of vaccines to generate immunoprotection and eliminate infectious diseases has been studied extensively in the past. However, the extent of success of vaccination depends on several factors such as nature of pathogen, vaccine delivery system and route of administration and immune system of the host. With the advances in recombinant DNA technology and identification of different cancer antigens, immunotherapy is now being explored to target the challenging cancer disease. Immunotherapy involves induction, enhancement or suppression of host’s immune system for treatment of a particular disease. Microparticle delivery system plays an important role by acting as self-adjuvant in form of particles and thus assisting immunotherapy.

There are two major approaches for vaccination: prophylactic or therapeutic. Prophylactic vaccines find their applications in the prevention of viral, bacterial or parasitic infectious diseases such as influenza, HIV, tuberculosis, malaria, pneumonia, polio, smallpox etc., which are caused by foreign antigens. However, in case of cancer which is caused by altered self-cells, vaccine formulation is a challenging task as it requires immune response against self-cell antigens without causing an auto-immune response. Prophylactic cancer vaccines can prevent the tumor development based on the
use of over-expressed or mutated proteins, mutated oncogenic growth factor receptors, heat-shock proteins or other tumor-associated antigens (1). Gardasil® (Merck) and Cervarix® (GSK) are the only two prophylactic anti-viral vaccines against human papilloma virus, responsible for cervical cancer until today.

In case of therapeutic approach, vaccines are given in order to trigger an immune response against existing residual tumor cells mostly in combination with surgery or chemotherapy and thus aiming at preventing or prolonging the relapse. Cancer chemotherapeutic agents can result in up-regulation of surface expression of MHC molecules, tumor-associated antigens, thus making them more susceptible to immune destruction. Moreover, cancer therapy utilizing vaccines in combination with local radiation, chemotherapy, and hormone therapy, can work better as compared to either of above-mentioned therapies or vaccine alone. Preliminary outcomes of clinical studies have shown prolongation of tumor relapse, retardation of tumor progression and better overall survival (2). Currently, there is only one therapeutic cancer vaccine, Provenge® (Dendreon) approved by FDA for treatment of prostate cancer. Various other clinical trials have been reported utilizing DNA/dendritic cell (DC)/viral vector-based vaccines depicting the continuous growth in the field of cancer immunotherapy (Anassi & Ndefo, 2011; Cheever & Higano, 2011a).

The microparticulate delivery system has several advantages over the usage of the antigens alone. Particulate antigens have been proven to be more immunogenic than soluble antigens (Chablani, Tawde, Akalkotkar, et al., 2012a; Ubale et al., 2013a). Improved uptake of the particles compared to the solution results in higher cytotoxic T-lymphocytes (CTLs) response against the cancer cells. The antigen presenting cells
(APCs) in the body easily phagocytose these microparticles recognizing it as an antigen and generate an immune response (Chablani, Tawde, Akalkotkar, et al., 2012a; X.-Q. Zhang et al., 2007). Further, they are drained into the nearby lymph nodes where they activate various other immune cells. Thus, the particulate delivery systems may mimic pathogens that are commonly recognized, phagocytosed and processed by professional antigen-presenting cells (APC) (24). The microparticulate drug delivery system can be used to assimilate various antigens in one delivery system that can reduce the number of doses as well as reduce the different vaccination regimen (15-16).

The American Cancer Society expects approximately 22,280 new cases of ovarian cancer in the USA by the end of 2016. According to the National Cancer Institute around 14,240 deaths are projected to occur due to ovarian cancer in the USA in 2016. Currently, ovarian cancer patients are treated by surgical removal of tumor cells followed by chemotherapy, radiation therapy or hormone therapy. Neither chemotherapy nor radiation therapy acts specifically against tumor cells nor hence they have serious side effects on normal cells. Due to known side effects, both therapies require dose and exposure time regulation. Evidently, all tumors are highly adaptive and have the ability to become insensitive towards chemo and radiation therapy (Casanovas, 2012). Even with the advancement in cancer therapy strategies, many patients still face the risk of cancer relapse (Schirrmacher, Feuerer, Beckhove, Ahlert, & Umansky, 2002). The aim of any cancer therapy is to inhibit micro-metastatic disease, avoid recurrence and enhance the long-term overall survival. Immunotherapy is a treatment strategy that uses the body’s immune system to help fight cancer. Cancer immunotherapy involves two steps, a) Stimulation of immune cells against cancer cells overexpressing certain protein and b)
Depleting the immunosuppressive immune cells. Most cancer cells overexpress certain proteins which are immunogenic and are a potential source of antigens for immunotherapy (Bolhassani, Safaiyan, & Rafati, 2011). Previously, we have explored tumor-associated antigens (TAAs) as immunogens for generating an immune response against cancer cells (Akalkotkar et al., 2012; Aravindaram, Wang, Yin, & Yang, 2014; Chablan, Tawde, Akalkotkar, et al., 2012a; Elliott & Head, 2013; Shariat, Badi, Jaafari, & Mortazavi, 2014; Tawde et al., 2012a).

Although these TAAs have been reported to produce a weak immune response, their immunogenicity can be enhanced using various adjuvant or immune-modulating drugs (Aravindaram et al., 2014; Mizukoshi et al., 2011; Win et al., 2012). A therapeutic prostate cancer (Provenge®) was introduced into the market in April 2010 by Dendron (Seattle, WA), which involves isolating dendritic cells (DCs) from prostate cancer patients and stimulating the in-vitro activated cells with a prostate-specific fusion protein and then re-introducing these DCs back into the patient. This procedure when carried out three times was found to marginally increase survival in prostate cancer patients by 4 months in clinical trials and the cost for the vaccine was $93,000 (Cheever & Higano, 2011b). Moreover, in most cancers, the antigens are not well characterized and often undergo mutations, thus limiting its use. The cancer vaccine developed in our lab consists of a pool of tumor-associated antigens (TAAs) prepared by lysing tumor cells. Vaccine microparticles were prepared by spray drying these TAAs along with a blend of enteric coating polymers for protection and sustained release effect. By using TAAs as the cancer vaccine, we ensure that an immune response will be generated against all the proteins overexpressed in the tumor cells.
1.1 Microparticle-based vaccination

There are several challenges in developing an effective oral vaccine, which includes the maintenance of vaccine integrity and stability, avoidance of immune tolerance, and induction of strong protective immunity. For an oral vaccine to be effective, first it must withstand gastro-intestinal degradation and release the antigenic material in a form that is absorbed in the intestine for subsequent interaction with the immune system. For this to occur, the antigen will be encapsulated in an enteric [hydroxy propyl methyl cellulose acetate phthalate (HPMCAS)] and sustained release polymer [β-cyclodextrin and ethyl cellulose] to make them insensitive to break down under acidic conditions in the stomach and release the antigen in a controlled manner, in the small intestine. Orally delivered vaccines, especially particulate antigens, are recognized by microfold (M) cells, which sample the antigen, and by dendritic cells and macrophages that are present in the Peyer’s patches (Chablani, Tawde, Akalkotkar, et al., 2012a; Pappo & Ermak, 1989). These microparticulate vaccines can be delivered via the transdermal route where the vaccine antigens are taken up by the dermal dendritic cells and thus lead to an effective immunization strategy. The particulate system in the order of micron size provides numerous benefits for vaccine delivery as they mimic the invading pathogens (6) However, larger particles provide longer duration of antigen release compared to the smaller counterparts and hence have a dramatic effect on immunogenicity.

The particulate Delivery system, in general, provides a lot of advantages compared to traditional methods of drug delivery. Some of them include:

- The capability of delivering via oral, transdermal and parenteral routes
- Can accommodate small and large molecules
- Multi-drug therapy using one particle
- Stable delivery system for bioactive molecules
- Easy manufacturing and scale up
- Eliminated cold-chain requirements

Numerous researchers have studied the concept of oral vaccine and currently, there are two oral vaccines in the market. The first one is the Polio Sabin™ oral vaccine by SmithKline Beecham Biologicals, and the other one is Dukoral™ oral vaccine for traveler’s diarrhea and cholera manufactured by SBL AB, Stockholm, Sweden. Apart from the usual advantages of oral vaccines in terms of patient compliance, ease of administration, lower cost of production and transportation, there is an added advantage of inducing both mucosal and systemic immunity. Recent studies have suggested that in order to produce a more robust immune response, both systemic and mucosal immunity have to be stimulated. Nevertheless, the oral delivery of proteins and vaccines face major hurdles such as the acidic environment of the stomach, the enzymatic environment of intestine and challenges regarding protein stability to achieve maximum bioactivity.

1.2 Oral Vaccines

**Current status and challenges of oral vaccination:** Oral vaccines for polio (Drutz & Ligon, 2000) and cholera (López-Gigosos, Plaza, Díez-Díaz, & Calvo, 2011), have been used very successfully with no toxicity issues. Experimental studies have shown that delivery of vaccines such as recombinant proteins with cholera toxin adjuvant, plasmid DNA encoding HIV immunogens, and whole inactivated viruses induced oral and
systemic humoral and cellular immune responses (Huang et al., 2008; Kataoka et al., 2004; T. Zhang, Hashizume, Kurita-Ochiai, & Yamamoto, 2009). Some of the current adjuvants on the market that act as delivery vehicles, such as liposomes, oil adjuvants, endotoxin adjuvants, and Freund’s adjuvants may help in targeting antigens to immune competent cells, but have disadvantages such as high costs of production (such as liposomes) or serious toxicity issues (Freund’s, endotoxin, oil-based adjuvants) (Banchereau & Steinman, 1998). However, research in oral vaccination along with adjuvants for cancer is minimal. Recently, four oral DNA vaccines have been tested against melanoma, breast, colon and lung carcinoma in different mouse models (Xiang, Luo, Niethammer, & Reisfeld, 2008). These vaccines were all evaluated for their ability to induce immunity against self-antigens resulting in suppression of tumor growth and dissemination. However, the limitation of DNA vaccines relates to the fact that these are specific for a single antigen, which limits the breadth of immune response. Also, these oral DNA vaccines were all tested in a prophylactic setting and few were capable of suppressing already established experimental tumor metastases in therapeutic animal models (Xiang et al., 2008)

Oral vaccine delivery is an attractive mode of immunization because of its ease of administration, low manufacturing cost, and patient compliance. However, the major hurdle in oral vaccine delivery is the protection of antigen from acidic and enzymatic degradation in the gastrointestinal tract. Another obstacle to be considered while designing an oral vaccine is the probability of oral tolerance (15). Low particle uptake and gastric degradation products of antigens can cause oral tolerance. One of the ways to avoid these issues is to formulate microparticles by using enteric coating polymers.
Intestinal Peyer’s patches are the predominant sites for uptake of such particles upon oral administration (16). The particle uptake depends on various factors such as size, charge, and hydrophobicity (17, 18). For oral delivery, it has been reported that particles of size less than 5 µm with a positive charge and hydrophobic nature can preferentially enter the Peyer’s patch of the small intestine (19). Orally delivered vaccines, especially particulate antigens are recognized and sampled by microfold (M) cells in Peyer’s patches. This is followed by transport of the particles to underlying follicles and to professional antigen presenting cells (APCs) such as dendritic cells and macrophages. As shown in Figure 1, these APCs can phagocytose the particles, process them and present them on both MHC Class I, through cross-priming, and MHC Class II molecules due to which both T and B-cells can be triggered.(20, 21).

Figure 1: Vaccine microparticle uptake by M cells of Peyer's patches in the small intestine after oral delivery
This strategically designed particulate vaccine incorporated enteric polymers to protect the breast cancer antigens from harsh gastric conditions as well as targeting ligands to enhance its uptake from M-cells of the Peyer’s patches in the small intestine. Polymeric microparticles offer several advantages such as activation of mucosal immunity, protection from gastric pH conditions, sustained release and higher uptake of vaccine by phagocytic cells (Akalkotkar et al., 2012; Chablani, Tawde, Akalkotkar, et al., 2012a; Hardy et al., 2013; P.-J. Lou et al., 2009; D T O’Hagan, 2001; Derek T O’Hagan & Singh, 2003). Microfold cells (M cells) are present in the Peyer’s patch of the intestine engulf vaccine microparticles. M cell targeting ligand Aleuria Aurantia Lectin (AAL) was incorporated to the vaccine formulation in order to enhance microparticle uptake.

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**Figure 2: Immune response triggered by vaccine**
M cells house numerous antigen presenting cells (APCs), which internalize the vaccine microparticles express the antigen on its surface as MHC I or II complex (Pickard & Chervonsky, 2010). Polymeric vaccine microparticles offer several advantages such as protection from gastric pH conditions, sustained release and higher uptake of vaccine by phagocytic cells (Akalkotkar et al., 2012; Chablani, Tawde, Akalkotkar, et al., 2012a; Hardy et al., 2013; P.-J. Lou et al., 2009; D T O’Hagan, 2001; Derek T O’Hagan & Singh, 2003). Antigen delivery by microparticles leads to better antigen presentation by APCs such as dendritic cells (X.-Q. Zhang et al., 2007). Dendritic cells (DCs) and macrophages are the major effector cells of the immune system. They form an important part of the linkage between innate and adaptive immune response (Figure 2). DCs internalize the microparticles and lyse the microparticles in order to express the antigens or their fragments on their surfaces (Andrianov & Payne, 1998; Hardy et al., 2013; L Thiele et al., 2001; Lars Thiele, Diederichs, Reszka, Merkle, & Walter, 2003). The dendritic cells are crucial for binding and priming the T cells against antigens expressed on its cells surface. In this study, we have evaluated the effect of delivery of antigen via microparticles on the dendritic cell activation and antigen presentation. Nitric oxide is produced in antigen presenting cells (APCs) like dendritic cells and macrophages by a nitric oxide synthase isoform (iNOS or NOS2), which is strongly up-regulated following an infection by an antigen (MacMicking, Xie, & Nathan, 1997). Following antigen expression, the T cells bind to the dendritic cells and this triggers a further adaptive immune response against the tumor-associated antigens. The antigen presenting cells release cytokines upon exposure to an antigen. Cytokines are small proteins that play an important role in cell signaling. TNF-α and IL 6 are examples
of cytokines released by the antigen presenting cells such as macrophages and dendritic cells. IL-6 is a cytokine that plays an essential role in differentiation of B cells into antibody-secreting cells. TNF-α plays an important role in apoptosis, cell survival, inflammation, immunity and also has anti-tumor properties (Horssen, Hagen, & Eggermont, 2006). Interferon-gamma (IFNγ) is a cytokine that is released by antigen-stimulated lymphocytes. It is an important activator of major histocompatibility complex I (MHC I). It promotes Th1 differentiation ultimately leading to the formation of cytotoxic CD8+ T cells (Whitmire, Tan, & Whitton, 2005). Interleukin 4 (IL4) is another cytokine released by lymphocytes that induce differentiation of Th0 to Th2 cells. It up-regulates the production of MHC II and suppresses the production of Th1 mediated immune response. The adaptive immune response is further classified as Humoral or Cellular immune response. The humoral immune response is mediated by antibody-producing B cells. The CD45 R protein is expressed by B cells and is a widely used identifier marker for the same. Similarly, CD25 is a protein commonly expressed by the T cells. The T cell-mediated immune response is specific for all cells expressing those tumor-associated antigens. The cytotoxic T cells or CD8+ T cells play a crucial role in killing the tumor cells. Along with CD8+ T cells, other major immune cells are the T helper cells or the CD4+ T cells. A CD4+ T cell is also very important immune cell as it helps further differentiation of cytotoxic T cells and also assists in the development of antibodies against the antigens. Hence a balanced humoral and cellular immune response will help in the faster killing of tumor cells. The CD62L+ T memory cells are a subset of cancer-fighting T cells. These cells will remain dormant till they encounter the same tumor-associated antigen. At the second encounter, the T memory cells recognize cancer
cells expressing the same antigens and create a faster and stronger immune response. In this study we will evaluate the effect of vaccine microparticles and other immune modulators on the entire immune system, beginning with the innate immune response that further leads to the development of a specific adaptive immune response.

1.3 Transdermal delivery of cancer vaccines

Various proteins and peptides have been approved or are being evaluated in clinical trials for the treatment of cancer. Due to limited oral bioavailability of such antigens, injectable routes of administration are currently being used. However, transdermal route of administration has recently gained a focus on vaccine delivery as it is a non-invasive route and it can overcome issues associated with parenteral delivery. However, its efficacy is restricted by a rate-limiting barrier of stratum corneum. Various methods are being used to breach this barrier facilitating permeability of skin to the macromolecules. These techniques include physical methods such as microneedles or chemical methods such as the use of penetration enhancers or laser ablation techniques.

Transdermal vaccine delivery is an attractive mode of immunization because of its ease of administration and requires no specially trained personnel and thus may eliminate many problems associated with needle injections (27). Transdermal delivery is considered as the best route for vaccine administration because of the skin-associated lymphoid tissue which comprises of Langerhans cells, dermal dendritic cells, lymph nodes and subsets of T-lymphocytes. The microparticles are taken up by these immune cells in the skin, which trigger mucosal as well as a systemic immune response (28). Langerhans cells are dendritic cells that activate T cells and induce a strong immune response and occupy around 20% of the skin’s area. They can induce immunity by either
endogenous antigen or exogenous antigen uptake. The endogenous antigen is processed
and presented by MHC Class I to CD8+ T cells and the exogenous antigen is presented
by MHC Class II to CD4+ T cells. Microparticles, when administered transdermally, can
be taken up by the antigen presenting cells (APCs). Also, the vaccine microparticles can
generate a better immune response when compared to the solution form. The vaccine
microparticles are formulated into microneedles for their ability to deliver the antigens
into the skin.

The skin provides a unique site for the vaccination purposes as it is easily accessible and
houses various immune cells for an efficient immune response against a range of
antigens. Skin serves as a barrier against various pathogens and is equipped with the skin
associated lymphoid tissues (SALT) to combat any insult from invading pathogens.

Various skin cells assist in the generation of an effective immune response. Keratinocytes
are the most predominant (95%) epidermal cells in the skin. They can be activated by
pathogens and result in the production of cytokines, which in turn recruits dendritic
cells/antigen-presenting cells to the site of action leading to initiation of the immune
response. Skin host’s special kind of dendritic cells, the Langerhans cells comprise of
only 2% of the total cell population in the epidermis but due to their extended dendrites
spread in the epidermal layer, they cover over 25% of the skin surface. These are
professional phagocytic cells efficient in immune surveillance and further signaling to the
T-cells present in their vicinity. Activated macrophages and T-cells drain into nearby
lymph nodes leading to an enhanced immune response.
1.4 Adjuvants

Adjuvanted cancer vaccines contain at least two components: a specific antigenic component and a nonspecific adjuvant component. Several adjuvants have been developed to augment the potency of cancer vaccines in order to generate appropriate types of response to control the disease, in particular, the induction of potent and long-lasting CD8+ T cells. These adjuvants have ranged from general immune stimulants, such as the live attenuated tuberculosis vaccine Bacille Calmette–Guérin (BCG) derived from Mycobacterium bovis, to molecularly defined compositions that trigger specific receptors. In this section, we discuss recent advances in the development of cellular or T-helper (Th) (associated with T-cell immunity) adjuvant products for therapeutic cancer vaccines. There have been various attempts to classify adjuvants and probably the most commonly applied classification system is based on the underlying mechanisms by which they act (Derek T. O’Hagan & De Gregorio, 2009; Schijns, 2000). However, adjuvants act by several different mechanisms, which makes their classification rather complex and subjected to regular redefinitions as additional innate pathways of immunity are discovered. Vaccine adjuvants are characterized in different classes of compounds such as alum salts and other mineral adjuvants, bacterial derivatives, vehicles, and cytokines. However, related compounds frequently have antagonizing immune-modulating properties; for example, the capacity to stimulate Th1 or Th2 immunity. Adjuvants such as MF59® (Novartis Vaccines and Diagnosis) and immune-stimulatory complexes (ISCOMs) enhance T-cell and antibody responses. More polarized Th1-cell responses are elicited by adjuvants that incorporate agonists of Toll-like receptor TLR3, TLR4, TLR7/8, and TLR9, while complete Freund's adjuvant (CFA) induces mixed Th1 and
Th17 responses (Coffman, Sher, & Seder, 2010). The toll-like receptor agonists are specific adjuvants that bind specifically to the Toll-like receptors present on the dendritic/macrophage cell surface as well as within the endolysosome (Figure 3). These adjuvants act in several ways: i) By inducing surface expression of antigen-presenting molecules such as MHC I and II on APCs, ii) By activating APC’s and up-regulating expression of co-stimulatory molecules such CD40, CD80, CD86 (Figure 21) and lastly iii) by enhancing the release of cytokine and chemokine. The mechanism of action of TLR agonists is described in further detail in chapter 5.

An enhanced immune response is generated as a result of a combination of these events. It has been established that APCs get activated via recognition of microbial products. APC’s like macrophages and DCs play a crucial role as a link between innate and adaptive immune response (Ubale, Gala, Zughai, & D’Souza, 2014a). DCs provide two crucial signals for optimum priming and activation of T cell (McAdam, Schweitzer, & Sharpe, 1998a). The two signals are i) Antigen presentation as a complex with MHC I or II and ii) co-stimulatory signals such as CD40 or CD86 which bind with CDD40L and CD28 on T cells respectively (Greenfield, Nguyen, & Kuchroo, 1998; Grewal & Flavell, 1998; PLANELLES, THOMAS, MARAÑÓN, MORELL, & LÓPEZ, 2003)
Figure 3: Toll-like receptor (TLR) signal transduction in an antigen-presenting cell. All TLR family members use the MYD88 (myeloid differentiation primary response protein 88) adaptor, except TLR3, which recruits TRIF (TIR domain-containing adaptor protein inducing IFNbeta)

We have evaluated the efficacy of combining these adjuvants along with breast cancer vaccine in inducing antigen expression as MHC class I and II complex and co-stimulatory molecules CD40 and CD86 for priming T cells. A Th2 type immune response is characterized by i) Expression of antigen along with MHC II, ii) Up-regulation of co-stimulatory molecules CD40 and iii) the release of cytokines such as IL-4, IL-5, IL-6, IL-
10, and IL-13. Similarly, A Th1 type immune response is characterized by i) Expression of antigen along with MHC I, ii) Up-regulation of co-stimulatory molecules CD80/86 and iii) the release of cytokines such as IL-2, IL-10, and IFN-γ. Several studies have reported that a balanced Th1/Th2 kind of immune response is required for prevention and treatment of cancer. Adjuvants have the ability to determine the quality and quantity of immune response. Adjuvants can direct the immune response to either Th1 or Th2 or a balanced Th1/Th2 kin of immune response.

In order to achieve a stronger cellular immune response, we have explored the option of combining cancer vaccines with various adjuvants in this study. We have divided the adjuvants into two kinds based on their mechanism of actions. Firstly we look at adjuvants that are already used in various marketed vaccines in USA and Europe, Alum and Addavax (like MF59). Both adjuvants are known to induce a strong humoral and cellular immune response. But their mechanism of action is yet unknown. Secondly, we look at combining cancer vaccines with adjuvants that are toll-like receptor agonists. The adjuvants being evaluated here include CpG (TLR 9 agonist), MPL (TLR4) and R848 (TLR7/8). A novel in vitro method to evaluate the immunogenicity of these combinations of vaccine and adjuvants is developed in this study. The aim of this part of my project is to screen adjuvants for enhancing the immunogenicity of cancer vaccines. As different adjuvants act by different mechanisms, each induces a different immune response upon combining with a cancer vaccine. For example, non-specific adjuvants such as Alum and MF59 would enhance uptake of antigens as well as induce dendritic cell activation due to its particulate nature. Whereas toll-like receptor agonists that act on specific receptors on dendritic cells would enhance immunogenicity by inducing class I
and II MHC molecule expression and cytokine release. Therefore by the end of this study, we will have a combination of cancer vaccine with an adjuvant with high immunogenicity. In future, the immunogenicity of vaccine and adjuvant combinations can be confirmed using an *in vivo* animal model.

**1.5 Aims:**

The aim of my thesis project was to develop a novel immune-based therapy for treatment of cancer by combining microparticulate cancer vaccines with various immune-modulators. We have used multiple approaches to enhance the immunogenicity of ovarian cancer vaccine: a) Use of cyclophosphamide to inhibit immune-suppressive regulatory T cells; b) Use of vaccine adjuvants that can enhance the overall potency of the formulated vaccines.

The latter part of my thesis discusses novel delivery systems such as Oral dissolving films (ODFs) for buccal and sublingual immunization and microneedles for transdermal immunization. Lastly, in my final aim, we have used a novel approach to develop a vaccine against gonorrhea infections using formalin fixed whole cell as the vaccine antigen. All of these aims are divided into different chapters to follow.
CHAPTER 3
FORMULATION AND DEVELOPMENT OF AN OVARIAN CANCER VACCINE

3.1 INTRODUCTION

Ovarian cancer is the most lethal gynecological cancer and the fifth most leading cause of cancer-related deaths in women globally and more specifically in the US. According to the data from Center for Disease Control (CDC), each year around 20,000 women in the US are diagnosed with ovarian cancer and 15,000 women sadly die each year due to ovarian cancer ("Cancer Facts & Figures - 2013"). The major reason for high mortality is that in more than 70% of women with ovarian cancer are diagnosed with advanced disease (Rosenberg, Yang, & Restifo, 2004). The overall one-year survival rate i.e. percentage of women who survive at least one year after the cancer is detected, excluding those who die from other diseases, of women with ovarian cancer is 75%. But Five-year survival rates for women with advanced disease range from 20% to 30% ("Cancer Facts & Figures - 2013").

The reason for late detection of ovarian cancer is that the symptoms are very vague in the primary stages. Many women with early-stage cancer of the ovary don't report any symptoms at all. Early symptoms can include pain in the lower abdomen or side, and/or a bloated, full feeling in the abdomen. Once cancer has grown out of the ovary, it can cause symptoms from the tumor growing anywhere in the area between the hip bones (the pelvis). This can cause lower tummy (abdominal) pain, back pain, passing urine more often than usual, constipation, pain during sex, or a swollen abdomen.
Women may also have irregular periods or bleeding after the menopause. Advanced ovarian cancer can cause even more symptoms because cancer has spread into the abdomen or elsewhere in the body. These can include a loss of appetite, feeling or being sick, constipation, tiredness, shortness of breath, or a noticeable swelling of the abdomen (Berkowitz, Rim, & Peipins, 2011).

There is a close correlation between the ovarian cancer stage and survival, various stages of ovarian cancer are been characterized by International Federation of Gynecology and Obstetrics Criteria (2002) which are summarized in Table 1 (Berkowitz et al., 2011).

Table 1: Ovarian cancer and its stages

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<td>Tumor limited to one ovary; capsule intact, no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings</td>
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<tr>
<td>IB</td>
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<tr>
<td>Tumor limited to both ovaries; capsules intact, no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings</td>
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<td>Stage II</td>
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<tr>
<th>Stage III</th>
<th>Tumor Involves One or Both Ovaries With Peritoneal Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>III A</td>
<td>Microscopic peritoneal metastasis beyond pelvis</td>
</tr>
<tr>
<td>III B</td>
<td>Macrosopic peritoneal metastasis beyond pelvis 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>Stage IV</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td></td>
</tr>
<tr>
<td><strong>Peritoneal metastasis beyond pelvis more than 2 cm in greatest dimension or regional lymph node metastasis, or both</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Distant Metastasis (Excludes Peritoneal Metastasis) to Liver Parenchyma or Other</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Visceral Organs or a Malignant Pleural Effusion</strong></td>
<td></td>
</tr>
</tbody>
</table>

An ovarian cyst, which forms on the surface of the ovary, is different than a noncancerous tumor and usually goes away without treatment. An ovarian cyst is not cancerous. Like most other cancers, ovarian cancer begins when normal cells in an ovary change and grow uncontrollably, forming a mass called a tumor. There are more than 30 types of ovarian cancers which are classified based on the type of cell they start from. There are three major types of ovarian cancer (Berkowitz et al., 2011):

1. **Epithelial Carcinoma:** It makes up 85% to 90% of ovarian cancers. This type of cancer begins in cells on the outer surface of the ovary.

2. **Germ cell tumor:** This uncommon type of ovarian cancer develops in the egg-producing cells of the ovaries. This type of tumor is more common for women ages 10 to 29.
3. **Stromal tumor:** This rare form of ovarian cancer develops in the connective tissue cells that hold the ovaries together and make female hormones.

The risk of developing ovarian cancer appears to be affected by several factors. The more children a woman has and the earlier in life she gives birth, the lower her risk for ovarian cancer. Certain genes defects (BRCA1 and BRCA2) are responsible for a small number of ovarian cancer cases. Women with a personal history of breast cancer or a family history of breast or ovarian cancer have an increased risk for ovarian cancer. Women who take estrogen replacement for 5 years or more seem to have a higher risk of ovarian cancer. Like other cancers, the chances of getting ovarian cancer are increased with increasing age (Stuart, 2003).

The first-line treatment for advanced ovarian cancer involves surgery followed by chemotherapy. Depending on the type and extent of spreading of cancer, following surgery is performed (Stuart, 2003):

- Removal of the uterus
- Removal of both ovaries and fallopian tubes
- Partial or complete removal of the omentum, the fatty layer that covers and pads organs in the abdomen
- Examination, biopsy, or removal of the lymph nodes and other tissues in the pelvis and abdomen
Chemotherapy is suggested after the surgery for the remaining disease if any. It is followed by radiation in many cases. This is the standard therapy being approved in the US (Stuart, 2003).

However, cancer relapses within relatively short periods of time even after treatment (Finn & Forni, 2002; Sheng & Huang, 2011). Moreover, chemotherapeutic treatments are toxic and/or of minimal therapeutic value. Therefore it is been realized that an immune intervention could be utilized to target residual disease and possibly dormant “cancer stem cells”, thus affording a more durable response compared with conventional chemotherapy with small molecules therapies, resulting in a novel approach, Cancer vaccines. Immune therapies thus complement chemotherapy, in a combination approach to maximize the impact on cancer. The approach is to significantly utilize T reg cells as a major roadblock to effective immunizations against Cancer.

Currently, there are a couple of clinical trials going on ovarian cancer vaccines in the US. One of them is in Phase 0 which is for recurrent ovarian, fallopian tube or primary peritoneal cancer. This vaccine consists of autologous dendritic cells (DC) loaded in vitro with lysate from autologous oxidized tumor cells administered intranodally alone or in combination with intravenous Bevacizumab and Cyclophosphamide. The second in clinical trials is a Phase 2 ovarian vaccine named Cvac by Prima BioMed Ltd. This is also a dendritic cell vaccine administered to ovarian cancer patients in first or second remission and to determine its potency to prevent cancer from reoccurring. Apart from these two vaccines several vaccines are underway in clinical trials and most of them have not progressed beyond phase I/II studies. Therefore, there is no ovarian cancer vaccine commercially available to date. Our approach addresses many
of the problems associated with the current vaccine therapies such as time involved in vaccine preparation, specific antigen isolation/purification including the high vaccine costs.

Previously in our laboratory, we have investigated whether vaccination with microparticles containing the ovarian cancer antigens can prevent/retard ovarian cancer growth. Oral vaccine delivery is an attractive mode of immunization because of its ease of administration, low manufacturing costs, and patient compliance. We demonstrate the efficacy of the microparticulate vaccine formulations which was evaluated in vivo in mouse tumor model, using the ID8 murine ovarian cancer cell line as a solid tumor model. This vaccine was given by oral, transdermal and subcutaneous routes and compared the effect of the route on the immunization study. Moreover, we have investigated the role of M-cells in particle uptake mechanism which is a crucial step in case of oral vaccination. We have used a microfold (M-cell) inducing agent, RANKL, Receptor Activator of NF-κB ligand and the enhancement in immune response was evaluated using microparticles with a model antigen, Ovalbumin with Cholera toxin as an adjuvant. We demonstrated the efficacy of vaccine microparticles containing whole cell lysate of ID8 ovarian cancer cells in retarding tumor growth in murine models. Spray drying process and the formulation used for this purpose could retain the immunogenicity of vaccine resulting in B-cell as well as T-cell response. Thus, the microparticulate vaccine provides a promising approach in terms of cost-effectiveness, ease of production and patient compatibility.

With our previous success with ID8 whole cell lysate vaccine, we decided to investigate various adjuvants for their ability to further potentiate the vaccine. As
discussed, an adjuvant is a substance that is added to a vaccine to increase the body's immune response to the vaccine. Vaccines containing adjuvants are tested for safety in clinical trials before they are licensed for use in the United States, and they are continuously monitored by CDC and FDA.

3.2 Materials and Methods

3.2.1 Materials

The murine ovarian cancer cell line, ID8 was kindly provided by Dr. Katherine Roby, Kansas University Medical Center, Kansas City, KS. Six to eight-week-old C57BL/6 female mice were purchased from Charles River Laboratories, Wilmington, MA. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), RPMI-1640 medium and Dulbecco’s phosphate buffer saline (DPBS) were purchased from Atlanta Biologicals, Atlanta, GA. Hydroxyl propyl methyl cellulose acetate succinate (HPMCAS), Hydroxyl propyl methyl cellulose (HPMC), Ethyl cellulose (EC) was purchased from AQOAT, FMC Biopolymers, Philadelphia, PA. Mouse plasma was obtained from Biochemed, Winchester, VA. Aleuria aurantia lectin (AAL) was obtained from Vector Labs, Inc., Burlingame, CA. Chitosan glycol, trehalose, and Insulin-transferrin-sodium selenite media supplement were obtained from Sigma, St. Louis, MO. MPL, R848 and cholera toxin from Vibrio cholera were purchased from Sigma Aldrich. AddaVax™ (similar to MF59), CpG oligonucleotide and Alhydrogel® 2% (Aluminum hydroxide gel) were obtained from InvivoGen (San Diego, CA). Flow cytometry cell markers, anti-mouse CD4 PE, anti-mouse CD8a FITC, anti-mouse NK and anti-mouse/human CD45R (B220) FITC were purchased from eBioscience, San Diego, CA. The goat anti-mouse HRP-IgG and anti-IgG subtypes were purchased from Bethyl
Laboratories, Montgomery, TX and Sigma, St. Louis, MO respectively. The AdminPen device was purchased from nanobioscience. All other materials used were of analytical grade.

3.2.2. Preparation of whole cell lysate of ID8 ovarian cancer cell line

The murine ovarian cancer ID8 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 4% fetal bovine serum, penicillin/streptomycin solution (Sigma, St. Louis, MO), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Insulin-transferrin-sodium selenite media supplement, Sigma, St. Louis, MO) (31, 32). They were maintained at 37⁰C in a humidified incubator with 5% CO₂ atmosphere until they were 90% confluent. The whole cell lysate was prepared using hypotonic lysis buffer (10mM Tris and 10mM NaCl). Briefly, the cells were washed with cold phosphate buffered saline (PBS), pH 7.4 six times. The flasks were then treated with hypotonic buffer and subjected to five 15 min freeze-thaw cycles at temperatures of -80⁰ C and 37⁰ C respectively to obtain the cell lysate (11). The lysate obtained was stored at -80⁰ C until used.

3.3.3 Preparation of vaccine / adjuvant microparticles

The vaccine formulation was prepared by using spray drying technique described elsewhere (33-42). Briefly, Cellulose Acetate Phthalate (CDP), hydroxyl propyl methyl cellulose acetate succinate (HPMC-AS) and Ethyl cellulose were dissolved in an alkaline solution (pH 8), followed by addition of chitosan glycol. Trehalose, tween 20 and AAL were added to the solution. Whole cell lysate obtained from ID8 cells (5% w/w) was added to this feed mixture and temperature was maintained at 4⁰ C throughout the
spraying. This aqueous solution was spray dried using Buchi B-290 Mini Spray Dryer (Buchi Corporation, New Castle, DE). The quantity of the polymers, antigen, and adjuvants for a batch of 1000 mg vaccine microparticles is given in table 2. The adjuvant particles (2% w/w loading) were also prepared separately using the same method. The microparticles obtained were stored in desiccators at -20°C. Each preparation was carried out in triplicates. Product yield was calculated as the weight percentage of the microparticles obtained as the final product in comparison to the total solid content of the materials sprayed.

Table 2: Formula for the vaccine / adjuvant microparticles and weights for a 1000 mg batch size of the vaccine microparticles.

<table>
<thead>
<tr>
<th>Antigen / Adjuvant</th>
<th>Percentages</th>
<th>1000 mg batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD</td>
<td>30%</td>
<td>300 mg</td>
</tr>
<tr>
<td>EC</td>
<td>30%</td>
<td>300 mg</td>
</tr>
<tr>
<td>HPMC-AS</td>
<td>25%</td>
<td>250 mg</td>
</tr>
<tr>
<td>Trehalose</td>
<td>5%</td>
<td>50 mg</td>
</tr>
<tr>
<td>Chitosan</td>
<td>5%</td>
<td>50 mg</td>
</tr>
<tr>
<td>Water</td>
<td>q.s.</td>
<td>q.s. to 100 ml</td>
</tr>
</tbody>
</table>

3.3.4. Microparticle recovery yield

Recovery yield of the microparticles after spray drying was calculated for all the batches formulated. Percent recovery yield was evaluated using the following formula:
Percentage Recovery Yield (%) = \frac{\text{Weight of microparticles after spray drying}}{\text{Weight of all ingredients before spray drying}} \times 100

3.3.5. Particle size distribution

The particle size of the optimized formulation was evaluated using the Spectrex Laser Particle Counter that works on the principle of laser diffraction. Two mg of the particles were suspended in 1 ml deionized water, vortexed well, and then analyzed by laser diffraction on the particle counter. Particle size was measured in triplicate for empty as well as antigen-loaded particles and contrasted.

3.3.6. Zeta potential measurement

Five micrograms of microparticles were suspended in 1 ml of deionized water, transferred to a zeta potential measurement cuvette, and measured using a Malvern Zetasizer. Zeta potential was measured in triplicate for the control formulation and contrasted with the antigen-loaded microparticles.

3.3.7. Scanning electron microscopy of the microparticles

Scanning electron microscopy (SEM) was performed to evaluate microparticle size distribution and surface morphology. Microparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum-coated with a thin layer (100-150Å) of gold, observed under 20kV by scanning electron microscope - Phenome benchtop SEM, Nanoscience Instruments, Phoenix, AZ.

3.3.8. Loading efficiency

To determine loading efficiency, 5 mg of the microparticles were accurately weighed and added to 1mL of 1X PBS, pH 7.4 at room temperature. Proteins were allowed to extract in PBS as the enteric particles dissolved at pH 7.0. The extracted
solution was analyzed for total lysate protein content using Biorad DC™ protein assay (Biorad, Hercules, CA) in comparison to placebo particles.

3.3.9. Fabrication of Ovarian cancer vaccine microparticles loaded microneedles

Table 3: Formula for the preparation of microneedles.

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Percent w/w</th>
<th>200 mg batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>10%</td>
<td>20 mg</td>
</tr>
<tr>
<td>Trhealose</td>
<td>25%</td>
<td>50 mg</td>
</tr>
<tr>
<td>Maltose</td>
<td>25%</td>
<td>50 mg</td>
</tr>
<tr>
<td>PVA</td>
<td>20%</td>
<td>40 mg</td>
</tr>
<tr>
<td>HPMC</td>
<td>20%</td>
<td>40 mg</td>
</tr>
</tbody>
</table>

Microneedles formulation was prepared as per formula mentioned in Table: 3. PVA, HPMC, Maltose, and Trehalose were added to a 1.7 mL microcentrifuge tube. The contents were dissolved in as less water as possible (e.g. 200 mg of total solid content can be dissolved in 600 µL water). To ensure maximum dissolution, the centrifuge tube was vortexed for 1 minute. Then approximately 1/5th quantity (of water that was added to dissolve the solids) of Ammonium hydroxide (NH₄OH) (6N purchased from Fisher Scientific, Pittsburgh, PA) is added to the microcentrifuge tube (here, 120 µL) and vortex again (2 minutes). The tube is kept aside for 2 hours to ensure complete dissolution of the
matrix polymers. Once the solution is clear, the weighed amount of blank or vaccine or adjuvant microparticles particles are added in the end. 150 – 200 µL of the final formulation is poured into the molds. Centrifugation is done in the fixed angle centrifuge in order to remove air bubbles and to force the formulation to go into the microneedles mold. The maximum speed is 2000 rpm which is achieved step wise, in order to avoid jerks in the rotation process for 10 mins. Speed was lowered gradually for the same reason as above. After this centrifugation step, 200 µL blank formulation i.e. formulation without vaccine microparticles is added to the top for the backing layer. Centrifugation is repeated as per the same method mentioned above for the backing layer. Once the backing layer is formed, the molds are placed in an incubator at 32°C for overnight drying. On the following day, the molds are placed in the vacuum desiccator until further used.

3.3.10. Cell culture

Murine dendritic cells (DC2.4) were grown in RPMI 1640 with L-glutamate supplemented with 10 % FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, 1 % sodium pyruvate and 1% nonessential amino acids. Culture flasks were incubated at 37°C with humidity under 5% CO₂. DC2.4 cell line is an immortalized murine dendritic cells generated from bone marrow-derived cells from the femur of the C57BL/6 mouse (Shen, Reznikoff, Dranoff, & Rock, 1997). DC2.4 clone was selected because it expresses the following antigen presenting and co-stimulatory molecules: MHC I, MHC II, DEC205, 33D1, B7-1 (CD80), B7-2 (CD86), CD32, CD54, mac-1(CR3), Moma-2 and Ly 6 A/E. This DC2.4 cell line has functional antigen presenting activity reported by Shen et al (Shen et al., 1997).
3.3.11 Quantification of antigen presentation molecule (MHC II) expression

Dendritic cells (DC2.4) were plated at 3 × 10^5 DC2.4 cells per well on a 48 well plate and incubated at 37°C for 24 h to adhere and stabilize. The adherent cells were pulsed with 250 µg vaccine microparticles (25 µg of antigen) along with the equal quantity of adjuvant microparticles in each well and incubated at 37°C for 16 h. Equal amount blank microparticles without any antigen or adjuvant were used as controls in every experiment. Additional plates of cells were pulsed with adjuvant microparticles only and were also used as controls. The pulsed DC2.4 cells were then washed to remove extracellular nanoparticles prior to staining with phycoerythrin (PE) labeled MHC II marker (10 µL of stain diluted to 5 mL PBS and then 100 µL of this stock solution per well) (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The adherent cells are removed using cell scraper. The stained DC2.4 cells were washed once with Hanks balanced salt solution (HBSS) (100 µL) and samples measurements were then acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).

3.3.12. Quantification of co-stimulatory molecules (CD80 CD86) expression

The expression of co-stimulatory molecules on dendritic cells was (DC2.4) determined as described above. Pulsed and control DC2.4 cells were stained with FITC labeled CD80, PE-labeled CD86, marker (10 µL of stain diluted to 5 mL with PBS and then 100 µL of this stock solution per well) (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The cells were then washed with Hanks balanced salt solution (HBSS) (100 µL) and samples measurements were acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA). At least 5000 events of live cell population were captured.
3.3.13. Nitric oxide release from DC2.4 cells

Freshly grown adherent DC2.4 cells were harvested, washed and re-suspended in Dulbecco's complete media, counted and adjusted to 10\(^6\) cell/mL. 250 µL aliquots were then dispensed into each well of a 48-well plate at final 250×10\(^3\) cell density prior to stimulation with either ovarian cancer vaccine microparticles and/or the adjuvant microparticles. The induced cells were incubated overnight at 37°C with 5% CO\(_2\) and supernatants were harvested. Nitric oxide release was quantified using the Greiss chemical method as previously described. Briefly, the Griess chemical method was used to detect nitrite (NO\(_2\)) accumulated in supernatants of induced DC 2.4 macrophages. Griess reagent was freshly prepared by mixing equal volumes of 1% sulfanilamide and 0.1% N-(1-naphthylethlenediamine) solutions. One hundred microliters of cell supernatants were transferred into a 48-well plate to which 100 µL of Griess reagent was added. The plate was mixed gently, incubated for 10 min at room temperature, and read at 540 nm using a microplate reader (EL312e; BIO-TEK Instruments, Winooski, VT). The optical densities were correlated to the concentration of nitrite. Nitrite was quantitated using the standard curve of sodium nitrite (1 mM stock concentration in distilled water further diluted to the highest standard at 100 µM followed by serial dilutions to 1.56 µM)(S. M. Zughaier et al., 2004).


The immunogenicity of the microparticulate vaccine was evaluated using C57BL/6 female mice model. Six to eight-week-old C57BL/6 mice were purchased from Charles River Laboratories, Wilmington, MA, and the animals were acclimatized for one week prior use. The animal experiments were carried out as per approved protocols by
Mercer University’s Institutional Committee for the Care and Use of Laboratory animals (IACUC).

Animals (n=8) were administered with microparticles as one prime dose followed by two boosters at week 2 and week 3. 2 days before the first dose, a low dose of 50mg/kg (non-cytotoxic dose) of Cyclophosphamide (Cyp) was given to all the mice intra-peritoneal to deplete the T-regulatory cells. Briefly, 10 mg of microparticles were suspended in 0.5 ml citrate buffer (pH 4.0) and administered orally using oral gavage. For delivering microparticles via the transdermal route, mice skin was shaved two days prior to vaccination. One day prior to vaccination, the remainder of the hair was treated with Nair Hair removal cream (Ewing, NJ) for 10 minutes and then wiped off with a cotton swab. The vaccine loaded microneedles prepared previously were administered in the skin previously treated. The microneedles patch was applied for 20 minutes which ensured the delivery of the vaccine transdermally.

3.3.15. Statistical analysis

All experiments were performed in quadruplets and were repeated four times. Mean values ± SD and P value (Student’s t-test unpaired, two –tail distribution) was determined individually for all experiments with Microsoft Excel software. A P value of less than 0.01 was considered to be statistically significant.

3.4. RESULTS AND DISCUSSION

3.4.1. Protein content of the ovarian cancer whole cell lysate

Whole cell lysate protein content was quantified using Bio-Rad DC total protein assay kit. The total protein content of the whole cell lysate prepared from 5 x10⁶ ID8
cells was 2-3 mg/mL. The whole cell lysate was used as a source of antigen to formulate vaccine loaded microparticles.

3.4.2. Characterization of Size, shape, and charge of microparticles

The mean particle size of particles was 4.18 ± 1.33 µm (Figure 4a). The size and shape of particles were confirmed using scanning electron microscopic images (Figure 4b). The particles had an irregular shape with a positive zeta potential of around 7 ± 1.5mV. According to a new study conducted at the University of North Carolina and Georgia Institute of Technology, microparticles shaped to resemble certain bacteria can more easily infiltrate cells. There are several other researchers who suspect that mimicking the distinctive shapes of fungi, bacteria, blood cells and even pollen could improve the ability of microparticles to deliver encapsulated material to the cells. We expect a higher internalization of these vaccine microparticles compared to the solution form of the same since they mimic the irregular shape of a bacteria or blood cell (Singh et. al. 2011). A higher internalization rate will lead to higher antigen delivery and quicker immune response.
Figure 4: Particle size chart of vaccine microparticles measured using a Malvern Nano-zs. The particles had a size range of 1-6 µm with a mean diameter of 4.18 ±1.33 µm.
3.4.3. Antigen loading analysis of vaccine microparticles

The antigen loading of the vaccine microparticle was determined by dissolving the particles in DPBS. As pH-dependent polymers dissolved at a pH of around 7, the complete content of the vaccine microparticles was released in DPBS and collected after centrifugation. The total protein content of the supernatant was evaluated using Biorad DC total protein assay. The loading efficacy of the vaccine microparticles was found to be 80±3.5 %w/w.

Figure 5: Scanning electron microscope image of polymeric microparticles depicting an irregular shape with a particle size in the range of 1-6 µm.
3.4.4. *In-vitro* antigen release from microparticles

Figure 6: Rate of release of antigen proteins microparticles was studied at stomach pH conditions (pH 3) for 30 minutes followed by intestine pH conditions (pH 5) to mimic pH conditions of the gastrointestinal tract. Protein concentration was evaluated using Biorad DC total protein assay.

Microparticles were prepared using enteric coating polymers such as ethyl cellulose and HPMCAS. Microparticles released about 30 %w/w of antigen at stomach pH conditions. Therefore about 70% antigen was still available in the matrix, which will be eventually taken up by the microfold cell in the Peyer's patches of the small intestine (Figure.6). The initial burst release is a common phenomenon observed in particulate
dosage forms due to the release of adsorbed antigen on the microparticulate surface. Protein and enzyme-free dissolution medium were used for this study to eliminate interaction with the antigen release profile.

3.4.5. Screening of adjuvants to potentiate the ovarian cancer vaccine

As multiple approaches are used to treat cancer, such as the surgery followed by the chemotherapy and, or radiation. Along with these conventional therapies, novel therapies such as the immunotherapies, therapeutic vaccines, monoclonal antibodies, gene therapy etc. have been researched and studies a lot. In our approach of the therapeutic vaccine, the patient's tumor after the surgery would be used to obtain the Tumor Associated Antigens (TAA’s) and then with further isolation, purification of these TAA, they are formulated into a dosage form and given back to the same patient. The principle idea behind the approach is to train the patient immune system to fight against the specific TAA’s. As discussed earlier, this ovarian cancer vaccine utilizes this principle to generate an immune response to prevent the reoccurrence of the tumor.

In the recent years, many approaches have been researched, one such previously done in our laboratory at the Vaccine Nanotechnology Laboratory at Mercer University was the use of interleukins along with the therapeutic vaccine to create a better immune response. We found that IL-12 and IL-2 helped in potentiating the ovarian cancer vaccine’s efficacy. With is a success we decided to further improvise the efficacy of the vaccine by the aid of adjuvants which is one of the main aims of my project: screening of adjuvants for improving the efficacy of the ovarian cancer vaccine. In this study, we have screen 7 different adjuvants - monophosphoryl lipid A (MPL), CpG, P4, cholera toxin (CT), R848, AddaVax™ (known as MF59) and alum for their ability to enhance immune
responses elicited by ovarian cancer vaccine when administered together. To assess antigenicity of ovarian cancer vaccine microparticles we employed murine dendritic cells as antigen presenting cells, in *in-vitro* cell-based assays and determined the expression of II molecules along with the expression of co-stimulatory molecules CD80 and CD86 when ovarian cancer vaccine is delivered with or without adjuvants. Following were the groups for our *in-vivo* screening experiments

Table 4: Different groups for the in-vitro screening of the adjuvants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>Blank polymeric microparticles</td>
</tr>
<tr>
<td>Vacc</td>
<td>Ovarian cancer (OC) vaccine microparticles</td>
</tr>
<tr>
<td>Vacc + MPL</td>
<td>OC vaccine microparticles + MPL</td>
</tr>
<tr>
<td>Vacc + CpG</td>
<td>OC vaccine microparticles + CpG</td>
</tr>
<tr>
<td>Vacc + CT</td>
<td>OC vaccine microparticles + CT</td>
</tr>
<tr>
<td>Vacc + R848</td>
<td>OC vaccine microparticles + R848</td>
</tr>
<tr>
<td>Vacc + MF59</td>
<td>OC vaccine microparticles + MF59</td>
</tr>
<tr>
<td>Vacc + Alum</td>
<td>OC vaccine microparticles + Alum</td>
</tr>
</tbody>
</table>

To understand the effect of adjuvants on the immunogenicity of OC vaccine microparticles, we incubated DCs with OC vaccine microparticles with a single adjuvant as shown in table 4.
Figure 7: Nitric oxide release from 250×10^3 DC 2.4 cells after 16 hours of incubation when pulsed with Vaccine and Vaccine + Adjuvant microparticles. (Vacc: Vaccine microparticles; Flag: Flagellin; CT: Cholera Toxin). The groups receiving Vaccine + MF59 and Vaccine + Alum showed significantly higher nitric oxide release when compared to vaccine only (*p < 0.05).

The empty or the blank microparticles stimulated significantly low levels of nitric oxide from DCs (Figure 7), indicating that microparticle matrix is relatively inert and will not contribute towards immune response. We found significantly higher release of nitric oxide from the cells of the groups which received Vaccine + MF59 and Vaccine + Alum
when compared to vaccine alone (*p < 0.05). The release of nitric oxide from other adjuvants in combination with the OC vaccine was also higher than the OC vaccine given along. Similarly, from Figure 8, we can see the expression of co-stimulatory molecules CD 80 and CD86 on DC 2.4 cells show a similar trend. The vaccine microparticles after uptake into the DC cells were processed and the antigen was expressed on Major Histocompatibility molecule II (MHC II) as seen in figure 7 and alongside there was an expression of co-stimulatory molecule CD80 and CD86 as shown in figure 8.

Figure 8: Antigen expression on MHC II molecules on $3 \times 10^4$ DC 2.4 cells post incubation (16 hrs) after being pulsed with vaccine microparticles and vaccine + adjuvant microparticles. (Vacc: Vaccine microparticles; Flag: Flagellin; CT: Cholera Toxin). The group receiving Vaccine + CT, Vaccine + MF59 and Vaccine + Alum showed significantly higher antigen presentation when compared to vaccine only (*p < 0.05).
We could see from figure 8 that three groups: receiving Vaccine + CT, Vaccine + MF59 and Vaccine + Alum showed significantly higher antigen presentation when compared to vaccine only (*p < 0.05). Even though other adjuvants help increase the antigen presentation when given along with the OC vaccine, these three adjuvants CT, MF59 and Alum showed higher antigen presentation.

Figure 9: Expression of co-stimulatory molecules CD 80 and CD 86 on 3 × 10^4 DC 2.4 cells post incubation (16 hrs) after being pulsed with vaccine microparticles and vaccine + adjuvant microparticles. (Vacc: Vaccine microparticles; Flag: Flagellin; CT: Cholera Toxin). The groups receiving Vaccine + CT, Vaccine + MF59 and Vaccine + Alum showed significantly higher CD80 and CD86 expression when compared to vaccine only (*P < 0.05).
We can see from figure 9 that groups receiving Vaccine + R848, Vaccine + MF59 and Vaccine + Alum showed significantly higher co-stimulatory molecule CD80 and CD86 when compared to the vaccine alone. From the in-vitro screening experiments, we have seen that adjuvants could help potentiate the effect of the ovarian cancer vaccine. As these in-vitro experiments show three very important markers in the generation of an immune response, the activation of innate immunity confirmed by nitric oxide release, the antigen presentation and co-stimulatory molecule expression which are vital for the activation of T-cell and further generation of an adaptive immune response. The encouraging results show that ovarian cancer vaccine when combined with adjuvants like alum, MF59, P4 could enhance the immune response significantly and we decided to carry out the further screening of the adjuvants when used in combination.

3.4.6. Multiple adjuvant effects of ovarian cancer vaccine

“Adjuvant systems” are combinations of adjuvants that act by different mechanisms and are specifically designed to stimulate a specific class or a balanced Th1/Th2 kind of immune response. GlaxoSmithKline (GSK) is the pioneer in the development of various adjuvant systems, such as ASO1 (MPL and QS21), ASO2 (o/w emulsion+ QS21+ MPL), ASO3 (DL-α-tocopherol, squalene, and polysorbate 80), ASO4 (MPL+alum). A few other notable adjuvant systems being explored include a combination of i) CpG and MF59 and ii) IC30 (poly-L-arginine) and CpG. All the aforementioned combinations were developed with an aim of inducing a balanced Th1/Th2 type immune response. So far, since no one has explored the advantages of combining alum and squalene-based adjuvants, the aim of our study was to evaluate the adjuvanticity of combining cancer vaccine along with both alum, MF59, and P4. From
the similar set of experiments where nitric oxide, expression of CD80 and CD86 along with antigen presentation (MHC II) of using this combination of adjuvant, we decided to use a combination of Alum + P4 and MF59 +P4 as a combination adjuvant system for our animal studies.

3.4.7. Immunization with vaccines suppress tumor growth

As this is a therapeutic vaccine study, the tumor was inoculated by injecting $10^7$ ID8 ovarian cancer cells subcutaneously in 4 – 6 week old female C57 mice. After one week of tumor inoculation, when the tumor volume was about 50 mm$^3$ in all mice, the immunization was carried out and tumor volume measurements were done every week using digital Vernier calipers. From figure 10 for oral immunization and figure 11 for transdermal immunization, the tumor grew rapidly in the animals which did not get any vaccine i.e. naïve group and the group which received the blank microparticles. This showed that the microparticulate matrix or the microneedle matrix in case of transdermal did not have any immunogenic effect and were inert. In both the cases, there was a significant tumor volume reduction in the all the immunized mice after week 5, until then end of the study (**$P < 0.001$). The tumor was 2.5 to 3 times larger in the unimmunized mice when compared to the immunized mice at week 8. Between the immunized mice, the 2 groups which were immunized with adjuvants (Vaccine + MF59 + P4 and Vaccine+ Alum + P4) showed no significant difference in tumor volume until week 5 with the non-adjuvanated vaccine only group. But post week 5, at week 6 and week 8 there was a significant tumor volume reduction in both the adjuvanted groups when compared to the non-adjuvanated group (*$P < 0.05$). This similar trend was observed in both oral and
transdermal immunization which indicated that there was no difference between these two routes of immunization.

Figure 10: Tumor volume reduction post vaccine administration. The vaccine was administered one week after the tumor inoculation and 2 prime doses were administered at week 2 and 3. The groups receiving the vaccine microparticles showed significantly reduced tumor volume when compared to groups receiving blank microparticles and naïve (**P < 0.001). Amongst the vaccinated groups, at week 6 and 8, the groups receiving Vaccine + Adjuvants showed significantly lower tumor volume growth to the groups receiving vaccine only (*P < 0.05).
Figure 11: Tumor volume reduction post vaccine administration transdermally. The vaccine was administered one week after the tumor inoculation and 2 prime doses were administered at week 2 and 3. The groups receiving the vaccine microparticles showed significantly reduced tumor volume when compared to groups receiving blank microparticles and naïve (**$P < 0.001$). Amongst the vaccinated groups, at week 7 and 8, the groups receiving Vaccine + Adjuvants showed significantly lower tumor volume growth to the groups receiving vaccine only (*$P < 0.05$).
3.4.8. Immunization with Ovarian Cancer Vaccine microparticles generated T-cell based immune response

Lymphatic organs such as spleen and lymph nodes were analyzed for T-cell populations. The cells were labeled with T-cell markers such as anti-mouse CD4 PE and anti-mouse CD8a FITC (eBioscience, San Diego, CA). The cells were analyzed for the specific cell population by flow cytometry. The cell count was compared with mice treated with blank microparticles. The groups where the vaccine was administered orally, we could see that there were high levels of CD8+ T-cells and CD4+ T-cells in the spleen and the lymph nodes when compared to the animals which received the blank microparticles. Moreover, the two groups which received the ovarian cancer vaccine along with the adjuvant combinations i.e. Vaccine + MF59 + P4 and Vaccine + Alum + P4 showed significantly higher CD8 + T-cells (Figure 12) and CD4+ T-cells (Figure 12) when compared to the animals which received the ovarian cancer vaccine alone (*P < 0.05).
Figure 12: Flow cytometry analysis showing cell population of CD8+ T cells in Spleen and Lymph nodes of the mice receiving vaccine and vaccine along with adjuvant combination administered orally. The groups receiving the vaccine along with the adjuvants showed significantly higher cell counts when compared to the group receiving vaccine alone (*p < 0.05).
Figure 13: Flow cytometry analysis showing cell population of CD4+ T cells in Spleen and Lymph nodes of the mice receiving vaccine and vaccine along with adjuvant combination administered orally. The groups receiving the vaccine along with the adjuvants showed significantly higher cell counts when compared to the group receiving vaccine alone (*p < 0.05).

In the transdermal administration of ovarian cancer vaccine using the microneedles, similar results were obtained as to the oral administration. As reported, there was a significant reduction in tumor volume growth in the mice which were immunized. Thus, the lymphatic organs such as spleen and lymph nodes were analyzed for T-cell populations. The cells were labeled with T-cell markers such as anti-mouse CD4 PE and anti-mouse CD8a FITC (eBioscience, San Diego, CA). The cells were analyzed for the specific cell population by flow-cytometry. The cell count was compared
with mice treated with blank microparticles. The groups where the vaccine was administered transdermally, we could see that there were high levels of CD8+ T-cells and CD4+ T-cells in the spleen and the lymph nodes when compared to the animals which received the blank microparticles. Moreover, the two groups which received the ovarian cancer vaccine along with the adjuvant combinations i.e. Vaccine+ MF59 + P4 and Vaccine + Alum + P4 showed significantly higher CD8+ T-cells (Figure 15) and CD4+ T-cells (Figure 14) when compared to the animals which received the ovarian cancer vaccine alone when administered transdermally (*p < 0.05).

![Transdermal - CD4 T cells](chart.png)

Figure 14: Flow cytometry analysis showing cell population of CD4+ T cells in Spleen and Lymph nodes of the mice receiving vaccine and vaccine along with adjuvant combination administered transdermally. The groups receiving the vaccine along with the adjuvants showed significantly higher cell counts when compared to the group receiving vaccine alone (*P < 0.05).
Figure 15: Flow cytometry analysis showing cell population of CD8+ T cells in Spleen and Lymph nodes of the mice receiving vaccine and vaccine along with adjuvant combination administered transdermally. The groups receiving the vaccine along with the adjuvants showed significantly higher cell counts when compared to the group receiving vaccine alone (*P < 0.05).

3.4.9. T-cell mediated tumor volume reduction

As cancer immunotherapy consists of multiple approaches that focus on harnessing and enhancing the innate powers of the immune system to fight cancer. Cancer immunotherapies can be divided into three major categories: (1) cytokines/immunomodulation agents, (2) monoclonal antibodies, and (3) cell-based therapies. Though monoclonal antibodies currently represent the largest class of
commercialized cancer immunotherapies, cell-based therapies are rapidly making headway. This class of patient-specific therapies involves collecting immune cells and, or, tumor from a cancer patient, engineering them (via genetic manipulation or peptide/adjuvant stimulation) to recognize and kill cancer cells, growing large numbers of these and reintroducing them into the same patient. Our approach has been to investigate the potential of activating the T-cell of the tumor patients to evaluate its efficacy for reducing the tumor.

T cells (also known as T lymphocytes) are found widely distributed within tissues and the tumor environment. They play a central role in cell-mediated immunity and can mediate long-lived, antigen-specific, effector and immune memory responses. These T-cells have a T-Cell Receptor (TCR) on the cell surface. The TCR is the transmembrane complex which confers the antigen-specific immunity thus with each receptor providing a single antigen-binding site. The TCR confers antigenic specificity on the T cell, by recognizing an antigen ligand comprising a short contiguous amino acid sequence of a protein that is presented on the target cell by a major histocompatibility complex (MHC) molecule. Along with the antigen presentation on the MHC I and MHC II molecule, the TCR also requires a co-stimulatory molecule (CD40, CD80, CD86) expression to complete the TCR binding. Following contact with their cognate peptides presented by MHC class I molecules, naive CD8+ cytotoxic T cells proliferate vigorously and acquire phenotypic and functional properties allowing them to act as effector T cells; these eliminate cells expressing the antigen, through apoptosis-inducing ligands or release of lytic granules. In addition, long-lasting memory T cells are generated that can self-renew,
allowing rapid expansion in the presence of the target antigen and providing a sustained
and durable response to it upon re-exposure.

In our study, we used the whole cell lysate as the vaccine antigen. Whole cell
tumor vaccines offer one major advantage over antigen/epitope-specific vaccines in their
ability to present multiple known as well as unknown antigens to both innate and
adaptive immune systems. In the current study, immunogenicity aspect of whole cell
lysate prepared was evaluated in microparticulate form. We then have 2 different routes
of administration, oral using the oral and transdermal using microneedles as the methods
of immunization. We have done an extensive screening of the adjuvants in order to
improvise the vaccine antigen and finally used a combination of adjuvant systems for our
in-vivo studies. As we saw in our in-vivo mice immunization study, the tumor grew in the
mice which got on the blank microparticles whereas in the groups which received the
vaccine, there was significantly lower tumor growth. Moreover, the animals which
received the vaccine along with the adjuvants, there was further lowered tumor volume at
week 6 and 8 when compared to animals which have the vaccine only. These are very
promising results, and to further get to know the mechanism by which the tumor volume
was reduced, post-sacrifice at the same time, the lymphatic organs such as the spleen and
the lymph nodes were isolated and T cell populations were measured using flow
cytometry. There was significantly higher CD4+ and CD8+ T cells in the vaccinated
groups as compared to the non-vaccinated group. The reduction in the tumor volume in
the immunized mice could thus be attributed to the high levels of the CD8+ T-Cell
present in those animals. Thus we could also attribute the ability of the vaccine to
generate an immune response. The major difference in the mice receiving vaccine alone
and the groups which received vaccine along with the adjuvants is the further tumor volume reduction and also significantly higher CD8+ T-cells. This higher level of CD8+ T-cells could be attributed to the adjuvants which might have activated the antigen presenting cells and help in the antigen presentation and co-stimulatory molecules which led to a higher TCR binding and T cell activation and thus the results. In terms of the route of delivery, we did not see any significant difference between the mice immunized orally or transdermally using microneedles.

3.4.10. Importance of adjuvants for the cancer vaccines

Even with the intense research in the cure of cancer over past three decades, cancer still remains one of the major health problems, impacting millions of people worldwide and suggesting the need for novel approaches to cancer treatment such as the immunotherapies. In the last decade, there has been intense research for improving these immunotherapies by utilizing various means such as cytokines (e.g. GM-CSF and IL-2), oncolytic viruses, checkpoint inhibitors, adjuvants etc. Since adjuvants showed success traditionally in infectious diseases vaccines, Dr. William Coley injects inoperable tumors with inactivated bacteria (Streptococcus pyogenes and Serratia marcescens)—the so-called Coley’s toxin. It was found out that anti-tumor effect of Coley’s toxin was due to the stimulation of multiple toll-like receptors (TLRs) (McCarthy, 2006). In another study, Rosenberg et al. reported that in a clinical trial with high numbers (440) of cancer patients, the efficacy of the cancer peptide vaccines was extremely low (2.6%) based on the selection of nonimmunogenic antigens or lack of powerful adjuvants capable of overcoming the immunosuppression in the cancer patients (Rosenberg et al., 2004). Gardasil® and Cervarix® are considered prophylactic cancer vaccines, as they have been
developed for preventing human papilloma virus (HPV) infection that is associated with almost 70% of cervical cancer cases. Both of them have adjuvants and are approved by FDA (Paavonen et al., 2009). Therefore, a potent adjuvant is a crucial component of cancer vaccines, as it can break the immunotolerance in the tumor microenvironment to aid in the elicitation of potent anti-tumor immune responses (Temizoz, Kuroda, & Ishii, 2016).

Our group at Mercer University has been working on developing particulate vaccines for cancers in recent years to address many of the problems associated with the current vaccine therapies. The vaccine microparticles can be prepared within 2-4 days using the patient’s own tumor tissue and administered to the patients before they leave the hospital. We also expect that in the future when sufficient tumor antigens have been identified and sub-typed in different ovarian cancer patients, a cocktail of these different ovarian cancer antigens can be entrapped into the particulate vaccine. Conceptually, this is particularly useful to vaccinate with a multitude of antigens in a single vaccine formulation thereby reducing the overall vaccine costs. Moreover, the particulate vaccine renders the delivery of antigens via non-living carrier system and thus addressing safety issues as in the case of live attenuated vaccines.

3.5. CONCLUSION

We have demonstrated the efficacy of vaccine microparticles containing whole cell lysate of ID8 ovarian cancer cells in retarding tumor growth in murine models. Spray drying process and the formulation used for this purpose could retain the immunogenicity of vaccine resulting in T-cell response. Thus, the microparticulate vaccine provides a promising approach in terms of cost-effectiveness, ease of production and patient
compatibility. Moreover, the immunization with oral and transdermal vaccine microparticles retarded tumor growth in the mice study. The efficacy of vaccine microparticles containing whole cell lysate of ID8 ovarian cancer cells in retarding tumor growth in murine models was demonstrated in this study based on routes of administration. Thus, the microparticulate vaccine when given via combination of oral and transdermal routes provides a promising approach in terms of cost-effectiveness, ease of production and patient compatibility. Vaccine with adjuvants when administered was found to result in higher tumor suppression in correlation to cellular responses.
CHAPTER 4
SCREENING OF ADJUVANTS FOR IMPROVING THE IMMUNOGENICITY OF MENINGITIS MICROPARTICULATE VACCINE

4.1. INTRODUCTION

*Neisseria meningitidis* (*N. meningitidis*) is primarily the single largest cause of meningitis and meningococcaemia globally (Stephens, Greenwood, & Brandtzaeg, 2007). Meningococcal infections continue to impact public health, especially in children and adolescents due to its rapid progression, high fatality rate and frequent occurrence of sequelae (Stephens et al., 2007). Meningococcal infections cause rapidly fulminant sepsis that can lead to death of previously healthy individuals within hours (Bjerre, Ovstebø, Kierulf, Halvorsen, & Brandtzaeg, 2000; Girard, Preziosi, Aguado, & Kieny, 2006). With the introduction of meningococcal conjugate vaccines and the prevalence of sophisticated intensive care combined with antimicrobial treatment, the mortality rate has been significantly reduced; nonetheless, the survivors often suffer from morbidity, tissue and neurological damage (Girard et al., 2006). Meningococcal capsular polysaccharides (CPS) are a major virulence factor and form the basis of preventive vaccines. To date, there are twelve distinct meningococcal serogroups designated based on the composition of CPS polymers, with serogroups A, B, C, W, Y, and X are responsible for the majority of disease (Jafri et al., 2013; S. M. Zughaier, 2011). The successful introduction of meningococcal serogroup A conjugate vaccine (MenAfriVac®) to Africa’s meningitis belt countries dramatically reduced the burden of disease (Dakar discussion group on priorities for research on epidemic meningococcal disease in Africa et al., 2013; Lee et
al., 2009) and induced herd immunity effect (Kristiansen et al., 2013). However, persistent low carriage of meningococcal serogroup A and other serogroups like Y and X are causing meningococcal infections in sub-Saharan Africa (Ba et al., 2014; Kristiansen et al., 2013; Lamelas et al., 2014). The emergence of other meningococcal disease-causing serotypes in Africa like serogroup W and X is a considerable concern and advocates for a wider vaccine serotype coverage (Boisier et al., 2007; Collard et al., 2013; Xie, Pollard, Mueller, & Norheim, 2013).

The quadrivalent meningococcal conjugate vaccines Nimenrix®, Menactra™ and Menveo® are commercially available. However, meningococcal conjugate vaccines require booster doses to maintain a protective immune response (Baxter et al., 2014), continuous refrigeration, and administration by needle use, in addition to the high cost of chemical conjugation and limitation of producing and distributing conjugate vaccines which are inherent to all currently licensed vaccines. We previously reported a novel meningococcal CPS vaccine nanoparticulate formulation that has an advantage over the current commercially available conjugate vaccines (Ubale et al., 2013a). The novel nanoparticulate formulation does not require chemical conjugation, it encapsulates meningococcal CPS antigens in a biodegradable material that slowly release antigens, thereby has antigen depot effect to enhance antigenicity. The novel vaccine formulation is inexpensive and can be stored as a dry powder with an extended shelf life that does not require the cold-chain which facilitates storage and distribution (Ubale et al., 2013a). We have previously shown that this meningococcal vaccine-loaded nanoparticles (CPS NP) are taken up by antigen-presenting cells (APCs) and induced a robust innate immune response, a prerequisite for inducing adaptive immunity (Ubale, Gala, Zughaier, &
D’Souza, 2014b). We further assessed the ability of the novel meningococcal vaccine nanoparticulate formulation to induce optimal antigen presentation (6). We observed the induction of the co-stimulatory molecules, CD80 and CD86 in macrophages and dendritic cells without the activation of the death receptor CD95 (FAS) on the antigen presenting cells, which if activated leads to apoptosis and suboptimal adaptive immune responses (Ubale et al., 2014b).

For decades Alum has been used as an adjuvant where it is incorporated in vaccine formulations to enhance the specific immune responses generated by the antigen. In recent years, many adjuvants are under consideration and few have been licensed for use in human. Adjuvants can potentiate the immune response by either increasing the antibody response or inducing cell-mediated immunity or both(Savelkoul, Ferro, Strioga, & Schijns, 2015). The use of adjuvants not only enhances immunogenicity but could also permit the reduction in the antigen dose delivered in the vaccine (Steinhagen, Kinjo, Bode, & Klinman, 2011). Adjuvants could be classified into two types: delivery systems and immune potentiators. Alum and MF59 are examples of antigen delivery system adjuvants(Li, Aldayel, & Cui, 2014; Morefield et al., 2005; D. T. O’Hagan, Wack, & Podda, 2007; Savelkoul et al., 2015). Aluminum-containing compounds were first discovered to have adjuvant activity in 1926 when an alum-precipitated diphtheria vaccine showed better antigenic properties compared to the standard diphtheria vaccine(Petrovsky & Aguilar, 2004). Adjuvants induce inflammation at the site of administration which subsequently leads to recruitment of APC’s(Hem & Hogenesch, 2007). MF59 is a squalene in water emulsion which is commercially been approved in Europe with more than 27 million doses of vaccine containing MF59 have been
administered (Schultze et al., 2008). Novartis Vaccines has developed an influenza vaccine using MF59 along with inactivated, subunit seasonal prophylactic vaccine which is commercialized as FluaD in Europe (“The Novartis Institutes for BioMedical Research (NIBR) is a global research organization committed to discovering innovative m - MF59-Adj-fact-sheet.pdf,” n.d.). MF59 has been widely used in the pandemic H1N1 vaccines such as Focetria® and Celtura®, Novartis (“The Novartis Institutes for BioMedical Research (NIBR) is a global research organization committed to discovering innovative m - MF59-Adj-fact-sheet.pdf,” n.d.). The safety and efficacy of MF59 have been established clinically with a large database (D. T. O’Hagan et al., 2007; Podda, 2001).

Immune potentiator adjuvants like Toll-like receptors (TLR) agonists, which activate TLR-mediated signaling pathways leading to robust innate immune responses. Monophosphoryl lipid A (MPL), bacterial flagellin, CpG oligonucleotide, and small molecule R848 are well characterized TLR agonists that induce TLR4, 5, 8 and 9 respectively (Kawai & Akira, 2011; Kuroda, Coban, & Ishii, 2013; Steinhagen et al., 2011; S. Zughaier, Steeghs, van der Ley, & Stephens, 2007).

The commercially available meningococcal vaccines Menactra™ (Sanofi Pasteur quadrivalent meningococcal conjugate MCV4 vaccine), Nimenrix® (GlaxoSmithKline quadrivalent conjugate meningococcal vaccine) and Menveo® (Novartis quadrivalent conjugate meningococcal vaccine) do not contain adjuvants in their formulation but are conjugated to a protein carrier (“[Product Monograph Template - Schedule D] - Nimenrix.pdf,” n.d.; “The Novartis Institutes for BioMedical Research (NIBR) is a global research organization committed to discovering innovative m - MF59-Adj-fact-sheet.pdf,” n.d.; “UCM131170.pdf,” n.d.). Here, we sought out to evaluate the effect of
adjuvants encapsulated in nanoparticles on the novel meningococcal CPS nanoparticulate (NP) vaccine formulation. In this study, we used meningococcal CPS serogroup A as a representative vaccine antigen to investigate immunogenicity in dendritic cells in-vitro since previous studies reported the immunogenicity of CPS–A in an in-vivo animal model (Berry, Lynn, Lee, Frasch, & Bash, 2002). We employed murine dendritic cells pulsed with meningococcal CPS NP in presence or absence of adjuvant NP in-vitro and measured dendritic cell maturation and antigen presentation markers. We observed that MF59 and Alum containing nanoparticles compared to TLR-based adjuvants, significantly enhanced dendritic cell maturation and antigen presentation markers CD80, CD86, CD40, MHC I and MHC II without increasing the expression of the death receptor CD95 on dendritic cells. Therefore, our data show that MF59 and Alum are superior to TLR ligands as adjuvants for improving the antigenicity of meningococcal CPS nanoparticulate vaccine.

4.2. MATERIALS AND METHODS

4.2.1. Materials

RPMI 1640 medium, Dulbecco's Modified Eagle medium, fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate and nonessential amino acids were obtained from Cellgro Mediatech (Herndon, VA). Dendritic cells (DC2.4) were given as a kind gift from Dr. Kenneth L. Rock (Dana-Farber Cancer Institute, Inc., Boston, MA, USA). The vaccine grade polysaccharide antigens were a kind gift received from Dr. Seshu Gudlavalleti (JN Medical Corporation, Omaha, NE, USA). MPL, R848 and cholera toxin from Vibrio cholera were purchased from Sigma Aldrich. AddaVax™ (similar to MF59), CpG oligonucleotide and Alhydrogel® 2% (Aluminum hydroxide gel)
were obtained from InvivoGen (San Diego, CA). Sterile and endotoxin free Bovine Serum Albumin (BSA) used to formulate the protein-based nanoparticles were purchased from Sigma-Aldrich. Antibodies used to stain murine MHC I, MHC II, CD80, CD86, CD40 and CD95 for flow cytometric analysis were purchased from eBioscience laboratories (San Diego, CA).

4.2.2. Preparation of albumin-based microparticles

The biodegradable nanoparticles were prepared as previously described method established by our laboratory using the Buchi Mini Spray Dryer B-191 (Bejugam, Gayakwad, Uddin, & D’Souza, 2013; Bejugam et al., 2008; Chablani, Tawde, & D’Souza, 2012; Shastri, Kim, Quan, D’Souza, & Kang, 2012b; Singh & Salnikova, 2014a; Tawde et al., 2012b; Ubale et al., 2013a, 2014b; Uddin et al., 2009a). Briefly, for a batch of 100 mg of vaccine microparticles (10% antigen loading) 10 mg of the capsular polysaccharide A (antigen) and 90 mg of pre-crosslinked BSA were mixed. Pre-crosslinked BSA solution is prepared by weighing the 90 mg BSA and dissolving it in 5 mL DI water in a 50 mL beaker. Once BSA is dissolved, glutaraldehyde (25% in DI water purchased from Fisher Scientific, Pittsburgh, PA) (200 µL for every 1gm of BSA) is added to the BSA solution. This 50 ml beaker is kept for stirring (300 rpm) overnight in a dark place at room temperature. The following day, excess glutaraldehyde was neutralized with sodium bisulfate (10 mg). At this point, the capsular polysaccharide A which is our antigen is added to the pre-crosslinked BSA prepared overnight. 100 mg this prepared formulation is dissolved in 10mL of DI water. This formulation was spray dried through a 0.5 mm nozzle (nozzle temperature: -5°C). The inlet temperature was 120°C with the aspirator at 100% and a flow rate of 20 mL/h to obtain the Meningitis vaccine
microparticles. The adjuvant particles (2% w/w loading) were also prepared separately using the same method. Particle characterization, polysaccharide content and recovery yield were calculated as previously described (Ubale et al., 2013a).

4.2.3. Cell culture

Murine dendritic cells (DC) 2.4 cells were grown in RPMI 1640 with L-glutamate supplemented with 10 % FBS, 50 IU/mL of penicillin, 50 µg/mL of streptomycin, 1 % sodium pyruvate and 1% nonessential amino acids. Culture flasks were incubated at 37°C with humidity under 5% CO₂.

4.2.4. Quantification of antigen presentation molecules (MHC I and MHC II) expression

Dendritic cells (DC2.4) were plated at 3 × 10⁵ DC2.4 cells per well a 48 well plate and incubated at 37°C for 24 h to adhere and stabilize. The adherent cells were pulsed with 250 µg CPS-loaded microparticles containing 25 µg of antigen along with the equal quantity of adjuvant microparticles in each well and incubated at 37°C for 16 h. Equal amount blank microparticles without any antigen or adjuvant were used as controls in every experiment. Additional plates of cells were pulsed with adjuvant microparticles only and were also used as controls. The pulsed DC2.4 cells were then washed to remove extracellular microparticles prior to staining with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled MHC I and MHC II markers respectively (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The adherent cells are removed using cell scraper. The stained DC2.4 cells were washed once with Hanks balanced salt solution (HBSS) and samples measurements were then acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).
4.2.5. Quantification of co-stimulatory molecules (CD80, CD86, and CD40) and CD95 expression

The expression of co-stimulatory molecules, as well as the expression of death receptor dendritic cells was (DC2.4), was determined as described above. Pulsed and control DC2.4 cells were stained with FITC labeled CD80, PE-labeled CD86, APC labeled CD40 or with FITC labeled CD95 marker (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The cells were then washed and samples measurements were acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).

4.2.6. Nitric oxide release from DC2.4 cells

Freshly grown adherent DC2.4 cells were harvested, washed and re-suspended in Dulbecco's complete media, counted and adjusted to 10^6 cell/mL. 250 µL aliquots were then dispensed into each well of a 48-well plate at final 250×10^3 cell density prior to stimulation with either nanoparticle doses containing meningococcal CPS and/or the adjuvant nanoparticles. The induced cells were incubated overnight at 37°C with 5% CO₂ and supernatants were harvested. Nitric oxide release was quantified using the Greiss chemical method as previously described. Briefly, the Griess chemical method was used to detect nitrite (NO₂) accumulated in supernatants of induced RAW264 macrophages. Griess reagent was freshly prepared by mixing equal volumes of 1% sulfanilamide and 0.1% N-(1-naphthylethylendiamine) solutions. One hundred microliters of cell supernatants were transferred into a 48-well plate to which 100 µL of Griess reagent was added. The plate was mixed gently, incubated for 10 min at room temperature, and read at 540 nm using a microplate reader (EL312e; BIO-TEK).
Instruments, Winooski, VT). The optical densities were correlated to the concentration of nitrite. Nitrite was quantitated using the standard curve of sodium nitrite (1 mM stock concentration in distilled water further diluted to the highest standard at 100 µM followed by serial dilutions to 1.56 µM)(S. M. Zughaier et al., 2004).

4.2.7. Cell viability measurements

The vital dye trypan blue (0.4%) stored in a dark bottle was used for measuring cellular viability as described before (Ubale et al., 2013a). Equal volumes of 0.4% trypan blue and cell suspension were mixed and allowed to stain for 3 min prior to quantification of viable cells.

4.2.8. Statistical analysis

All experiments were performed in quadruplets. Mean values ± SD and P value (Student’s t-test unpaired, two –tail distribution) was determined individually for all experiments with Microsoft Excel software. A P value of less than 0.01 was considered to be statistically significant.

4.3. RESULTS

4.3.1. Adjuvants nanoparticles enhance antigen presentation by dendritic cells pulsed with meningococcal CPS vaccine nanoparticles

Antigen presentation by matured dendritic cells to T lymphocytes is essential for vaccine-induced protective immune responses. We previously demonstrated that the novel meningococcal CPS-loaded vaccine nanoparticles induced dendritic cell maturation markers CD80 and CD86 (Ubale et al., 2014b). Here we expanded our observation to assess the effect of adjuvants on enhancing antigen presentation by increasing the expression of major histocompatibility class I and II as well as the co-stimulatory
molecules CD40, CD80 and CD86 on mature dendritic cells. Although capsular polysaccharides as carbohydrate antigens induce T cell-independent responses (Avci & Kasper, 2010). Studies documented that bacterial capsular polysaccharides from Strep tococcus pneumoniae (Velez, Lewis, Kasper, & Cobb, 2009) and Bacteroides fragilis are presented by MHC II (Cobb & Kasper, 2005). Hill et al observed that Neisseria meningitidis serogroup X CPS induced the expression of CD86 and MHC II in murine DC (Hill et al., 2011). However, it is not known whether meningococcal capsular polysaccharides can utilize MHC II for antigen presentation when cross-linked to albumin-based nanoparticles. We examined if meningococcal CPS nanoparticles along with various adjuvants would retain the ability to induce dendritic cell maturation and enhance antigen presentation markers. To this end, we screened six different adjuvants encapsulated in nanoparticles formulation: monophosphoryl lipid A (MPL), CpG, cholera toxin (CT), R848, AddaVax™ (known as MF59) and alum for their ability to enhance immune responses elicited by meningococcal CPS NP vaccine when administered together. To assess antigenicity of CPS NP we employed murine dendritic cells as antigen presenting cells, in in-vitro cell-based assays and determined the expression of MHCI and II molecules along with the expression of co-stimulatory molecules CD40, CD80 and CD86 when CPS NP vaccine is delivered with or without adjuvants. Here we report that MF59 and Alum adjuvants when added with CPS NP, significantly ($P < 0.01$) and synergistically enhanced the expression of the antigen presenting molecules MHC I, MHC II on dendritic cells (Figure 16). In contrast, adjuvants NP alone (tested without CPS NP), did not induce significant antigen presentation markers on dendritic cells (Fig 1). Although all adjuvants tested in this study showed some enhancement of antigen
presentation markers when combined with CPS NP as an antigen, MF59 and Alum showed synergistic effect (Figure 16).

Figure 16: Antigen-induced expression of MHC II complex on murine dendritic cells. Murine dendritic cells (3 × 10^5) were pulsed with CPS-loaded nanoparticulate vaccine CPS NP, (antigen dose: 25 µg/3 × 10^5 cells) alone or with various adjuvants (adjuvant...
dose: 25 µg/3 × 10^5 cells) for 16 hours. The expression of antigen-presenting receptor MHC II (A) and MHC I (B) were detected using flow cytometer following staining with FITC MHC II and FITC MHC I markers. The yellow bars represent the group receiving CPS NP along with the adjuvant particles, the red bars represent the group receiving the adjuvant nanoparticles alone and the blue bar is the empty nanoparticles referred to as Blank. Error bars represent the standard deviation from the mean of four independent experiments. P values were calculated using Student’s t-test unpaired, two – tail distribution comparing to the blank NP and nanoparticulate vaccine only,**P < 0.01 were significant.

4.3.2. Antigen-induced expression of co-stimulatory molecules (CD40, CD80, and CD86)

Co-stimulatory molecules provide the crucial second signal to naïve T cells for its activation and proliferation leading to subset differentiation and effector functions required to elicit adaptive immune responses. Since effective antigen presentation by mature dendritic cells to T cells requires the expression of co-stimulatory molecules CD40, CD80 and CD86, we assessed their expression in dendritic cells pulsed with CPS NP with or without adjuvant NP. We found that MF59 and Alum adjuvants when combined with CPS NP, significantly (P < 0.01) and synergistically enhanced the expression of co-stimulatory molecules CD40, CD80, and CD86 on dendritic cells (Figure 17). In contrast, adjuvants NP alone (tested without CPS NP), did not induce significant upregulation of these co-stimulatory molecules on dendritic cells (Fig 17). All
adjuvants tested in this study showed some enhancement of co-stimulatory markers expression when combined with CPS NP as antigen, but MF59 and Alum are found to be synergistic to CPS NP. Of note, dendritic cells pulsed with CPS NP combined with the TLR8 ligand R848 as adjuvant showed a significant increase in CD40 expression (Fig 17 C) but not CD80 and CD86 (Fig 17 A-B) when compared to CPS NP alone ($p < 0.01$). Although, R848 containing NP alone did not enhance CD40 expression. Taken together, our data suggest that MF59 and Alum are superior to TLR ligands as adjuvants for improving antigenicity of meningococcal CPS nanoparticulate vaccine.
CD80 Expression

CD86 Expression

CD40 Expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Fluorescence Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>4,000</td>
</tr>
<tr>
<td>CPS NP</td>
<td>10,000</td>
</tr>
<tr>
<td>MPL</td>
<td>15,000</td>
</tr>
<tr>
<td>CpG</td>
<td>20,000</td>
</tr>
<tr>
<td>CT</td>
<td>25,000</td>
</tr>
<tr>
<td>R848</td>
<td>30,000</td>
</tr>
<tr>
<td>MF59</td>
<td>35,000</td>
</tr>
<tr>
<td>Alum</td>
<td>40,000</td>
</tr>
</tbody>
</table>

CPS NP + Adjuvant NP vs. Adjuvant NP

** Significant difference
Figure 17: Expression of co-stimulatory molecules CD80 and CD86 on dendritic cells.

Murine dendritic cells (DC 2.4) (3 × 10^5) were pulsed with CPS-loaded nanoparticulate vaccine CPS NP (antigen dose: 25 µg/3 × 10^5cells) alone or with various adjuvants (adjuvant dose: 25 µg/3 × 10^5cells) for 16 hours. The expression of co-stimulatory molecules CD80 (A), CD86 (B) and CD40 (C) on dendritic cells were detected using flow cytometer following staining with FITC CD80, PE CD86, and APC CD40 markers. The yellow bars represent the group receiving CPS NP along with the adjuvant NP and the red bars represent the group receiving adjuvant NP alone. Error bars represent the standard deviation from the mean of four independent experiments. P values were calculated using Student’s t-test unpaired, two – tail distribution comparing to the blank NP and nanoparticulate vaccine only,**P < 0.01 were significant.

4.3.3. Surface expression of CD95 on dendritic cells pulsed with meningococcal CPS-loaded nanoparticles in presence of various adjuvants.

We previously reported that meningococcal CPS as vaccine antigen-induced the expression of death receptor CD95 on the surface of macrophages in a dose-dependent manner. We showed that with a high dose of CPS antigen, but not the low dose, induced high expression of CD95 and subsequently reduced cell viability (Ubale et al., 2014b). High expression of the death-inducing receptor CD95 on APCs and its binding ligand
CD95L (Fas/FasL) causes the activation of the death pathway leading to apoptosis of the APC, consequently compromising antigen presentation and the ability to induce optimal protective immune responses (Ubale et al., 2014b). In order to verify that adjuvants combined with meningococcal CPS NP did not induce the death pathway, we determined the expression of CD95 in pulsed dendritic cells and assessed cellular viability. Using meningococcal CPS NP dose at 25 µg combined with adjuvant NP dose at 25 µg, no significant increase in the level of CD95 expression on pulsed dendritic cells is observed when compared to CPS NP alone (Figure 18). Moreover, at this concentration of CPS NP combined with adjuvants, the viability of dendritic cells was similar and no significant cell death is observed (Figure 18). The concentration of CPS NP combined with adjuvants was constant across experiments and the data showed that MF59 and Alum like TLR-based adjuvants, induced similar levels of CD95 expression in dendritic cells without reducing dendritic cell viability. The data indicate that this dose (25 µg of CPS NP and adjuvants) helps achieve significant higher antigen presentation and co-stimulation, without activating CD95/CD95L mediated cell death. In this proof-of-concept study, our data is limited to serogroup A CPS. Zughaier observed that CPS serogroup B induced the expression of CD95/CD9L genes in THP-1 monocyctic cells (S. M. Zughaier, 2011). Whether other meningococcal CPS serogroups are able to induce CD95/CD95L is under current investigation.
Induction of CD95 and viability of antigen presenting cells pulsed with meningococcal vaccine-loaded nanoparticles along with adjuvants. Murine dendritic cells (3 × 10^5) were pulsed with CPS NP (antigen dose: 25 µg/3 × 10^5 cells) alone or with various adjuvants (adjuvant dose: 25 µg/3 × 10^5 cells) for 16 hours. The expression of CD95 on dendritic cells was detected using flow cytometer following staining with FITC CD95 marker. Error bars represent the standard deviation from the mean of four independent experiments. The cell viability of DC2.4 cells pulsed with CPS NP and various adjuvants was assessed with trypan blue and depicted by the red line (read on the secondary axis on right), there is no significant reduction in the cell viability with different combinations of adjuvants along with the CPS NP.
4.3.4. Release of Nitric oxide as an innate immune precursor

In the further assessment of adjuvant effects on the antigenicity of meningococcal CPS NP, we assessed nitric oxide release from dendritic cells. Nitric oxide (NO) is an innate immune marker which is released after the uptake and processing of the vaccine antigens and the adjuvants. Nitric oxide also being an important innate immunity marker reflecting the recognition antigen and stimulation of dendritic cells. A higher level of NO release indicates a stronger activation of dendritic cells. We observed that MF59 and Alum NP when combined with meningococcal CPS NP significantly ($p < 0.01$) induced higher amounts of nitric oxide release from pulsed dendritic cells when compared to CPS NP alone (Figure 19). However, the TLR8 ligand R848 when combined with meningococcal CPS NP also induced high amounts of nitric oxide release from dendritic cells ($p < 0.05$) compared to CPS NP or to R848 NP alone (Figure 19). In contrast, the blank nanoparticles which did not contain any antigen or adjuvant did not stimulate the expression of nitric oxide from the murine dendritic cells indicating that the nanoparticle matrix was inert and did not contribute to the innate immune response.
Figure 19: Nitric oxide release from dendritic cells. Murine dendritic cells (DC 2.4) (250 × 10^3) were pulsed with CPS-loaded nanoparticulate vaccine CPS NP (antigen dose: 25 µg/250 × 10^3 cells) along with various adjuvants (adjuvant dose: 25 µg/250 × 10^3 cells) for 16 hours. Nitric oxide release was quantified in the supernatants using the Griess method. Error bars represent the standard deviation from the mean of three independent wells. This is a representative result of four independent experiments. There is a significant increase in nitric oxide release by groups receiving CPS NP along with MF59 and alum was compared to CPS NP group and blank NP (**P < 0.01).

4.3.5. Adjuvant induced percent change in antigenicity

When dendritic cells were pulsed with CPS NP combined with adjuvant NP a significant increase in the antigen presentation markers (Fig 16) and the co-stimulatory molecules
(Fig 17) was observed when compared to the CPS NP only. In order to compare the overall effect of adjuvants NP on the antigenicity of CPS NP in dendritic cells, we calculated the percent increase in activity compared to CPS NP alone (Table 5). We found at least a 200% increase in the expression of antigen presentation markers and co-stimulatory molecules when M59 or Alum NP were combined with CPS NP. However, at these concentrations neither CPS NP nor adjuvants, increased the expression of CD95. Together, our data show that MF59 and alum are superior to TLR-based ligands in inducing antigen presentation markers and co-stimulatory molecules in dendritic cells pulsed with meningococcal CPS nanoparticulate vaccine.

Table 5: Percent activity increase in antigen presentation markers induced by meningococcal nanoparticulate vaccine with adjuvants compared to vaccine nanoparticles alone.

<table>
<thead>
<tr>
<th></th>
<th>MHC I</th>
<th>CD80</th>
<th>CD86</th>
<th>MHC II</th>
<th>CD40</th>
<th>CD95</th>
<th>Nitric Oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPS NP</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>CPS NP + MPL</td>
<td>132.4</td>
<td>149.8</td>
<td>116.2</td>
<td>149.9</td>
<td>125.2</td>
<td>117.8</td>
<td>128.2</td>
</tr>
<tr>
<td>CPS NP + CpG</td>
<td>94.3</td>
<td>171.5</td>
<td>137.9</td>
<td>132.9</td>
<td>134.2</td>
<td>115.9</td>
<td>162.9</td>
</tr>
<tr>
<td>CPS NP + CT</td>
<td>141.3</td>
<td>193.5</td>
<td>157.4</td>
<td>196.6</td>
<td>138.4</td>
<td>125.9</td>
<td>135.5</td>
</tr>
<tr>
<td>CPS NP + R848</td>
<td>179.8</td>
<td>197.3</td>
<td>191.2</td>
<td>143.5</td>
<td>201.2</td>
<td>129.0</td>
<td>195.6</td>
</tr>
<tr>
<td>CPS NP + MF59</td>
<td>254.4</td>
<td>288.0</td>
<td>223.2</td>
<td>221.2</td>
<td>189.8</td>
<td>118.2</td>
<td>262.9</td>
</tr>
<tr>
<td>CPS NP + Alum</td>
<td>236.9</td>
<td>272.0</td>
<td>261.1</td>
<td>222.9</td>
<td>240.9</td>
<td>116.0</td>
<td>276.0</td>
</tr>
</tbody>
</table>
4.4 DISCUSSION

Upon vaccine administration, effective antigen presentation is a prerequisite for an optimal adaptive immune response. In this study, we show that the meningococcal CPS vaccine nanoparticulate formulation when administered along with MF59 or Alum adjuvant nanoparticles induced significantly higher antigen presentation and co-stimulatory molecules markers on dendritic cells. The biodegradable albumin-based nanoparticles serve as a depot, thereby slowly releasing the antigen and adjuvant which enhances priming and activation of the antigen-presenting cells.

Alum has been employed as an adjuvant for decades and been shown to increase the efficacy of vaccines by generating long-lasting B memory response and protective neutralizing antibodies (Chiarella, Massi, De Robertis, Signori, & Fazio, 2007; Li et al., 2014; Morefield et al., 2005). Recently MF59 has been licensed in Europe as an adjuvant for the influenza vaccine (D. T. O’Hagan et al., 2007). Both Alum and MF59 enhance immune responses to vaccine antigens by different mechanisms. The actual mechanism by which alum potentiates the antigenicity of vaccine antigens remains unclear but Alum is known to induce Th2 dependent immune response (Savelkoul et al., 2015). Studies reported that Alum adjuvant effect was mediated by the induction of reactive oxygen species release and the activation of the NLRP3 inflammasome in antigen presenting cells (Eisenbarth, Colegio, O’Connor, Sutterwala, & Flavell, 2008). In contrast, MF59 adjuvant effect is NLRP3-independent (Seubert et al., 2011) and reported to induce a robust innate immune response at the site of vaccination. MF59 as an emulsion, induces a large influx of immune cells leading to cytokines and chemokines to release that further
recruit key immune cells to the site of vaccination. This process of amplified chemotaxis is reported to enhance antigen uptake and presentation (D. T. O’Hagan, Ott, De Gregorio, & Seubert, 2012). Since robust innate immune response to vaccine antigen is crucial for the induction of protective adaptive immune response, TLR-based ligands have been investigated for their adjuvant activity. The mechanism of action for TLR-based ligands is dependent on their ability to engage TLR signaling pathways leading to the release of proinflammatory mediators including chemokines which then potentiate the response to vaccine antigen (Steinhagen et al., 2011; S. Zughaier et al., 2007). In our study here, we observed that MF59 and Alum are superior to TLR-based adjuvant in their ability to potentiate antigen presentation markers in dendritic cells. This is possibly due to the physicochemical properties of these adjuvants. Unlike MF59 and alum that are particulate in nature, TLR-based ligands are soluble, thereby can be cleared or neutralized by host factors faster than particulate adjuvants (Steinhagen et al., 2011). Of note, we observed that the TLR8 ligand R848, when delivered in combination with meningococcal CPS NP, induced the expression of CD40 significantly higher than other TLR ligands used in this study. R848 adjuvant NP alone did not induce high CD40 or CD80 and CD86. Our unpublished data indicated that meningococcal CPS polymers induced high gene expression of TLR8 in human macrophage-like monocytic THP-1 cells. Therefore, it is possible that CPS NP increased the expression of TLR8 and when TLR8 ligand R848 is added combined with CPS led to this significant upregulation of CD40 expression in dendritic cells.

Adjuvants enhance antigen presentation by increasing the expression of MHC I and MHC II and co-stimulatory molecules thus potentiating the interactions between
mature dendritic cells and T cells. In our study, various adjuvants nanoparticles, in particular, MF59 and Alum, when combined with meningococcal nanoparticulate vaccine increased the activation of dendritic cell maturation markers CD80, CD86 and CD40 which are important for effective antigen presentation. These co-stimulatory molecules are expressed on antigen-presenting cells (APCs) and bind to CD28 in naïve T cell, delivering a crucial signal for T cell activation together with the MHC I or MHC II that present antigens by binding to T cell receptor (TCR) on naïve T cell leading to differentiation and effector function (Chen & Flies, 2013; Saito, Yokosuka, & Hashimoto-Tane, 2010). Both of these signals are necessary for an effective dendritic cell-T cell communication. We observed that MF59 and Alum adjuvants have a synergistic effect with meningococcal vaccine nanoparticles as they increase the expression of antigen presentation and co-stimulation markers by 2 to 3 fold. Since the main correlate of protection for the meningococcal vaccine is the opsonic bactericidal antibody titers, an antibody-mediated Th2 immune response is desired (Bröker & Fantoni, 2007). The expression of co-stimulatory molecules is necessary for the generation of high avidity neutralizing antibody response, which is critical for preventive vaccine efficacy (Chen & Flies, 2013).

In support of our observation that Alum and MF59 seem to be most promising adjuvants for the novel meningococcal vaccine nanoparticles, several studies reported on the efficacy of MF59 as an adjuvant for subunit vaccines (D. T. O’Hagan et al., 2007). The presence of MF59 helps to recruit APC’s to the site of injection and hence enhance influenza antigen uptake. MF59 also enhances cross-reactive immune response and generates a strong immune memory needed to maintain the humoral immunity. For
decades aluminum-based adjuvants (aluminum sulfate, aluminum phosphate and aluminum hydroxide) have been used in various vaccine formulations such as diphtheria, hepatitis B, tetanus, pertussis, influenza, human papilloma virus and other (Eifan, Shamji, & Durham, 2011; Francis & Durham, 2004; Krause et al., 2014; Miller, Roque, & Clegg, 2014; Thakur, Kaur, & Kaur, 2014). Therefore, MF59 and Alum adjuvants are well tolerated and their adjuvant effect is well established. Although, aluminum hydroxide deposition in macrophages that persist in intramuscular inoculation site was suggested to cause granulomatous lesion known as macrophagic myofasciitis (Authier et al., 2006; Crucitti & Tsai, 2011; Gherardi & Authier, 2012).

Assessing antigenicity using dendritic cells in-vitro is a limitation of our study. However, this method is informative and predictive of in-vivo responses since dendritic cells are the major antigen presenting cells that interact and respond to antigens and adjuvants. Therefore, using dendritic cells to screen a collection of different adjuvants in-vitro provide a very efficient approach to identify and select the most synergistic and active adjuvants to be used in future in-vivo studies.

4.5. CONCLUSION

MF59 and alum adjuvant nanoparticles when combined with meningococcal CPS nanoparticulate vaccine, are superior to TLR-based ligands in enhancing antigen presentation markers and co-stimulatory molecules in dendritic cells. Enhancing antigen presentation by adjuvants potentiates dendritic cell interactions with effector immune cells leading to desired protective immune responses.
5.1. INTRODUCTION

Measles is a highly contagious infectious disease caused by the measles virus and was responsible for high worldwide morbidity, with conservative estimates in the millions of patients, and potential mortality prior to the introduction of the measles vaccine (Moss & Griffin, 2012; Clements & Cutts, 1995). The attenuated measles vaccines were introduced in the 1960s after the successful isolation and culture of the measles virus in tissue culture by John Enders and then subsequent attenuation by Maurice Hilleman (Clements & Cutts, 1995; Goodson & Seward, 2015). With the advent of these vaccines, tremendous progress has been achieved in reducing the number of deaths due to measles. Nonetheless, patient exposure has been steadily increasing, culminating in a record outbreak of measles in 2015 (“What You Should Know About the Latest Measles Outbreaks,” n.d.). This is because the measles virus is one of the most highly contagious, directly transmitted pathogens, where outbreaks can occur in populations in which fewer than 10% of people (infants and adults) are susceptible (Stalkup, 2002).

With the current vaccination regimen, two doses of the measles vaccine are currently prescribed one for infants before the age of 12 months and a second booster vaccine administered in between 5 to 10 years of age (Goodson & Seward, 2015; Diane E. Griffin, Pan, & Moss, 2008). In the case of the measles vaccine, both humoral and
cell-mediated immune responses are important in fighting measles virus infection (D. E. Griffin, Ward, & Esolen, 1994; Diane E. Griffin, Lin, & Pan, 2012). The humoral response is critical in controlling viral replication and conferring protection, while the cell-mediated immune response is necessary for overcoming acute measles infection by eliminating infected cells (Diane E. Griffin et al., 2012). Thus for controlling an epidemic outbreak in the developed and developing countries, a non-invasive, easy to transport and administer vaccine is needed which correspondingly can activate both arms of the immune system and provide maximum protection (Garly & Aaby, 2003; D. E. Griffin & Pan, 2009). This therapy has largely reduced the incidence of measles epidemics in infants, although in recent years there has been a pronounced increase in outbreaks that have caused global alarm. It should also be noted that debate over the role of thimerosal in the etiology of autism and the related spectrum of disorders may have led to a reduction in measles vaccinations and could be partly responsible for the significant recurrence of measles in this age range (Godlee, Smith, & Marcovitch, 2011; Kalkbrenner, Schmidt, & Penlesky, 2014; Madsen et al., 2002; Taylor, Swerdfeger, & Eslick, 2014). Therefore, currently, a significant research emphasis has been placed on the development of novel vaccine delivery systems that improve patient compliance through the use of non-invasive dosage forms that eliminate thimerosal in order to prevent outbreaks within the unvaccinated population, thus raising herd immunity to a more effective level. (Goodson & Seward, 2015; Diane E. Griffin et al., 2008).

An orally disintegrating film (ODF) formulation is a thin film prepared using hydrophilic polymers which dissolve rapidly on the tongue in the mouth (H. Lou et al., 2014). The buccal cavity provides a large surface area for rapid disintegration, release of
the therapeutic entity, subsequent absorption and provides a potentially good site for antigen delivery. The mucosal surface of the buccal cavity can be an ideal site for vaccination because of its easy accessibility and offers good antigen presentation (Amorij et al., 2012; Cui & Mumper, 2002). The buccal cavity is rich in dendritic cells similar to Langerhans cells, which are a type of antigen-presenting cells (APC). Also, a high density of T lymphocytes and mucosal-associated lymphoid tissue like tonsils, salivary glands, Waldeyer’s ring, and pharyngeal lymphoid tissue are present in the buccal mucosa (Amorij et al., 2012). Hence, buccal immunization utilizing an ODF can help to elicit both mucosal and systemic immunity. ODF formulations are also very robust and enable the accurate delivery of selected antigens with ease of administration reflected in patient compliance for both pediatrics and adults (Kraan et al., 2014; Liew, Tan, & Peh, 2012; Kweon, 2011). Moreover, ODF formulations may also provide safe delivery in infants since the rapid dissolution may alleviate dysphagia and associated choking concerns (Alayoubi et al., 2016).

Mucosal vaccines have been more recently been evaluated and have shown great success, suggesting that the approach can be further evaluated in the future (Tawde et al., 2012c). Previous studies have shown that microparticulate vaccine for delivery via the mucosal surfaces such as the oral cavity has elicited a significantly higher immune response compared to an equivalent solution or suspension formulations (Bhowmik et al., 2011b; Chablani, Tawde, Akalkotkar, et al., 2012b; Shastri et al., 2012a). Thus in this study, we have formulated the measles vaccine encapsulated in microparticles intended to be delivered via the buccal route. These microparticles are made from a biodegradable material that slowly releases antigens, thereby having an antigen depot effect to enhance
immunogenicity (D’Souza et al., 2012; Lai & D’Souza, 2007; Uddin, Bejugam, Gayakwad, Akther, & D’Souza, 2009b). The advantage of utilizing the enteric polymer matrix is that if the vaccine microparticles are not taken up from the buccal cavity, they will still elicit an immune response after being taken up from the GI track. Antigen presentation to the immune cells such as the antigen presenting cells have been suggested to significantly improve response when given in a microparticulate form (Ubale, Gala, Zughaier, & D’Souza, 2014c). Vaccine microparticles formulated as a dry powder possess extended shelf life and doesn't require cold-chain facilities for storage and commercial distribution and along with its formulation in a new dosage form such as the ODF (Ubale, D’Souza, Infield, McCarty, & Zughaier, 2013b) becomes compliant with a global distribution.

In order to overcome these challenges such as needle and syringe free delivery, and to enable ease of administration, we have developed a novel orally disintegrating film (ODF) formulation for microparticulate measles vaccine delivery that is readily adaptable to large-scale production. Moreover, the ODF will facilitate the first line of defense by providing mucosal exposure and triggering immunity through both systemic and local immune response towards the measles virus. In this study, we have formulated measles vaccine in a microparticulate form by using the spray drying technique. These microparticles were incorporated into an optimized orally disintegrating film to produce the ODF measles vaccine formulation for delivery via the buccal route. The ODF formulation of the embedded microparticles can also be readily taste masked to improve compliance, although we have not assessed those parameters in this proof of concept study (Figure 20).
Figure 20: Summary of the method of production of the orally disintegrating film (ODF) loaded with the vaccine microparticulate measles vaccine, and further the immunization studies.

We have tested this novel ODF vaccine formulation in the juvenile porcine model and found that significantly increased levels of antibody have been produced in contrast to naïve juvenile porcine controls. We have also demonstrated antigen presentation and induction of innate immunity in response to our vaccine *in vivo*. Thus, our data demonstrate that a novel measles vaccine delivered in a flexible, orally disintegrating film (ODF) formulation can induce an efficient immune response that may be translated for global clinical applications.
5.2. MATERIALS AND METHODS

5.2.1. Materials

Live attenuated measles virus (Edmonston Zagreb Strain), M-VAC™ in lyophilized form was obtained from Serum Institute of India Ltd., Pune, India. Sterile and endotoxin–free bovine serum albumin (BSA) used to formulate the protein-based microparticles was purchased from Sigma-Aldrich. LYCOAT® RS 720, Neosorb P60W used to make the ODF, was a kind gift from Roquette America Inc., Geneva, IL. Dendritic cells (DC2.4) were given as a kind gift from Dr. Kenneth L. Rock (Dana-Farber Cancer Institute, Inc., Boston, MA, USA). Antibodies used to stain murine MHC I, MHC II, CD80 and CD40 for flow cytometric analysis were purchased from eBioscience laboratories (San Diego, CA). RPMI 1640 medium, Dulbecco's Modified Eagle medium, fetal bovine serum (FBS), penicillin/streptomycin, sodium pyruvate and nonessential amino acids were obtained from Cellgro Mediatech (Herndon, VA).

5.2.2. Preparation of microparticles using BSA

The biodegradable microparticles were prepared following a method previously developed in our vaccine nanotechnology laboratory at Mercer University using the Buchi Mini Spray Dryer B-191 (Bejugam et al., 2013, 2008; Chablani, Tawde, & D’Souza, 2012; Shastri et al., 2012b; Singh & Salnikova, 2014a; Tawde et al., 2012b; Ubale et al., 2013a, 2014b; Uddin et al., 2009a). Briefly, a 1% solution of sterile BSA in sterile water was prepared and kept overnight for cross-linking with glutaraldehyde (200 µL for every 1gm of BSA). Excess glutaraldehyde was neutralized with sodium bisulphate the following day. Live attenuated measles virus (Edmonston Zagreb Strain) solution (5% w/w) was added to the solution and spray dried through a 0.5 mm nozzle
(nozzle temperature: -5°C) at a flow rate of 20 mL/h to obtain the measles vaccine loaded microparticles (Ubale et al., 2013a).

5.2.3. Microparticle recovery yield

Recovery yield of the microparticles after spray drying was calculated for all the batches formulated. Percent recovery yield was evaluated using the following formula:

\[
\text{Percentage Recovery Yield (\%)} = \frac{\text{Weight of microparticles after spray drying} \times 100}{\text{Weight of all ingredients before spray drying}}
\]

5.2.4. Particle size distribution

The particle size of the optimized formulation was evaluated using the Spectrex Laser Particle Counter that works on the principle of laser diffraction. Two mg of the particles were suspended in 1 ml deionized water, vortexed well, and then analyzed by laser diffraction on the particle counter. Particle size was measured in triplicate for empty as well as antigen-loaded particles and contrasted.

5.2.5. Zeta potential measurement

Five micrograms of microparticles were suspended in 1 ml of deionized water, transferred to a zeta potential measurement cuvette, and measured using a Malvern Zetasizer. Zeta potential was measured in triplicate for the control formulation and contrasted with the antigen-loaded microparticles.

5.2.6. Scanning electron microscopy of the microparticles

Scanning electron microscopy (SEM) was performed to evaluate microparticle size distribution and surface morphology. Microparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum-coated with a thin layer (100-
150Å) of gold, the microparticles were examined at 20 kV by a scanning electron microscope Phenome benchtop SEM, Nanoscience Instruments, Phoenix, AZ.

### 5.2.7. Cytotoxicity study

The toxicity of the measles vaccine microparticles toward murine RAW264 macrophages was examined in three replicates by the Alamar Blue assay (Shastri et al., 2012a; Ubale et al., 2013b). Briefly, $2.5 \times 10^5$ cells were plated in each well of a 48 well plate and vaccine microparticles ranging in concentration from 50 µg to 500 µg, n=3 for each concentration, were added to each well. Atropine sulfate was used as a positive control at a concentration of 10 mg/ml. The readings were normalized with the blank microparticles. After 24 hours, 10 µl of a 10× solution of Alamar Blue was added to each well and plates were incubated for 4 hours at 37°C following which the fluorescence was using BioTek Synergy H1 plate reader, Winooski, VT.

### 5.2.8. Quantification of antigen presentation molecules (MHC I and MHC II) expression

Dendritic cells (DC2.4) were plated at $3 \times 10^5$ cells per well in a 48 well plate and incubated at 37°C for 24 h to adhere and stabilize. The adherent cells were pulsed with 250 µg measles vaccine microparticles containing 25 µg of antigen in each well and incubated at 37°C for 16 h. Equal amounts of blank microparticles without any antigen were used as controls in every experiment. The pulsed DC2.4 cells were then washed to remove extracellular microparticles prior to staining with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labeled MHC I and MHC II markers respectively (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The stained DC2.4 cells were
washed with Hank’s Balanced Salt Solution (HBSS) and samples measurements were then quantified on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).

5.2.9. Quantification of co-stimulatory molecules (CD40 and CD80)

A similar method was used for the quantification of co-stimulatory molecules CD40 and CD80, DC2.4 were plated at $3 \times 10^5$ cells per well in a 48 well plate and incubated at 37°C for 24 h to adhere and stabilize. The adherent cells were pulsed with 250 µg measles vaccine microparticles containing 25 µg of antigen in each well and incubated at 37°C for 16 h. An equal amount of blank microparticles without any antigen were used as controls in every experiment. The pulsed DC2.4 cells were then washed to remove extracellular microparticles prior to staining with FITC labeled CD80 and, APC labeled CD40 (eBioscience laboratories, San Diego, CA) for 1 h at 4°C. The cells were then washed and samples measurements were acquired on BD Accuri C6 flow cytometer (BD Bioscience, San Jose, CA).

5.2.10. Nitric oxide release from DC2.4 cells

Freshly grown adherent DC2.4 cells were harvested, washed and re-suspended in Dulbecco's complete media, counted and adjusted to $10^6$ cell/mL. 250 µL aliquots were then dispensed into each well of a 48-well plate at final 250×$10^3$ cell density prior to stimulation with measles vaccine microparticles and blank microparticles which served as blank control. The induced cells were incubated overnight at 37°C with 5% CO₂ and supernatants were harvested. Nitric oxide release was quantified using the Greiss chemical method as previously described (Ubale et al., 2014b). Briefly, the Griess chemical method was used to detect nitrite (NO₂) accumulated in supernatants of induced DC 2.4 cells. Griess reagent was freshly prepared by mixing equal volumes of 1%
sulfanilamide and 0.1% N-(1-naphthylethylenediamine) solutions. One hundred microliters of cell supernatants were transferred into a 48-well plate to which 100 µL of Griess reagent was added. The plate was mixed gently, incubated for 10 min at room temperature, and read at 540 nm using the EL312e microplate reader (BIO-TEK Instruments, Winooski, VT). The optical densities were correlated to the concentration of nitrite. Nitrite was quantitated using the standard curve of sodium nitrite (1 mM stock concentration in distilled water further diluted to the highest standard at 100 µM followed by serial dilutions to 1.56 µM) (S. M. Zughaier et al., 2004).

5.2.11. Preparation of the orally disintegrating film (ODF)

The orally disintegrating films were cast based on the composition presented in table 7. Briefly, the film-forming polymer Lycoat RS720 was dispersed in a solution containing pre-calculated amounts of water, Neosorb P60W and Tween 80 under mechanical stirring. Measles vaccine microparticles previously prepared were uniformly dispersed into the previous suspension. This sample was then allowed to stand still until air bubbles formed during preparation dissipated. Thereafter, the ODF formulation loaded with vaccine microparticles was casted on a plastic support (byco-charts from BYC Gardner GmbH, Germany) using an automatic Erichsen Control Coater KCC101 equipped with a 200 µm wire wound rod applicator. The film was dried in a humidity chamber and when dry was cut into 3 x 4 cm film strips (H. Lou et al., 2014).

5.2.12. Physicochemical characterization of the ODF

Film thickness was measured using a Mitutoyo screw-type micrometer with an accuracy of ± 3µm (Kanagawa, Japan). Tensile strength, Young’s Modulus, and percent
elongation were determined using Stable Micro Systems’ TA.XTplus Texture Analyzer (Surrey, England, U.K.) and the equations below:

\[
Tensile \text{ Strength (MPa)} = \frac{\text{Force at Breaking (N)}}{\text{Film Cross Sectional Area (mm}^2)\}
\]

\[
Young's \text{ Modulus (MPa)} = \frac{\text{Force at Breaking (N)} \times \text{Initial Film Length (mm)}}{\text{Film Cross Sectional Area (mm}^2) \times \text{Change in Film Length (mm)}}
\]

\[
Elongation \% = \frac{\text{Change in Film Length (mm)}}{\text{Initial Film Length (mm)}} \times 100
\]

5.2.13. Porcine methods

The porcine studies were conducted in two domesticated, crossbred swine in the Purdue Translational Pharmacology (PTP) CTSI Core Facility under an approved Purdue University Animal Care and Use protocol (1112000407) (Roth et al., 2013). The PTP facility is registered with the USDA and OLAW and is housed in the School of Veterinary Medicine at Purdue. The domesticated pigs, approximately 5 months of age (20 kg; which have previously shown reveal PK properties similar to pediatric populations (Kulkarni et al., 2012; Roth et al., 2013) arrived at the Animal Housing Facility at Purdue and were acclimated for 7 days prior to any procedure. Pigs were housed for 8 weeks with access to food and water on a 12 hour light cycle. Animals were fasted overnight prior to dosing. Animals were sedated with a combination of Telazol,
Ketamine, Xylazine (TKX 50mg/mL) and maintained on 1-3% Isoflurane via a nose cone. Predose blood samples (1 mL) were taken via jugular stick and placed in a vial containing EDTA. Each of the animals received 25 ODF films in the buccal pouch. The pouches were applied in sets of 2 and once they dissolve the next set of pouches were administered. Once all 25 films were inserted and dissolved animals were removed from anesthesia and allowed to recover in a dark room. The ODFs were dosed at day 0 and two weeks later to provide a boost to the immune system. Animals were fed upon standing. Upon initiation of the study, the blood samples (1 ml) were collected into a vial containing EDTA at 2, 4, and 6 weeks after initial dosing for immunological evaluation and contrasted with naive pigs. Upon collection, each blood sample was stored at -4°C for approximately 30 min until they were processed to separate plasma via centrifugation at 3400 rpm for 10 min. Plasma was siphoned off and placed in an Eppendorf tube for storage at -80°C until shipping.

5.2.14. Quantification of serum antibody using ELISA

The blood samples were collected prior to each dose of vaccination. Serum was isolated and analyzed for specific IgG titers for measles virus ELISA (Enzyme-Linked Immunosorbent Assay) (Plans et al., 2015). ELISA was performed by an overnight coating of the live attenuated measles virus (vaccine antigen) on poly-L-lysine coated high binding 96 well plate (100µg/well in coating buffer) at 4 °C. The plate was washed with washing solution (Tris 50mM, NaCl 0.14M, Tween-20 0.05%) and blocked with 4% non-fat dry milk (Biorad, Hercules, CA) for 2 hrs at 37 °C. After washing, the plate was then incubated with 1: 100 dilution of serum from vaccinated pigs. After 2 hrs of incubation followed by washing, HRP-tagged secondary anti-pig goat IgG (AbD
Serotec®, Raleigh, NC) was added to each well, incubated for 1 hr and then washed.

TMB substrate reagent (3, 3’, 5, 5”-tetramethyl benzidine) (BD OptEIA™, BD Biosciences, CA) was added and the plate was again incubated for 30 min at 37°C. The reaction was stopped by addition of 4N H₂SO₄. The plate was read and the absorbance values quantified at 450 nm using BioTek Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT).

5.2.15. Statistical analysis

All experiments were performed in quadruplets unless otherwise noted. Mean values ± SD and P value (Student’s t-test unpaired, two–tail distribution) was determined individually for all experiments with Microsoft Excel software. A p value of less than 0.05 was considered to be statistically significant.

5.3. RESULTS

5.3.1. Formulation and characterization of measles microparticulate vaccine

3.3.1.1. Microparticles formulation and recovery yield:

The yield of spray drying process was within a range of 85%–95% (w/w) (Table 6). The loss during microparticle preparation can be attributed to microparticles sticking to the cylinder and cyclone of the spray dryer.
Table 6: Physical characteristics of the measles vaccine microparticles. The recovery yield (%) after the spray drying process, particle size, and the zeta potential were measured in triplicates and mean and the standard deviation are reported.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recovery yield (%)</strong></td>
<td>92.76 ± 4.5</td>
</tr>
<tr>
<td><strong>Particle Size (µm)</strong></td>
<td>3.65 ± 1.89</td>
</tr>
<tr>
<td><strong>Zeta Potential (mV)</strong></td>
<td>-32.65 ± 2.4</td>
</tr>
</tbody>
</table>

5.3.1.2. Particle size analysis

The particle size distribution of novel vaccine microparticle formulations from two different batches of empty particles and measles antigen-loaded microparticles was investigated using Spectrex laser counter (Spectrex Corporation). There was no significant difference in size between empty and measles vaccine loaded microparticles ~90% of which were between 1-5 µm with an average particle size of 3.65 ± 1.89 µm (Table 1).

5.3.1.3. Zeta potential measurements

Zeta potential is indicative of the surface charge of the particle. A high positive or negative charge indicates good stability and suspendability of the particle when reconstituted in media as it avoids agglomeration (Ubale et al., 2013b). The zeta potential measurements of empty (unloaded) and antigen-loaded microparticle suspensions in
deionized water were in the range of -30 to -35 mV with the mean of -32.65 ± 2.4 mV and did not differ significantly from each other (Table 6).

5.3.1.4. Scanning Electron Microscopy

The surface morphology of the formulated microparticles was investigated using scanning electron microscopy. The surface of the microparticles was irregular shaped and rough (Figure 21). The different shapes of the microparticles may be helpful for biodistribution and uptake by macrophages (Agarwal et al., 2012; Agarwal & Roy, 2013; Glangchai, Calдорera-Moore, Shi, & Roy, 2008).

Figure 21: Scanning electron microscope (SEM) image of the measles vaccine microparticles.

5.3.1.5. Cytotoxicity study

To assess the cytotoxicity of the formulated vaccine-loaded microparticles on macrophages, we employed the Alamar Blue assay (Shastri et al., 2012a; Ubale et al.,
The results of the cytotoxicity study indicated that the formulation was not toxic to murine macrophages RAW264 at doses ranging from 50 to 500 µg per well (Figure 3). The viability of the cell population exposed to different doses of microparticles did not differ significantly from the cell populations not exposed to microparticles indicating that the microparticles were not toxic to the cells (Figure 22). Atropine sulphate was used as a positive control and as expected showed highly decreased viability in comparison to the negative control i.e. cells alone. The results indicate that measles vaccine-loaded microparticles are not toxic to macrophages.

Figure 22: Albumin based microparticles are not toxic to macrophages. Briefly, RAW264 murine macrophages treated with increasing doses of microparticles and incubated overnight. Microparticles by themselves were not toxic to RAW264 cells as compared to untreated cells. The cytotoxicity was analyzed by the Alamar Blue assay that uses the reducing power of living cells to quantitatively measure cell viability. The experiment was performed in triplicate.
5.3.1.6. Antigen presentation on dendritic cells

The primary requirement for a vaccine to show its effect by activating the dendritic cell after the antigen processing and expressing the antigen on the major histocompatibility class I and II molecules. Antigen presentation by matured dendritic cells to T lymphocytes is essential for vaccine-induced protective immune responses. Thus, we assessed the expression of the MHC class I and MHC class II molecules on the surface of dendritic cells when exposed to the vaccine microparticles. Our results show that there is a significant increase in the expression of both MHC I and MHC II molecules when exposed to vaccine microparticles as compared to blank microparticles (Figure 23).
Figure 23: Antigen induced expression of MHC I and MHC II complex on murine dendritic cells is presented. Briefly, murine dendritic cells ($3 \times 10^4$) were pulsed with measles vaccine microparticles (antigen dose: 25 µg/$3 \times 10^4$cells) for 16 hours. The expression of antigen presenting receptor MHC I (Red bars) and MHC II (Green bars) were detected using flow cytometer following staining with FITC MHC I and FITC MHC II markers. Error bars represent the standard deviation from the mean of four independent experiments. P values were calculated using Student’s t-test unpaired, two – tail distribution comparing to the blank MP and microparticulate vaccine,**P < 0.01 were significant.
5.3.1.7. **Co-stimulatory molecules expression on dendritic cells**

Co-stimulatory molecules provide the crucial second signal to naïve T cells for its activation and proliferation leading to subset differentiation and effector functions required to elicit adaptive immune responses (Fuse, Tsai, Rommereim, Zhang, & Usherwood, 2011; McAdam, Schweitzer, & Sharpe, 1998b; Ubale et al., 2014c). Since effective antigen presentation by mature dendritic cells to T cells requires the expression of co-stimulatory molecules such as CD40 and CD80, we assessed their expression in dendritic cells pulsed with measles vaccine microparticles and blank microparticles. We found that measles vaccine microparticles enhanced the expression of co-stimulatory molecules CD40 and CD80 on dendritic cells when compared to blank microparticles (Figure 24).
Figure 24: The results from the expression of co-stimulatory molecules, CD 40 and CD 80 on dendritic cells is illustrated. Murine dendritic DC 2.6 cells (3 × 10^4) pulsed with measles vaccine microparticles (antigen dose: 25 μg/3 × 10^4 cells) for 16 hours. The expression of co-stimulatory molecules CD40 (blue bars) and CD80 (yellow bars) on dendritic cells were detected using flow cytometer following staining with APC CD40 markers and FITC CD80. Error bars represent the standard deviation from the mean of four independent experiments. P values were calculated using Student’s t-test unpaired, two – tail distribution comparing to the blank MP and microparticulate vaccine,**P < 0.01 were significant.
5.3.1.8. Nitric oxide release from the dendritic cells

Nitric oxide (NO) is an innate immune marker which is released after the uptake and processing of the vaccine antigens by the dendritic cells. Nitric oxide also being an important innate immunity marker reflecting the recognition antigen and stimulation of dendritic cells. A higher level of NO release indicates a stronger activation of dendritic cells. We observed a significantly higher level of NO released by the dendritic cells when compared to the blank microparticles (Figure 25). This also suggests that BSA crosslinked polymer matrix used to make the microparticles is not immunogenic and the innate immune response generated can be attributed to the measles antigen present the vaccine loaded microparticle group.
Figure 25: Nitric oxide release from dendritic cells was measured. Briefly, murine dendritic cells (DC 2.4) \((250 \times 10^3)\) were pulsed with measles vaccine microparticles (antigen dose: 25 µg/250 \times 10^3\text{cells}) for 16 hours. Nitrite accumulation in the supernatants was determined using the Greiss reagent. There is a significant release of nitric oxide release from the measles vaccine microparticles when compared to blank microparticles. Error bars represent the standard deviation from the average of two independent determinations per well. The data shown are representative of three independent experiments. *\(p\) value < 0.05 were calculated as above.
5.3.2 Formulation and characterization of measles microparticulate vaccine

5.3.2.1. Vaccine loaded ODF characterization

Pharmaceutical films must endure a different type of mechanical stress encountered during manufacture, packaging, travel, and consumer handling. The physical characteristics of the vaccine loaded ODF are listed in Table 7. Briefly undamaged films with an average width of 30 mm and thickness of 79 μm were placed between two rubber grippers approximately 21 mm apart. Stress was applied at 10 mm/min until rupture. Studies were conducted with 4 replicates. Mechanical properties of the vaccine loaded ODF are listed in Table 4. The transverse section of the vaccine loaded ODF is shown in Figure 26 where the white spots are the vaccine microparticles which are uniformly dispersed throughout the matrix of the film.

Table 7: Composition of the measles vaccine microparticulate loaded orally disintegrating film (ODF)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Ratio (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measles vaccine microparticles</td>
<td>0.2</td>
</tr>
<tr>
<td>LYCOAT® RS 720</td>
<td>23</td>
</tr>
<tr>
<td>Neosorb P60W</td>
<td>5</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 8: Physical characterization of the measles ODF vaccine.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>2.0 cm</td>
</tr>
<tr>
<td>Width</td>
<td>3.0 cm</td>
</tr>
<tr>
<td>Thickness</td>
<td>0.079 mm</td>
</tr>
</tbody>
</table>

Table 9: Mechanical properties of the films are listed as the average and the standard deviation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tensile Strength (MPa)</td>
<td>15.8 (2.4)</td>
</tr>
<tr>
<td>Young’s Modulus (MPa)</td>
<td>739 (192)</td>
</tr>
<tr>
<td>Elongation (%)</td>
<td>2.2 (0.4)</td>
</tr>
</tbody>
</table>
5.3.3. Immunization studies

To assess the ability of the measles vaccine loaded on to the ODF, the ODF was given to pigs via the buccal route. We chose the juvenile porcine model to test our vaccine formulation because the oral mucosa of pigs resembles that of human more closely than any other animal in terms of structure and composition (C. A. Squier, Cox, Wertz, & Downing, 1986; Christopher A. Squier, Cox, & Wertz, 1991). Moreover, pigs also have non-keratinized buccal mucosa similar to that of human (Hoogstraate et al., 1996; Junginger, Hoogstraate, & Verhoef, 1999). The presence of measles specific
antibody in the serum samples was assessed using ELISA as mentioned. The increase in the quantity of measles specific antibody was compared to the serum collected prior to dosing (naïve pigs). There was a significant increase in antibody titers after 2 weeks of the first dose. After the second dose at 2 weeks, a further increase in the antibody titer was observed in weeks 4 and 6. The antibody levels remained significantly higher at all the time points (week 2, 4 and 6) when contrasted with predose levels (Figure 27).

Figure 27: IgG levels post-immunization was assessed in the juvenile porcine model and contrasted with predose levels in naïve pigs. Briefly, blood samples were collected before each dose and serum were separated. The serum antibody levels were measured using ELISA. There is a significant increase in the serum IgG levels when compared between serum IgG level pre-dosing and post dosing (**P < 0.01). Error bars represent the standard deviation from the average of two independent determinations per well. The data shown are representative of three independent experiments (**P < 0.01).
5.4. DISCUSSION

There is an imperative need for the development of novel vaccine delivery strategies in order to improve the patient compliance and effective immunization on large-scale populations. Orally disintegrating films (ODF) containing measles vaccine microparticles are one such method of a noninvasive immunization strategy. Being by design a fast melting formulation (few seconds), the ODF dissolves at the first contact with saliva and facilitates the coating of the buccal surface enhancing the delivery of the vaccine into the buccal mucosae. ODF also assures dosing accuracy and elicits the induction of an effective immune response. As the buccal mucosae are rich in immune cells such as dendritic cells, Langerhans cells etc., it is a very convenient and patient compliant site for vaccination. This is supported by data including that of Lundholm et al. demonstrating that pDNA administered to the oral cheek of mice using a jet injection induced the production of IgA signifying a mucosal immune response (Lundholm, Asakura, Hinkula, Lucht, & Wahren, 1999). In addition, Wang et al. reported that mucosal delivery of a melanoma vaccine in a hamster model helped treat oral melanoma and distant skin lesions (Wang et al., 2001). All of these studies demonstrated that buccal immunization is possible and can be very effective at the same time.

Despite tremendous achievements in global measles control, the virus still circulates, causing infection, severe disease, and death. Since measles virus is highly contagious, high levels of population immunity are required in order to interrupt measles virus transmission (Goodson & Seward, 2015). This poses a challenge for healthcare practitioners to immunize large populations, as the current vaccine is invasive and requires medical professionals for its administration (Brniak, Maślak, & Jachowicz,
2015; Diane E. Griffin et al., 2008; Stalkup, 2002). This problem could be overcome using an ODF measles vaccine as it can be administered by the individual themselves. Thus the cost associated with administration and cold chain storage could be minimized and large population could be immunized at an affordable cost.

Microparticles offer a novel delivery system for the vaccines and have more recently been utilized for both infectious disease and cancer vaccines (Bhowmik et al., 2011b; “Nanoparticulate Vaccine Delivery Systems,” n.d.; Shastri et al., 2012a; Singh & Salnikova, 2014b; Tawde et al., 2012c; Ubale et al., 2013b; Uddin, Bejugam, Gayakwad, Akther, & D’Souza, 2009c). The microparticles are made from a biocompatible and biodegradable polymer matrix which protects the antigen (measles virus) in a stable form. The microparticulate matrix is not toxic to normal cells at higher concentrations and does not induce an immune response. These microparticles due to their size are easily taken up by the antigen presenting cells and thus help in better antigen presentation to the immune system (Ubale et al., 2014c). Any adjuvants, such as alum, MF59, etc. can also be encapsulated in these microparticles to improve the efficacy of the vaccine and generate a robust immune response (Ubale et al., 2013b).

In our study, we found that the measles vaccine when formulated as microparticles and incorporated into the ODF formulation as an oral delivery system can induce significantly higher antigen presentation to the MHC I and II molecules and also the corresponding co-stimulatory molecules CD40 and CD80 when compared to the blank formulation. This ensures that the ODF, after dissolving in the oral cavity is coating the buccal mucosa allowing the vaccine microparticles to be taken up by the APCs and stimulate both the arms of the immune system via the Th1 and Th2 pathways. Due to this
activation, we found significant high antibody titer in the juvenile porcine immunization model study (Roth et al., 2013). Thus from this exploratory study to determine if the juvenile porcine buccal mucosa can serve as a surrogate for human response and an alternative to invasive vaccines using the ODF formulations, we plan to test other vaccines against influenza, meningitis etc. in similar formulations in the future. We also plan to further develop the current measles vaccine ODF to increase loading and optimize efficacy in a readily translatable formulation.

5.5. CONCLUSION

The novel in vivo approach to formulating microparticulate measles vaccine in an orally disintegrating film for delivery via the buccal route in a juvenile porcine model has revealed a promising mode of immunization strategy against measles in a commonly accepted surrogate model for human buccal delivery (Roth et al., 2013). As mentioned earlier, this mode of immunization can be used for large-scale immunizations to prevent transmission of measles virus and epidemics. Moreover, it is patient compliant due to its ease of administration and can be given to populations of all ages from infants to adults. In this exploratory study, we conducted immunization studies with the ODF measles vaccine in the juvenile porcine model and found a significant increase in the antibody titers along with induction of innate immune response.
CHAPTER 6
FORMULATION AND DEVELOPMENT OF A NOVEL GONORRHEA VACCINE FOR TRANSDERMAL DELIVERY

6.1. INTRODUCTION

Gonorrhea is a sexually transmitted bacterial infection caused by the Gram-negative diplococcus Neisseria gonorrhoeae (Gc). Gonorrhea is one of the most common infectious diseases worldwide, with significant immediate and long-term morbidity and mortality. According to CDC, Gonorrhea is the second most commonly reported sexually transmitted notifiable disease in the United States. The WHO estimated that the global burden of gonorrhea in adults was 106 million cases in 2008, a 21% increase compared to 2005. In sexually active adolescents and adults, Gc causes clinically in apparent mucosal infections (most common in women), symptomatic urethritis and cervicitis, upper urogenital tract infections, and pelvic inflammatory disease. Extra-genital rectal and pharyngeal infections occur frequently and coinfections with other sexually transmitted pathogens are common. Systemic or disseminated gonococcal infections (DGI) are infrequent (0.5–3%), occur mainly in women, and include a characteristic gonococcal arthritis-dermatitis syndrome, supportive arthritis, and rarely endocarditis, meningitis or other localized infections. Complications from Gc infections are frequent, debilitating, and disproportionately affect women. Gonorrhea also dramatically increases the acquisition and transmission of human immunodeficiency virus (HIV).
6.1.1. Antibiotic resistance to Gonorrhea

The gonorrhea infection is being treated with antimicrobials. Gonorrhea treatment is complicated by the ability of N. gonorrhoea to develop resistance to antimicrobials (1). N. gonorrhoea is a microbe with a natural competence throughout the vital cycle, the absorption of drug resistance chromosomal genes can be an efficient and widespread process [2]. The constant effect of antimicrobials on Neisseria (during treatment of gonorrhea or other diseases) can lead to the selection of resistant strains, which arise due to spontaneous gene mutations and/or acquisition of resistance genes (parts of genes) [3].

In 2007, the emergence of fluoroquinolone-resistant N. gonorrhoea in the United States prompted CDC to cease recommending fluoroquinolones for treatment of gonorrhea, leaving cephalosporins as the only remaining class of antimicrobials available for treatment of gonorrhea in the United States. Reflecting concern about emerging gonococcal resistance, CDC’s 2010 STD treatment guidelines recommended dual therapy for gonorrhea with a cephalosporin plus either azithromycin or doxycycline.

Consequently over the years, on the basis of experience with other microbes that have developed antimicrobial resistance rapidly, a theoretical basis exists for combination therapy using two antimicrobials with different mechanisms of action (e.g., a cephalosporin plus azithromycin) to improve treatment efficacy. Antibiotic resistance is widespread and has developed rapidly with each successive treatment regimen.

Alarmingly, with the advent of resistance to extended-spectrum cephalosporins, we have now reached the point where untreatable disease can be anticipated in the near future [6]. Although rapid effective treatment of gonorrhea decreases long-term sequelae and can
eliminate the effect on HIV transmission [7], expansion of multi-drug resistant Gc is a global threat to public health and amplifies the urgent need for novel prevention methods.

6.1.2. Interaction of Gonorrhea cocci with the immune system

The Gc interacts with the immune system and prevents the generation of an adaptive immune response. Gc can interact with various immune cells to elicit innate inflammatory responses and suppress Th1/Th2-mediated specific immune responses (Figure 28). (A) Phagocytosis by macrophages results in activation of NLRP3 inflammasomes, the production of IL-1 and activation of PMNs, and activation of cathepsin B, which leads to pyronecrosis of APC (Duncan et al., 2009). (B) Interactions with DCs lead to up-regulation of PDL-1 and PDL-2, which induce apoptosis of cells bearing PD1. This up-regulation also causes the release of IL-10, which has immunoregulatory properties and stimulates type 1 regulatory T cells (Tr1) (Zhu et al., 2012). (C) Interaction with CD4+ T helper cells (or B cells) induces secretion of IL-10, TGF-β, and IL-6 (Liu, Islam, Jarvis, Gray-Owen, & Russell, 2012). IL-10 and TGF-β suppress the activation of Th1 and Th2 cells both directly, and through the activation of Tr1 cells. TGF-β and IL-6 drive the development of Th17 cells which secrete IL-17 and IL-22, leading to the recruitment or induction of innate defenses such as PMNs and antimicrobial peptides. Gc is able to resist destruction by PMNs and antimicrobial peptides while concomitantly suppressing the development of adaptive immune responses such as Gc-specific antibodies that could enhance phagocytosis and intracellular killing by phagocytes and bacteriolysis through the classical complement pathway (Jerse, Bash, & Russell, 2014). Thus exploring a transdermal microparticle vaccine formulation that confers protection in patients, induces herd immunity, and provides significant
advantages over the conventional antibiotic therapy will have high public health impact in the United States.

Figure 28: Mechanisms of interaction of N. gonorrhoea with cells of the immune system.


6.1.3. Vaccines strategies for gonorrhea

Development of an effective gonorrhea vaccine is likely to have significant benefits given the impact of gonorrhea on human health. Only two vaccines for GC have entered into clinical trials. The first was a crude killed whole cell vaccine, which was studied in a controlled experiment in a population of Inuit in northern Canada with high incidence and prevalence of GC infection. There was no evidence of protection, even though the vaccine even though the vaccine was said to be well tolerated and induced an antibody response in over 90% of vaccine recipients. There were other studies, which showed some success, but the major problem remains the prevention of generation of an
adaptive immune response. This the main aim of vaccine development strategies has been to identify specific antigens and secondly generation of a protective immune response.

6.1.4 Transdermal immunization

The skin provides a unique site for the vaccination purposes as it is easily accessible and houses various immune cells for an efficient immune response against a range of antigens. Skin serves as a barrier against various pathogens and is equipped with the skin associated lymphoid tissues (SALT) to combat any insult from invading pathogens. Various skin cells assist in the generation of effective immune response (Gao, Pan, Chen, Xue, & Li, 2008). Keratinocytes are the most predominant (95%) epidermal cells in the skin. They can be activated by pathogens and result in the production of cytokines, which in turn recruits dendritic cells/antigen-presenting cells to the site of action leading to initiation of the immune response. Skin host’s special kind of dendritic cells, the Langerhans cells. Langerhans cells comprise only 2% of the total cell population in the epidermis but due to their extended dendrites spread in the epidermal layer, they cover over 25% of the skin surface. These are professional phagocytic cells efficient in immune surveillance and further signaling to the T-cells present in their vicinity. Activated macrophages and T-cells drain into nearby lymph nodes leading to an enhanced immune response. Currently, most of the vaccines are administered via the subcutaneous or intramuscular route. These have been highly effective in generating a protective immune response but they remain to be invasive, painful and require a skilled professional for vaccination. In an attempt to minimize some of these issues scientists have explored the potential of delivering vaccine antigens intradermally using microneedles. Microneedles, as the name indicates, are micron-sized needles, which upon
insertion into the skin result in the formation of aqueous conduits forming a passage for the vaccine antigens towards the immune-competent skin layers (Figure 2). Due to their short needle length, they avoid contact with the nerve endings in the dermis thus remain to be a painless mode of immunization. Recently FDA approved Intanza™ (by Sanofi Pasteur), an intradermal influenza vaccine that incorporates a 1.5 mm needle attached to a pre-filled syringe loaded with flu antigens. It has been shown to be efficacious when compared with an IM flu vaccine thus bringing a switch from hypodermic needles to “micro”-needles for immunizations. This opens a new avenue of vaccine delivery through an effective, painless and patient-friendly route of administration. The success of immunization via skin using microneedles inspired us to evaluate the potential of delivering gonorrhea vaccine through this route.

In our study, we have utilized formalin fixed dead gonorrhea bacteria as the vaccine antigen. This was formulated into microparticles for delivery. These microparticles were formulated into microneedles for transdermal immunization and the antibodies generated were quantified using ELISA.

6.2 METHOD

6.2.1. Preparation of the N. Gonorrhea vaccine antigen

The N. gonorrhea cells were grown in culture medium. When confluent, the media was removed and a 10% solution of formalin was added and kept overnight. This will lead to fixation of the cells in their native form which were used as the antigen for the vaccine.
6.2.2. Preparation of the N. Gonorrhea vaccine loaded microparticles

The biodegradable microparticles were prepared following a method previously developed in our vaccine nanotechnology laboratory at Mercer University using the Buchi Mini Spray Dryer B-191 (Bejugam et al., 2013, 2008; Chablani, Tawde, & D’Souza, 2012; Shastri et al., 2012b; Singh & Salnikova, 2014a; Tawde et al., 2012b; Ubale et al., 2013a, 2014b; Uddin et al., 2009a). For a batch of 100 mg of vaccine microparticles (10% antigen loading) 10 mg of the formalin-fixed whole cell of N. Gonorrhea (5 ml of 2 mg/mL stock solution) and 90 mg of pre-crosslinked BSA were mixed. Pre-crosslinked BSA solution is prepared by weighing the 90 mg BSA and dissolving it in 5 mL DI water in a 50 mL beaker. Once BSA is dissolved, glutaraldehyde (25% in DI water purchased from Fisher Scientific, Pittsburgh, PA) (200 µL for every 1gm of BSA) is added to the BSA solution. This 50 ml beaker is kept for stirring (300 rpm) overnight in a dark place at room temperature. The following day, excess glutaraldehyde was neutralized with sodium bisulphate (10 mg). At this point, the formalin-fixed whole cell of N. Gonorrhea which is our antigen is added to the pre-crosslinked BSA prepared overnight. 100 mg this prepared formulation is dissolved in 10mL of DI water. This formulation was spray dried through a 0.5 mm nozzle (nozzle temperature: -5°C). The inlet temperature was 120°C with the aspirator at 100% and a flow rate of 20 mL/h to obtain the N. Gonorrhea vaccine microparticles.

6.2.3. Microparticle recovery yield

Recovery yield of the microparticles after spray drying was calculated for all the batches formulated. Percent recovery yield was evaluated using the following formula:
Percentage Recovery Yield (%) = \frac{\text{Weight of microparticles after spray drying} \times 100}{\text{Weight of all ingredients before spray drying}}

6.2.4. Particle size distribution

The particle size of the optimized formulation was evaluated using the Spectrex Laser Particle Counter that works on the principle of laser diffraction. Two mg of the particles were suspended in 1 ml deionized water, vortexed well, and then analyzed by laser diffraction on the particle counter. Particle size was measured in triplicate for empty as well as antigen-loaded particles and contrasted.

6.2.5. Zeta potential measurement

Five micrograms of microparticles were suspended in 1 ml of deionized water, transferred to a zeta potential measurement cuvette, and measured using a Malvern Zetasizer. Zeta potential was measured in triplicate for the control formulation and contrasted with the antigen-loaded microparticles.

6.2.6. Scanning electron microscopy of the microparticles

Scanning electron microscopy (SEM) was performed to evaluate microparticle size distribution and surface morphology. Microparticles were mounted onto metal stubs using double-sided adhesive tape. After being vacuum-coated with a thin layer (100-150Å) of gold, the microparticles were examined by a scanning electron microscope Phenome benchtop SEM, Nanoscience Instruments, Phoenix, AZ.
6.2.7. Fabrication of N. Gonorrhea vaccine microparticles loaded microneedles

Table 10: Formula for the preparation of microneedles

<table>
<thead>
<tr>
<th></th>
<th>Percent w/w</th>
<th>200 mg batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine Microparticles</td>
<td>10%</td>
<td>20 mg</td>
</tr>
<tr>
<td>Trhealose</td>
<td>25%</td>
<td>50 mg</td>
</tr>
<tr>
<td>Maltose</td>
<td>25%</td>
<td>50 mg</td>
</tr>
<tr>
<td>PVA</td>
<td>20%</td>
<td>40 mg</td>
</tr>
<tr>
<td>HPMC</td>
<td>20%</td>
<td>40 mg</td>
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</tbody>
</table>

Microneedles formulation was prepared as per formula mentioned in Table: 10. PVA, HPMC, Maltose, and Trehalose were added to a 1.7 mL microcentrifuge tube. The contents were dissolved in as less water as possible (e.g. 200 mg of total solid content can be dissolved in 600 µL water). To ensure maximum dissolution, the centrifuge tube was vortexed for 1 minute. Then approximately 1/5th quantity (of water that was added to dissolve the solids) of Ammonium hydroxide (NH₄OH) (6N purchased from Fisher Scientific, Pittsburgh, PA) is added to the microcentrifuge tube (here, 120 µL) and vortex again (2 minutes). The tube is kept aside for 2 hours to ensure complete dissolution of the matrix polymers. Once the solution is clear, the weighed amount of blank or vaccine microparticles particles in the end. 150 – 200 µL of the final formulation is poured into the molds. Centrifugation is done in the fixed angle centrifuge in order to remove air
bubbles and to force the formulation to go into the microneedles mold. The maximum speed is 2000 rpm which is achieved stepwise, in order to avoid jerks in the rotation process for 10 mins. Speed should be lowered gradually for the same reason as above. After this centrifugation step, 200 µL blank formulation i.e. formulation without vaccine microparticles is added to the top of the backing layer. Centrifugation is repeated as per the same method mentioned above for the backing layer. Once the backing layer is formed, the molds are placed in an incubator at 32°C for overnight drying. On the following day, the molds are placed in the vacuum desiccator until further use.

6.2.8. In-vivo transdermal immunization for gonorrhea vaccine

The immunogenicity of the microparticulate vaccine was evaluated using Swiss Webster (CFW) female mice model. Six to eight-week-old Swiss Webster (CFW) mice were purchased from Charles River Laboratories, Wilmington, MA, and the animals were acclimatized for one week prior use. The animal experiments were carried out as per approved protocols by Mercer University’s Institutional Committee for the Care and Use of Laboratory animals (IACUC).

In the first animal study (proof-of-concept), the mice were immunized subcutaneously on the back with 10 mg of vaccine particles using a 26.5G needle. One prime dose at day 0 and two booster doses were given at week 1 and 2 were administered. The mice were monitored and blood samples were collected at every 2-week interval starting from day 0, week 2, 4, 6, 8 and 10. The antibody was quantified using ELISA mentioned in next section.
For delivering microparticles via the transdermal route, mice skin on the back was shaved two days prior to vaccination. One day prior to vaccination, the remainder of the hair was treated with Nair Hair removal cream (Ewing, NJ) for 10 minutes and then wiped off with a cotton swab. The vaccine loaded microneedles prepared previously were administered in the skin previously treated. The microneedles patch was applied for 20 minutes which ensured the delivery of the vaccine transdermally. One prime dose at day 0 and two booster doses were given at week 1 and 2 were administered. The mice were monitored and blood samples were collected at every 2-week interval starting from day 0, week 2, 4, 6, 8 and 10. The antibody was quantified using ELISA mentioned in next section.

6.2.9. Quantification of serum antibody using ELISA

The blood samples were collected prior to each dose of vaccination. Serum was isolated and analyzed for specific IgG titers for N. gonorrhea ELISA (Enzyme-Linked Immunosorbent Assay) (Plans et al., 2015). Briefly, ELISA was performed by an overnight coating of the formalin-fixed whole cell of N. gonorrhea (vaccine antigen) on poly-L-lysine coated high binding 96 well plate (100µg/well in coating buffer 200 µL) at 4 ºC. The plate was washed with washing solution (200 µL) (Tris 50mM, NaCl 0.14M, Tween-20 0.05%) and blocked with 4% non-fat dry milk (200 µL) (Biorad, Hercules, CA) for 2 hrs at 37 ºC. After washing, the plate was then incubated with 1: 100 dilution of serum from mice. After 2 hrs of incubation followed by washing, HRP-tagged secondary anti-mouse goat IgG (AbD Serotec®, Raleigh, NC) (100 µL / well) was added to each well, incubated for 1 hr and then washed with washing solution. TMB substrate reagent (3, 3’, 5, 5”-tetramethyl benzidine) (BD OptEIA™, BD Biosciences, CA) (100
µL / well) was added and the plate was again incubated for 30 min at 37ºC. The reaction was stopped by addition of 4N H₂SO₄ (100 µL / well). The plate was read and the absorbance values quantified at 450 nm using BioTek Synergy H1 microplate reader (BioTek Instruments Inc., Winooski, VT).

6.2.10. Determination of T-cell and B-cell based immune response in lymphatic organs

The single cell suspension of the spleens and lymph nodes was made using 40 µm cell strainer. The viability of the cells was checked using trypan blue exclusion method by TC10™ automated cell counter (Biorad, Hercules, CA). 1 ml of viable cells at the concentration of 1 × 10⁶ cells/ml is taken in a 1.7 mL Eppendorf tube. The anti-mouse CD4 PE and anti-mouse CD8a FITC (eBioscience, San Diego, CA) was added to cells at the concentration of 10 µL / ml. The tube was protected from light and hence stored in a dark place. The tube were incubated with the marker for 30 ± 5 minutes over ice. After the incubation, the cells were spun and washed 2 times for 30 seconds using Hanks – ive buffer (200 µL). Then the cells are resuspended in Hanks + ive buffer (200 µL) and stored on ice in a dark place. Meanwhile, the flow cytometer, BD Accuri™ C6 Plus (BD Accuri Cytometers, Ann Arbor, MI) was started and warmed up. The gate for live cells was set with the stock cells. 5000 events were recorded in the gate for each CD4 and CD8 on the flow cytometer.

6.2.11. Statistical analysis

All experiments were performed in quadruplets unless otherwise noted. Mean values ± SD and P value (Student’s t-test unpaired, two–tail distribution) was determined individually for all experiments with Microsoft Excel software. A p value of less than 0.05 was considered to be statistically significant.
6.3 RESULTS

6.3.1. Formalin-fixed whole cell N. Gonorrhea vaccine antigen

The N. gonorrhea cells were grown and cultured as mentioned previously. Post reaching the 70% confluency, they were treated with formalin overnight to ensure, the complete killing of the bacterial cells at the same time, maintaining the morphology of the bacterial cell. The bacterial surface proteins are conserved and preserved in their native form by this treatment of formalin. From the scanning electron micrograph (Figure: 29) we could see the cells maintaining the cellular structure post-treatment with formaldehyde. This is very important, as it is the source of all the surface expressed proteins which could be potential antigenic sites. At the current time, there are no identified antigens candidates for the gonorrhea vaccine, conserving all the possible antigenic epitopes on the bacterial surface in their native form and presenting it to the antigen presenting cells of the immune system, there is a chance of generating an immune response.
6.3.2. Yield of the microparticles using the spray dryer

The yield of spray drying process was within a range of 85%–95% (w/w) (Table 11). The loss during microparticle preparation can be attributed to microparticles sticking to the cylinder and cyclone of the spray dryer.
Table 11: Physical characteristics of the measles vaccine microparticles. The recovery yield (%) after the spray drying process, particle size, and the zeta potential were measured in triplicates and mean and the standard deviation is reported.

<table>
<thead>
<tr>
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<th>Mean ± SD</th>
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<tr>
<td>Recovery yield (%)</td>
<td>91.56 ± 5.3</td>
</tr>
<tr>
<td>Particle Size (µm)</td>
<td>3.65 ± 1.89</td>
</tr>
<tr>
<td>Zeta Potential (mV)</td>
<td>-32.65 ± 2.4</td>
</tr>
</tbody>
</table>

6.3.3. Particle size analysis

The particle size distribution of novel vaccine microparticle formulations from two different batches of empty particles and measles antigen-loaded microparticles was investigated using Spectrex laser counter (Spectrex Corporation). There was no significant difference in size between empty and measles vaccine loaded microparticles ~90% of which were between 1-5 µm with an average particle size of 3.65 ± 1.89 µm.

6.3.4. Zeta Potential Measurements

Zeta potential is indicative of the surface charge of the particle. A high positive or negative charge indicates good stability and suspendability of the particle when reconstituted in media as it avoids agglomeration (Ubale et al., 2013b). The zeta potential measurements of empty (unloaded) and antigen-loaded microparticle suspensions in deionized water were in the range of -30 to -35 mV with the mean of -32.65 ± 2.4 mV and did not differ significantly from each other (Table 11).
6.3.5. Surface morphology of the microparticles

The surface morphology of the formulated microparticles was investigated using scanning electron microscopy. The surface of the microparticles was irregular shaped and rough (Figure 30). The different shapes of the microparticles may be helpful for biodistribution and uptake by macrophages (Agarwal et al., 2012; Agarwal & Roy, 2013; Glangchai et al., 2008).

Figure 30: Scanning electron microscope image of polymeric microparticles depicting an irregular shape with a particle size in the range of 1-5 µm.

6.3.6. Measurement of antibodies post subcutaneous immunization

Once the formulation of the microparticles containing the formalin fixed *N. gonorrhea* whole cell as the antigen was characterized for surface charge, size, and morphology, we decided to carry out a proof-of-concept *in-vivo* study. Six to eight week-old Swiss Webster (CFW) mice were used in this study and there were three groups – Negative control, which did not receive blank microparticles without any vaccine. This
group served as the control. The second group was the vaccine suspension (GnH Sol) given subcutaneously, which served as a control for the microparticles. Finally, the third group received the vaccine formulated into microparticles (GnH MP). There were six animals in each group and the experiments were carried out as per the approved IACUC from Mercer University. Figure 31 shows the antibody titers measured at week 2, 4, 6, 8, 10 measured using ELISA. There was one prime on day 0 and two booster’s doses of vaccine given at week 1 and 2. The groups receiving the vaccine i.e. GnH Sol vaccine suspension and GnH MP vaccine microparticles showed significantly higher antibody titers when compared to the Negative control group i.e. blank microparticles at week 4, 6, 8 and 10 (*P < 0.05). From this experiment, we could show the initial results that the formalin fixed dead gonorrhea bacteria was able to generate an immune response and antibodies were produced in response to the vaccine.

Figure 31: N. gonorrhoeae vaccine induced serum IgG level
![N. gonorrhoeae vaccine induced serum IgG level](image-url)

The groups receiving GnH Suspension and GnH microparticulate vaccines showed significantly
higher serum IgG levels when compared to the bank microparticles which serve as negative control after week 4 (n=6) (*P<0.05). Both the groups receiving the vaccine, showed significantly elevated levels of antibody at week 4, 6, 8 and 10 when compared to the negative control (*P<0.05).

From the promising results from the initial study, we decided to further investigate if these microparticulate vaccines could be formulated into microneedles for transdermal vaccine delivery. Thus, we then formulated the microneedles loaded with the gonorrhea vaccine loaded microparticles.

6.3.7. Characterization of the microneedles

The maltose, trehalose based microneedles containing the gonorrhea vaccine loaded microparticles were prepared using the mold casting method. The microneedles were 600 µm in length and were pyramid shape (Figure 32). The microneedle array consisted of 100 microneedles arranged in the 10 × 10 configurations.
Figure 32: Scanning electron microscopy (SEM) image of the N. Gonorrhea vaccine microparticles loaded microneedles.

6.3.8. Microneedle based transdermal delivery of vaccines

The microneedles were fabricated in a step-by-step method, where the microneedle formulation along with the vaccine microparticles were added first and thus the vaccine was in the needle portion of the microneedles. The Backing layer of the microneedles was prepared by the same polymer mixture except for the vaccine microparticles. This would ensure that when dosing, the microneedles were in the skin and the entire dose is been delivered in the transdermal region of the skin.
Figure 33: N. Gonorrhea specific antibody measurement in serum via ELISA. The groups receiving vaccine showed significantly higher serum IgG titers when compared to the controls – blank microparticles and blank microneedles after week 2. The group which received the GnH vaccine microparticles in microneedles showed significantly higher antibody titers than the other 2 vaccine groups at week 6 and 8 (n=6) (*P<0.001; #P<0.05).

Similar to the previous study, we had six to eight-week-old Swiss Webster (CFW) mice (n=6 per group) with the same dosing regimen of one prime dose at day 0 followed by two booster doses at week 1 and 2. There were five groups in this study, first the naïve groups which received nothing and served as our control. The second group received the blank microneedles without any vaccine (Blank MN), this group also served as the microneedle control. The third group was the gonorrhoea vaccine suspension given
subcutaneous (GnH Susp SubQ). The forth group was the gonorrhoea vaccine suspension loaded into the microneedles (GnH Susp MN) via transdermal delivery. The last group was the gonorrhoea vaccine loaded microparticles loaded in the microneedles (GnH MP MN). Blood samples were collected bi-weekly and the serum antibody titters were measured using ELISA as done previously. The groups receiving the vaccine (group 3, 4 and 5) showed significantly higher serum IgG titers when compared to the controls – blank microparticles and blank microneedles after week 2 (Figure 33). These titers further increased over the next few weeks and remained significantly higher than the control groups. Further, the group which received the GnH vaccine microparticles in microneedles (GnH MP MN) showed significantly higher antibody titers than the other 2 vaccine groups at week 6 and 8 (n=6) (*p<0.001; #p<0.05). This higher titers could be attributed to the vaccine encapsulated in the microparticulate form as opposed to the suspension form in the other two groups. From various studies done in past, it has been shown that the particulate vaccines show significantly higher response when compared to its solution equivalent group. This is mainly due to the slow release of the vaccine antigen from the polymer matrix of the microparticle and also the higher uptake of the microparticles as opposed to the solution as shown by Chablani et. al. (Chablani, Tawde, Akalkotkar, et al., 2012b). There was a reduction in the antibody titers at week 10 because of lack of infection and no further dosing past the booster.

We further wanted to check the levels of the immune cells such as the CD4+ and CD8+ T cells in the spleens at week 10. These would give a resource of information on the mechanism of the vaccine action in-vivo.
Figure 34: Vaccine-induced antigen-specific CD4+ and CD8+ T-cells counts in the splenocytes at week 10 after immunization. Groups receiving the vaccine showed significantly higher CD4+ and CD8+ T cells than compared to the controls – naïve and blank microneedles (*p<0.05).

As we can see from figure 34, there were significantly higher antigen-specific CD4+ and CD8+ T cells present in the spleens of the mice which received the vaccine when compared to the unvaccinated group. However, we could see a significant difference between the three vaccinated groups. These results suggest that the vaccine induced the immune response however, there was no significant difference on the route of administration of the vaccine since the two groups of the three were transdermal immunization and third was subcutaneous administration. Similar results have been seen.
that the vaccine-induced but the CD4 and the CD8 T cell in a study conducted by Prauznitz et al in an influenza vaccine being delivered by microneedles via the transdermal route. These findings suggest that transdermal route is of particular importance in activating both the humoral and the cell-mediated effects of the immune system.

Many challenges are inherent in the development process of the gonorrhea vaccine including the time that would be taken for the clinical trials and other resources which could be in the billions of dollars. In the long run, the development of vaccines to prevent infections has been of significant public health advantage. This approach proves to be cost-effective for many diseases in the past. Researchers like Ann Jerse, say that unlikely that a gonorrhea vaccine will be 100% effective, it is predicted that an efficacy level of 70% would reduce a considerable amount of disease and transmission worldwide. This could of significant importance in combating the antibiotic resistances being developed in the bacteria. A comprehensive effort on many fronts to make gonorrhea vaccine a reality. On one front, the continuous research on understanding the mechanisms of pathology and host responses should be carried out, this would help determine the mechanism of immune defense against the GC infection. This would also give insights on the mechanism of antibiotic resistance that has been developed. Secondly, a consistent effort on vaccine development has to be carried out. In this project, we have looked at utilizing the entire cell surface and its antigenic sites as the antigen. This approach is particularly important at the present time, as there are no identified antigenic sites or regions on the surface being identified currently. With these preliminary results, the stage is set for future experiments such as to check the
opsonization power of the antibodies produced by the vaccine. This would ultimately prove the efficacy of the vaccine and the antibodies being produced by them. Lastly, a live challenge study which would mimic the real world situation would be the final endpoint. This would lead to proving the efficacy of the vaccine and also establishing the fact that the vaccine was able to generate an adaptive immune response. This study is of particular importance in many ways, one proving the efficacy of the vaccine and second being that it is very difficult to conduct human clinical trials of gonorrhea vaccines. This live challenge study would help in pre-clinical screening and optimization of the potential vaccines against the disease.

6.4 CONCLUSION

The gonorrhea vaccine microparticles were prepared using a novel approach of formalin fixed dead gonorrhea bacteria as the antigen. Since the bacteria is formalin fixed, all the surface proteins, antigenic domains are conserved in their native form which are presented to the antigen presenting cells. We have shown the generation of antibodies post vaccination in mice both via the subcutaneous and microneedle-based transdermal route. The vaccination generated both CD4+ and CD8+ T cell-based immune response, which is an important factor for the success of a vaccine. The microparticulate based vaccine delivery system is a promising strategy for the delivery of the gonorrhea vaccine.
CHAPTER 7

SUMMARY

Cancer immunotherapy is currently one of the most promising avenues to manage and treat cancer. Cancer is currently the most deadly and painful terminal disease to affect humans globally. The FDA has approved Provenge® for men with metastatic castrate-resistant prostate cancer. This event had reignited enthusiasm for the field of active immunotherapy. Most cancers can be successfully treated with surgical removal of the cancerous tissue and delivery of chemotherapeutic agents and radiation. However, chemotherapeutics have severe side effects or have unfavorable pharmacokinetic profiles and thus prove only moderately effective causing more damage than good. Scientists and researchers believe that activation of the immune system against antigens found on tumor cells will initiate a body-wide search for tumor cells without the side effects of standard chemotherapy. Another key advantage is that if cancer reappears, the immune system’s memory should be able to mount a rapid assault on the tumor. Considering the advantages of cancer immunotherapy, the aim of my project was to develop a novel immunotherapy strategy for treatment of ovarian cancer. Ovarian cancer is one of the most lethal gynecological cancers after breast cancer and needs more attention for its treatment. Immunotherapies such as T-cell based, antibody-based and also therapeutic vaccines are being investigated. We have shown the use of the whole cell lysate as a potential therapeutic vaccine candidate for ovarian cancer. With the addition of adjuvants, such as Alum and MF59, the immune response potentiated and resulted in higher tumor volume reduction when compared to the vaccine alone. The results suggest, the CD8 T cells based tumor volume reduction as we found significantly higher CD8+ T
cells in the adjuvanated group. One of the limitations at the current time is the lack of knowledge and identification of specific tumor-associated antigens (TAA’s) in ovarian cancer. With identified and specific TAA’s, the development of a specific vaccine could be possible. This would potentially eliminate the auto-immunity cases with the whole cell lysate. The future studies are set for identification of these specific TAA’s and combining with other tumor prevention strategies which would lead to better cancer management and improving the quality of life.

The second important novel strategy we used in the development of the gonorrhea vaccine is the use of formalin fixed dead whole cell bacteria – *N. gonorrhea* as the vaccine antigen. At the present time, since there are no vaccines available for gonorrhea and the increasing antibiotic resistance against the pathogen, the CDC, and the NIH have shown urgency to develop a vaccine against the bacteria. In our approach, we preserve the entire bacterial cell structure containing its antigenic sites in their native form. This would help in generation of an immune response to specific antigenic moieties present on the bacterial surface and help generate a vaccine. We incorporated this formalin fixed dead whole cell into microparticles and carried a preliminary proof-of-concept study in mice and found that there were antibodies generated against the bacteria. Further, we tested the potential of incorporating the gonorrhea vaccine into microneedles for transdermal immunization. The microneedles were dissolving in nature made up of maltose and would dissolve after been applied on the skin delivering the vaccine. In the transdermal immunization study, we found similar results where there were high antibody titers along which higher CD4+ and Cd8+ T cells in the vaccinated mice. These preliminary studies suggest the generation of the antibodies by the antigen when
delivered in the microparticulate form. With these results, the stage is set for further studies on investigating the opsonization capacity of the antibodies. This would provide the valuable information on the antibodies generated post immunization. Lastly, a challenge study would be the ultimate proof of the efficacy of the vaccine. These studies are being planned and the Vaccine Nanotechnology Laboratory at Mercer University would be conducting them.

Adjuvants have been used since the 1920’s and have been evolving over the years for their enhancement effect of the vaccines. Some of the features involved in the adjuvant selection are the antigen, the species to be vaccinated, the route of administration and the likelihood of side-effects. In my research projects, we looked at the effect of adjuvants on potentiating the efficacy of few vaccine antigens such as the ovarian cancer vaccine, the meningitis vaccine, and the gonorrhea vaccine antigens. Our screening of adjuvants was based on the antigen presentation and co-stimulatory molecule presentation on the antigen presenting cells which could lead to higher T cell priming and a lasting effect. We also looked at the safety of the adjuvants in terms of their dose and measure the induction of autophagy at higher doses led to sub-optimal immune response. As the regulatory requirements for vaccine adjuvants are very stringent, a lot of pre-clinical research on the safety, tolerability and the dose is being extensively carried out. Adjuvants systems i.e. a combination of adjuvants would be the future of vaccine adjuvants due to their combination effect.

The microparticulate delivery system offers a range of advantages from better uptake into the antigen presenting cells which is the most important step for immunization. Due to the sustained release of the vaccine antigen from the microparticle,
the release kinetics of the antigen, the processing of the antigen in the APC’s impacts the immune response. From the stability and patient compliance standpoint, these solid microparticles provide a significant advantage. Other novel immunization strategies such as Oral dissolving films and microneedles are very promising immunization systems. The idea of a vaccine on the strip or on a Band-Aid would make immunizations more patient compliance, and accessible in the future.

In conclusion, the entire project was to develop solid dosage forms which are stable, patient compliance and safety for the immunizations. Also, the vaccine efficacy and other mechanistic studies were done to enhance the immune response.
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