Mucosal Immunization for Cancer: Opportunities and Challenges

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of Master of Science in the Department of
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ABSTRACT

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Abstract

Cancer continues to be a large health and economic burden, despite advances in diagnostics and therapy. Cancer immunotherapy research and development of novel cancer vaccine strategies continues to grow, and new immunotherapy options offer considerable promise for patients. Research has identified more than four hundred tumor-associated antigens, yet only one cancer vaccine is FDA approved and on the market for established cancer. Since mucosal tissues are often the site of cancer development and metastasis, vaccine systems that induce tumor-specific mucosal immune responses are worthy of investigation. Mucosal immunization has the ability to induce tumor-specific immune responses in non-mucosal (systemic) sites while also inducing mucosal immune responses that are characterized by effector cells that home to and reside in mucosal tissues. The purpose of this review is to discuss recent preclinical advances with the use of mucosal immunization for the induction of protective anti-cancer immunity and discuss critical factors related to mucosal immunization, such as the route of immunization and adjuvants. Additionally, we will discuss challenges associated with translating effective mucosal vaccines for tumors from preclinical to clinical use. Finally, we will discuss the importance of preclinical and clinical studies to determine if mucosal immunization is critical for therapeutic benefit against tumors that arise at or metastasize to mucosal tissues.

Dedication

To London.

Contents

1. Introduction

Even with advances in diagnostics and treatment, cancer remains a major problem, with few options to prevent malignant progression. Tumors arising from mucosal tissues make up more than half of new cancer diagnoses and a large percentage of cancer deaths. In 2014, there was an estimated 224,210 new cases of lung cancer, accounting for 13.5% of all new cancer cases. Additionally, in 2014, breast cancer and colon/rectal cancer cases accounted for 14% and 8.2%, respectively, of all new cancer diagnoses. These cancers arise from mucosal tissues, and some patients have disease recurrences and progression even with standard of care therapy [1]. There is a clear need for advanced treatment options that may slow progression and prevent recurrence of these mucosal cancers.

The basis of a cancer vaccine is to use a tumor antigen to train the immune system to target and kill cancer cells [2]. One of the earliest reports of an association between immune stimulation and decreased cancer growth is Coley, 1891, who reported decreased carcinoma growth in women actively infected with erysipelas [3]. Since then, many tumor associated antigens have been discovered, and many tumor vaccines have been studied. Some antigens arise from overexpressed normal human genes, mutated genes, and genes inserted by infectious agents. Some antigens that arise from carcinogens, like tobacco and ultraviolet light, contain up to thousands of

nonsynonymous mutations [4]. Within these carcinogenic genes, there are likely antigens and antigenic peptide epitopes that could be recognized by the human immune system [5, 6]. There have been a total 403 reported tumor antigenic peptides identified in 19 tumor types with more being discovered at a steady pace [5]. In breast cancer, for example, human epidermal growth factor receptor (HER-2) is overexpressed in 20-30% of breast cancers [7]. Caspase 5 (CASP-5) is mutated in colorectal, gastric and endometrial carcinomas [8]. A specific type of Human Papillomavirus (HPV) causes cervical cancer by integrating HPV DNA into the patient genome [9]. Despite the identification of human tumor antigens that can potentially be targets for T cells , one questions why there is only one FDA-approved cancer vaccine for people. The one FDA-approved cancer vaccine is sipuleucel-T (Provenge®) which is an antigen-presenting cell vaccine for castrate-resistant prostate cancer. With Provenge, a patient's white blood cells are harvested and cultured with a recombinant protein consisting of human prostatic acid phosphatase (PAP), an antigen and biomarker of prostate carcinoma, fused with granulocyte-macrophage colony stimulating factor (GM-CSF), a growth stimulating cytokine [10]. These "loaded" antigen-presenting cells are then put back in the host [11]. Patients receiving the vaccine had a 22% reduction in risk of death compared to placebo [12]. There are currently about 60 open studies on clinicaltrials.gov testing cancer vaccines,

so clearly effort is being made to take advantage of the large database of tumor associated antigens (searched "cancer vaccine" April 30, 2015).

A review article was recently published comprehensively describing cancer vaccine approaches that have progressed to clinical studies [13]. Since many cancers first appear or metastasize to mucosal tissues, vaccination methods able to induce tumor-specific immune responses that specifically target mucosal tissues may provide enhanced treatment of cancers in mucosal tissues. This review will focus on preclinical studies that evaluate the use of mucosal immunization to induce cancer-specific immune responses and discuss opportunities and challenges associated with the use of mucosal immunization for cancer.

2. The Mucosal Immune System

The mucosal immune system is the compartment of the immune system that protects the mucosal surfaces of the body [14]. It is composed of lymphoid tissues connected with specific mucosal surfaces, such as the gut, urogenital tract, oropharynx and bronchi. Since many cancers develop at or metastasize to mucosal tissues of the host, activation of tumor-specific immune responses within the mucosal immune system may be one strategy to enhance vaccine-induced protection against tumors as compared to protection provided by vaccines delivery by injection (i.e. intravenous, subcutaneous, intramuscular, intradermal). To take full advantage of the mucosal immune system for cancer vaccines, a brief introduction of the mucosal immune system is required.

2.1 Inductive Tissues of the Mucosal Immune System

The inductive sites of the mucosal immune system represent the organized lymphoid tissue where naive lymphocytes encounter presented foreign antigens and antigen-specific immune responses are initiated [15]. In the gut, and nasal mucosa, there is a network of arranged lymphoid tissues termed gut-, nasopharynx-associated lymphoid tissues, termed GALT, and NALT [16, 17]. In these mucosal locations, these mucosa-associated lymphoid tissues (MALTs) are inductive sites for the mucosal immune system [18]. For example, Peyer's Patches are inductive sites of the mucosal immune system in the small intestines [19, 20]. Once an antigen is transported across the epithelium

at inductive sites, the antigen can processed and presented by dendritic cells to naïve T cells that support the induction and expansion of antigen-specific B and T cell responses[16, 18]. In some mucosal tissues, such as the genital tract, there are no local aggregates of lymphoid tissue; thus loaded antigen presenting cells must traffic to draining lymph nodes to induce antigen-specific immune responses [21].

2.2 Effector Tissues of the Mucosal Immune System

Following induction of antigen-specific immune cells in the mucosal immune system, antigen-specific B cells, $CD4^+$ and $CD8^+$ T cells circulate to the mucosal immune system effector sites, which include the lamina propria layers of the gut, upper respiratory tract, urogenital tract and secretory glands such as the mammary, salivary and lacrimal glands, to execute antigen-specific effector function[22]. A hallmark of the mucosal immune system is production of IgA in mucosal sites due to the induction of IgA-producing plasma cells which develop and expand with support from CD4+ Th1 and CD4+ Th2 cells [16]. Mucosal epithelial cells produce polymeric Ig receptor that binds polymeric IgA and IgM and transports polymeric immunoglobulins through the cell for release into secretions at the mucosal surface[23]. Monomeric IgA may be important in anti-tumor immune responses due to its ability to recruit phagocytic cells that target tumor cells [24]. Beyond the induction of secretory IgA and $CD4^+$ T cell responses, the

mucosal immune system is also a potent inducer of $CD8^+$ cytotoxic T lymphocytes after mucosal immunization or mucosal infection, with influenza for example [25, 26].

2.3 Homing of Antigen-Specific Effector Cells to Mucosal Tissues

The mucosal immune system utilizes tissue-specific homing modifications that enhance migration of antigen-specific effectors cells to mucosal tissues 27. Once naïve T and B cells expand into antigen-specific lymphocytes, they develop surface homing receptors that control migration of antigen-specific lymphocytes to specific sites28. For example, lymphocytes primed to gut antigens express the integrin $\alpha 4\beta 7$ and binds to the mucosal vascular addressin MAdCAM-1 thus luring these cells back to the lamina propria of the colon and small intestines, primed and ready for action1, 29-31. Dendritic cells in mucosal tissues provide the signals that imprint the effector cells to develop mucosal homing properties31. These mucosal homing cells have been shown to be important in protecting against gastrointestinal infections in mice. For example, CD8+ T cells that express $\alpha 4\beta 7$ are effective at clearing rotavirus infection, while CD8+ T cells that do not express $\alpha 4\beta 7$ are not 32, 33. Homing to mammary glands also requires interaction between $\alpha 4\beta 7$ and MadCAM-134. Mucosal immunization strategies able to induce tumor-specific effector cells expressing $\alpha 4\beta 7$ may provide a mechanism to

enhance immune-mediated clearance of tumors within tissues of the gastrointestinal tract and the breast.

Antigen-specific effector cells also migrate to mucosal tissues other than the gastrointestinal tract or the breast. In the lung, the chemokine receptor CCR4 contributes to the ability of effector and memory T cells to migrate to the lung. These lung-migrating T cells are more effective at protecting against an influenza challenge than gut and skin dendritic cell-activated T cells35. Similar to the imprinting of gut-homing properties on T cells by the Peyer's Patch dendritic cells31, imprinting of lung-homing properties is also controlled by dendritic cells located within the lung35. In the urogenital tract and salivary glands, homing of effector cells requires $\alpha 4\beta 1$ interaction with VCAM-1 21, 34. Since dendritic cells from specific mucosal tissues imprint the mucosal-homing capacity in antigen-specific effector cells31, 35, immunization by systemic routes is not likely to induce antigen-specific effector cells with mucosal-homing properties. Development of mucosal immunization strategies for cancers in mucosal tissues may enhance the induction of tumor-specific effector cells able to preferentially migrate to the tumor and improve the immunogenicity and efficacy of cancer vaccines when compared to cancer vaccines delivered by non-mucosal routes.

3. Mucosal Cancer Vaccines: Opportunities and Challenges

Most cancer vaccine approaches are predominantly for the induction of cytotoxic T cells[13]. Cytotoxic T cells have been shown to be active in the killing of tumor cells due to their high specificity and effectiveness [36, 37]. Thus the goal of cancer vaccines is to induce tumor specific CD8+ T cells [13]. These CD8+ T cells are supported by CD4+ T cells, specifically during the priming and maintenance phase of CD8+ T cells [38] and during the effector phase of an anti-tumor response [39]. Mice depleted of CD4+ T cells were unable to reject a tumor challenge [40]. CD4+ T cells' anti-tumor effect has been associated with interferon-γ, which has cytotoxic activity on tumor cells, up-regulation of MHC expression and induction of inhibitors of angiogenesis by tumor cells [41]. An effective cancer vaccine will induce potent protective immune effector responses (i.e. anti-tumor CD8, etc.), but the use of mucosal immunization could induce anti-tumor CD8+ and CD4+ T cell responses with mucosal homing properties. This unique ability of mucosal immunization and may confer superior protection against tumors at mucosal tissues not induced with current systemic cancer vaccines. Below we will discuss opportunities and challenges that face the development of mucosal vaccines for cancer.

3.1 Opportunities

As mentioned above, there is one FDA approved vaccine for established cancer: sipuleucel-T for castrate-resistant prostate cancer [42]. Vitespen was approved in April, 2008 for patients with metastatic melanoma [43, 44]. With the exception of sipuleucel-T and vitespen, there has been a lack of success with the development of vaccines that provide effective induction of anti-tumor immunity and resolution of tumors. The lack of efficacy of cancer vaccines in human studies may be due to multiple factors including low immunogenicity of tumor-associated antigens due to immune tolerance to self-antigens [45], secretion of immunoinhibitory cytokines and regulation of host immune response due to immune checkpoint pathways as discussed in a recent review on cancer vaccines[13]. Current clinical trials testing cancer vaccines are utilizing peptides and adjuvant, dendritic cells, and whole tumor cells (NCT00722228, NCT01863108, NCT01702792, NCT01532960) and all are administered via a systemic route. While a tremendous amount of effort has been put into the development of efficacious cancer vaccines for many years [13], the consistent lack of success suggests that something is missing. For cancers in mucosal tissues this missing piece may be a requirement for mucosal immunization to maximize the induction of antigen-specific effector cells capable of homing back to the tumor cells within the mucosal tissues.

3.1.1 Preclinical Cancer Vaccine Mouse Models of Cancer

Mouse models of experimental cancer vaccines have demonstrated that mucosal immunization is an efficacious route to administer tumor vaccines to induce responses against cancers of mucosal and non-mucosal tumors [46-50]. There are benefits and limitations to the use of mouse cancer models as surrogates to human cancers, but the principles of mucosal immunization and immunity evaluated in mouse cancer vaccine models re valuable to human cancer vaccine development. Mouse models of cancer represent an opportunity to study a cancer vaccines ability to induce an immune response, as well as a protective response. Mouse models vary from implantation of tumor cell lines expressing model antigens, like ovalbumin, tumor cell lines expressing proteins from oncogenic viruses (i.e. human papilloma virus) to transgenic mouse mice overexpressing a natural tumor antigen that spontaneously generate cancer. The antigen used in mouse cancer vaccine models dramatically impacts the immunogenicity and efficacy of the cancer vaccine.

3.1.2 Foreign Tumor Antigens

Tumor vaccine studies in mice often utilize a tumor challenge consisting of injection of a mouse tumor cell line into the host. If the vaccine-induced immune responses are able to kill the tumor cells, the tumor cell line will not grow or will be cleared resulting in long-term survival of the mouse. If the vaccine-induced immune

responses are unable to provide immunological control of the tumor growth, the tumor cells will proliferate leading to tumor masses and humane euthanasia. Many tumor cell lines used in mouse tumor challenge models have been engineered to express a foreign antigen that is used as the tumor antigen in the candidate cancer vaccine. One of the most commonly used tumor model systems in cancer vaccine studies is the ovalbumin (OVA)-expressing cancer line. Mouse melanoma, B16, and mouse lymphoma, E.G7, cell lines have been developed to express OVA, and thus OVA has been used as the surrogate tumor antigen to test the efficacy of cancer vaccine strategies [46, 47, 49, 50]. In another model of foreign cancer antigens, TC-1 mouse cell lines expressing human papillomavirus 16 E6 and E7 proteins have been used as the tumor challenge[48, 51]. Since OVA and HPV 16 E6-E7 are antigens foreign to mice, antigen-specific immune responses to these antigens are readily induced by effective vaccine strategies.

3.1.3 Self Tumor Antigens

A second class of mouse models for cancer vaccine studies include transgenic mice that naturally develop cancers due to overexpression of a tumor antigen. This is an important distinction from the tumor models that utilize injection of tumor cells expressing a foreign antigen since the tumor antigen used in the transgenic tumor model animals is a "self" antigen and the host immune system has been regulated by tolerance mechanisms to not mount an immune response against self-antigens. Use of these

transgenic mouse tumor models in cancer vaccine studies will require the cancer vaccine to not only break tolerance to the tumor antigen, but also induce a response that kills the tumor cells while not inducing robust immune-mediated damage to normal non-cancerous tissue. One example of a mouse cancer model that expresses natural tumor antigens are the transgenic *HER-2/neu* mouse models that express an activated rat *c-neu* on the FVB/n or BALB/c background [5253]. There is another similar mouse model of HER-2/neu breast cancer in which FVB/n mice have been engineered to express an unactivated rat *c-neu* [54]. Though these animals were engineered to express rat *c-neu*, these mice were born with this antigen and are therefore tolerized to the antigen, thus a vaccine that uses HER-2/neu epitopes as the antigen will need to break tolerance so that a protective immune response can be induced. Beyond breast cancer, there are transgenic ovarian cancer models, expressing Mullerian inhibiting substance type II receptor (MISIIR) and benign colorectal cancer models, expressing a mutant gene named multiple intestinal neoplasia (*Min*), which would fit into this category of natural tumor antigens [55, 56]. The use of transgenic mice that express ovalbumin as a self-antigen provide a unique model to take advantage of the extensive immunological reagents for OVA to evaluate the ability of cancer vaccines to break immunological tolerance against a self-antigen [57, 58] .

3.1.4 Mucosal Immunization against Mucosal Tumors

The ability of mucosally-administered vaccines to induce antigen-specific lymphocytes that actively migrate to mucosal tissues supports the rationale of testing mucosally-delivered cancer vaccines for their ability to kill tumors at mucosal sties. Several reports have discussed the ability of mucosally-administered vaccines to induce clearance of tumors at mucosal sites. Using an orthotopic mouse model of TC-1 epithelial cells expressing HPV E6 and E7 implanted at the base of the tongue, Sandoval et al. reported that both intranasal and intramuscular immunization with E7 peptide plus Shiga Toxin B subunit adjuvant, induced epitope-specific immune responses in the spleen but only intranasal immunization induced epitope-specific immune cells in the cervical and mediastinal lymph nodes, and only intranasal immunization inhibited tumor growth [48]. In a metastasis study, intranasal immunization but not intramuscular was the only immunization route to inhibit lung tumor growth despite both routes of immunization inducing comparable levels of epitope-specific T cells in the spleen [48]. When TC-1 cells also expressing HPV E6-E7 were implanted orthotopically into the vagina, both intranasal and subcutaneous immunization with E7 peptide with Resiquimod (R-848), heat labile toxin, and CpG coadministered as adjuvants conferred long term protection, although only subcutaneous immunization induced complete regression of an established tumor [51]. In another TC-1 expressing HPV E6-E7 model,

intravaginal and subcutaneous immunization with E7 peptide and heat labile toxin and CpG were effective at inducing antigen-specific CD8+ T cells in the bladder, and reducing othotopic bladder tumor growth, while, intranasal immunization with the same vaccine formulation was not [59]. In a different cancer model, gastric immunization with an attenuated *Salmonella typhimurium* containing a plasmid coding for murine prostate stem cell antigen was shown to induce prostate tumor protective immunity in mice [60]. In an intravenous B16-OVA tumor lung metastasis challenge, sublingual immunization with OVA peptide plus αGalCer as the adjuvant was effective at reducing tumor foci in the lung [49]. In a transgenic mouse model of spontaneous intestinal adenomas that progress to carcinomas, intrarectal immunization with a vaccinia virus expressing human carcinoembryonic antigen was effective at reducing adenoma incidence and preventing progression to carcinoma; intrarectal immunization was also effective at inducing systemic antigen-specific IgG titers, CD4+ and CD8+ T cells as well as mucosal IgA, and CD8+ T cells [61]. Some papers only evaluated a mucosal route of immunization and did not directly compare results to a parenteral route of immunization. What can be said is that there is a clear benefit of mucosal immunization against tumor challenges at mucosal sites.

3.1.5 Mucosal Immunization against Subcutaneous Tumors

Mucosal immunization with cancer vaccines may also provide an alternative method of immunization to treat cancers that develop in systemic tissues. For example, nasal immunization with the OVA epitope SIINFEKL combined with cholera toxin (CT) as the mucosal adjuvant induced epitope-specific CTL responses that were as effective as intravenous immunization with peptide-pulsed dendritic cells for the control of E.G7-OVA growth in a prophylactic subcutaneous tumor challenge model [47]. Nasal immunization with SIINFEKL and mutant CT also induced epitope-specific CTL responses in the spleen [62]. Of particular interest was the observation that intranasal subcutaneous immunization with the identical antigen and adjuvant used for intranasal immunized failed to induce OVA-specific CTL responses.[47] This observation is similar to the report that subcutaneous immunization with SIINFEKL plus incomplete freund's adjuvant (IFA) induced transient CD8+ effector cells that failed to expand and were incapable of killing target cells [63]. Nasal immunization with a mixture of HPV E6 and E7 peptides and mutant cholera toxin as adjuvant also induced peptide-specific CD4 and CTL response in the spleen and protection against a subcutaneous TC-1 tumor challenge[64]. Further supporting this observation is another publication reporting intranasal immunization with E6 and E7 peptides adjuvanted with a bacterial flagellin (FlaB) induced strong antigen-specific CD8+ T cells in the spleen and cervical lymph

nodes, and strong protective immunity in mice [65]. Intrarectal vaccination with recombinant vaccinia virus expressing human carcinoembryonic antigen or recombinant vaccinia virus expressing LacZ reduced subcutaneous tumors with a colorectal cancer line [61]. Collectively, these results suggest that nasal immunization with CTL epitope peptides and adjuvant effectively induces epitope-specific CTL able to protect against systemic tumor growth. Additionally, the intranasal immunization with peptide antigens may provide an alternate method of immunization that is superior to subcutaneous immunization for the induction of protective, anti-tumor immunity.

3.2 Challenges

3.2.1 Mucosal Adjuvants

Many of the preclinical studies reporting the ability of mucosal immunization with peptide antigens to induce epitope-specific $CD4^+$ and/or $CD8^+$ responses used toxin based adjuvants such as cholera toxin [47], their derivatives[64] or shiga toxin B [48] Despite the potent adjuvant activity of these toxins, their use is associated with numerous adverse effects in both preclinical and clinical studies. In preclinical studies, intranasal administration of toxin adjuvants can facilitate trafficking of the vaccine antigen and accumulation of the antigen in the olfactory bulb and the central nervous system [69-71]. Cholera toxin used as a nasal vaccine adjuvant is also associated with the induction of excessive inflammation and IgE responses [72]. In clinical studies, intranasal

immunization using E. coli heat labile toxins as vaccine adjuvants was associated with the development of Bell's palsy, or facial nerve paralysis [73, 74]. While the potential toxicity of toxin-based adjuvants used with preclinical mucosal tumor vaccine studies would seem to limit the translatability of mucosal immunization against tumors, there are a variety of non-toxin adjuvants that have been tested in human nasal vaccine studies that may be useful in the development of mucosally-administered cancer vaccines for clinical use. Monophosphoryl lipid A (MPL) has been used as an adjuvant with an intranasal Norwalk virus vaccine and its use enhanced the induction of IgA secreting cells that expressed homing surface molecules (α4β7), in addition to being a safe adjuvant [75]. Endocine, a lipid-based nasal adjuvant, has been used safely as a human nasal antigen, and was shown to induce dose-dependent T cell responses, as well as mucosal and systemic immune responses [76]. Nanoemulsions have been shown to be an effective nasal technology for cancer as discussed above [68], and there is data suggesting their use in humans is safe and well tolerated, and potent at inducing systemic and mucosal immunity following mucosal immunization [77, 78]. Among TLR adjuvants, TLR3 agonists have been shown to be safe and effective mucosal adjuvants in humans. In influenza, rintatalimod, and polyI:polyC(12)U are effective adjuvants at inducing systemic and mucosal anti-influenza immune responses [79, Overton, 2014 #102, 80]. The latter, polyI:polyC(12)U also called Ampligen has been suggested as a potential adjuvant for cancer

immunotherapy, due to its immunostimulatory behavior which may be beneficial in overcoming tolerance to tumor self-antigens [81]. In vaccine safety studies, proteasome adjuvants have been used nasally and are well tolerated by humans, and promotes a vaccine-induced immune response [82]. Use of other nasal adjuvants has been reported to be effective, as well as safe in humans. Thus the potential for mucosal immunization as a route of immunization for cancer vaccines is not limited by adjuvant selection. The adjuvants described above are safe and effective in humans for the induction of infectious agent-specific responses. More importantly, these adjuvants allow for induction of mucosal responses with homing, which may be beneficial in the context of cancer vaccination.

3.2.2 Mucosal Antigens

Finding an adjuvant that is immunogenic, and non-toxic is important, as is the antigen and dose of antigen. A large number of cancer vaccine studies in mice have utilized peptide antigens delivered with an adjuvant. For mucosal immunization, published reports have used a wide variety of antigen doses. In two papers that report on the benefit of mucosal immunization against E.G7-OVA, different types and doses of antigen induced similarly protective responses. Porgador et al. used the CTL epitope of OVA, SIINFEKL, nasally and Wakabayashi et al. used the entire OVA protein orally [47, 50]. Other papers reporting benefits of mucosal immunization against cancer use peptide

antigen doses that range from 60 μg to 10 mg nasally and orally, and up to 100 mg subcutaneously [46, 47, 50, 51, 67]. Other antigen formulations delivered mucosally are effective at inducing anti-tumor responses. Dendritic cells (also referred to as nature's adjuvants) transfected with mRNA are effective at inducing cancer-specific immune responses in cancer patients [83-86]. In mice, intranasal immunization with OVA mRNA nanoparticles induces antigen-specific CD8+ cells in the spleen, and reduces E.G7-OVA subcutaneous tumor growth [87]. There are antigen-only vaccine systems that have been used mucosally and have demonstrated efficacy, including the recombinant adeno-associated virus expressing major capsid protein L1 from HPV 16. Intransasal immunization with this rAAV induced high titers of L1-specific vaginal mucosal, and systemic L1-specific antibody titers; antibody titers were detected for up to 60 weeks after immunization [88]. Beyond just antigen selection, the question of tolerance must be addressed. In a mouse study aiming to induce anti-self-antibody responses, three essential requirements to break tolerance were identified: (1) a potent pattern recognition receptor, (2) an adjuvant that keeps the PRR at the site of injection and (3) presence of foreign sequences in the vaccine protein.[89] While a challenge to human mucosal cancer vaccine formulation will be the antigen selection, there are options beyond the basic peptide plus adjuvant system. The goal should be to induce the best response, and the results of these papers show that responses can be induced with low amounts of antigen (60 μg, [47]). Finding an

optimal and effective mucosal vaccine formulation is important because when comparing routes, our lab has shown that mucosal immunization requires up to three-times the dose of systemic immunization (unpublished data). Even in cancer vaccines, there is a clear requirement for more mucosal immunization administrations to induce comparable CTL activity to subcutaneous immunization [46].

3.2.3 Mucosal Delivery of Cancer Vaccines in Humans

Finally, another factor that must be considered when developing a mucosal vaccine for cancer is the route of mucosal immunization. As mentioned, the mucosal immune system has adapted to induce effector and memory cells that home to and reside in mucosal tissues. This is an important benefit to consider when trying to drive a response that targets a cancer at a mucosal site. Anatomically, a sublingual or nasal vaccine should induce effector cells that home to and target the oral and nasal mucosa. A sublingual or nasal vaccine may also induce a response that localizes to the upper and lower respiratory tracts, possibly providing additional benefit against a lung tumor [49]. A gastric or rectal vaccine may be beneficial at inducing effector cells that home to and reside in the gastrointestinal tract and target gastric, or colorectal cancers. Finally, vaginal immunization may prove to be useful at targeting urogenital tumors, such as cervical cancer. There are many reasons to suggest mucosal immunization as an effective

route for cancer vaccine delivery, but there are many factors to consider when developing a mucosally-delivered cancer vaccine.

Based on the work reviewed here, there are scientific opportunities to use mucosal immunization to increase the immunogenicity and protection induced by a cancer vaccine in humans. There are, also, inherent challenges to mucosal immunization in humans. Aside from antigen type and dose, adjuvant type and dose, more work needs to be done to better understand mucosal immunization routes in humans. A current example of widely used mucosal immunization in humans is FluMist®, the live attenuated influenza vaccine. This vaccine uses 0.2 mL of total vaccine liquid, with about half sprayed into each nostril (FluMist ® package insert). This nasal spray induces serum antibodies, mucosal antibodies and influenza-specific T cells (FluMist® package insert). This product shows that it is not impossible to have a marketed nasal vaccine for humans, but to utilize this route in humans for cancers, considerations must be made about route. There is some clinical data suggesting that nasal drops, and intranasal mist were very effective at inducing systemic and mucosal immune responses compared to oral spray which was not effective at inducing a mucosal response [90]. Further, nasal spray has shown to be more consistent and reproducible delivery method compared to nasal drops [91]. Pulmonary immunization is another possible route of mucosal immunization. Pulmonary immunization with a dry measles vaccine in macaques was

not effective at inducing neutralizing antibodies compared to responses induced by an injected vaccine or a nebulized aerosol [92]. Contrasting this is work reporting an intratracheal immunization with a diphtheria toxoid powder plus chitosan in guinea pigs induces greater systemic neutralizing antibodies compared to a subcutaneous vaccine adjuvanted with alum [93].

To induce gastrointestinal mucosal responses, studies have shown efficacy of oral and intestinal immunization. Human patients given oral inactivated cholera vaccine induced specific IgA responses in the human gastric mucosa of Helicobacter pylori infected patients, compared to uninfected patients [94]. Small intestinal immunization has been shown to induce vaccine specific gastric IgA secreting cells, and duodenal antibody secreting cells [95]. Gastric immunization resulted in gastric and duodenal vaccine specific B cells.

With regards to other mucosal immunization routes used in humans, there is data suggesting there may be a benefit that can be exploited for cancer vaccines. In a comparison of oral, rectal and vaginal immunization for the induction of cholera-specific immune responses, all three routes were effective at inducing serum IgG and salivary IgA. Rectal immunization was most effective at inducing responses in rectal secretions, but not female genital tract secretions, but vaginal immunization induced antigen-specific responses in the cervix and vagina [96]. This suggests that there is a clear site

specific immune response dependent on the site of immunization. In a paper that compared oral and rectal immunization with Salmonella Typhi Ty21a, both routes were effective at inducing effector cells that expressed $\alpha 4\beta 7$ [97]. Oral immunization induced superior salivary and vaginal responses, while rectal induced superior nasal, rectal and lacrimal responses. No differences were found in serum and intestinal responses. While only one vaccine has been FDA approval for mucosal delivery, there is early clinical data suggesting mucosal immunization may be an effective method at inducing mucosal site specific immune responses to target mucosal tumors.

Finally, work needs to be done to have standardized mucosal route delivery systems. Non-human primates provide an opportunity to test a nasal delivery system and measure resulting immune responses. While work has been done to better understand the distribution of nasally instilled liquid in mice [98], there are no standardized methods for nasal delivery in experimental animals. Using macaques as a model for the human nasal cavity, some researchers have used nasal immunization in macaques, and administered 315 μL of liquid [99]. This volume, compared to the 100 μL of FluMist ® liquid placed in each nostril of humans shows a wide range in volumes of nasal vaccines. This variation in volume is just one reason why considerable work would need to be done to normalize and optimize nasal delivery. A nasal vaccine's

potency and immunogenicity cannot be assessed unless the nasal delivery methods and routes are optimized.

4. Conclusions

There is a clear scientific justification that mucosal immunization may be the missing link to cancer vaccine development. The induction of mucosal responses that home to and reside in mucosal tissues has the potential to be very effective at targeting primary mucosal tumors, as well as mucosal metastases. To translate the preclinical work that provides this scientific justification, there are obstacles that must be addressed. Antigen dose, and type is the first part of an effective vaccine formulation. The tumor-associated antigen database continues to evolve and expand, and this provides a great opportunity to begin to developing a cancer vaccine. The adjuvant is the next component that presents challenges. With toxin based adjuvants being classical and effective mucosal adjuvants, considerable work needs to be done to find equally effective, but less toxic adjuvants for mucosal delivery in humans. The delivery route, and volume must then be evaluated. There is human data confirming the route of immunization drives mucosal responses in different mucosal sites. Taken together, the field of mucosal immunity is providing evidence to suggest the cancer vaccine field should consider mucosal routes of immunization to improve the vaccine immunogenicity, and thus cancer patient outcomes and protection.

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