INTRANASAL HPV PEPTIDE VACCINE FORMULATION INDUCES POTENT CYTOTOXIC CD8 T CELL IMMUNITY FOR THE TREATMENT OF GENITAL HPV TUMORS

Gloria Sierra

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INTRANASAL HPV PEPTIDE VACCINE FORMULATION INDUCES POTENT CYTOTOXIC CD8 T CELL IMMUNITY FOR THE TREATMENT OF GENITAL HPV TUMORS

By

Gloria Sierra, B.S.

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INTRANASAL HPV PEPTIDE VACCINE FORMULATION INDUCES POTENT CYTOTOXIC CD8 T CELL IMMUNITY FOR THE TREATMENT OF GENITAL HPV TUMORS

A DISSERTATION

Presented to the Faculty of
The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by
Gloria Sierra
Houston, Texas
August, 2021
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I would like to thank my advisor, Jagan Sastry, whom provided constant support and guidance throughout the years. Thank you for having an open door policy and always being available to help me make sense of my thoughts. For continuously being patient and encouraging when I needed it the most, in particular this last year as we navigated the pandemic. Thank you for all the cheer-leading and motivation to do better and show passion for my work.

I would also like to thank the members of my advisory committee, past and present, who helped me ask the right questions. I am grateful to have been surrounded by all the brilliant and helpful scientists and staff at MD Anderson and GSBS that have helped me get to where I’m at today, it is truly an honor to be surrounded by this incredible network of people.

I want to thank my family for their support while keeping me grounded. I am grateful for my parents who have always stressed the value of a good education. Although neither of them had the opportunity to finish high school, they made sure that all my siblings and I received a college education. Their hard work and sacrifices have definitely paid off, and for that, I am grateful.

I would also like to thank my hard working and loving husband, for always being my biggest believer; for believing in me whenever I would doubt myself, and for always helping me put things in perspective. Thank you for your patience as I figured out how to navigate these past few years.
Lastly, I’d like to thank the GSBS for providing such an inviting and caring environment for its students. I’m grateful for the friends I’ve made at GSBS along the way, and the opportunities I’ve had to grow my leadership and teambuilding skills, particularly as a member and leader of the Association of Minority Biomedical Researchers (AMBR).
Human Papillomavirus (HPV) induced cancers continue to affect millions of women worldwide, with the five year survival rate hovering just under 60% in some demographics. Therefore there is an unmet need to develop effective, yet, easily administered therapies to treat established HPV genital lesions. Even though immune checkpoint therapy (ICT) is a promising treatment option in some HPV+ cancers, the high cost and associated toxicities are still major concerns for their widespread application. HPV cancers are textbook candidates for therapeutic vaccination intervention because they’re driven by the expression of viral oncoproteins E6 and E7, which serve as ideal tumor specific antigen targets. An effective therapeutic vaccine should be able to overcome tumor mechanisms of immune-evasion and immune-suppression, while inducing a robust anti-tumor mediated response. In this dissertation I investigated a novel therapeutic HPV peptide vaccination strategy; by incorporating two diverse acting adjuvants for induction of strong cytotoxic effector immunity, and utilizing the intranasal mucosal route of immunization to ensure efficient trafficking to the genital
mucosal tumors. Overall, I hypothesized that intranasal HPV peptide vaccination employing the combination of TLR9 and NKT cell ligands, (CpG-ODN and α-GalCer, respectively) will induce potent systemic and mucosal antigen-specific CD8 T cell response, specifically at the female reproductive tract (FRT) to eliminate HPV genital tumors.

Utilizing an orthotopic vaginal tumor model in mice, I obtained evidence demonstrating the efficacy of the therapeutic HPV peptide vaccine containing α-GalCer and CpG-ODN (TVAC), in terms of inducing sustained and efficient tumor regression in nearly 85% of treated mice. The therapeutic efficacy correlated with significant CD8 T cell responses and increased ratios of cytotoxic effector to immune suppressive populations (regulatory T cells and myeloid derived suppressor cells) in the tumor microenvironment. Treatment with TVAC was also effective against tumors implanted in the flank, representing a systemic HPV tumor model. These results support the feasibility and benefits of utilizing intranasally delivered therapeutic vaccines formulated with combinations of diverse acting adjuvants, such as the TVAC tested in this investigation, as a potential strategy for clinical development to treat established genital HPV tumors.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papillomavirus</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C Virus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>HHV-8</td>
<td>Human herpesvirus-8</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>Human T-Cell leukemia virus type 1</td>
</tr>
<tr>
<td>MCPyV</td>
<td>Merkel cell polyomavirus</td>
</tr>
<tr>
<td>MCC</td>
<td>Merkel Cell Carcinoma</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and Neck Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>URR</td>
<td>Upstream Regulatory Region</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural Killer T cell</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant Natural Killer T cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid Derived Suppressor Cells</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particles</td>
</tr>
<tr>
<td>AAHS</td>
<td>Amorphous aluminum hydroxyphosphate sulfate</td>
</tr>
<tr>
<td>TIL(s)</td>
<td>Tumor infiltrating lymphocytes/leukocytes</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>SLP</td>
<td>Synthetic long peptides</td>
</tr>
<tr>
<td>FRT</td>
<td>Female reproductive tract</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissues</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissues</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal associated lymphoid tissues</td>
</tr>
<tr>
<td>CALT</td>
<td>Conjunctiva associated lymphoid tissue</td>
</tr>
<tr>
<td>VALT</td>
<td>Vaginal associated lymphoid tissues</td>
</tr>
<tr>
<td>BALT</td>
<td>Bronchus associated lymphoid tissue</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-Liable Toxin</td>
</tr>
<tr>
<td>MPL</td>
<td>Monophosphoryl lipid A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytolytic T lymphocyte</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>IVIS</td>
<td>In Vivo Imaging System</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>Tg</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>ILN</td>
<td>Iliac Lymph node</td>
</tr>
<tr>
<td>CLN</td>
<td>Cervical Lymph Node</td>
</tr>
<tr>
<td>ICC</td>
<td>Intracellular Cytokine</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>Alpha-galactosylceramide</td>
</tr>
<tr>
<td>CpG-ODN</td>
<td>Cytosine and guanine with phosphodiester backbone Oligodeoxynucleotides</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>N-9</td>
<td>Nonoxyl-9</td>
</tr>
<tr>
<td>TVA</td>
<td>Therapeutic Vaccine with α-GalCer</td>
</tr>
<tr>
<td>TVC</td>
<td>Therapeutic Vaccine with CpG-ODN</td>
</tr>
<tr>
<td>TVAC</td>
<td>Therapeutic Vaccine with α-GalCer and CpG-ODN</td>
</tr>
<tr>
<td>SAIF</td>
<td>Small animal imaging facility</td>
</tr>
<tr>
<td>Ns</td>
<td>Not Significant</td>
</tr>
<tr>
<td>V2</td>
<td>Vaccine 2</td>
</tr>
<tr>
<td>GzmB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
</tbody>
</table>
CHAPTER 1. INTRODUCTION

HPV induced cervical cancers are the second leading cause of death in women worldwide. Despite the availability of preventative vaccines, millions of people who have already been exposed to the virus, or are ineligible/unable to receive the vaccine are still at risk of developing cancer and requiring treatment. Therefore there is an unmet need for the development of an easily administered therapy for the treatment of HPV induced cancers. HPV driven cancers constitutively express viral gene products (e.g. E6 and E7) necessary to induce oncogenic transformation, thereby making them ideal targets for treatment using a therapeutic vaccine.

Therapeutic vaccines are used to boost or modulate the immune response to treat existing ailments such as viral infections or cancers [1]; in contrast to prophylactic vaccines which offer protection against initial infection [2]. Similar to prophylactic vaccines, however, therapeutic vaccines are often made up of two components to induce an effective immune response; the first is an immunogen such as a bacterial toxin or pathogen-encoded antigen, foreign to the host, the second component is an adjuvant, or substance which enhances the body's immune response to the co-administered immunogen/antigen [3]. Different classes of adjuvants capable of modulating and inducing immune responses in different sets of immune cells are available, and therefore the selection of safe yet effective adjuvants is a key aspect for the development of a successful vaccine. In addition, therapeutic vaccines must be effective at inducing the proper immune response at the site of infection or cancer. This dissertation investigates the potential of two diverse-acting adjuvants, α-GalCer and CpG-ODN, as components
of a therapeutic peptide vaccine for the treatment of HPV genital tumors. I also investigated different routes of vaccine delivery for determining the most effective regimen for therapeutic efficacy against HPV genital cancer.

In the following chapters, I will first review the role of HPV and other viruses in the development of cancer, how HPV, in particular, evades immune recognition permitting cancer progression, and the overall burden HPV induced cancers have globally. I will also discuss the various therapeutic approaches that have been developed to treat HPV induced cancers, and how the compartmentalization of the mucosal immune system can play a role in therapeutic efficacy. Lastly, I will discuss the main questions and rationale of my research.

1.1 HPV and Cancer

Cancer is defined by the National Institute of Health (NIH) as a group of diseases in which abnormal cells divide without control with the potential to invade nearby tissues. They are often categorized by the type of tissue the cancer originates from, and the primary site of the disease. Carcinomas and sarcomas are cancers originating in the epithelial layer and supportive/connective tissues of the body, respectively, while leukemia and lymphomas are cancers of the bone marrow and glands or lymph nodes. A fifth category of cancer classification includes a mixed type of cancer, in which the growths are found in multiple categories.

It is difficult to identify the exact cause of cancer, however, in 1971 Alfred G. Knudson formulated the two-hit hypothesis which describes the notion that two "hits" or defects in the DNA-encoding tumor suppressor genes were required to induce the
hereditary cancer, retinoblastoma [4]. Although we now know that there are more factors at play for the development of cancer, this theory indirectly led to the identification of major oncogenes, or genes that, under certain situations, can transform a healthy cell into a tumor cell. The process of cancer transformation not only depends on the mutation of tumor suppressor genes, but requires a combination of characteristics termed the Hallmarks of Cancer which were describe by Hanahan and Weinberg [5]. These hallmarks include self-sufficient growth signals, insensitivity to anti-growth signals, evading programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, deregulated metabolism, and evading the immune system.

Several external or environmental factors have been found to directly increase the likelihood of cancer. These include smoking, tobacco use, poor diet, lack of physical activity, exposure to radiation, and infections [6, 7]. In fact, approximately 15% of all cancers are caused by viral infections [8, 9]. There are currently seven recognized viruses which are known to cause cancer, termed oncogenic viruses. These viruses and their associated cancers are listed in Table 1 [9]. Oncogenic viruses have the capacity to prompt unregulated cell replication and uncontrolled growth. In certain cases, an oncogenic virus is able to embed their genetic material into the host genome, allowing for the cell’s transformation into cancer. Although HIV does not directly cause cancer and is not listed in this table, it is important to note that, because it causes immunodeficiency, it increases the risk of cancer by reducing the body’s ability to fight off or control other oncogenic viruses or infections [10-12].
Table 1 Association of viruses and cancer. The seven known oncogenic viruses alongside the type(s) of cancer(s) they are associated with [9].

<table>
<thead>
<tr>
<th>ONCOGENIC VIRUS</th>
<th>CANCER TYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUMAN PAPILLOMA VIRUS (HPV)</td>
<td>Cervical, Penile, vulvar, vaginal, head and neck squamous cell carcinoma (HNSCC)</td>
</tr>
<tr>
<td>EPSTEIN-BARR VIRUS (EBV)</td>
<td>Stomach cancers, nasopharyngeal cancers, and Burkitt or Hodgkin lymphomas.</td>
</tr>
<tr>
<td>HUMAN HERPES VIRUS 8 (HHV-8)</td>
<td>Kaposi Sarcoma,</td>
</tr>
<tr>
<td>HUMAN T LYMPHOTROPIC VIRUS TYPE I (HTLV-1)</td>
<td>Non-hodgkin lymphoma (adult T cell leukemia/lymphoma), and lymphocytic leukemia</td>
</tr>
<tr>
<td>HEPATITIS B VIRUS (HBV)</td>
<td>Liver Cancer</td>
</tr>
<tr>
<td>HEPATITIS C VIRUS (HCV)</td>
<td>Liver Cancer, also increases the risk of non-Hodgkin lymphoma and head and neck cancers.</td>
</tr>
<tr>
<td>MERKEL CELL POLYOMA VIRUS (MCPYV)</td>
<td>Merkel cell carcinoma (MCC)</td>
</tr>
</tbody>
</table>

Currently, there are only three FDA approved vaccines for protection against these oncogenic viruses. They include vaccines for the prevention of HBV, HPV, and EBV [13]. Despite their availability, however, vaccination rates for each of these viruses vary greatly depending on region and among virus [7].

This dissertation will focus on human papillomavirus (HPV) as it is the most common sexually transmitted disease, is responsible for nearly 100% of cervical cancers worldwide, 70% of vaginal and vulvar cancers, 60% of penile cancers, 90% of anal cancers, and approximately 60-70% of newly diagnosed oropharynx cancers [14]. In the next section, the virology and oncogenic mechanisms of HPV will be described, followed by the virus’ global impact.
1.1.1 Virology of HPV

HPV is an 8kb double-stranded DNA virus containing 8 protein-coding genes. There are currently over 200 identified strains of HPV, each having less than 10% homology with the rest [15, 16]. The HPV DNA can be categorized into three sections based on the genes it encodes or the function it provides: the noncoding upstream regulatory region (URR), the early protein coding region which of the virus encodes the replication machinery (E1, E2, E4, E5, E6, and E7), and the late protein encoding region which encodes the capsid proteins (L1 and L2) [17]. The virus’s replication cycle is dependent on the differentiation of the epithelial cells it infects. Initial infection begins at the basal layer of the epithelial layer where the virus gains access to these cells through naturally occurring micro-lesions in the epithelia, and begins early gene expression. The expression of E6 and E7 genes helps drive the epithelial cells into the S-phase, creating an ideal viral replication environment during host cell proliferation. As new cells develop in the basal epithelia, infected cells move into the upper epithelial layers, where the virus begins the production of the late genes required for capsid assembly and eventually progeny release [18]. HPV is not a lytic virus, meaning that it does not need to kill, or lyse the cell it infects to release its progeny. It is released when the epithelial cell it infects, reaches the top of the epithelium and is shed naturally, avoiding virus-induced necrosis and inflammation. This is one of the many ways HPV is able to remain undetected in its host and will be discussed in further detail in the next section [19].

There are 14 identified oncogenic or “high-risk” strains of HPV. HPV16 and HPV18 are the most common “high-risk” strains and are responsible for approximately
70% of cervical cases around the world [20, 21]. Two of the most common “low-risk strains”, which induce genital warts, but do not lead to cancer, are HPV6 and HPV11, accounting for over 90% of genital warts cases [22]. It is predicted that over 80% of the population has been or will become infected with at least one strain of HPV in their lifetime, however, the majority of these cases are asymptomatic and cleared naturally by the immune system [18]. When a host becomes infected with what is considered a “high-risk” HPV strain, and is unable to clear the virus (chronic infection), changes to the virus’s genome can lead to integration of the viral genome into the host cell DNA [23, 24]. Continuous and overexpression of HPV E6 and E7 are the main drivers of HPV induced cancers; as previously mentioned, these proteins are capable of driving the host cell into uncontrolled proliferation. E6 induces the degradation of p53, a tumor-suppressing molecule, while E7 binds to and inhibits retinoblastoma (Rb) from binding with E2F, a protein, which when is unbound, leads to cell cycle activation and proliferation (Fig. 1) [25]. With both of these proteins overexpressed in the host cell, the healthy cells transform into cancerous ones.
Figure 1. HPV E6 and E7 mechanisms of oncogenic transformation. HPV16 E6 and E7 are the main drivers of oncogenic transformation. E6 is known to bind and degrade p53, a major tumor suppressor gene within the host cell, while E7 competitively inhibits the binding of pRB and E2F. By doing so, the unbound E2F protein is able to induce cell cycle activation and proliferation. Figure created with BioRender.com
1.1.2. Mechanisms of HPV immune evasion

The immune system is a complex network of cells, tissues, and organs that aids the body to fight off infections. It is comprised of the innate and adaptive immune systems that work together to control and eliminate pathogens, such as HPV, and can also prevent re-infection. The innate immune system is a rapid, non-specific defense mechanism that includes physical barriers such as the skin, defense mechanisms such as secretions and mucus, and innate immune cells. Innate immune cells, including but not limited to natural killer (NK) cells, natural killer T (NKT) cells, macrophages and dendritic cells (DCs), are capable of recognizing microbial substances through the use of pattern recognition receptors (PRRs). These PRRs, in response to an invading pathogen, activate downstream signaling pathways to promote a protective inflammatory immune response through the release of cytokines such as IFNγ, TNF-α, and granulocyte macrophage colony-stimulating factor (GM-CSF).

The adaptive immune system is a highly regulated and antigen specific part of the immune system that includes B cells and T cells. While CD4 T cells recognize peptides presented on MHC class II and are referred to as “helper T cells”, CD8 T cells recognize peptides on MHC class I molecules and are often referred to as cytotoxic T lymphocytes (CTLs), as they are able to directly kill infected or cancerous cells. CD4 helper T cells aid in tailoring the immune response to the type of pathogen encountered. They release a multitude of cytokines to either prime B cells for antibody release and pathogen neutralization or activate dendritic cells, a type of antigen presenting cell (APC) to educate and activate CD8 T cells for cell-mediated killing.
Dendritic cells are known as the main bridge between the innate and adaptive immune systems since they are able to take up, process, and present antigens to the adaptive immune cells, inducing antigen-specific immunity. Dendritic cells require stimulatory signals in order to present co-stimulatory molecules required for CD8 T cell activation. These signals can be from inflammatory cytokine responses, or CD4 T cell responses [26].

HPV can avoid both innate and adaptive immunity in approximately 10% of infected individuals through several specific mechanisms briefly described here. Further details can be found in the following reviews [27, 28].

During the initial infection stage, the virus is able to enter the basal epithelial cells through naturally occurring micro lesions, and by maintaining low copy numbers, it avoids immune detection [27]. As the infected basal cells grow and enter the differentiation phase of the epithelium, the virus significantly upregulates viral gene expression and DNA replication, wrapping up its replication process as the infected cell reaches its terminal differentiation phase at the top of the epithelium, naturally dying and shedding the HPV viral progeny as it is released. By circumventing virus-induced cell death and the associated inflammation, HPV is able to avoid triggering the influx of APCs to the infection site [27]. Additionally, because HPV is localized to the epithelium, it avoids the blood stream and lymphatic system where it can be detected by the host. Studies also indicate that high-risk HPV strains downregulate interferon gene responses necessary for antiviral and innate immune response, although this is still not thoroughly understood [29].
As previously mentioned, approximately 10% of HPV infections with high risk strains that remain undetected experience viral mutations and result in oncogenic transformation [30]. Failure to induce cellular immunity against infected cells, and viral genome integration or deregulation are vital steps that cause HPV infections to lead to cancer. As the cancer progresses, regulatory T cells (Tregs) and Myeloid Derived Suppressor Cells (MDSCs) significantly increase within the affected site, abrogating CD8 T cell responses, and further aiding tumor growth [31].

1.1.3. Global burden of HPV and promise of a vaccine

HPV driven cancers make up four percent of total cancer diagnosis every year, amounting to a total of almost 700,000 cases, with the majority of patients being women diagnosed with cervical cancer [32]. Frequencies and impact of HPV induced cancers vary greatly between countries of different economic statuses. While they make up less than three percent of cancer diagnoses in Australia and the United States, they account for 26% in sub-Saharan African countries [33]. Even within the United States, economic and racial disparities depict a correlation with those who are diagnosed with HPV cervical cancer [34]. Studies indicate that a lack of regular screenings, access to affordable medical care, and vaccination rates contribute to these disparities in the US and around the world [33, 35].

The prophylactic vaccine Gardasil9 is currently offered and approved for the prevention of HPV infection, significantly reducing the risk of HPV-induced cancers by up to 90% [36, 37]. This vaccine and its predecessors were originally targeted at
protecting women against cervical cancer, but because HPV infects and is transmitted by men and women, it is now recommended that both males and females of adolescent age be vaccinated against HPV in order to reduce transmission and protect against cervical, penile, anal, vaginal, vulvar, and oral cancers [36, 38-40]. The Gardasil9 vaccine is composed of virus-like particles (VLPs) corresponding to the major capsid proteins on the surface of the virus, L1, which are immunogenic on their own. It induces immunity and provides protection against the seven most common oncogenic HPV strains, HPV16, 18, 31, 33, 45, 52 and 58, plus the two strains responsible for genital warts, HPV 6 and 11 [41]. Admixed with aluminum salts, amorphous aluminum hydroxyphosphate sulfate (AAHS), studies indicate that even just a single dose is sufficient to significantly reduce the risk of cancer [42, 43].

Despite the vaccine's availability, global HPV infections remain high. Originally released in 2006, delays in vaccine uptake and availability rendered millions of adults ineligible for the vaccine because it is not recommended for those with preexisting infections.

The current standard of care for patients diagnosed with cervical cancer varies depending on the severity and spread of the disease but typically involves the surgical removal of the affected area plus a combination of either chemotherapy or radiation therapy. This approach often comes with toxic side effects, lowering the quality of life for affected individuals [44-46]. In addition, the overall five year survival rate of women treated for cervical cancer is still less than 60% in some demographics [21]. Therefore, there is an unmet need for the development of more effective, yet low-cost and easily
administered treatment options for HPV induced cancers without the use of chemotherapy and radiation therapy.

1.1.2. Therapeutic alternatives for HPV induced cancers

Over the years, various approaches have been taken to treat HPV-induced cancers, with the overall goal of shifting away from chemotherapy and radiation therapy. In the following section I will briefly describe some of these methods including checkpoint inhibitors, cell based vaccines and therapeutic vaccines. It is important to reiterate that the currently available prophylactic vaccine described in the preceding section is not an effective treatment for existing HPV infections, as it only protects against initial infection. As previously mentioned, the prophylactic vaccine induces immunity against the surface proteins of the virus, allowing it to neutralize the virus prior to entering and infecting the host cell [21, 36]. These late genes encoding the surface proteins, along with a portion of the early genes are often lost during viral DNA integration into the host genome [23, 24]. Therefore, the majority of the following therapeutic approaches focus on inducing or enhancing cellular immunity against HPV proteins E6 and E7 as they are the primary drivers of cell transformation and cancer. In addition, these proteins are constitutively expressed in HPV induced cancers, and targeting these virus-specific proteins avoids off-target effects. Recent studies have also identified the constitutive expression of the E5 protein in a large percentage of cancers, signifying that it could serve as a third antigenic target of interest [47].
One promising method of enhancing cellular immunity for the treatment of HPV induced cancers is the use of checkpoint inhibitors. Studies indicate that patients progressing from early stage HPV disease to higher grades express increased levels of PD-1 and PDL-1 on the infiltrating T cells and DCs, significantly dampening the anti-tumor immune response [48]. Utilization of checkpoint inhibitors to block these dampening functions of PD-1 and PDL-1, allows the infiltrating CD8 T cells to continue killing infected cells [49]. Many ongoing clinical trials are testing the potential of these and other checkpoint inhibitors such as CTLA-4, alone and in combination with traditional HPV therapies and are listed in detail in the following review [50].

In addition, cell-based therapies for treatment of advanced and non-responding cancers have also been receiving growing interest. Cell therapies include adoptive T cell transfer, dendritic-cell vaccines, CAR-T cell therapies, and treatment with engineered TCR T cells. These methods all involve the isolation of cells of interest, such as dendritic cells or T cells from a patient, performing ex-vivo manipulations to increase therapeutic potential, and infusing these cells back into the same patient.

For example, DCs pulsed with recombinant HPV proteins, stimulated ex vivo and transferred into cervical cancer patients increase antigen specific immunity in the blood [51-53]. Although this method showed efficacy for inducing CTL response systemically, a clinical response was not observed [52].

A more direct approach involves the isolation of tumor infiltrating lymphocytes (TILs) from cervical cancer patients, and clonally expanding those specific to the patient’s tumor through the use of cytokines and HPV peptides, or genetically modifying them for
antigen specificity. This method is a highly personalized form of medical treatment and has shown great clinical response in patients [54, 55].

While these therapies are currently used in clinics and have shown promising results, they are often very time consuming and costly. In the following chapter, I will describe the multiple types of therapeutic vaccines that have been developed and proposed for HPV induced cancers, while including few examples that have been tested in clinical trials.

1.2. Therapeutic Vaccines

Therapeutic vaccines are designed to boost or modulate immune response to treat existing ailments such as viral infections or cancers, in contrast to prophylactic vaccines which offer protection against initial infection [2]. Several types of therapeutic vaccines have been developed for the treatment of HPV induced cancers. These vaccines, as previously mentioned are aimed at inducing cellular immunity against the HPV proteins E6 and E7 as they are the main drivers of oncogenic transformation. One major distinction between these therapeutic approaches is in the antigen formulation used. These include the use of viral vectors, proteins, peptides, and DNA for the delivery of antigens. The following section will describe each of these approaches along with some examples and benefits and disadvantages of each.

1.2.1. Live vector based vaccines

Viral and bacterial vector based vaccines utilize recombinant vectors encoding HPV target antigens to infect host cells with the main goal to get recognized by APCs.
Once within the cell, viral and bacterial vectors are able to produce the target antigens or peptides, promoting antigen presentation. In order to increase immunogenicity of this approach and ensure the infected cell is recognized by the immune system, the vectors typically also encode stimulatory factors such as IL-2 [56, 57] or pathogen associated molecular patterns (PAMPS) [58]. Upon successful infection and translation of the encoded antigens, the vector activates downstream sequence of events, inducing an inflammatory response against the target antigen.

One approach, utilizing live attenuated *Listeria monocytogenes* encoding HPV16 E7 protein linked to listeriolysin O (TG4001) showed promising pre-clinical results, however, clinical safety and efficacy are still undergoing in cervical cancer patients with recurring disease [56]. Another group utilized a modified vaccinia virus Ankara vector for the production of E6 or E7 proteins of HPV along with IL-2, which has shown success in inducing antigen specific CD8 T cell responses in patients [56]. Additionally, vaccinia vectors, adenovirus vectors, and semliki forest virus vectors have also been used for the development of therapeutic HPV vaccine candidates [56, 59, 60].

Although effective measures are taken to ensure safety profile of these vaccines, utilizing live viral vectors continues to pose safety concerns as the risk of DNA mutation, integration and pathogenic potential in immunocompromised patients is a genuine risk [61]. In addition, because live attenuated viruses are also slightly immunogenic, there is the possibility of the patient’s immune system recognizing the viral antigen and inducing immunity against the treatment itself [62].
1.2.2 Protein and peptide based vaccines

Protein and peptide based vaccines have long been proposed for the treatment of HPV induced cancers [63]. This approach consist of delivering recombinant proteins or peptides from HPV to be taken up, processed and presented by DCs, inducing an antigen specific CD8 T cell response. Interests remain high in these categories, in particular peptide based vaccines, due to the low cost of production, long term stability, and high safety profiles. This approach does not carry concerns associated with the use of vector or DNA based vaccines, however, they do require the use of adjuvants due to low immunogenicity. Co-administration with adjuvants such as chemokines, toll-like receptor ligands, or lipids have shown to increase immunity against these peptide, resulting in enhanced CD8 T cell responses [63]. One hurdle of utilizing peptides for immune induction is the specificity of different epitopes to MHC types. Not all peptides have high affinity or compatibility to certain MHC types, therefore depending on the peptides of choice, these vaccines can be specific to different HLA haplotypes. One method to circumvent this is the use of long overlapping peptides covering the entire protein sequence. This ensures complete coverage of the proteins of choice while avoiding oncogenic risks of delivering the entire protein. Additionally, whole protein administration typically results in longer peptide presentation on MHC class II molecules, shifting the immune response towards a CD4 T helper immune response [64]. A Synthetic Long Peptide (SLP) vaccine which utilizes HPV16 E6 and E7 peptides co-administered subcutaneously with Montanide ISA-51 adjuvant is currently in clinical trials for HPV driven gynecological and oral cancers [65].
1.2.3. DNA vaccines

DNA and RNA based vaccines are another emerging field of interest as they utilize genetic material with the capability to produce full-length protein or peptides, allowing for natural processing and presentation by APCs[66]. Precautions and modifications to the full-length protein approach must be taken, however, to prevent the risk of causing cellular transformation [67]. DNA and RNA constructs are economically feasible for large scale production, but DNA holds better stability than RNA [68]. DNA vaccines are poorly immunogenic and require the use of adjuvants, similar to protein and peptide vaccines [68].

One example of a DNA vaccine encoding a full-length protein construct shuffled the gene sequence of HPV E6 and E7 in a way that disabled the oncogenic potential of translated proteins, but still allowed for processing of the proteins and proper epitope presentation for efficient E6 and E7 induced immunity [69]. Although this approach has proven to induce robust immunity even in pre-clinical models, clinical outcome was not as robust as anticipated [70].

1.2.4. Genome editing

The latest approach and last one that will be covered in this chapter is the use of genome editing tools to reduce the expression of the driving oncogenes. Pre-clinical models in vitro and in vivo have shown the power of using the CRISPR/Cas9 genome editing system in order to cleave the HPV16/18 E7 oncogene embedded in the host DNA, allowing for cell-induced apoptosis and reduction of tumor growth [71, 72].
Because primary HPV lesions are found at mucosal sites, it’s important but often overlooked, for a therapeutic vaccine to induce an immune response at these locations. Many pre-clinical studies are tested against subcutaneously implanted HPV gene expressing tumor models and show promising results, however, lack investigations focusing on immune induction in the genital mucosa [73-76]. A more thorough understanding of the mucosal immune system can benefit the advancement of therapeutic HPV vaccines.

1.3. Mucosal immunology and Vaccines

The majority of HPV induced cancers are localized to the genital mucosa, with an increasing percentage occurring in the oral mucosa. Therefore, therapeutic efforts must ensure not only a robust immune response, but also proficient cellular immunity at the genital tract, or female reproductive tract (FRT). As previously mentioned, the oncogenic proteins of HPV E6 and E7 are the primary targets of therapeutic vaccine approaches. Although several strategies have proven effective for the induction of a cellular immune response against these antigens, clinical outcome has not reflected pre-clinical expectations. Increasing tumor infiltration is a current challenge facing many of the previously described vaccination approaches. In the following section I will describe the compartmentalization of the mucosal immune system and prospective benefits of mucosal vaccinations.
1.3.1. Compartmentalization of the Mucosal Immune System

The mucosal immune system is a highly compartmentalized portion of the overall immune system. It functions independently from the systemic immune system and is responsible for discriminating harmful pathogen encountered from non-pathogenic or commensal bacteria at the mucosal membranes throughout the body [77, 78]. It consists of the gastrointestinal, genital, and respiratory tracts which are constantly being exposed to foreign microbes through the functions of gas exchange, nutrient absorption and reproduction, therefore the overall nature of the mucosal immune system is highly tolerogenic, avoiding unnecessary inflammatory immune responses to commensal bacteria and nutrients. Despite the tolerogenic nature of the mucosal immune system, the mucosal epithelial layers are lined with what is an equivalent to the systemic lymph nodes specific for mucosal immunity [78]. The Mucosal-associated lymphoid tissues (MALT) are situated adjacent to mucosal tissues and make up a complex network of lymphoid structures similar to lymph nodes of their corresponding mucosal tissue [79]. They are classified into several compartments including the Gut-Associated Lymphoid Tissues (GALT), the nasopharynx-associated lymphoid tissues (NALT), the conjunctiva-associated lymphoid tissue (CALT), the vaginal associated lymphoid tissue (VALT), and the bronchus-associated lymphoid tissues (BALT). Presence of these structures can vary between species, however, their functions are similar, and are the sites where antigen-specific immune responses are initiated. Each of these components make up portions of the common mucosal immune system, which is able to induce immune activation at one mucosal site, disseminate and arise at a distal site [80]. Many studies
have found that the route for mucosal immunization can heavily influence the resulting immune response [81-83]. Oral, vaginal, rectal, and nasal vaccinations have all been investigated and will be briefly discussed below.

Currently, there are only six mucosal vaccines approved for human use targeting the prevention of the following infections; cholera, rotavirus, salmonella, and influenza. All are administered orally except for Flumist®, which protects against influenza and is the only intranasal vaccine currently available for human use [84]. The once orally administered polio vaccine that aided in the eradication of the disease in the United States is no longer commercially available after a handful of cases reporting the live attenuated virus reverting its virulence and causing vaccine-associated paralytic poliomyelitis [85]. Protection against polio is now achieved through the intramuscular immunization using an inactivated polio vaccine (IPV), eliminating the risk of utilizing a live virus for immunization.

The ease and non-invasive nature of oral and nasal vaccine administration make these routes attractive for mass-scale vaccination, however, route selection depends on intended immune outcome. While oral immunizations have the capacity to induce immunity at the oral tissues and gastrointestinal tract, nasal immunizations have been shown to induce immunity in the nasal tissues, respiratory tract, and interestingly, studies have demonstrated immune response at the female reproductive tract (FRT) after intranasal vaccinations [86]. Vaginal and rectal vaccinations induce less disseminated responses but are capable of inducing immunity at the genital tissues and gut, respectively. While most mucosal vaccinations induce both systemic and mucosal
immunity, the reverse is not always true. Intraperitoneal vaccinations repeatedly failed to induce any mucosal immune response [87-89]. These characteristics are important to note when developing drugs to combat infections like HPV which reside primarily in the mucosal microenvironment.

One of the benefits of utilizing the mucosal route of immunization is mucosal imprinting. Mucosal imprinting refers to the ability of mucosally derived DCs to induce upregulation of mucosal-specific homing receptors on activated T cells. Studies show that mucosal DCs derived from the lung or gut, are able to induce upregulation of related integrin and homing receptors such as CD49a and α4β7, respectively [90, 91], therefore mitigating the localization of the resulting immune responses. Imprinting mechanisms have been investigated for various immunization routes, but the majority of detailed studies have been on GALT and gut-homing mechanisms. It's been shown that GALT DCs, specifically CD103+ DCs from the lamina propria are the only cells able to generate gut-tropic CD8+ effector cells in vitro. This indicates that the priming APC is the primary factor responsible for mucosal imprinting, and not so much the location of the priming [91].

1.3.3. Mucosal Adjuvants

Similar to systemically delivered vaccines such as intramuscular or subcutaneous vaccines, immune response is greatly enhanced with the use of adjuvants. Mucosal adjuvants have historically been non-toxic derivatives of bacterial toxins such as cholera toxin (CT), bacterial DNA, virus like particles, and cytokines/chemokines. Although all are able to induce robust immune responses, not all are safe for use in humans. A
commonly used derivative of heat-labile enterotoxin (LT), for example proved to be toxic in humans when used in clinical studies [92]. In another instance, administration of CT via the intranasal route showed evidence of it trafficking into the brain, therefore also deemed unsafe [93].

Bacterial DNA such as CpG-ODN, has however shown successes in clinical trials when delivered intranasally and orally with no apparent toxicities [94]. CpG-ODN is a TLR9 agonist, and is capable of inducing APC activation, increasing CD80/86 levels, and skewing immunity towards a CD8 mediated response [94, 95]. Monophosphoryl lipid A (MPL) is another TLR agonist that binds to TLR4 on APCs and has recently been approved by the FDA for intramuscular immunization of the prophylactic vaccine against HPV infections-Cervarix [96]. Several pre-clinical trials which contain MPL have resulted in robust immune responses when delivered intranasally [97]. A more thorough review of mucosal adjuvants and their use in clinical trials can be found here [98, 99]

1.3.4. α-GalCer as a mucosal adjuvant

α-Galactosylceramide (α-GalCer), a NKT cell ligand which was originally derived from a marine sponge, has shown great use as a potent NKT cell agonist. It is taken up by APCs, and presented on CD1d molecules, inducing the rapid activation of NKT cells. This activation results in a rapid and robust release of pro-inflammatory cytokines including IFN-γ, IL-2, and IL-4, which in turn causes activation of NK cells, CD8 cells, B cells, and DCs [100, 101]. Although α-GalCer has been deemed safe in the clinics, one concern with its use is the anergy induced in NKT cells after repeated systemic
administration [102, 103]. Our lab, and others, has shown evidence that mucosal administration in the form of intranasal or oral delivery of α-GalCer circumvents NKT cell anergy, allowing for repeated activation and a stronger induced immune response against co-administered antigens [104-107]. Although systemic administration of α-GalCer showed a good safety profile in humans, mucosal route of administration has yet to be tested in [108]. Nonetheless, interests in its use for anti-tumor efficacy remains high. Multiple adjuvant related studies have shown enhanced immunity, and sometimes even synergistic increases of immune response after the use of α-GalCer in combination with a second adjuvant. In particular, several studies have shown that α-GalCer combined with a TLR agonist results in significantly higher DC activation, cytokine release and overall immunity [109-111]. An example in our own lab, showed that mucosal immunization with the combination of TLR-9 and α-GalCer is able to induce robust antibody mediated immunity against HIV proteins [105]. Because of the robust potential of α-GalCer and CpG-ODN as therapeutic adjuvants, I set out to investigate whether mucosal intranasal administration of these two adjuvants harbored enhanced therapeutic potential against HPV genital tumors.

Utilizing an orthotopic HPV vaginal tumor model, I tested the therapeutic potential of these adjuvants by co-administering them with a set of HPV16 peptides.
CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Understanding how route of vaccination and adjuvants interact with one another is critical for the development of a successful therapeutic vaccine. Despite the availability of prophylactic vaccines against HPV, thousands of people who are ineligible/unable to receive the vaccine are still at risk for HPV induced cancers. Therefore, there is still a need to develop safe, yet effective treatment strategies that are not only easily distributed, but are also cost-effective. Based on the reasons described in the previous chapter, this dissertation investigates the potential of an intranasal therapeutic HPV peptide vaccine employing the combination of adjuvants ($\alpha$-GalCer and CpG-ODN) for the treatment of HPV genital tumors. The peptides chosen for this vaccine were originally identified in a study from our lab which realized that immune memory to these select peptides correlated with recurrence free survival in women treated for cervical neoplasia [112]. They include HPV16 Q19D (E744–62, QAEPRAHVYNIVTFCCKCD); R9F (E749–57, RAHVYNIVTF); Q15 L (E643–57, QLLRREVYDFAFRLD); and V10 C (E649–58, VYDFAFRDLC).

Therefore, I set out to investigate the following hypothesis:

Intranasal vaccination employing the combination of TLR9 and NKT cell ligands, (CpG-ODN and $\alpha$-GalCer) will induce potent systemic and mucosal antigen-specific CD8 T cell immunity, specifically at the female reproductive tract (FRT) to eliminate HPV genital tumors. In order to address this hypothesis, I developed the following aims:
• **Aim 1:** Test whether intranasal vaccination employing the combination of adjuvants, relative to each adjuvant alone, will induce strong CD8 T cell response to the co-administered antigen at multiple systemic and mucosal tissues.

• **Aim 2:** Determine whether intranasal delivery of HPV peptides together with the adjuvant combination will be effective in treating established HPV+ vaginal tumors.

• **Aim 3:** Investigate whether the tumor location and route of vaccination will influence therapeutic efficacy of the HPV peptide vaccine.
CHAPTER 3. MATERIALS AND METHODS

3.1 Animals

Female C57BL/6J mice (6–10 weeks) were purchased from the Jackson Laboratories (Bar Harbor, ME, USA) and were maintained in a pathogen-free environment at the institutional animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animals Care International. Animals were anesthetized with isoflurane for blood draws and hormonal administration, and with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) administered by intraperitoneal (IP) route for tumor implantation and N9 Treatment. All animal procedures were conducted, including euthanasia in compliance with the University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC) guidelines (Project ID: 00000858-RN02).

3.2 Cell Line and Reagents

The TC-1-luciferase (TC-1–Luc) tumor cell line is of lung fibroblast origin from C57BL/6 mice that was transfected to stably express the E6 and E7 oncogenes of HPV-16 as well as the H-Ras oncogene. This cell line additionally expresses firefly luciferase. This cell line was a kind gift from Drs. T.-C. Wu and C. Hung (Johns Hopkins School of Medicine, Baltimore, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 50 units/mL of penicillin–streptomycin, and 50 µg/mL gentamycin.
The four vaccine peptides Q19D (E744–62, QAEPDRAHVYNIVTFCKCD); R9F (E749–57, RAHVYNIVTF); Q15 L (E643–57, QLLRREVYDFARDL); and V10 C (E649–58, VYDFAFRDLC), were purchased from Elim Biopharma (Hayward, CA, USA). Stock solutions were prepared in a mixture of 10% dimethyl sulfoxide (DMSO) and 90% 1 × PBS at a concentration of 10 mg/mL. The α-GalCer adjuvant was purchased from DiagnoCine (Hackensack, NJ, USA) and dissolved in DMSO (Sigma, St. Louis, MO, USA) to prepare a stock solution at a concentration of 1 mg/mL. The CpG-ODN 1826 adjuvant was obtained from InvivoGen (San Diego, CA, USA) and dissolved in sterile endotoxin free water to prepare a stock solution at a concentration of 10 mg/mL. Endotoxin-free ovalbumin (OVA) protein was purchased from InvivoGen (San Diego, CA, USA) and reconstituted at 20 mg/mL in PBS. Anti-CD8 blocking antibody was purchased from BioXCell (Lebanon, NH, USA). The α4–1BB monoclonal antibody (LOB12.3) with <1 endotoxin unit/mg was purchased from BioXcell (Lebanon, NH, USA) and administered three times (350 µg/dose) via IP route on days 5, 8, and 11 after tumor implantation as previously described [11]. The APC-labeled tetramer of H-2 Db-restricted CD8 T cell epitope peptide R9F from the E7 oncoprotein of HPV-16, (E749–57, RAHYNIVTF) was procured from the MHC tetramer production facility at Baylor College of Medicine (Houston, TX) and was used for the detection and analysis of HPV antigen-specific CD8 T cell responses in different tissues by flow cytometry.

The estradiol was obtained by Sigma and diluted to a stock concentration of 1mg/ml in peanut oil (Sigma) by first dissolving 20mg estradiol in 1ml of 100% ethanol (Sigma) and adding 19mls of peanut oil. Medroxyprogesterone acetate (Depo-Provera)
was obtained from TCI and was used at a 10mg/mL solution in PBS. Nonoxyl-9 was obtained from Abcam and stored in a de-humidifier chamber at room temperature.

3.3 In Vivo Tumor Experiments

For vaginal tumor experiments, female C57BL/6J mice (6–10 weeks) were first hormonally synchronized by administering 0.1µg estradiol in peanut oil and 2mg Medroxyprogesterone acetate (DepoProvera) in PBS subcutaneously on two consecutive days (Fig. 2). One week later, cytology of vaginal smears was analyzed to ensure mice were in diestrus as described by Decrausaz et al [113]. On the same evening after collecting cytology samples, 20µL of 10% Nonoxyl-9 (N-9) was instilled in the vaginal cavity of mice anesthetized with the ketamine/xylazine mixture and were left over night. The following morning, each mouse was again anesthetized with ketamine/xylazine mixture and the vaginal cavity was washed five times with 80µL PBS for the removal of all N-9. After washes were complete, $2 \times 10^4$ TC-1-Luc cells in sterile PBS were instilled in the vaginal tract. Mice are kept in the supine position with their lower halves slightly raised until anesthesia wears off. Treatment with N-9 induces micro-lesions in the vaginal epithelial walls, mimicking the naturally occurring micro-lesions in humans necessary for HPV to access and infect basal epithelial cells. In this vaginal tumor model, the micro-lesions generated provide an anchoring surface for the tumor cells to embed within the vaginal epithelia. Tumor growth was monitored semiweekly using Xenogen In Vivo Imaging System (IVIS) and expressed as average luminescent signal in select Region of Interest (ROI) (p/sec/cm2/sr).
For the experiments testing the flank tumor model, $2 \times 10^5$ TC-1 cells in sterile PBS were injected subcutaneously in the left dorsal flank. Tumor size (length x width) was measured 3 times per week using calipers and reported as tumor area (mm$^2$).

![Diagram](image)

**Fig. 2. Vaginal tumor implantation and vaccination scheme.** Mice are hormonally synchronized one week prior to tumor implantation, on days -8 and -7 by administering Estradiole and Depo-provera subcutaneously 24 hours apart. One day prior to tumor implantation vaginal washes are collected to ensure diestrus synchronization and treated with 10% nonoxyl-9, overnight. The next morning, on day 0, the vaginal cavities are washed multiple times to remove the N-9, and 20,000 TC-1 Luc cells are then instilled into the vaginal cavity. Mice are immunized on days 5 and 11 after tumor implantation, and after successful tumor implantation has been confirmed, and imaged weekly.

**3.4 Vaccination Treatment**

For intranasal vaccinations, five mice at a time were put under ketamine/xylazine anesthesia and were maintained in the supine position while the listed doses of HPV E6/E7 peptides (100 µg each) plus either α-GalCer (2 µg), CpG-ODN (10 µg), or the
combination of both adjuvants were carefully administered in a drop-wise fashion using a micropipette as previously described [114, 115]. More specifically, the total volume of each vaccine (41ul for TVC, 42ul for TVA, and 43ul for TVAC) was divided into four doses (ranging from 10.25ul for TVC to 10.75ul for TVAC) and each dose was administered in a slow, dropwise fashion to each of the five mice, this allowed each mouse to rest in between doses. Vaccinations were administered on days 5 and 11 post-tumor implantation. For vaginal tumor-bearing mice, tumor size was first assessed by luciferase imaging on day 5 to ensure successful tumor formation, and tumor bearing mice were grouped according to tumor size in order to obtain similar average starting tumor size per treatment. Mice with a starting tumor size exceeding 20,000 ROI (p/sec/cm²/sr) were evaluated separately and are referred to as “large vaginal tumors” in the results section.

3.5 Isolation of Lymphocytes

For isolation and characterization of vaginal and flank tumor infiltrating lymphocytes (TIL), mice were implanted with $3 \times 10^4$ TC-1–Luc cells in 10 µL, and $30 \times 10^4$ TC-1-Luc cells in 200µL of PBS/Matrigel mixture, respectively, at a 2:1 ratio for the analyses of tumor-infiltrating leucocytes (TIL). Based on the prior survival analyses of the different treatments/vaccinations, one week after the second vaccination was selected as the time point to sacrifice mice for the TIL analyses studies. After CO₂-based euthanasia, each tumor was collected and diced into $< \frac{1}{2}$ cm pieces and digested in a mixture of 1 mg/mL collagenase D + 0.1 mg/ml DNase for 45 min at 37 °C before being
passed through a 45 µm strainer. Lymphocytes were enriched through discontinuous percoll gradient centrifugation and stained for flow cytometry analysis as previously described [114, 116].

For isolation and characterization of lymphocytes from the female reproductive tract (FRT), the vagina, uterus, and uterine horns were collected and cut into small pieces. Diced tissue was then incubated in 5 mM EDTA for one hour, followed by digestion with collagenase D (1 mg/mL) for 1h at 37 °C. Tissues were then passed through a 45 µm strainer and purified by discontinuous percoll gradient centrifugation [117].

Lastly, for isolation of lymphocytes from other lymphoid organs, including spleen, CLN, ILN, these soft tissues were directly passed through a 45um strainer. Splenocytes were pelleted and treated with red blood cell (RBC) lysis solution (ACK from Thermo Fisher) for one minute before being washed with and re-suspended in cell media.

3.6 CD8 Depletion
For in vivo CD8 depletion, mice were administered 100 µg of aCD8 mAb from BioXCell (Lebanon, NH, USA) via the intraperitoneal (IP) route one day prior to the first vaccination dose, and every three days after that until the completion of the experiment. The CD8 depletion was monitored in the blood throughout the course of the experiment as described in the results section.
3.7 NK Depletion

For in vivo NK cell depletion, mice were administered 200 µg of PK136 mAb from BioXCell (Lebanon, NH, USA) via the intraperitoneal (IP) route one day prior to the first vaccination, and once every week until the completion of the experiment. Proper depletion of NK cells was monitored in the blood throughout the course of the experiment as described in the results section.

3.8 Adoptive Transfer of OT-I Cells

The ovalbumin (OVA)-specific OT-I TCR transgenic (Tg) CD8 T cells were obtained from lymph nodes of untreated OT-I mice (CD45.1+) and 1 × 10^6 cells were transferred to congenic C57BL/6J mice (CD45.2+) intravenously in 200 µL of sterile PBS. Mice were immunized intranasally with 5 µL of reconstituted endotoxin free OVA alone or in the presence of α-GalCer and/or CpG-ODN adjuvants one day after adoptive transfer and sacrificed one week after vaccination.

3.9 Flow Cytometry Analysis

Single cell suspensions from tumors, tissues or PBMC were fixed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) and then stained with antibodies to different surface and intracellular markers obtained from Biolegend (San Diego, CA, USA), BD Biosciences (Franklin Lakes, NJ, USA), eBioscience (San Diego, CA, USA), and Life Technologies (Carlsbad, CA, USA). The CD1d tetramer reagent specific for NKT cells was obtained from NIH tetramer core facility
at Emory University (Atlanta, GA, USA). Flow cytometry data were collected on a five-
laser, BD Biosciences LSR II cytometer and analyzed using FlowJoTM Software for
Windows, version 10 (Becton, Dickinson and Company, Ashland, OR, USA).

3.10 Statistical Analysis

All statistical analyses were performed using Graphpad Prism version 8 for
Windows. Statistical significance was determined using either ordinary one-way ANOVA
plus multiple comparisons, or the Brown–Forsythe and Welch ANOVA plus multiple
comparisons to test for differences between groups. Statistical significance for survival
analysis was calculated using the Mantel–Cox log rank test where indicated. p value of
<0.05 was considered significant in each comparison. All figures depict average data
values with SEM.
CHAPTER 4. RESULTS

4.1. Introduction:

We previously reported that in the TC-1 luc preclinical HPV vaginal tumor model, therapeutic vaccination with synthetic peptides corresponding to the E6 and E7 oncoproteins of HPV-16 and α-GalCer adjuvant delivered by the intranasal route in mice, reduced tumor growth rate but required α4–1BB antibody immunotherapy to induce sustained tumor regression and significant survival advantage [114]. The anti-tumor efficacy from this combination of intranasal vaccination and immunotherapy correlated with the induction of highly cytotoxic CD8 T cells expressing multiple granzyme effector molecules.

Toxic side effects and the high costs associated with immunotherapy, however, are concerns with this treatment approach [118-120]. Therefore, I tested an alternate strategy for effective induction of antigen-specific CD8 T cell response by incorporating the toll-like receptor (TLR) 9 ligand CpG-ODN along with α-GalCer, as diverse acting, but clinically relevant adjuvants.

The rationale for the adjuvant selection is that α-GalCer adjuvant activates antigen presenting cells (APCs) through the stimulation of NKT cells, resulting in the release of cytokines such as IFN-γ and IL-2 which act back on the APCs, inducing maturation and increasing antigen presentation [121]. Meanwhile CpG-ODN directly stimulates APCs by binding to TLR-9 present on the cell surface, inducing antigen-specific CD8 T cell immunity [122]. These two activation mechanisms recognized as alternative (NKT-mediated), and classical licensing (mediated by TLR-ligands) of APCs, are expected to
activate distinct chemokine signaling to recruit diverse sets of naïve CD8 T cells for enhanced adaptive immunity [26].

I hypothesized that intranasal vaccination employing the combination of TLR9 and NKT cell ligands, (CpG-ODN and α-GalCer) will induce potent systemic and mucosal antigen-specific CD8 T cell immunity, specifically at the female reproductive tract (FRT) to eliminate HPV genital tumors.

In order to test this hypothesis, I developed the following aims:

- **Aim 1**: Test whether intranasal vaccination employing the combination of adjuvants, relative to each adjuvant alone, will induce strong CD8 T cell response to the co-administered antigen at multiple systemic and mucosal tissues

- **Aim 2**: Determine whether intranasal delivery of HPV peptides together with the adjuvant combination will be effective in treating established HPV+ vaginal tumors.

- **Aim 3**: Investigate whether the tumor location and route of vaccination will influence therapeutic efficacy of the HPV peptide vaccine.

4.2: **(Aim 1)** Test whether intranasal vaccination employing the combination of adjuvants, relative to each adjuvant alone, will induce strong CD8 T cell response to the co-administered antigen at multiple systemic and mucosal tissues

In this section, I first addressed the hypothesis that intranasal vaccination utilizing the combination of α-GalCer with CpG-ODN, relative to each adjuvant alone, will result in higher induction of CD8 T cells specific to the co-administered antigen at multiple systemic and mucosal tissues, including the FRT (Aim 1.1). I then addressed the
hypothesis that the resulting CD8 immune response will exhibit stronger cytolytic characteristics in mice receiving the combination of adjuvants in comparison with either adjuvant alone.

Data obtained from these studies were included in recently published manuscript (Seirra et al. 2020)

4.2.1 Determine the potential of the intranasal vaccination using α-GalCer and/or CpG-ODN adjuvants for promoting trafficking of CD8 T cells to the FRT (Aim 1.1)

In order to determine whether the proposed intranasal therapeutic peptide vaccination strategy will be effective to treat established HPV genital tumors, I first tested for successful induction of cellular immunity to the FRT. Specifically, I investigated whether vaccination using the combination of adjuvants, relative to each adjuvant individually, will increase CD8 T cell responses at the FRT, in particular antigen specific CD8 T cells. For this, I have adapted the ovalbumin (OVA)-specific OT-I TCR transgenic (Tg) CD8 T cells as a model system because of the convenience and efficiency to monitor induction and localization at multiple tissue sites.

In this approach, mice were adoptively transferred with 1x10^6 OT-I cells intravenously one day prior to intranasal administration of endotoxin-free ovalbumin (OVA) alone or along with α-GalCer, or CpG-ODN, or the combination of α-GalCer and CpG-ODN. Mice were sacrificed one week after for flow cytometry analysis of isolated lymphocytes from the spleen, and FRT along with vaccine-draining cervical lymph nodes (CLN), FRT-draining inguinal lymph nodes (ILN). Data shown in Fig. 3A illustrates the
percentages of OT-I cells (CD45.2+ and Va2+) in each of the different groups of mice and clearly establishes the effectiveness of the vaccine containing the two adjuvants, α-GalCer and CpG-ODN, together verses each adjuvant alone in terms of significantly higher percentage of OT-I cells in each of the tissues analyzed.

These data demonstrate that intranasal immunization with OVA using either α-GalCer or CpG-ODN increased the percentage of OT-1 cell in the spleen, cervical lymph nodes (CLN), inguinal lymph nodes (ILN) and the female reproductive tract (FRT) when compared to mice receiving OVA alone. Immunization using the combination of adjuvants further increased the percentages of OT-I cells significantly, in all the tissues examined (Fig 3B-E).
Figure 3. Intranasal immunization utilizing the combination of α-GalCer and CpG-ODN adjuvants induces significant increase of antigen specific CD8 T cells in systemic and mucosal tissues. Adoptively transferred OT-I cells were analyzed by flow cytometry in spleen, FRT, CLN, and ILN one week after intranasal immunization with OVA alone or in combination with α-GalCer and CpG-ODN adjuvants individually or together. Representative gating strategy from the spleen is shown (A); OT-I cells are gated from CD8+ cells as double positive for CD45.1 and Vα2 (left panels of B-E). Representative flow plots of OT-1 cells in each tissue are shown in B-E (left panels of B-E), and average values for the frequencies in each tissue are summarized in the bar graphs in the far right panels for each tissue. Statistical significance was calculated using ordinary one-way ANOVA with multiple comparisons and the Brown–Forsythe and Welch ANOVA with multiple comparisons, p < 0.05 (*), p < 0.005 (**), p < 0.0005 (**), p < 0.00005 (**).
4.2.2 Determine the therapeutic potential of CD8 T cells generated after intranasal delivery of α-GalCer and CpG-ODN, alone or in combination at both systemic and mucosal tissues (Aim 1.2)

Although infiltration of CD8 T cells to the tumor is generally considered indication of positive antitumor efficacy [123-125], functionality, in terms of killing capacity of the tumor infiltrating CD8 T cells is important for effective tumor eradication. Therefore, I next examined the functionality of the antigen specific CD8 T in the spleen and FRT of vaccinated mice by using intracellular cytokine (ICC) staining for Granzyme B expression, a marker for cytotoxicity.

Results from this analysis indicate that immunization with OVA plus the combination of α-GalCer and CpG-ODN produced significantly higher percentages of Granzyme B expressing antigen specific CD8 T cells (OT-1 cells), as well as the total CD8 T cell population in the spleen and FRT (Fig. 4A, B, respectively).
Fig. 4. Intranasal immunization employing the combination of α-GalCer and CpG-ODN adjuvants significantly increases the frequencies of functional antigen-presenting cells as well as overall CD8 T cells in the spleen and FRT. Adoptively transferred OT-I cells were analyzed by flow cytometry in the spleen and FRT one week after immunization with OVA alone, or with the combination of α-GalCer and CpG-ODN. Average values for the frequencies of OT-I cells expressing GzmB (A), and overall frequency of CD8 T cells expressing GzmB (B) in the spleen and FRT of mice were compared between the treatments using t-test, \( p < 0.005 \) (**), \( p < 0.0005 \) (***).
These results demonstrate that intranasal immunization using the combination of α-GalCer and CpG-ODN adjuvants is an effective strategy to induce a robust and functional immune response in both systemic tissues, such as the spleen, and in the mucosal tissues, like FRT.

Based on these results, I tested whether therapeutic HPV peptide vaccination employing the combination of α-GalCer and CpG-ODN adjuvants will induce anti-tumor efficacy against HPV genital tumors.

4.3. (Aim 2) Determine whether intranasal delivery of HPV peptides together with the adjuvant combination will be effective in treating established HPV+ vaginal tumors.

The therapeutic vaccine comprised of synthetic peptides corresponding to the E6 and E7 oncoproteins of HPV-16, immunity which correlated with disease-free survival in women treated for CIN2/3 neoplasia[112]. Therefore, I tested the efficacy of vaccination using these peptides along with α-GalCer and/or CpG-ODN adjuvants for therapeutic efficacy in the orthotopic HPV vaginal tumor model. For simplicity sake the different vaccine compositions from here on out will be referred to as therapeutic vaccine containing α-GalCer (TVA), therapeutic vaccine containing CpG-ODN (TVC), and therapeutic vaccine containing α-GalCer and CpG-ODN (TVAC).

Some of the results from this following section were also reported in a recently published manuscript (Sierra et. Al 2020)

In the orthotopic HPV vaginal tumor model, mice were hormonally synchronized and implanted with 20,000 TC-1Luc cells as described in the methods. Five days after
tumor induction, mice were imaged to ensure successful implantation of the tumor and sorted in order to ensure a similar average starting tumor size in the control group as well as in each of the treatment groups (TVA, TVC, or TVAC). Therapeutic vaccines were administered intranasally on days 5 and 11 after tumor implantation. Tumor size was monitored using the In Vivo Imaging System (IVIS) 200 in the institutional Small Animal Imaging Facility (SAIF) and reported as average radiance (p/s/cm²/sr) within a 2x2cm circular region of interest (ROI) placed over the FRT on the imaging software Living Image.

Tumor size was measured on a weekly basis and survival was charted up to 90 days post-tumor implantation (Fig. 5A, 5B). Mice treated with TVA or TVC exhibited tumor regression in a minor subset (20% and 33% respectively), but overall significantly better survival when compared to the untreated mice, or mice treated with the adjuvants alone. On the other hand, the majority (85%) of mice treated with TVAC, showed complete tumor regression, and significantly higher survival rates in comparison to either TVA or TVC treated groups.
A. Survival Analysis

Survival curves showing percent survival over days post tumor challenge for different treatment groups:
- Untreated
- TVA
- TVC
- TVAC

B. Graphs showing average ROI vs. days post tumor challenge for:
- Untreated
- TVA (α-GalCer)
- TVC (CpG-ODN)
- TVAC (α-GalCer + CpG-ODN)
Fig. 5. Intranasally administered TVAC is effective at treating established orthotopic HPV vaginal tumors. Female C57BL/6J mice (n = 10 to 22) were hormonally synchronized and challenged with $2 \times 10^4$ TC-1-Luc cells into the vaginal cavity. Intranasal vaccinations using HPV peptide vaccine formulated with either α-GalCer, CpG-ODN, or both α-GalCer and CpG-ODN (TVA, TVC, or TVAC, respectively) were administered on days 5 and 11 after tumor cell implantation; control groups included untreated mice, or mice immunized with the mixture of adjuvants without peptides (adjuvants alone). Survival analysis was recorded between each treatment group as well as the appropriate controls (A). Tumor size was measured using luciferase expression (ROI units) and plotted over time. The numbers of mice with complete tumor regression over total per group (minimum 10 mice per group) are shown in each panel for the different treatment groups (B). Significance in survival proportions was measured using the log-rank test. $p < 0.05$ (*), $p < 0.00005$ (****),
4.3.1. Characterization of antitumor immune responses in mice treated with the different vaccines

Because positive modulation of the tumor micro-environment, specifically enhanced levels of tumor infiltrating CD8 T cells have historically correlated with better treatment-mediated outcome in patients [126], I analyzed the tumor infiltrating leukocytes (TILs) in mice with vaginal orthotopic HPV tumors after the various treatments on day 18 post-tumor implantation. This time point was chosen based on the substantial differences in tumor size evident between treatment groups (Fig. 6A).

The TILs were isolated as described in the methods section and analyzed by polychromatic flow cytometry using 14 different antibodies to detect various cell surface and intracellular markers. A typical gating strategy used for the flow cytometry analyses is shown in Fig. 6B.
Fig. 6. **Strategy for characterization of tumor infiltrating lymphocytes.** Tumor bearing mice were sacrificed one week after the second vaccination (V2) for characterization of tumor infiltrating leukocytes. Tumor size significantly varies at this time point and thus was chosen to distinguish immunological differences between treatment groups (red oval) (A). A generalized gating strategy is shown to depict characterization of the tumor infiltrating leukocytes (B). The first set of gates were set to detect cells based on size and granularity, followed by exclusion of dead cells and detection of CD45+ leukocytes. Subsequently, the live CD45+ cells were differentiated as T cells based on the expression of CD3, followed by further characterization of different CD4+ and CD8+ T cells subsets along with functional markers.
We found that TVAC treated mice showed consistently higher frequencies of CD8 T cells when compared to those treated with TVA and TVC, as well as the untreated control groups (Fig. 7A). More importantly, significantly higher levels of antigen specific CD8 T cells were detected using the HPV16 E7 peptide tetramer in the TVAC treated mice, relative to other groups (Fig 7B). Furthermore, granzyme B (GzmB) positive CD8 T cells as well as E7-specific CD8 T cells, representative of cytotoxic functionality, were found at higher frequencies in TVAC treated mice, relative to those in all other groups (Fig. 5A, 5B, left panels, respectively).

In addition to the CD8 T effector cells, I also investigated the frequencies of suppressor cell populations (MDSCs and Tregs) within the tumor. Overall, a trend for higher density of CD8 T cells (measured in terms of cells/tumor weight) was observed in mice receiving TVAC when compared to TVA, TVC, or untreated controls, while the suppressor cells showed the opposite trend (Fig. 7C). This translated into highest proportion of CD8 T cells relative to Tregs and MDSC combined in the TVAC treated animals, relative to those in other groups (60% CD8 in TVAC vs ~40% in TVA/TVC) as shown in the pie charts (Fig. 7D).
Fig. 7. Increased frequencies of effector CD8 T cells, relative to suppressor cells are correlates of protection by TVAC in mice with HPV genital tumors. Highest percentages of total and cytotoxic (GzmB+) populations of CD8 T cells as well as HPV E7 antigen specific CD8 T cells were observed within the tumors of TVAC treated mice, relative to those treated with TVA and TVC or untreated controls (B). Densities of CD8 T cells and suppressor cells (Tregs and MDSCs) in the tumors of mice in the different groups were plotted numbers per tumor weight (C). Individual frequencies of tumor infiltrating CD8 T cells, Tregs, and MDSCs among the three populations together reveal the dominance of CD8 T cells over the two suppressor together in the tumors of TVAC treated mice, relative to other groups (D). Statistical significance was calculated using a one way ANOVA, p<0.05 (*), p<0.005 (**), p<0.0005 (***)
4.3.2. Comparative analyses of immune correlates for antitumor efficacy between

TVAC and the combination of TVA and anti-4-1BB immunotherapy

In comparing the antitumor efficacy of TVAC with TVA plus anti-4-1BB treatment (TVA + α4-1BB) against established vaginal TC-1 tumors [114], there was no observed significant difference between these two treatments in terms of tumor regression and tumor-free survival (Fig. 8A). Therefore, I next investigated, by flow cytometry, the tumor infiltrating lymphocytes isolated from mice treated with TVA + α4-1BB and TVAC one week after the second vaccination. Although comparable levels of total and HPV E7 antigen specific CD8 T cells were induced in both treatment groups (Fig. 8B), we did not detect the highly cytotoxic CD4 and CD8 T cell subsets called ThEO, and TcEO cells, respectively, in TVAC treated mice, which were the defining feature of the efficacy of the TVA + α4-1BB treatment (Fig. 8C, 8D). We observed a trend for higher frequencies of total and antigen-specific CD8 T cells expressing Granzyme B or IFN-γ in TVAC treated mice, but the differences did not reach significance (Fig. 8E). Mice treated with TVA + α4-1BB, however, did show significantly higher levels of liver enzymes in the blood-Aspartate transaminase (AST) and alanine aminotransferase (ALT)- which serve as indicators of systemic toxicities attributed to immune checkpoint therapy, relative to those receiving TVAC [119, 120, 127-129].
Figure A: Survival Analysis

Figure B: CD8 T cells and Tetramer+ cells

Figure C: CD8 TcEO FOL

Figure D: Untreated, TVAC, TVA+4-1BB

Figure E: IFNγ + GzmB +

Figure F: Tetramer+ GzmB

Figure G: Double positive GzmB IFNγ

Figure H: AST, ALT
Fig. 8. Comparable antitumor efficacy for TVAC and TVA + α4-1BB treatments.

Female C57BL/6J mice with vaginal TC-1 tumors were treated with TVAC or TVA + α4-1BB as described in the methods and monitored for survival (A). TIL analysis was carried out to compare CD8 T cell frequencies as well as antigen specific CD8 T cells (B). TcEO cells (classified as EOMES⁺ KLRG-1⁺ CD8 cells) were found to be a unique feature of TVA + α4-1BB treatment (C, D). Functionality of overall CD8 T cells (E, G) and HPV E7 antigen specific CD8 T cells (F) were also compared between these two treatments. Liver enzyme levels were recorded in blood three days after each vaccination and reported as units of aspartate transaminase (AST) or alanine aminotransferase (ALT) per liter (L). Significance in survival proportions was measured using the log-rank test; ns=not significant. Statistical significance between different treatment groups was calculated using a One-Way ANOVA with multiple comparisons. p<0.00005 (** **), p<0.0005 (**), p<0.005 (**), p<0.05 (*).
4.3.3. Investigate the contribution of CD8 T cells for the anti-tumor efficacy of TVAC treatment

Since the data in Fig. 7 showed induction of CD8+ T cells as an important correlate for the efficacy of TVAC, I tested whether and to what extent depletion of CD8 T cells would abrogate the protection. Vaginal TC-1 tumor-bearing mice treated with TVAC were administered an isotype control or anti-CD8 depletion antibody every three days starting one day prior to tumor induction, and untreated mice served as an additional control (Fig. 9A). Blood collected over time was analyzed to confirm depletion of CD8 T cells, Day 17 post tumor induction flow plots are depicted as representative graphs (Fig. 9B). I observed near complete depletion of CD8 T cells (99.6% decrease relative to control) in mice receiving the CD8 depletion antibody, which correlated to significant abrogation of the anti-tumor efficacy of TVAC in terms of significantly lower survival due to tumor growth when compared to the isotype control (Fig. 9C, 9D).

These results indicate that the CD8 T cell response generated by the intranasal administration of TVAC is essential for tumor clearance.
A. 1. PBS Control  
2. HPV Peptides + α-GalCer+CpG (IgG Control)  
3. HPV Peptides + α-GalCer + CpG (αCD8)

B. Untreated Isotype Control αCD8

C. Average Tumor Growth

D. Survival Analysis
Fig. 9. CD8 T cells are essential for TVAC efficacy against vaginal TC-1 tumors in mice. Tumor bearing mice were vaccinated intranasally as previously described with TVAC on days 5 and 11, and either treated with a CD8 depletion antibody or IgG control once every three days. An untreated group served as an additional control (A). CD8 depletion was monitored in the blood over the course of multiple time points using FACS. Representative FACS plots gated on live CD3 population show complete CD8 depletion only in mice receiving depletion antibody at one week post-tumor implantation (B). Tumor growth and survival were monitored in mice and plotted over time (C). Significance in survival proportions was measured using the log-rank test. $p < 0.005 (**), p>0.05$ (ns).
4.3.4-Role of NK and NKT cell subsets in TVAC efficacy

The composition of TVAC includes the adjuvant α-GalCer known to promote antigen presentation via the activation of NKT cells, which are important for innate immune surveillance via direct cytotoxic functions. Therefore, I investigated their role in the observed efficacy of TVAC against the vaginal TC-1 tumor model.

NKT cells are classified into at least two main subsets, type 1 NKT cells and Type 2 NKT cells. Type 1 NKT cells, which are more commonly referred to as invariant NKT (iNKT), express the invariant Vα14-Jα18 in mice and Vα24-Jα18 in humans, and are generally identified in flow cytometry as CD3 intermediate and NK1.1+[130]. They are strongly reactive to α-GalCer and can be identified using α-GalCer loaded CD1d tetramer reagents.

Type 2 NKT cells, however, are described as the more evasive population of NKT cells due to the fact that they do not express a semi-invariant TCR chain like the iNKT cells and can’t be identified with α-GalCer loaded CD1d tetramer [131], instead they have diverse TCRs but are still CD3+, NK1.1+. Under normal conditions, these cells harness anti-inflammatory capabilities, however, recent studies have indicated that CpG-ODN can shift the capabilities of type 2 NKT cells to an inflammatory response with anti-tumor capabilities [132]. Based on this information, I hypothesized that TVAC comprising both α-GalCer and CpG-ODN adjuvants may be engaging type 2 NKT cells for their potential contribution towards the observed anti-tumor efficacy.

Interestingly, one of the primary reasons early clinical trials using α-GalCer showed dampened results in humans is due to the reduced frequencies of iNKT cells in
comparison to mouse models[108]. Given that type 2 NKT cells are more prominent than iNKT in humans [133], engagement of these cells could potentially lead to better clinical outcomes. By gating on CD3+NK1.1+ cells in the flow cytometry analyses, I attempted to identify these cell within the TC-1 tumors of untreated mice and those administered the different vaccine formulations (Fig 10A).

Tumor bearing mice were treated with TVA, TVC, or TVAC and sacrificed three days after the second dose. This time point was chosen due to the rapid response of NKT cells after intranasal stimulation where the peak response is typically at three days [107]. I observed slight increase in the levels of iNKT cells (Type 1 NKT cells) in the tumors of TVAC and TVA treated mice, (Fig 10B) with TVAC treated mice exhibiting the highest number of IFN-γ expressing iNKT cells among the different groups of mice(Fig. 10C). The frequency of type 2 NKT cells increased within the tumor in TVC treated groups, however, no differences in the numbers of IFN-γ positive cells was recorded in the tumors of different groups of mice examined (Fig 10D, 10E).
Fig. 10. Detection and characterization of NKT cell subsets in the vaginal TC-1 tumors of vaccinated mice. Tumor bearing mice were treated on days 5 and 11 as previously described with either TVA, TVC, or TVAC. Three days after the second vaccination, lymphocytes were collected and analyzed according to the gating strategy shown (A). The CD1d tetramer positive cells were classified as type I NKT (iNKT) cells, while CD1d tetramer negative were classified as type 2 NKT cells. Frequencies of each subset and functionality in terms of IFN-γ expression are shown (B-E).
In order to evaluate the potential contributions of NK and NKT cells subsets for the anti-tumor efficacy of TVAC, I conducted a NK depletion study. Because there is no single antibody to deplete specifically the NKT cells, I used the PK136 antibody that depletes both NK and NKT cells subsets. The tumor bearing mice were treated with TVAC as previously described, on days 5 and 11, along with IP administration of the PK136 antibody or a control IgG one day prior to each TVAC treatment (days 4 and 10 post-tumor implantation) and weekly thereafter. Blood was collected weekly and analyzed to ensure NK depletion. Representative flow plots depict specific NK depletion in the blood and are plotted as averages per antibody treatment (Fig. 11A, 11B). Survival and average tumor growth were monitored over time (Fig. 11C, 11D), and individual mouse tumor growth is also shown (Fig. 11E)

Despite near complete depletion of NK cells subsets, I observed only marginal decrease in TVAC-mediated protection, in terms of tumor growth and survival percentage. These results suggest minor contribution by the NK and NKT cells subsets towards the observed antitumor efficacy of TVAC against vaginal TC-1 tumors, the genital HPV tumor model.
Fig. 11. Depletion of NK cells does not significantly influence the efficacy of TVAC against orthotopic vaginal TC-1 tumors. Vaginal tumor bearing mice were administered either PK136 antibody or control IgG via the IP route, one day prior to each vaccination, and weekly thereafter. TVAC was administered intranasally as previously described on days 5 and 11. Blood was analyzed weekly to ensure proper NK depletion, and representative flow plots as well as numeric summaries are shown (A, B). Mice were monitored for survival (C) and imaged weekly to monitor tumor growth (D, E). Statistical significance was calculated using a two-sided t-test, p<0.05(*)
4.4. (Aim 3) Investigate the effect of tumor location and route of vaccine administration on the therapeutic efficacy of TVAC

Based on the potent antitumor efficacy observed for TVAC administered by the intranasal route against established HPV orthotopic tumor model of vaginal /TC-1 tumors, I tested whether the vaccine is equally efficacious against subcutaneously implanted flank tumors (aim 3.1), and whether the vaccine will be effective if delivered by systemic subcutaneous route (aim 3.2).

4.4.1 Determine the efficacy of intranasally administered TVAC against subcutaneous HPV tumors.

Up to 23% of Stage IV HPV genital cancers are reported to spread into other parts of the body [134], therefore I set out to determine whether intranasally administered TVAC would also be effective in treating tumors at non-mucosal location by injecting the TC-1 tumor cells subcutaneously on the flank.

Naïve female C57Bl6/J mice were implanted with 200,000 TC-1 Luc cells subcutaneously in the flank, and immunized on days 5 and 11 as described in the methods. Mice were administered either TVA, TVC, or TVAC by the intranasal route and monitored for tumor growth and survival (Fig. 12A). Tumor size was measured three times per week using calipers and plotted as area (mm$^2$) over time.

I observed nearly 30% of the TVAC treated mice with subcutaneous flank tumors exhibit tumor regression and improved survival relative to any other treatment (Fig. 12B). This level of antitumor efficacy for the intranasal TVAC treatment is lower than the 70% observed for mice with mucosal vaginal tumors (Fig.7).
Fig. 12. Intranasal delivery of TVAC shows limited efficacy in subcutaneously implanted TC-1 tumors. Groups of 57BL/6 female mice were injected with $2 \times 10^6$ TC-1Luc cells subcutaneously on the flank and treated by the intranasal route with either TVA, TVC, or TVAC on days 5 and 11 after tumor induction (A), a separate group of untreated mice served as control group. Survival rates (B), and tumor growth were monitored over time (C) and also expressed as individual mice in each group (D).
In order to determine the immune correlates for the observed partial protection of TVAC against the subcutaneous tumors, tumor infiltrating lymphocytes were collected for flow cytometry analysis one week after the last treatment.

The frequencies of overall and antigen specific CD8 T cells were significantly increased in the tumors of mice treated with TVAC when compared to untreated control mice, similar to the data from the analyses of TILs from mice with vaginal tumors (Fig. 13A, 13B). Furthermore, granzyme B expressing total and antigen specific CD8 T cells were also at higher frequencies in mice receiving TVAC, relative to that in the untreated mice (Fig. 13C, 13D). This data indicates that intranasal administration of TVAC is effective at inducing a robust immune response in flank tumors, but was insufficient to afford the level of tumor regression matching that in the vaginal tumor model (Fig. 7). Therefore, I investigated for changes in the frequencies of immune suppressive Tregs and MDSCs and compared the effector to suppressor cell ratios in the different groups of mice treated for subcutaneous flank tumors. Although the CD8 T cells in the flank tumors were found at higher percentages in the TVAC treated mice when compared to the untreated and TVC treated mice, the density of CD8 T cells in the flank tumors were similar in TVA and TVAC treated mice (Fig. 13E). Treg and MDSC populations were also similar in TVA and TVAC groups, with lower levels found in the untreated and TVC treated groups (Fig. 13F, 13G). Unlike the trend we saw in the TVAC administered mice with vaginal tumors, a stronger CD8 T cell response was not observed when compared to the suppressor cell populations, resulting in no improvement of the ratio of effector (CD8 T cells) to total fractions of suppressor cells (Tregs and MDSCs) (Fig. 13H).
Fig. 13. Tumor infiltrating lymphocytes in the flank tumors of mice treated with TVAC by the intranasal route. Groups of 57BL/6 female mice were injected with $2 \times 10^6$ TC-1Luc cells subcutaneously on the flank and treated by the intranasal route with either TVA, TVC, or TVAC on days 5 and 11 after tumor induction (A), and a separate group of untreated mice served as control group. Single cell suspensions from flank tumors collected one week after completion of the last immunization were analyzed by flow cytometry. Frequencies of total and antigen specific CD8 T cells (A and B) as well as respective functional subsets (C and D) were enumerated in different groups of mice. Densities of CD8, Treg, and MDSCs populations in terms of numbers of cells/tumor weight were plotted (E). The ratios of CD8 to cells to each of the suppressor cell subsets as well as total suppressor cells were shown (F). Statistical significance was calculated using one-way ANOVA, $p < 0.05$ (*), $p < 0.005$ (**), $p < 0.0005$ (***)}, not significant (ns).
Early diagnosis and treatment of tumors offers higher rates of survival [135, 136]. It is therefore possible that delayed treatment would most likely results in the accumulation of higher numbers of immune suppressive cells, including MDSCs and T regulatory cells in the tumor microenvironment. Because the experimental protocol for subcutaneously implanted flank tumors involved injection of a higher dose of tumor cells (200,000), relative to that for the vaginal tumors (20,000), I hypothesized bigger flank tumor size prior to the start of the treatment/vaccination regimen (day 5) allowing for greater accumulation of immune suppressive cells resulting in the observed lower efficacy of the therapeutic vaccine, TVAC, relative to that in the vaginal tumor model studies.

To test this hypothesis, specifically MDSC accumulation as a major reason for the reduced efficacy of TVAC against flank TC-1 tumors, I utilized anti-Gr-1 antibody to deplete MDSCs. The antibody was administered by the intraperitoneal route weekly, starting two days after tumor implantation to mice treated with TVAC and control untreated mice (Fig. 14A). Blood was analyzed by flow cytometry at several time points to ensure depletion of MDSCs (Fig 14B, 14C). Tumor growth and survival were recorded over time (Fig. 14D, 14E, 14F).

I observed that depletion of the MDSCs did not provide any significant advantage in survival or reduction of tumor size/growth rate in either the TVAC treated or control groups of mice. This indicates that MDSC accumulation prior to treatment may not be contributing to the diminished TVAC efficacy in the flank implanted TC-1 tumors. Alternately, Tregs and or the combination of MDSC and Tregs together directly or
through soluble factors could be at overwhelmingly higher frequencies/densities in the tumor for the total and antigen-specific cytotoxic T cell populations to impart antitumor efficacy.
Fig. 14. MDSCs depletion fails to increase efficacy of TVAC in subcutaneously implanted flank TC-1 tumors. Tumor bearing mice were treated with anti-GR-1 antibody (IP) weekly, starting two days after tumor implantation followed by TVAC (IN) on days 5 and 11 as previously describe in the methods section (A). Proper MDSC depletion was ensured via flow cytometry at several time points throughout treatment; representative gating strategies are shown for the detection of MDSCs in the blood and are shown for each treatment group (B, C). Tumor growth was monitored over time (D, E), and survival was recorded (F). Significance in survival proportions was measured using the log-rank test. P>0.05 (ns).
Thus, the timing of vaccine administration with respect to tumor burden could potentially be a confounding factor for the weaker immune protection by the TVAC. In fact, a comparison of tumor size determined by the luciferase expression based imaging analyses, revealed a significantly larger starting size for the subcutaneous flank tumors relative to the vaginal tumors correlating with the relative differences observed for the vaccine-mediated efficacy (Fig. 15B). Because subcutaneously implanted tumors with less number of cells in the inoculum tend to spontaneously regress in immune competent mice, reducing the number of tumor cells for initiating the tumor growth was not an option. Therefore, I tested initiating vaccine treatment on day 1 after subcutaneous injection of tumor cells, followed by the second dose delivered on day 5, thus keeping the intervals between the two vaccinations the same as in the previous experiment (Fig. 15A). Importantly, the size of the subcutaneously growing flank tumors at day 1 was comparable (based on luciferase expression) to that of vaginal tumors at day 5 (when the immunization was initiated) (Fig. 15B). In this vaccination regimen, TVAC-treated mice exhibited effective control of tumor growth in about 75% of mice with significant survival and these outcomes are comparable to that seen for vaginal tumors treated with this vaccine TVAC (Fig. 15C, 15D). Furthermore, TIL analysis revealed that treatment of the smaller subcutaneously flank tumors treated on day 1 resulted in significantly higher concentrations of CD8 T cells (per gram of tumor), when compared to that in the larger flank tumors (treated on day 5) (Fig. 15E).
A. Induce Tumor

B. Tumor starting size

C. Small SC Survival Analysis

D. Untreated

E. #CD8 T cells /tumor weight

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**Fig. 15. Flank and vaginal tumors matched for pre-treatment size are responsive to intranasally delivered TVAC.** Groups of C57BL/6 female mice were injected with 2x10^6 TC-1Luc cells subcutaneously on the flank and treated by the intranasal route with either TVA, TVC, or TVAC on days 1 and 6 post-tumor induction (A). Vaginal tumor size on day 5 compared to that of flank tumors on days 1 versus day 5 (B). Tumor bearing mice were followed for survival and tumor growth (C, D). Density of CD8 T cells in the tumor one week after second dose of TVAC are compared between mice with smaller flank tumors receiving TVAC treatment on day 1 (small SC TVAC) versus the in mice with larger flank tumors that began treatment on day 5 (Large SC TVAC) (E). Statistical significance was calculated using One-Way ANOVA test with multiple comparisons. p>0.05 (ns), p<0.005 (**), p<0.00005 (***)

4.4.2. Determine the importance of mucosal intranasal route of immunization for the efficacy of TVAC against vaginal TC-1 tumors (Aim 2.2)

The mucosal immune system is a compartmentalized portion of the overall immune system. Because HPV is primarily transmitted via mucosal surfaces, a major rationale for adapting mucosal intranasal route of immunization is to not only induce robust antigen specific CD8 T cell response, but also to promote their trafficking to the female reproductive tract for efficient tumor clearance. While data obtained from intranasal delivery of TVAC demonstrated strong efficacy against vaginal HPV tumors, it is not clear whether systemic route of vaccine administration would be inferior or equally effective at protecting against vaginal HPV tumors.

To begin testing this, the OT-I transgenic mouse model was adapted to first monitor distribution of antigen specific CD8 T cells across multiple systemic and mucosal tissues after systemic intramuscular immunization regimen. A total of 1x10^6 OT-I cells were adoptively transferred into congenic C57Bl/6 mice followed one day later by treatment with OVA plus the combination of α-GalCer and CpG-ODN adjuvants either by the mucosal intranasal or systemic intramuscular route. Frequencies of OT-I cells were determined at various tissues one week later.

While a significantly higher frequency of OT-I cells was observed in the spleens of mice receiving the systemic, relative to the mucosal route of immunization (Fig. 16A), there was no significant difference in the proportions of these antigen specific CD8 T cells within the FRT (Fig. 16B). Furthermore, the cytotoxic functionality, in terms of granzyme B expression, of these CD8 T cells within the FRT was also not significantly
different between mice receiving the systemic versus intranasal immunization (Fig. 16C), suggesting that both routes of vaccination are equally efficient in inducing functional antigen-specific CD8 T cell responses within the female reproductive tract.
Fig. 16. Mucosal and systemic routes of immunization induce similar antigen specific immune response at FRT. Mice were adoptively transferred with 1x10^6 OT-I cells one day prior to being treated with either intranasal or systemic administration of OVA or OVA + α-GalCer + CpG-ODN. Percentages of OT-I cells were examined in the spleen and FRT of treated mice (A, B). Granzyme B expressing CD8 T cells were examined within the FRT (C). Statistical significance was calculated using the one-way ANOVA test with multiple comparisons. p>0.05 (ns) p<0.005 (**), p<0.0005 (***)
Based on the data showing the potency of systemic immunization to induce antigen-specific cytotoxic CD8 T cell responses in the FRT, I also compared mucosal and systemic routes of TVAC delivery to treat vaginal HPV tumors. Since it is known that multiple doses of α-GalCer delivered via systemic route of immunization induce NKT cell anergy, and thereby impede development of adaptive immunity to co-administered antigens [107], for subcutaneous immunization mice were dosed only once with TVAC (containing the α-GalCer along with CpG-ODN) on day 5.

Delivery of TVAC by either SC or IN route was equally effective at treating the HPV vaginal tumors as observed in terms of tumor growth and survival (Fig. 17A). Analysis of TILs suggested that intranasal immunization was more efficient at inducing CD8 antigen specific T cells in the tumor, when compared to SC immunization (Fig. 17C, 17D). Despite this difference, overall efficacy in terms of survival advantage was not statistically different in mice receiving the TVAC by the two different routes.

This data indicates that the therapeutic HPV peptide vaccine containing α-GalCer and CpG adjuvants (TVAC) is equally effective when delivered in two doses by the intranasal route or in one dose by the subcutaneous route at eliciting anti-tumor immunity and efficacy.
Fig. 17. Single dose of SC TVAC is effective at treating vaginal tumors. Vaginal tumor bearing mice were immunized on days 5 and 11, intranasally, as previously described, or only on day 5 via the subcutaneous route and monitored for tumor growth and survival (A, B). Tumor infiltrating lymphocytes were analyzed on day 18 post-tumor implantation and are reported as frequency of parent population (C, D). Statistical significance was calculated using one-way ANOVA with multiple comparisons. p<0.005 (**).
CHAPTER 5: DISCUSSION AND FUTURE DIRECTIONS

Data from this dissertation demonstrates the antitumor efficacy of mucosal intranasal vaccination regimen comprised of peptides corresponding to immunogenic sequences in the HPV-16 oncoproteins E6 and E7 and the combination of α-GalCer and CpG-ODN adjuvants (TVAC) in an established preclinical HPV genital tumor mouse model. This is particularly significant because sustained tumor regression-free survival was achieved solely by vaccine-induced cytotoxic effector immunity successfully overcoming the immunosuppressive tumor microenvironment. Importantly, the therapeutic vaccine formulation was effective without the need to employ immune checkpoint therapy that often suffers from systemic toxicities [128, 137]. Despite many efforts to overcome the toxicities [138, 139], treatment related costs, widespread availability/accessibility, and ease of administering ICT to patients in resource-limited areas of the world are the major hurdles. In this regard, the needle-free intranasal vaccination regimen employing clinically relevant adjuvants to promote immunogenicity demonstrated to be efficacious in this investigation may be an ideal choice for the treatment of HPV+ cervical cancer. Our data demonstrates that in the absence of ICT, intranasally delivered TVAC exerted significant induction of antigen-specific CD8 T cells resulting in substantial increase in the ratios of effector CD8 T cells to the immunosuppressive Tregs and MDSCs within the TME.

Additionally, serum levels of liver enzymes AST and ALT that serve as surrogates for toxicity measurements were comparable between mice with vaginal HPV tumors in the untreated control group and those treated with the therapeutic HPV peptide vaccine
containing the combination of α-GalCer and CpG-ODN adjuvants. However, these measurements of liver toxicity in mice receiving the α4–1BB ICT were significantly above the normal range (Fig. 7).

We reported earlier that the significant antitumor efficacy afforded by the therapeutic HPV peptide vaccination employing α-GalCer adjuvant and supplemented α4-1BB ICT was the induction of a unique subset of Eomesoderm in expressing CD4 and CD8 T cells with high cytotoxic potential (ThEO and TcEO cells, respectively) owing to their capacity for producing multiple granzymes [140]. Even though TVAC treated mice did not exhibit induction of this novel T cells subsets, the frequencies of total and antigen-specific CD8 T cells as well as the antitumor efficacy in terms of tumor regression-free survival were comparable. It is possible that the therapeutic efficacy of TVAC may be also due to the engagement of other cytotoxic effector mechanism including the contributions from NK cells and/or NKT cells, a possibility that needs to be evaluated in future studies. Since the tumor killing functions of the CD8 T cells were not directly assessed in either of the treatments (α4-1BB supplemented single-adjuvant vaccine and TVAC), it is difficult to surmise whether enhanced cytotoxic potential of CD8 T cells was an important feature of efficacy in the TC-1 vaginal tumor model.

The unique therapeutic potential of TVAC is likely due to the ability of the adjuvant combination, relative to each adjuvant alone, in the vaccine to impart dual licensing of dendritic cells and promoting induction of stronger CD8 T cell responses. While CpG-ODN is known to induce dendritic cell (DC) activation through Toll-Like Receptor (TLR) 9 signaling, referred to as classical licensing, α-GalCer engages NKT cells to promote
stron maturation of DCs, a phenomenon referred to as alternative licensing [26, 141]. It is suggested that DC receiving the signals from dual licensing can induce strong recruitment and activation of naïve CD8 T cells through the actions of two independent sets of chemokines [122, 142, 143]. It is therefore possible that the combination of α-GalCer and CpG-ODN adjuvants, relative to each adjuvant alone, and intranasal delivery enabled promotion of strong antigen-presentation in mucosal DCs, resulting in the induction and recruitment of high levels of antigen-specific CD8 T cells to the tumor. While studies in this investigation were limited by less efficient isolation of adequate numbers of mucosal dendritic cells from the nasal associated lymphoid tissues, it is known that higher DC activation is directly correlated with enhanced antigen presentation and thus CD8 response. Within the tumors of mice treated with TVAC, higher frequencies of overall CD8 T cell as well as functional subsets of HPV-specific CD8 T cells expressing granzyme B were observed, and the number of CD8 T cells exceeded those of the immunosuppressive Tregs and MDSCs together. This data showing significant induction of functional CD8 T cells in mice treated with TVAC is in line with reports in the literature for their role in antitumor efficacy, and proved to be the primary driver of protective immunity in the vaginal TC-1 model, as CD8 depletion completely abrogated vaccine efficacy (Fig. 4) [144]. Compatibility of different TLR agonists with α-GalCer for enhanced DC-mediated activation of CD8 T cells has been described in vitro using co-cultures of DC and T cells and after subcutaneous delivery in vivo [109, 110, 122, 145-147]. However, my results show for the first time the effectiveness of α-GalCer in combination with TLR9 agonist for intranasal route of vaccination to promote induction
of functional antigen-specific CD8 T cell responses in systemic and mucosal tissues, and more importantly for curative efficacy against HPV genital tumors. These results are in line with previous data from our group that showed intranasal delivery of vaccines is an effective strategy to drive significant levels of antigen-specific CD8 T cell responses at the genital mucosa [117].

Intranasally delivered TVAC was also effective at treating subcutaneously implanted tumors in the flank supporting its wider utility in cases where the cancer has spread out of the genital mucosal tissues. Albeit, the efficacy of TVAC was significantly reduced against larger size tumors, despite the induction of strong CD8 T cell response, implicating that the larger flank tumors may have significantly accumulated suppressor cells, as commonly seen in patients [19]. Interestingly, however, depletion of MDSCs alone was not enough to overcome immune suppression and restore antitumor efficacy of TVAC (Fig. 14). These results suggest contribution of additional immune suppressive populations like Tregs in the tumor microenvironment and a combined depletion of both MDSCs and Tregs at the start of tumor induction could improve antitumor efficacy of the vaccination regimen. It is therefore, important and informative to determine whether the frequency and/or density of functional populations of total and antigen specific CD8 T cells within the tumor are sufficient for inducing tumor regression. In this aspect, recent investigations of MDSCs have identified subsets with differing immunosuppressive potentials, therefore, inclusion of additional markers for characterization of these subsets and determining functionality of tumor-infiltrating MDSCs would be beneficial to further understand the effector : suppressor cell populations within the HPV tumors in future
studies [148]. Because surface markers and gating strategies alone cannot discriminate some MDSC subsets from monocytes and neutrophils, one method of identifying MDSC functionality is the inclusion of a marker to detect Arginase [149]. Arginase is an enzyme produced by immunosuppressive MDSCs which depletes L-Arginine, inhibiting T-cell function [150]. In addition, in-vitro studies co-culturing purified suppressor MDSCs with increasing number of effector cells is an alternate option for further investigating the effector to suppressor cell ratios within the TME. Additionally, analysis for the expression of immune checkpoint inhibitory receptors/ligands (such as PDL-1/PD-1) within the tumor microenvironment as well as comparison of the ratios of CD8: MDSC + Treg cells in relation to tumor size would be instrumental to further refine the intranasal vaccination strategy described in this investigation.

Another important aspect of the intranasal route of TVAC delivery that included delivering multiple doses of the vaccine formulation containing the α-GalCer and CpG-ODN adjuvants to inducing immunity at the FRT is the ability to circumvent NKT cell anergy, which results from multiple doses of α-GalCer if delivered by systemic immunization [151, 152]. Even though this investigation did not specifically determine repeated activation of iNKT cells after each vaccination, previous reports from our lab have showed the increase of antigen specific T cell immunity resulting from multiple mucosal doses of α-GalCer as a result of repeated NKT cell stimulation [105, 107]. It would be useful to also investigate the effect of administering multiple doses of α-GalCer and CpG-ODN by the intranasal route on different NKT cell subsets. In this regard it is known that type 2 NKT cells are predominant among the different NKT cells subsets in
humans and may be an essential cell population for overcoming immune suppression in tumors [132, 133]. Interestingly, recent literature reports suggest that targeting these type 2 NKT population through the use of CpG-ODN can shift their immune suppression phenotype to an inflammatory anti-tumor response [153, 154].

Although NK depletion studies using the anti-PK136 antibody did not significantly decrease the efficacy of TVAC in the vaginal tumor model (Fig. 10), we cannot conclude that NK1.1 expressing cells (including NKT cells) are unnecessary for TVAC efficacy. In fact, if NKT cells, in particular, did not contribute to TVAC efficacy, we would expect TVC to induce similar anti-tumor results as TVAC since α-GalCer specifically induces anti-tumor immunity through NKT cell engagement and activation. One explanation as to why NK1.1 depletion antibody resulted in minimally reduced TVAC efficacy is due to the NK1.1 negative NKT cell subsets. Studies show that circulating and thymic immature NKT cells do not express NK1.1, and NKT cells activated in vitro, also downregulate NK1.1 [155]. Therefore, it is possible that immature NKT cells contributed to the TVAC efficacy in mice treated with PK136 antibody.

Several groups are currently developing models to further study NK and NKT cells separately, and understandably it is a difficult task due to the many shared phenotypic markers and functions. One group described the development of an NKT specific monoclonal antibody that could be utilized in future studies for further investigation of NKT cells vs NK cells in TVAC efficacy [156].

While the majority of results from this investigation established the advantages for inducing protective immunity of a 2-dose regimen of TVAC delivered by the mucosal
intranasal route, one dose of TVAC delivered via the subcutaneous systemic (SC) route also exhibited anti-tumor efficacy against genital HPV tumors (Fig. 17). However, one caveat to concluding equal potency of systemic vaccination to induce protection against the vaginal mucosal tumors is that the SC vaccination was administered close to the hind limbs, and therefore, it is likely that a combination of the lymphatic drainage pattern and nearby inflammatory tumor environment aided in directing the antigen-specific immune responses to the FRT [157]. It is therefore, important to test the efficacy of TVAC delivered at a different SC location such as the back of the neck and analyzing the draining lymph nodes as well as vaginal tumor infiltrating lymphocytes.

Overall, results from this investigation demonstrated the efficacy TVAC against established vaginal tumors and smaller size flank tumors via induction of strong total and antigen-specific cytotoxic effector CD8 T cells. Both the vaccine and the immunization regimen were however less optimal for treating much advanced bigger size tumors where additional strategies to overcome immune suppression may have to be explored. Given this, it is tempting to speculate that mucosal intranasal delivery of TVAC could be an option to treat pre-cancerous cervical intraepithelial lesions (CIN) that are detected during a pap smear testing procedure. However, standard practice after a CIN (stage I) diagnosis is to wait and determine whether the infection and pre-cancerous growth resolves itself within a few months. If the growth is not naturally resolved, it progresses to CIN II/III stages and those patients undergo surgery for the removal of the affected area, which pose quality of life concerns. In this setting it could be advantageous to implement therapeutic vaccination such as the TVAC developed in this investigation as
an early treatment option. Unfortunately, there are no currently available preclinical models for pre-cancerous lesions. In this regard, a recent study described the potential of a new, infection-based HPV tumor model for HPV head and neck squamous cell carcinomas which could be promising for use as a vaginal tumor model as well [158]. HPV infections naturally take several years to develop, thereby allowing the tumor microenvironment to initiate immune-suppression mechanisms, otherwise not established in orthotopic models such as the one utilized here. Additionally, some critics argue that inflammation is induced during the tumor implantation process, aiding whatever therapeutic treatment administered, however, an infection-based model such as the one described above could circumvent these concerns. In future studies, intranasal administration of TVAC in primates would be necessary to ensure proper and robust Th1 immunity at the FRT prior to promoting this therapeutic approach in clinics. It would also be interesting to compare with the therapeutic HPV synthetic long peptide (SLP) vaccine, which has shown some level of efficacy against high-grade vulvar lesions but was ineffective against invasive cervical cancer in clinical trials[159-161].

CHAPTER 6: REFERENCES


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