An injectable, spontaneously assembling biomaterial-based cancer vaccine platform

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An injectable, spontaneously assembling biomaterial-based cancer vaccine platform

A dissertation presented

by

Aileen Weiwei Li

to

The School of Engineering and Applied Sciences

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An injectable, spontaneously assembling biomaterial-based cancer vaccine platform

Abstract

Cancer immunotherapy has the potential to provide a cure for many patients. Therapeutic cancer vaccination can stimulate the immune system to eradicate tumor cells while sparing normal tissues, and establish long-term memory to prevent tumor recurrence. However, an effective cancer vaccine has remained elusive.

In this thesis, we developed an injectable biomaterial-based cancer vaccine. Mesoporous silica micro-rods (MSR or MPS) were shown to spontaneously assemble to form a 3D scaffold in vivo, and the surface of the MSRs can be chemically modified to modulate immune cell infiltration. When formulated with GM-CSF and CpG, the MSR vaccine modulated host dendritic cell (DC) activation and trafficking. A single injection of the MSR vaccine against a gonadotropin-releasing hormone (GnRH) decapeptide elicited highly potent anti-GnRH response that lasted for over 12 months and was more effective compared to traditional bolus strategies. Moreover, a MSR vaccine directed against a Her2/neu peptide within the Trastuzumab binding domain showed immunoreactivity to native Her2 protein on tumor cell surface. A facile strategy by modifying the MSRs with PEI was demonstrated to further enhance CTL responses.
against tumor antigens. Impressively, using a HPV-E7 expressing tumor model, we demonstrated that a single injection of the MSR-PEI vaccine completely eradicated large established tumors in over 80% of mice. Finally, when immunized with a pool of recently sequenced B16 melanoma neoantigen peptides, the MSR-PEI vaccine induced therapeutic tumor growth control and synergy with anti-CTLA4 therapy.

The potency, flexibility, and simplicity of the MSR-based vaccine platform may overcome many of the current barriers to unlocking the potential of cancer vaccines. The potency of this approach can effectively drive immune responses against libraries of cancer specific mutations and synergize with other immunotherapies. The flexibility and modularity of this strategy are expected to drastically simplify the construction of cancer vaccines. The simplicity of this approach is highly amenable to clinical translation. Overall, the MSR-based vaccine can serve as an effective and generalizable platform to modulate host immune cell functions for, among others, cancer, infectious diseases and reproductive biology.
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Aileen W. Li

July, 2017
To my parents
Chapter 1: Introduction

1.1 Background and Motivation

Cancer immunotherapy

Cancer is characterized by an accumulation of genetic alternations that result in defects in circuits that govern cell proliferation, apoptosis and homeostasis. These alternations also result in mutations that differentiate them from their normal untransformed counterparts, which can be recognized and cleared by the immune system (“immune surveillance”). The role of the immune system in cancer was first probed in the early 20th century. The pioneering work by the New York surgeon William Coley described that, in some cases, bacterial toxins could stimulate immune-mediated tumor clearance. However, the role of the immune system did not become a hallmark of cancer until the recent demonstration that tumor specific targets (tumor antigens) exist and can be recognized by the immune system. We now appreciate that the immune system participates in controlling tumor growth, and tumor cells can develop sophisticated mechanisms to escape immune surveillance.

The overall goal of cancer immunotherapy is to effectively find and eradicate tumor cells while sparing normal tissues, and generate immunological memory to prevent tumor recurrence. An effective anti-cancer immune response requires several steps that need to be initiated, amplified, propagated and expanded iteratively (Fig1.1). Chen and Mellman coined these steps as “the cancer immunity cycle”. First, tumor
antigens must be released and efficiently captured by antigen presenting cells (APCs) (step 1). These antigens must be processed and presented by APCs in the context of MHC-I and MHC-II molecules (step 2). Subsequently, APCs need to migrate to secondary lymphoid organs to prime naïve T and B cells (step 3). In order to generate anti-cancer specific T and B cells and lead to immune-stimulation rather than tolerance, APCs must be accompanied by “activation signals” such as co-stimulatory molecules and type 1 cytokines. Upon stimulation, naïve CD4+ and CD8+ T cells differentiate and expand into tumor-specific T cells with specific effector functions (step 4). The potency of the immune response is determined by the specificity of the recognition, the number and the magnitude of response of the effector T cells and the balance between effector T cells and regulatory T cells. Simultaneously, B cells differentiate into anti-tumor specific antibody secreting cells, which can produce antibodies that mark tumor cells for destruction by other innate cells such as macrophages and NK cells. Next, the activated T cells traffic to the site of the tumor and migrate into the tumor tissue (step 5), where it can recognize tumor cells via T cell receptor (TCR) and peptide-MHC binding (step 6). Upon recognizing tumor cells, CD8 T cells release Granzyme B and kill the target tumor cell; CD4 T cell secretes a variety of anti-tumor cytokines that mediate killing and modulate the tumor tissue (step 7). As the tumor cells are killed, its cellular contents are released and picked up by additional APCs, further increasing the breadth of the immune response (step 1).
Current approaches to cancer immunotherapy

A number of approaches currently exist to affect the cancer immunity cycle. Attempts to prime tumor specific T cells largely involve the use of therapeutic vaccines as vaccines have shown efficacy against many viral and bacterial diseases (cancer...
vaccines are reviewed in detail in the next section). Therapeutic cancer vaccines could, in theory, stimulate the immune system to eradicate tumor cells (by priming APCs with tumor specific antigens) while sparing normal tissues, and establish long-term memory to prevent tumor recurrence\textsuperscript{11}. A potent cancer vaccine has remained elusive\textsuperscript{10,12}. The primary challenges associated with therapeutic cancer vaccines are (1) the identification of highly specific tumor specific antigens can be presented on MHC molecules with high affinity\textsuperscript{1}, and (2) methods to induce optimal APC processing and maturation to prime effective T cells while suppressing regulatory T cell activities\textsuperscript{13}. While exogenous vaccines involve introducing a pre-selected pool of tumor antigen to APCs, endogenous vaccines can mobilize antigens directly from the tumor itself\textsuperscript{14}. Harnessing tumor antigens embedded directly in the tumor tissue bypasses the need for antigen identification, selection and delivery. Though the exact mechanisms are unclear, recent evidence showed that a number of anti-cancer drugs could elicit effector T cell response \textit{de novo} or reactivate pre-existing immunity\textsuperscript{15}. For example, conventional chemotherapeutic agents such as doxorubicin\textsuperscript{16}, cyclophosphamide\textsuperscript{17} and paclitaxel\textsuperscript{18} can enhance the antigenicity of malignant tumor cells and increase their visibility to immune surveillance. Tumor cells that succumb to these anti-cancer drugs can release cell death-associated molecules, which can serve as adjuvants to stimulate APCs in the tumor tissue\textsuperscript{15}, initiating the “immunity cycle”. Additionally, radiation therapies can increase the rate of somatic mutation in tumor cells, making them more visible for immune destruction\textsuperscript{19,20}.

Other than through vaccination, tumor specific effector T cells can be introduced \textit{in vivo} after \textit{ex vivo} expansion of existing tumor specific T cells or genetic
modification. Adoptive cell therapy (ACT) is a treatment that uses the patient’s own tumor specific T cells\textsuperscript{21}. These T cells can be harnessed from tumor-infiltrating lymphocytes (TILs), expanded \textit{ex vivo} with IL-2, and reinfused into the patient\textsuperscript{22,23}. ACT has shown tremendous promise, as 50\% of melanoma patients receiving this therapy showed objective regression\textsuperscript{21}. Since ACT is a highly personalized therapy as high avidity effector T cells can only be isolated from a subset of patients, there is a need to generate broadly reactive effector T cells that recognizes shared tumor antigens. One promising demonstration of genetically engineered T cells is the chimeric antigen receptor T cells (CAR-Ts). In CAR-Ts, the patient’s own T cells are transfected with a construct encoding an antibody recognizing a tumor antigen linked to the TCR signaling domains\textsuperscript{24}. CAR-T cells can directly recognize whole tumor surface antigens, bypassing the need for the tumor antigens to be expressed in the context of MHC molecules. Clinical evidences for CAR-Ts in hematological malignancies are extremely promising, as many types of blood cancers are essentially eliminated by CAR-T constructs\textsuperscript{25,26}. The next generations of CAR-Ts included additional co-stimulatory molecules and “safety breaks” to dampen off-target toxicity. Future generations of genetically modified T cells will likely build in more sophisticated sensing and responding mechanisms, include methods to eliminate off-target effects and enhance their \textit{in vivo} persistence and infiltration into solid tumors\textsuperscript{27,28}.

Pre-existing tumor-reactive T cells have been shown to exist in a number of cancers, such as melanoma. The activity of these T cells can be rescued or reactivated using drugs that dampen T cell exhaustion (T cell “checkpoint blockade” therapies)\textsuperscript{29}. CTLA-4 is a molecule recruited to the plasma membrane surface on activated T cells
and binds to a family of costimulatory molecules, CD80/CD86, expressed on activated DCs. Blocking this interaction could essentially take the break off activated T cells and prolong their effector function. Ipilimumab is a monoclonal antibody that binds to CTLA-4. In a seminal trial, it was demonstrated that Ipilimumab showed clear survival benefit in late-stage metastatic melanoma patients. Due to the clinical success of Ipilimumab, a major effort has been committed to evaluating other T-cell immune modulators. Agonist antibodies against activating molecules on T cells, such as CD28 and OX40, and blocking antibodies against negative co-stimulatory molecules, such as TIM-3 and VISTA, have shown preclinical success, and combinations of checkpoint inhibitors may increase the overall therapeutic responsive rate.

Another example of T cell checkpoint blockade therapies is the PD-1/PD-L1/2 axis, which modulates the immune-suppressive activity of tumor cells. PD-1 is a negative regulator on activated T cells that binds to its ligand PD-L1/2 on target tumor cells, thereby rendering the T cell unresponsive. Blocking the PD-1/PD-L1/2 interaction using a mono-therapy showed significant response rate and unprecedented durability in a number of cancers. In melanoma, an anti-PD-1 antibody showed ~30% overall response rate and 2-10 months duration of response. Looking across a broad range of human cancers, including melanoma, lung, colon, gastric and head and neck cancers, the patient’s response rate to PD-1/PD-L1/2 therapy ranges from 10-40%. Recent evidence from analyzing individual responsive and unresponsive tumors showed that the abundance of mutations in the tumor (“neoantigens”) is directly correlated with the response to PD-1 blockade. Therefore, generating neoantigen
reactive T cells will likely increase the sensitivity to checkpoint blockade therapies and provide synergy.

**Therapeutic cancer vaccines**

Therapeutic cancer vaccines have the potential to stimulate tumor antigen specific adaptive immune responses, which could lyse cancerous tissues and establish long-term immunological memory. A successful cancer vaccine requires a number of important factors: (1) the identification of tumor-specific and tumor-associated antigens that are exclusively expressed on cancerous tissues, (2) the efficient processing and presentation of these antigens by mature APCs via surface MHC-I and MHC-II molecules and (3) the presence of co-stimulatory molecules, stimulatory cytokines and lack of inhibitory cues on the APCs. Together these steps, in theory, could lead to the induction of antigen specific type-1 T-helper (T\(_{H1}\)) CD4\(^+\) T cells, CD8\(^+\) killer T cells (CTLs), and high affinity, high titer antibodies.

Effective vaccines likely require a coordinated regulation of a DC network and optimally exploit the migratory and sampling functions of DCs. DCs function as sentinels that constantly move around the periphery and sample foreign proteins before relocating to secondary lymph organs to present the antigens to T cells. They are attractive vehicles to use in vaccines because they are the most efficient APCs of the innate immune system\(^{10}\). DCs are derived from the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow and are differentiated two main categories: conventional DCs (cDCs) and plasmacytoid DCs (pDCs)\(^{39}\). Conventional DCs include myeloid lineage DCs (CD11c\(^+\)CD11b\(^-\)) and lymphoid lineage DCs
cDCs are specialized in antigen presentation and initiating primary T-cell responses. In particular, CD8α+ DCs are characterized by their ability to cross-present extracellular antigens onto the MHC-I molecule and stimulate CTLs. On the other hand, pDCs do not typically stimulate T cells into cycles but rather produce large amounts of type I interferon upon stimulation. After stimulation, subsets of DCs are mobilized to the draining LNs (dLNs), where they either directly stimulate T cells, or transfer the acquired antigens to other LN resident DCs for further processing and presentation. In the latter case, this inter-DC antigen transfer function can drastically amplify the antigen presentation signal across a large network of LN resident DCs. This concerted effort by a broad set of DCs result in a robust adaptive response. One approach to target DC subsets is conjugating the antigen to a monoclonal antibody against a specific DC receptor, such as DEC205 and CLEC9A.

DCs are capable of conventional processing of exogenous antigens (MHC-II pathway) and endogenous antigens (MHC-I pathway), and cross-presentation where exogenously captured antigens are presented on MHC-I molecules. These capabilities allow mature DCs to efficiently stimulate CD4+ and CD8+ T cells simultaneously. Optimally activated DCs need three signals to stimulate T cell proliferation: (1) high levels of antigen derived peptides presented in the context of MHC-I and MHC-II molecules, which can engage T cell receptors (TCRs) on naïve T cells, (2) upregulation of costimulatory molecules such as CD80, CD86 and CD40 to engage on their cognate receptors on T cells to induce T cell survival and proliferation, and (3) secretion of type-1 cytokines such as IL-12 that can promote T cell differentiation into effector phenotypes (Fig. 1.2). Stimulation of danger sensing mechanisms such as toll-like
receptors (TLRs) or nod-like receptors (NLRs) in DCs is typically used to activate DCs. DCs that lack any of the three signals could fail to induce T cell proliferation, T cell apoptosis or anergy, or T cell tolerance.

Figure 1.2. Priming of naive T cells by resting, mature, and activated dendritic cells (DCs). The fate of newly activated T cells is determined largely by the activation status of the presenting DC. Stimulation of naive T cells by resting DCs results in cell death or anergy. Mature DCs induce the clonal expansion of T cells but not their differentiation (no effector function).

DC vaccines have been tested in clinical trials of various cancers such as melanoma, prostate cancer, glioblastoma and renal cell carcinoma with varying
degrees of success, but the overall response rate to DC vaccines has remained low\textsuperscript{10}. DC vaccines using tumor specific peptides such as NY-ESO-1 and gp100, adjuvanted with CpG, ISCOMATRIX or IFA have showed tumor specific CTL responses and some clinical response, suggesting that vaccinating against self-antigens is possible\textsuperscript{49-51}. The effector response from these vaccines was shadowed by the presence of inhibitory receptors, such as CTLA4, on the effector T cells and the generation of regulatory T cells (Tregs). Compared to short peptides, DC vaccines with long peptides, whole protein or mRNAs encoding the desired antigen showed better responses\textsuperscript{52}. Overall, the challenges associated with peptide or protein based vaccines include that it is difficult to achieve a broad response against various unknown tumor antigens, the antigens (especially peptides) have short circulation half-life in vivo, and the presence of regulatory cues possibly generated from improper DC activation. To generate a broad immune response, DC vaccines with whole, irradiated autologous tumor cells have been evaluated. One notable example of this approach is one using GM-CSF producing tumor cells (GVAX). GVAX induced anti-tumor responses in several independent preclinical models\textsuperscript{53}. When evaluated in clinical settings, GVAX enhanced lymphocyte infiltration in the tumor in a majority of the patients and showed modest efficacy\textsuperscript{54, 55}. One challenge with using autologous whole tumor cells is the inclusion of immune-tolerizing cells, such as myeloid derived suppressor cells (MDSCs), in the vaccine, which may induce T_{reg} priming. More recently, with the advancement in genomics, sequencing and bioinformatics, it is now possible to identify specific somatic mutations associated with the tumor (neoantigens)\textsuperscript{56}. RNA vaccines using a pool of neoantigens with predicted MHC binding capacity showed protective response.
in preclinical models\textsuperscript{57}. A DC neoantigen vaccine showed early clinical promise as it induced neoantigen-specific immunity and promoted a diverse neoantigen TCR repertoire in patients with advanced melanoma\textsuperscript{58}.

**Engineering approaches to cancer immunotherapy**

The field of materials engineering has gone beyond its traditional application in medical device engineering, and biomaterials are now the basis of many drug and cell delivery, drug targeting, and tissue regeneration therapies\textsuperscript{59}. It is now feasible to engineer sophisticated and multifaceted technologies for cellular and molecular targeting and delivery, and these technologies are likely to integrate synergistically with current cancer immunotherapies. This section will discuss the potential advantages of material engineering and recent key advances in material engineering to develop novel vaccines for cancer immunotherapy.

Materials based technologies have a number of attractive features that are likely to augment current cancer vaccine strategies\textsuperscript{60-62}. These technologies can be multifunctional and are designed to package multiple immune-modulating agents into a single material system; doing so allows one to efficiently deliver multiple moieties into a cell and gain understanding of how different agents modulate immune cells. Materials can promote cell-cell crosstalk and enhance both innate and adaptive immunity. They can target specific organs and deliver payloads to cells within these organs, potentially increasing the potency of the payloads while reducing systemic toxicity. Lastly, they can prolong the in vivo bioactivity of immune-modulating agents by protecting them against degradation. Recent advances can be broadly divided into using synthetic
scaffolds to host and direct immune cell function, or micro- and nanoparticles to directly target immune organs and cells (Fig1.3).
Figure 1.3. Three major engineering approaches to cancer vaccine are depicted: scaffold vaccines incorporated with chemo-attractants, immunostimulants, and tumor antigens serve as an in situ reservoir for DC maturation and uptake of tumor antigens. DCs matured in the 3D structure of the scaffold traffic to the lymph node and initiate antitumor immune responses. Alternatively, micro- or nanoparticles carrying tumor antigens or immunostimulants target lymph node resident DCs, which contain populations with potent cross-priming capabilities, thus triggering DC crosspresentation of tumor antigens and priming of tumor-specific T cells. These particles can also be targeted to the tumor directly, to stimulate maturation of intratumoral DCs. Trafficking of matured DCs to the lymph node results in antigen presentation and induction of antitumor immunity.
Micro- and nanoparticle strategies

Engineered nanoparticles that directly target immune cells in various tissues such as draining lymph nodes (dLN), mucosal sites and the tumor provide an alternative approach to recruiting and programming immune cells with a biomaterial. At the site of interest, nanoparticles can target specific cell types and subcellular compartments. Nanoparticles can be synthesized using a wide range of materials, and immune-modulatory agents can be incorporated into the particles prior to delivery by exploiting the chemical and physical properties of the specific material.

LN targeting nanoparticle systems for cancer vaccination have shown significant promise in preclinical models. DC subsets exist in high concentrations in LNs, and targeting the LN can enhance antigen processing and DC-T cell crosstalk. The efficiency of nanoparticle localization to the dLN is controlled mostly by their size, as a number of papers have demonstrated that nanoparticles 20-50nm in diameter transport more efficiently into lymphatic capillaries and their draining lymph nodes (~60%) compared to 100nm or larger nanoparticles (<10%)\textsuperscript{63-67}. Once in the dLN, nanoparticles can release their payloads to enhance multiple immune processes such as antigen cross-presentation\textsuperscript{65, 68-70}. The release and presentation of the payload can be controlled by the incorporation method\textsuperscript{71-73}. Nanoparticles that release encapsulated IL-12 and TNFα into the dLN in a controlled and sustained manner have been shown to prolong a polarized systemic T\textsubscript{H}1 response. This is likely a result of prolonging immune cell action by protecting the cytokines from degradation until release\textsuperscript{69}. Interbilayer-crosslinked multilamellar vesicle (ICMV) nanoparticles encapsulating both ovalbumin and MPLA elicited greater antigen specific CD8\textsuperscript{+} T cell and sera antibody response.
than soluble forms of ovalbumin mixed with MPLA. More strikingly, this work demonstrated that incorporating the MPLA within the layers of the nanoparticle increased the OVA specific CD8+ response by over three fold compared to incorporating the MPLA simply on the outside of the nanoparticles. Finally, conjugation of CpG and peptides to hydrocarbon lipid tails that bind to the fatty acid binding pocket of serum albumin prolonged CpG and antigen retention at the dLN more than 10 fold compared to the unconjugated forms. These lipid-conjugated vaccines significantly slowed tumor growth at doses that were ineffective for unconjugated vaccines. Altogether, these data suggest that not only can nanoparticles efficiently deliver immune-modulatory payloads to APCs in the dLN, but also indicate the design of payload incorporation may significantly alter the resulting immune response.

Complementary to delivering antigens and adjuvants to target cells in the LNs, the materials used to fabricate nanoparticle carriers can possess inherent adjuvant effects, acting as “danger signals”. Synthetic materials such as PLGA, silica, poly(propylene sulfide) and poly (ethylene glycol) have been shown to activate the complement pathway and pattern recognition receptors, and their adjuvant effects can be harnessed in a vaccine system. For example, nanoparticles synthesized from a-Al2O3, in contrast to Alum, were shown to induce autophagy and effective cross-presentation of the loaded antigen. Mice vaccinated with a-Al2O3, nanoparticles conjugated to autophagosomes derived from tumor cells, without additional adjuvants, showed drastically enhanced percentage of IFNγ secreting CD8+ cells and decreased lung metastasis in a 3LL lung tumor model.
In addition to dLN targeting, nanoparticles can be used to target mucosal tissues, where many primary tumors originate. Mucosal tissue targeting for cancer immunotherapy has focused on respiratory tissues\textsuperscript{81-83}, but there is an increasing body of work targeting the gastrointestinal (GI) track and vaginal mucus\textsuperscript{84, 85}. One key consideration for pulmonary vaccination is the biocompatibility of the material. Studies have shown that having a targeting motif built into the nanoparticles significantly reduces local pulmonary inflammation, and that materials with hydrophilic properties are better tolerated than hydrophobic materials. Intranasal vaccination using a number of nanoparticle systems, including cholesterol bearing pullulan (CHP) polysaccharide nanogels, interbilayer-crosslinked multilamellar vesicle nanoparticles and poly(propylene sulfide) nanoparticles have resulted in significantly enhanced antigen specific CD8\textsuperscript{+} T cell and an overall T\textsubscript{H}1 biased response in the lungs\textsuperscript{81, 82}. One vaccine using amphiphilic poly(γ-glutamic acid) nanoparticles encapsulating ovalbumin as a model antigen significantly reduced the number of lung metastasis of B16-OVA cells, compared to bolus ovalbumin vaccination\textsuperscript{86}.

Targeting the tumor directly as a vaccine strategy has two advantages. First, it may bypass the need for predicting and discovering novel tumor associated antigens, as it uses the tumor directly as the antigen depot. Immune tissue targeting nanoparticles, in contrast, require a source of antigens, but it may be difficult to successfully predict the array of defined protein or peptide antigens that are needed for a potent cancer vaccine as a tumor mutates\textsuperscript{87}. Second, it could break tumor associated immune suppression, as pro-inflammatory cytokines and adjuvants can be targeted to immune and stromal cells in the tumor microenvironment that mediate suppression\textsuperscript{88}. 
Tumor targeting materials can be introduced systemically and locally. Liposome nanogels carrying IL-2 and a TGF-β inhibitor, when delivered systemically, led to increased retention of both the cytokine and small molecule drug in the tumor. When evaluated in a B16 lung metastasis model, the nanogel formulation significantly enhanced survival and decreased the number of metastases compared to the bolus formulations. The prolonged presentation and release of immune-stimulating agents also enhanced CD8+ T cell and NK cell activity in the tumor. Peri- and intra-tumoral injection of immune-modulatory agents carrying nanoparticles could also reduce the toxicity elicited from the agents, as the nanoparticle will diminish diffusion of the agent from the injection site. For example, intratumoral injection of bolus anti-CD137 and IL-12, or anti-CD40 and CpG led to significant systemic toxicity as demonstrated by increased serum IL-6, TNF-α, enlarged spleens and weight loss; this is likely due to the leaking of these agents from the tumor into systemic circulation. However, tethering these agents onto liposome nanoparticles prevented their leakage from the tumor microenvironment, thereby reducing systemic toxicity while retaining their bioactivity.

**Synthetic scaffold strategies**

Material scaffolds that incorporate key physical and chemical cues to create synthetic microenvironments may be widely useful to program immune cells directly in vivo. This approach can harness the potent stimulatory function of specific subsets of immune cells and stimulate both innate and adaptive responses that cannot otherwise be achieved by ex vivo vaccines. Macro-porous three-dimensional scaffolds that allow cell infiltration and subsequent emigration from the scaffold provide control over cell-
cell and cell-material interactions\textsuperscript{96-99}. These scaffolds can serve as a site to expose immune cells with biochemical and physical cues to direct cell function\textsuperscript{88}. One of the first demonstrations of in vivo immune cell modulation with a biomaterial used a non-porous polymeric rod implant\textsuperscript{100}. These centimeter-long rods were loaded with CCL19 and tumor associated antigens, and implanted subcutaneously. Dermal and migratory APCs such as Langerhans cells were attracted to the rods, and the devices led to tumor prevention and regression. Subsequently, a poly(lactide-co-glycolide) (PLGA) based scaffold system that contained more than 90% open and interconnected macro-pores was demonstrated to enhance immune cell infiltration into the scaffold\textsuperscript{101, 102}. PLGA is an FDA approved and biodegradable material that has been used widely in suture materials. GM-CSF was released from the scaffold in a sustained manner over a period of more than 30 days. Immature myeloid DCs migrated towards the gradient of GM-CSF and populated the scaffold. A single implant of the complete vaccine, composed of GM-CSF, CpG oligonucleotide condensed with polyethyleneimine and melanoma tumor lysate, led to >80% melanoma protection. More impressively, two implantations of this vaccine led to complete melanoma regression in more than 40% of the mice. Tumor efficacy in this vaccine system is correlated with an increased CD8\textsuperscript{+} T effector cell to CD4\textsuperscript{+}Foxp3\textsuperscript{+} T regulatory cell ratio in the scaffold site and increased production of pro-inflammatory type 1 cytokines in the tissue surrounding the scaffold. Based on these results, this vaccine has moved to a first-in-human phase I clinical trial in patients with metastatic melanoma\textsuperscript{103}.

A number of recent technologies have sought to generate injectable scaffolds and antigen depots in situ to reduce surgical burden and widen the application pool of
scaffold-based vaccines\textsuperscript{104, 105}. For example, one vaccine system incorporates immune-modulatory agents into chitosan and hydroxyapatite, which are co-injected with a crosslinking solution containing tripolyphosphate\textsuperscript{106}. When the two components are mixed in situ, a hydrogel is formed that can slowly release the immune-modulatory agents, leading to a more prolonged antibody response. Since it is likely that macropores are important in inducing potent anti-tumor immunity due to the ability of immune cells to reside within these material while being manipulated, significant effort has been put into engineering injectable scaffolds with interconnected macro-pores. For instance, the hydrogel polymer alginate was polymerized in a partially frozen state, allowing the ice crystals to form throughout the hydrogel. Once the hydrogel is completely polymerized, the ice crystals were thawed, leaving behind macro-pores throughout the polymer\textsuperscript{104}. The shape-memory properties of this hydrogel make it injectable via a needle and recruit APCs in vivo. Using irradiated whole tumor cells as the antigen source, this vaccine system showed superior prophylactic and therapeutic efficacy compared to a bolus irradiated tumor cell vaccine\textsuperscript{96}. The degradation profile in vivo of these types of materials can be readily manipulated by altering the base polymer forming the hydrogel\textsuperscript{107}; a long-term presence of the scaffold may not be desirable in some applications.

**Motivation**

There are a number of challenges associated with current biomaterials based approaches to recruit and prime host DCs \textit{in situ}. First, as these biomaterials are typically on the scale of several millimeters, surgical manipulation is required for
placing many of these systems into the patient. This limits their application in regions that are not equipped with surgery units, requires highly trained personnel to administer the vaccine and increases hospital cost. Second, most of these systems utilize a single bulk material, and immune-modulating drugs are incorporated during material fabrication. This approach may lead to low incorporation efficiency and high variability. Additionally, as all components are incorporated into a single bulk material, the incorporation of one component likely affects the incorporation of others. Here, we propose an injectable and spontaneously assembling material vaccine platform. This vaccine platform will allow for modular construction of materials containing immune-stimulating drugs, and be injected into patients using clinically used needles.

1.2 Hypothesis

An injectable, spontaneously assembling scaffold can recruit host dendritic cells, locally program them to generate potent antigen specific antibody and cellular responses, and elicit an effective therapeutic anti-tumor immunity.

1.3 Specific aims

The above hypothesis will be tested using four specific aims:

Aim 1: Develop an injectable and spontaneously assembling scaffold that can allow for cell infiltration in vivo

Aim 2: Recruit and locally program host DCs to generate antigen specific adaptive immunity against a model antigen ovalbumin

Aim 3: Evaluate the efficacy of the developed scaffold in eliciting a desired immune response in vivo.

Aim 4: Assess the vaccine platform’s clinical feasibility and potential for widespread use in cancer therapy.
Aim 3: Evaluate the vaccine system developed in aims 1 and 2 for enhancing single-shot humoral responses against peptide antigens

Aim 4: Modify the vaccine system with polyethyleinemine to enhance CD8 T cell responses and generate anti-tumor immunity

1.4 Significance

This work will expand the applications of biomaterials based strategies for cancer immunotherapy. The overall approach here builds on previous work to modulate host DC function using an implantable macroporous scaffolds delivering autologous tumor lysates. Significant advances are proposed towards the development of injectable macroporous biomaterials and effective cancer vaccines using a defined source of tumor antigens.

The goal of this work is to engineer a simple and effective approach to overcome many of the current barriers to unlocking the potential of cancer vaccination. This thesis proposes that effective immune activation can be achieve through controlling material-cell interactions and drug delivery from the material. Moreover, the simplicity of the proposed solution enables its wide applicability and clinical translation potential. In addition to cancer vaccination, the general platform developed in this thesis may be used as a local delivery vehicle for cells, proteins and small molecules, and as a therapeutic technology in the context of viral infections, reproductive medicine and regenerative medicine.
More generally, this body of work will increase our insight into controlling immunity. Biomaterials offer sophisticated control over cell behavior and the spatial-temporal presentation of bioactives. They hold great potential to harness various aspects of the immune system. In turn, the effectiveness of biomaterials can also rely on its interaction with the immune system (e.g., transplantation, synthetic organs). This thesis aims to provide a deeper analysis of the relationship between a synthetic material and the immune system. The knowledge gained from these studies will provide insights into designing smarter biomaterials to exploit wanted immune activities while avoiding adversary ones.
1.5 References


Chapter 2: Development of an injectable, spontaneously assembling scaffold

Adapted from:


and


* Equal contributions

2.1 Introduction

Various immunotherapies have shown significant clinical success and preclinical promise, and are rapidly becoming standard approaches for cancer treatment and management\(^1\). However, in most cases it remains difficult to generate sufficiently robust immune responses to achieve lasting therapeutic success. Biomaterials may be useful to enhance the effectiveness of vaccines and other immunotherapies\(^2-8\). The design and fabrication of porous materials has been intensively investigated to pursue new material properties for a variety of applications including cell/tissue engineering and regenerative medicine\(^9-11\). Recently, it has been proposed that in vivo modulation of host cell populations can be achieved using 3D biomaterials with spatiotemporal control of biochemical and mechanical cues\(^3, 12-14\). However, 3D biomaterials are
typically fabricated in vitro, requiring surgical placement in the body, and their preformed structures could limit the capability of host cells to organize themselves.

In the first part of this chapter, we propose an approach in which host immune cells are recruited and modulated in vivo by 3D scaffolds that spontaneously assemble from mesoporous silica rods (MSRs) of high aspect ratio. Here, we hypothesize that long aspect ratio micro-rods will spontaneously assemble to form a defined 3D scaffold. The low stacking density resulted from the long aspect ratio rods will enable the rods to form inter-particle space sufficient for cell trafficking.

Mesoporous solids have been extensively studied as biosensors, therapeutic drug carriers and imaging agents in various disease applications such as tissue engineering, diabetes and cancer. Mesoporous solids with regular arrays of uniform channels were first described in 1992\textsuperscript{15, 16}. This class of materials is characterized by large surface area, ordered pore systems typically around \(~15\) Å, and well-defined pore distributions\textsuperscript{17}. Three most well studied representatives of this class of material are MCM-41 with a hexagonal arrangement of the mesopores, MCM-48 with a cubic arrangement of the mesopores and SBA-15 with 2D hexagonal structures\textsuperscript{17, 18}. While it is challenging to obtain SBA-15 particles with small size, they generally have wider pore sizes (up to 30 nm) compared to MCM-41 and MCM-48 particles. The large pore volume and long-range order make SBA-15 particles ideal to make long aspect micro-sized particles\textsuperscript{19}.

SBA-15 is commonly synthesized by the co-condensation of tetram-alkyoxysilanes (TEOS) in the presence of a structure directing agent (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer) in acidic
conditions\textsuperscript{20, 21}. This synthesis has a number of advantages. First, since the organic functionalities are direct components of the silica matrix, so pore blocking is not a problem\textsuperscript{17}. Second, the organic units are generally more homogeneously distributed. Additionally, SBA-15 has been shown to have good biocompatibility\textsuperscript{22-24}, supporting its development as a versatile platform for clinical applications.

In the second part of this chapter, we explore the effect of the mesoporous silica particle’s surface chemistry on its ability to modulate immune cell function. A number of studies have demonstrated that various surface modification of nano and macro systems play a significant role in its interaction with the host immune system. For example, surface hydroxyl group content on aluminum oxyhydroxide nanoparticles was shown to influence maturation of human dendritic cells and monocytes\textsuperscript{25}. Surface modifications of amorphous silica particles with -COOH, -NH\textsubscript{2}, -SO\textsubscript{3}H, or -CHO groups reduced endosome rupture, IL-1\textbeta and reactive oxygen species (ROS) production by human monocytes as compared to unmodified particles\textsuperscript{26}. The effect of surface chemistry of mesoporous silica particles on immune cell function remains largely unexplored. However, uptake of unmodified mesoporous silica nanoparticles by macrophages resulted in generation of reactive oxygen species, increased levels of nitric oxide (NO) and production of pro-inflammatory cytokines such as IL-1\textbeta, IL-6 and TNF-\textalpha\textsuperscript{27}.

The production of pro-inflammatory cytokines by immune cells is the result of stimulating pattern recognition receptors (PRRs)\textsuperscript{28, 29}. Phagocytosis of particulates such as crystalline silica particulates (SiO\textsubscript{2}), calcium phosphate and PLGA was shown to result in the activation of the NOD-like receptor, Nlrp3, an intracellular PRR\textsuperscript{28, 30, 31}. This
crystal-induced Nlrp3 activation was shown to be a result of lysosomal perturbation rather than direct sensing of the crystal structure\cite{30}. Upon activation, Nlrp3 forms an intracellular multi-protein complex that regulates the production of highly inflammatory cytokines such as IL-1β and IL-18, which leads to the recruitment of innate immune cells to the affected tissue to assist in clearing of the particulate materials\cite{30}.

In the second part of this chapter, we investigated whether modifying the surface groups of the MSR scaffold can modulate its inflammatory properties and the profile of immune cell infiltration. Poly(ethylene-glycol) (PEG), which is considered nontoxic and nonimmunogenic, is among the most widely used polymers to decrease immune recognition and minimize nonspecific uptake\cite{32,33}. Modifying materials with the integrin-binding ligand Arg-Gly-Asp (RGD) is widely used to enhance cell adhesion and previous work demonstrate that DCs express several integrins that bind to RGD\cite{34}. Therefore, we hypothesized that functionalizing the MSR scaffold with PEG would reduce its immunogenicity and thus decrease immune cell infiltration, whereas modifying the MSR scaffold with PEG-RGD would enhance immune cell adhesion and infiltration. We also investigated the role of the Nlrp3 inflammasome in immune cell infiltration and activation.

2.2 Materials and methods

Synthesis of mesoporous silica micro-rods

MSRs were prepared following the previous reports with a slight modification\cite{35}. To synthesize high aspect ratio MSRs (88 μm × 4.5 μm), typically, 4 g of P123 surfactant (average Mn ~5800, Aldrich) and 46 mg of ammonium fluoride (NH₄F, 98%, sigma-
aldrich) were dissolved in 150 g of 1.6 M HCl solution and were stirred with 8.6 g of tetraethyl orthosilicate (TEOS, 98%, Aldrich) at 40 °C for 20 h, followed by aging at 100 °C for 24 h. To prepare lower aspect ratio MSRs (37 μm × 3.2 μm), the synthesis is conducted without adding ammonium fluoride. To extract the surfactant, the as synthesized particles were refluxed for 10 h in 1% HCl in ethanol. The pore-filled MSRs were prepared following a previous report with a slight modification48. One gram of high aspect ratio MSRs was impregnated with 1.4 mL of TEOS under gentle agitation. Aqueous HCl (pH 1) was added drop-wise and mixed with MSRs. The mixture powder was aged at 40 °C for 3 h and water and ethanol formed during hydrolysis were removed by evaporation at 80 °C. The impregnation procedure was repeated 4 times. The monolith-type MSRs were prepared by pressing 5 mg of high aspect ratio MSRs in a mold of 8 mm diameter using a laboratory press.

**Surface modification of MPS/MSRs**

Pristine MSRs were used for surface modification. MSRs were first functionalized with (3-Aminopropyl)triethoxysilane (APTES) (Sigma Aldrich) by stirring 15 ug of APTES with 100mg of pristine MSR in ethanol for 18 hours at room temperature. Particles were washed extensively in ethanol and dried overnight at 70 °C. Extent of modification was measured using a zeta potential analyzer (Malvern Zen3600) and surface amines were quantified using fluorescamine and Lavapep (GelCompany) according to manufacturer’s manual.

To PEGyate the MSRs, 100mg of APTES modified MSRs were reacted with 100 μmol NHS-MS(PEG), (Thermo Scientific). Reaction was allowed to proceed for 2 h and
particles were washed extensively with PBS. 100 mg APTES-modified MSRs were then reacted with 100 μmol PEGylated bis(sulfo succinimidyl)su berate BS(PEG9) (Life Technologies) and subsequently with 3 molar excess of GGGGRGDSP/GGGGRDGSP (Peptide 2.0). MSRs were then washed extensively with and PEGylation and PEG-RGD/RDG reaction efficiencies were quantified by assessing residual surface amines using Lavapep. RGD and RDG peptide content was assayed using micro-BCA (Thermo Scientific). All materials were tested to be endotoxin free using an LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific).

Transmission electron microscopy

MSRs were diluted to 0.1mg/ml in 200-proof ethanol (Sigma Aldrich) and dripped on Formvar/Carbon TEM grids (Ted Pella) and air-dried for 1-2 hours at room temperature. The MSRs were then imaged using the JEOL 2100 transmission electron microscope.

Nitrogen adsorption and desorption

To remove adsorbed water from the surface, MSRs were first dried under low pressure (~50mmHg) and at 150 °C for 8 hours. Nitrogen sorption isotherms of MSRs were then measured on a Micromeritics ASAP 2000 apparatus at liquid nitrogen temperature (77 K). Surface area was determined by Brunauer-Emmett-Teller (BET) method. Pore size distribution was calculated from the adsorption branch of the isotherm using the Barret–Joyner–Hallenda (BJH) model.
**Loading and *in vitro* release of GM-CSF**

1 μg of GM-CSF was loaded onto 1 mg of MSRs for 1 h at 37 °C. The MSRs were centrifuged at 2000g for 10 minutes and the supernatant was collected for the initial time point. The MSRs were then resuspended in release media composed of RPMI supplemented with 1% penicillin/ streptomycin and 1% heat-inactivated FBS. Supernatant containing released GM-CSF was collected periodically. The release of GM-CSF was measured in two different ways; by measuring radioactivity in release media using radio-labeled GM-CSF (125I-GM-CSF) or by enzyme-linked immunosorbent assay (ELISA) using incorporation of normal (non-radioactive) GM-CSF, in order to investigate the release of bioactive GM-CSF. The amount of 125I-GM-CSF released was determined at each time point by counting the radioactivity of the collected media in a gamma counter. Bioactive GM-CSF levels in the collected media were measured using ELISA (R&D systems).

**Bone marrow derived dendritic cells (BMDC) isolation and culture**

Bone marrow cells were isolated from female C57Bl/6J mice between 6 and 15 weeks old. Cells were cultured in RPMI based media (Sigma Aldrich) supplemented with essential amino acids, 10% heat inactivated FBS (Sigma-Aldrich), 1% penicillin/streptomycin, 50μM β-mercaptoethanol and 20 ng/ml murine GM-CSF (Peprotech) and cultured at 37C and 5% CO2 [18]. Non-adherent dendritic cells were harvested and used for experiments between day 7 and 8 of differentiation. Differentiation was confirmed using the CD11c, CD11b and MHC II surface markers.
In vitro stimulation of BMDCs with MSR particles

On day 7 or 8 of BMDC differentiation, non-adherent cells were collected, counted and seeded in tissue culture plastic wells at 2.5x10^6/ml. BMDCs were allowed to attach for 1 hour and MSRs were added directly to the culture for an additional 18 hours. Cells were stained with PE anti-CD86 (eBioscience) to measure their activation. For experiments measuring IL-1β production, BMDCs were primed with 20ng/ml LPS (Sigma-Aldrich) immediately before adding the MSRs. Cells were cultured for 18 hours and supernatant was collected for cytokine analysis.

Mice

Unless otherwise noted, all in vivo studies were carried out using female C57BL/6J mice (Jackson Laboratories) between 6 to 10 weeks old at the beginning of the experiment. Nlrp3−/− mice were obtained from Millennial Pharmaceuticals. The generation of Nlrp3−/− mice was previously described; the mice were backcrossed onto the C57BL/6 genetic background for at least four generations. The Nlrp3−/− mouse colony was maintained at Harvard University. All animal studies were performed in accordance with NIH guidelines, under approval of Harvard University’s Institutional Animal Care and Use Committee.

Surface marker staining and flow cytometry analysis

After single cell suspensions were made, cells were stained with a live/dead dye followed by fluorophore-conjugated antibodies for 15 minutes on ice. Cells were washed 3 times in cold staining buffer containing 1% BSA and 0.1% sodium azide and
analyzed using multi-colored flow cytometry (BD). APC-conjugated CD11c, and FITC-conjugated CD11b stains were conducted for DC and leukocyte recruitment analysis. APC-conjugated CD11c, FITC-conjugated MHC II, and PE-conjugated CD86 stains were conducted for DC maturation analysis. Myeloid cells/neutrophils were stained using Pacific Blue conjugated Ly6C, FITC conjugated Ly6G and PE-CY7 conjugated CD11b. Macrophages were stained using PE conjugated F4/80 and FITC conjugated CD11b. All antibodies were purchased from eBiosciences and used according to the manufacturer’s protocols. Live cells were determined using a fixable live/dead dye (eBiosciences) or 7-AAD (Biolegend). Live cells were first gated on forward and side scatter followed by the live/dead channel. Fluorophore gating was then performed on isotype control.

In vivo MSR scaffold injection

Five (5) mg of sterile MSRs were lyophilized, resuspended in sterile PBS (150ul) and injected into the left flank, midpoint between the inguinal lymph node and the axillary lymph node, of mice.

Scaffold harvesting and cell infiltration analysis

Scaffolds were excised and tissues were processed through mechanical disruption, washed and suspended in PBS. The resulting cell suspension was then filtered through a 40 μm cell strainer. Cells and small remaining MSR particles were pelleted and counted. The portion of cells in the mixture of cells and small silica particles was assessed using side scatter, forward scatter and a live/dead dye in flow cytometry (BD
LSRII. Number of live cells in the scaffold was calculated as the count of cell and particle mixture multiplied by the percentage of live cells determined using flow cytometry.

In vivo tissue processing for cytokine analysis
Tissue samples between 1 mm and 3 mm from the scaffold were extracted from the animals and digested using the Tissue Protein Extraction (T-Per) Reagent (Pierce), and homogenized via brief sonication. Cell debris was pelleted with centrifugation. The supernatant was analyzed using Bio-Plex Pro™ mouse cytokine 23-plex immunoassay (Bio-Rad) and ELISA (eBioscience), according to the manufacturer’s instructions.

MSR scaffold size measurement
Animals were injected with 5 mg of blank MSRs. Subcutaneous nodule size was quantified over time by measuring the nodule length, width and height using a caliper.

Hematoxylin and Eosin staining and analysis
Animals were injected with MSR with 1 μg GM-CSF and the scaffold was explanted on day 3 and 7 post injection. Scaffolds were paraffin embedded, sectioned and stained with Hematoxylin and eosin.

Statistics
All values in the present study were expressed as mean ± S.D. Sample sizes were calculated, using InStat software, to allow the statistical significance of differences of
50% or greater to be determined. The specific sample size required depended on the experiment. Statistical analysis was performed using GraphPad Prism and Microsoft Excel. Sample variance was tested using the F test. For samples with equal variance, the significance between the groups was analyzed by a, two-tailed, student’s t test. For samples with unequal variance, a two-tailed Welch’s t-test was performed. In all cases, a P value of less than 0.05 was considered significant.

2.3 Results

MPS/MSRs spontaneously assemble to form 3D scaffold in vivo

We hypothesized that rod-shaped mesoporous silica particles with high aspect ratio could non-specifically assemble, or coalesce to form structures with significant interparticle spaces (pores) upon subcutaneous injection in vivo (Fig2.1). If interparticle pores generated by particle assembly are bigger than the size of cells, host cells could potentially infiltrate into that space.
To test this idea, MSRs with a hexagonal mesoporous structure were synthesized through the silica sol-gel reaction in the presence of a pore-directing agent, Pluronic block copolymer P123. The MSRs were, on average, 88 μm in length and 4.5 μm in diameter as measured by SEM (Fig 2.2 A), and had cylindrical mesopores as measured by TEM (Fig 2.2 B). The N2 adsorption/desorption isotherms exhibited a type IV isotherm with a hysteresis loop, demonstrating the mesoporous characteristic of the MSR (Fig 2.2 C) with 10.9 nm pore size (Fig 2.2 D). The BET surface area and the total pore volume were 703 m² g⁻¹ and 1.33 cm³ g⁻¹, respectively. SEM imaging of MSRs on a model substrate demonstrated random particle assembly, with interparticle spaces of tens of micrometers (Fig 2.2 A).

Figure 2.1. A schematic representation of in vivo spontaneous assembly of mesoporous silica rods (MSRs) and recruitment of host cells for maturation. A PBS dispersion of MSRs is injected into subcutaneous tissue of mice to form a pocket. After diffusion of PBS from the pocket, in situ spontaneous assembly of MSRs, analogous to the random assembly of thrown matchsticks, results in the formation of three-dimensional interparticle spaces where host cells can be recruited and educated by the payloads in MSRs. Educated cells may then emigrate from the structure to interact with other immune cells.
To test if the MSRs could be randomly assembled in vivo, MSRs dispersed in PBS were injected via needle into the dorsal flank of mice. A large bump was formed immediately after injection due to the volume of the buffer. The outward diffusion of the dispersion buffer occurred in less than 30 min, and led to the disappearance of the initial bump. At around 4 h, a small nodule began to form (Fig2.3 A). The size of the nodule increased rapidly after injection (Fig2.3 B). To examine if the injected MSRs

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**Figure 2.2. Characterization of MPS/MSRs.** (A) SEM image of MSRs of 88 μm in length and 4.5 μm in diameter. (B) TEM image of MSRs of 88 μm in length and 4.5 μm in diameter. (C) Nitrogen sorption isotherms of MSRs of 88 μm in length and 4.5 μm in diameter (D) BET pore size distribution from adsorption branch of the MSRs.
were assembled, MSRs conjugated covalently with rhodamine dye were injected, and the nodule was isolated after 24 h. Fluorescent microscopy revealed a random assembly of MSRs into 3D structures with interparticle spaces (Fig2.3 C), as observed with SEM in vitro (Fig2.2 A).

![Figure 2.3. MPS/MSRs spontaneously assemble to form scaffold in vivo.](image)

(A) Images of subcutaneous nodules formed by the unmodified and surface modified MSRs scaffolds after 7 days. (B) Nodule size measurements after injection of blank MSRs in time course of hours (n=3). (C) Fluorescent image of cross-section of the retrieved nodule after injection of rhodamine B-labeled MSRs.

**MPS/MSR scaffold can recruit host immune cells in vivo**

The ability of host cells to infiltrate the interparticle pores of injected MSR scaffolds in vivo was examined. MSRs were injected into subcutaneous tissue of mice, and the nodule was retrieved at designated time points. The injection of MSRs did not induce a noticeable wound in the skin of the mice. The histology of nodules retrieved on day 3 demonstrated high cellular infiltration into the interparticle spaces and almost no collagen deposition nor fibroblast infiltration (Fig2.4 A). Nodules retrieved at day 7 (Fig2.4 B) were analyzed with SEM, confirming they were composed of a high number
of cells that completely occupied the structure (Fig 2.4 C). Removal of the cells, followed by SEM imaging revealed the underlying structure formed by the injected MSRs (Fig 2.4 D). The isolated cells showed more than 90% viability as determined using flow cytometry after staining with propidium iodide (Fig 2.4 E).
Figure 2.4. MPS/MSRs recruit host cells in vivo. (A) H&E staining of sectioned nodule retrieved at day 3 after subcutaneous injection. *: representative crosssection of MSRs. #: surrounding fibrotic tissue. (B) Localization of MSRs as a nodule in the dorsal flank of a mouse one day after subcutaneous injection (left) and the retrieved nodule (right). (C) Isolation of MSR-scaffold and cells from the nodule (left) and SEM photomicrograph (right) demonstrating a high number of recruited cells. Yellow outline represents a visible MSR. Red arrows indicate the representative cells. (D) SEM image of MSR-scaffold after removal of most recruited cells. (E) Representative bright-field optical microscope of the isolated cells (left), fluorescent image of the cells after live-dead staining (middle, green: live cells, red: dead cells), and propidium iodide (PI) flow cytometry analysis of retrieved cells from the nodule (right).
As interparticle pores formed through the spontaneous assembly of particles with elongated shapes, we hypothesized that longer MSRs with higher aspect ratio would lead to the formation of larger spaces than particles with lower aspect ratio, thus providing more room for cells to infiltrate. Higher (88 × 4.5 μm in length and diameter, denoted as “Higher AR”) and lower (37 × 3.2 μm in length and diameter, denoted as “Lower AR”) aspect ratio MSRs were synthesized (Higher AR shown in Fig2.2A and Lower AR shown in Fig2.5A) and injected subcutaneously, and the numbers of recruited cells were analyzed on day 7 post injection. As hypothesized, higher aspect ratio MSRs led to 2.5-fold more cells residing in the structures than lower aspect ratio MSRs (Fig. 2.5B). Fifty three million cells were recruited to structures formed from the high aspect ratio particles (20 mg). To determine whether the number of recruited cells is overestimated due to background cell counts, we extracted MSRs from mice that had been injected only 20 min earlier. The cell number was 22 times fewer than that found after 24 hours, and 374 times fewer than after 5 days, indicating cells measured in the MSR scaffolds were recruited over time, and not contaminating cells from the surrounding tissue.
To determine the role of mesopores and macropores in recruiting cells, we compared cell recruitment using two control materials based on MSRs. One was pore-filled silica microrods with the same aspect ratio and morphology, but almost no mesoporosity compared to the pristine MSRs measured using TEM (Fig.2.6 A) and BET (Fig.2.6 B). The other control was monolith-type (pressed) MSRs lacking interparticle macropores (Fig.2.6 C) while maintaining the intrinsic mesopores of MSRs (Fig.2.6 D). The numbers of host cells recruited at day 3 into the same mass of both pore-filled silica microrods and pressed MSRs were significantly lower than pristine MSRs (Fig.2.6 E). This result clearly indicates that the interparticle macropores formed by MSR assembly are crucial to recruit a high number of cells. This result also indicates that a constant mass of MSRs is more effective than non-porous silica rods, likely because a
higher number of MSR particles is present at constant mass due to their lower density, resulting in more interparticle macropores.

**Figure 2.6.** The effect of mesopores and inter-particle pore space on cell recruitment *in vivo*. (A) TEM images of MSRs (left) and pore-filled silica microrods (right). (B) Pore size distribution calculated from the adsorption branch of MSRs and pore-filled silica microrods. (C) SEM image of pressed MSRs (inset: photograph of pressed MSRs). (D) N2 isotherms and the corresponding pore size distribution of pressed MSRs. (E) (j) Number of total recruited cells in 5 mg of pore-filled, pressed, and pristine MSRs, respectively, at day 3 post injection (n=4). Values represent mean and s.d. * represents p<0.05.

The biodegradability of biomaterials is an important issue in the body. It is known that mesoporous silica can degrade into silicic acid in vitro and in vivo over time. To indirectly investigate in vivo degradation and clearance of the MSR scaffolds in injection site, we measured the size of the subcutaneous nodule over time.
after injection of blank MSRs (Fig2.7 A). The nodule increased in size until day 7, and was reduced to an almost unmeasurable size after 25 days. Fluorescent imaging of sections of the nodule retrieved at day 7 after injection of fluorescent protein-adsorbed labeled MSRs showed the random assembly of MSRs surrounded by the recruited cells (Fig2.7 B), while the sectioned nodule at day 28 showed very few MSRs (Fig2.7 C). These results indicate that the injected MSRs were significantly degraded in vivo after subcutaneous injection. There was no significant toxicity or inflammation in liver, kidney, or other organs of the mice injected with MSRs. We did not observe any difference in animal behavior after injection compared to naïve animals. Animals appeared to tolerate well the injections, and made no efforts to disturb the injection site, suggesting it did not present significant discomfort or pain.

**Figure 2.7. In vivo clearance of MPS/MSR scaffold.** (A) Nodule size measurement after injection of blank MSR in time course over weeks (n=3). (B,C) Confocal images of sectioned nodules retrieved at (B) day 7 and (C) day 28 post-injection, respectively. The injected MSRs were labeled with AF488 and loaded with GM-CSF (1 μg). The cryosections were stained with DAPI and rhodamine-phalloidin. Values represent mean and s.d.
Collectively, these results demonstrate that simple, injectable MSRs provide a material platform for infiltration of large numbers of host immune cells. As the macroporous structure is spontaneously generated in vivo, this spontaneously assembling scaffold allows one to bypass ex vivo synthesis of the scaffold and to minimize geometric and spatial constraints of a preformed scaffold.

The effect of surface modification of MPS/MSRs on BMDCs in vitro

The surface of MPS/MSRs can be functionalized with amine, thiol, chloro and phophonate groups using standard silane procedures. PEG and PEG-RGD modifications were performed using amine functionalized MSRs (Fig2.8).

Figure 2.8. Schematics of MSR surface modification. MSRs were functionalized with APTES, and the amine groups were then modified with methoxyl terminated PEG\textsubscript{12} (NHS-MS(mPEG)\textsubscript{12}) to achieve PEG modified MSRs, or methoxyl terminated PEG\textsubscript{9} (BS(PEG)\textsubscript{9}) followed by RGD/RDG peptides for PEG-RGD/RDG modified MSRS.

MPS/MSRs were first functionalized with primary amine groups using (3-Aminopropyl)triethoxysilane (APTES); modification was confirmed using
fluorescecamine and the resulting material had 0.13 µmol of APTES per mg of MSR. APTES modified MPS/MSRs were then modified with PEG or PEG-RGD groups using standard amine reactive N-hydroxysuccinimide esters (NHS) reaction. The extent of PEG and PEG-RGD modification was 76.2 +/- 0.3% and 78.1 +/- 0.6%, respectively (Fig2.9 A). No change in the bulk structure and lengths of the MSRs after modification was observed. Nitrogen adsorption/desorption analysis revealed that the modified materials possessed a typical type IV isotherm with a well-defined presence of mesopores (Fig2.9 B). The PEG and PEG-RGD modified MSRs had a slightly decreased and narrow BJH pore size distribution (around 10 nm) and significantly decreased total surface area and pore volume compared to APTES-only modified MSRs (Fig2.9 C). The decrease in surface area and pore volume was likely due to space occupied by the PEG polymers and peptides. Additionally, TEM confirmed an aligned and open pore structure in the modified MSRs (Fig2.9 D).
As we are interested in using the MPS/MSR scaffold to induce DC activation in vivo, we first evaluated the effect of the modified MPS/MSRs on DC maturation and activation in vitro. Bone marrow derived DCs (BMDCs) were harvested and stimulated...
with 100µg/ml of unmodified and surface modified MSRs. After 18 hours, BMDCs were collected and co-stained with the DC marker CD11c and activation marker CD86 or MHC-II. Unmodified (-Hydroxyl) and various functionalized MPS/MSRs led to significant CD86 up-regulation compared to the PBS control (Fig2.10 A). In a separate set of experiments, BMDCs were stimulated with 20µg/ml of unmodified and PEG or PEG-RGD modified MSRs. PEG modified MSRs led to even higher CD86 expression (Fig2.10 B). Incorporating RGD in PEG modified MSRs reduced CD86 expression as compared to PEG modified MSRs (Fig2.10 B). In a separate experiment, the effect was found to be not sequence specific, as no significant difference was observed between PEG-RGD modified and PEG-RDG modified MSRs (Fig 2.10 C).

**Figure 2.10. Effect of surface modified MSRs on BMDC activation marker expression.** (A), (B) and (C) Flow cytometry analysis of CD11c, MHC-II and CD86 expression on BMDCs after 18 hours of stimulation with surface modified MSRs. In (A), data was expressed as percentage of BMDCs positive for either CD86 (dark) or MHC-II (light). In (B) and (C), data was expressed as percentage of BMDCs double positive for CD11c and CD86. Data are shown as mean and s.d with n=3. * indicates p < 0.05.
The effect of the surface modified MSRs on DC cytokine production was also examined as activated DCs also secrete pro-inflammatory cytokines. BMDCs were first primed with 20ng/ml of LPS and stimulated with the surface modified MSRs. A panel of inflammation related cytokines from DCs stimulated with unmodified and PEG modified MSRs was first analyzed (Fig2.11).

Among the 18 cytokines analyzed, IL-1α and IL-1β were significantly increased in PEG modified MSRs compared to unmodified MSRs, with IL-1β secreted at higher quantities. Secretion of IL-1β by DCs after stimulation with various concentrations of PEG modified, PEG-RGD modified and PEG-RDG modified MSRs was then measured with ELISA. Consistent with what was observed from the DC activation study, PEG

Figure 2.11. Effect of surface modified MSRs on BMDC cytokine production. Bioplex analysis of 18 inflammation related cytokines in supernatant of BMDCs primed with 20 ng/ml LPS and stimulated with unmodified and PEG modified MSRs. Data are shown as mean and s.d with n=4
modified MSR significantly enhanced IL-1β production in a dose dependent manner, while PEG-RGD modified and PEG-RDG modified MSRs decreased the effect as compared to PEG-MSRs (Fig2.12 A). One of the most important regulators of IL-1β production is the Nlrp3 inflammasome, which is a member of the NLR family capable of sensing danger-associated molecular patterns (DAMPs). To investigate whether the Nlrp3 inflammasome was responsible for the enhanced IL-1β production after stimulation by the MSRs, BMDCs from Nlrp3−/− mice were stimulated with 5 μg/ml unmodified and surface modified MSRs, and no secretion of IL-1β was found after stimulation regardless of the surface groups (Fig2.12 B).

Figure 2.12. Effect of surface modified MSRs on BMDC IL-1β production. (A) ELISA analysis of IL-1β concentration in BMDC supernatant after 18 hours of stimulation with various concentration of surface modified MSRs and (B) ELISA analysis of IL-1β concentration in Nlrp3−/− or WT BMDC with 5 μg/ml MSR (mean and s.d, n=3 or 4). * indicates p < 0.05. ns indicates no significance.
The effect of surface modification of MPS/MSRs on host immune cell recruitment in vivo

The inflammatory response to the unmodified and modified MSRs was next analyzed in vivo. Unmodified MSRs, PEG MSRs, PEG-RGD MSRs and PEG-RDG MSRs were injected subcutaneously into the flank of mice. As previously described, the MSRs spontaneously assembled after injection, forming a 3D scaffold that allowed for cell infiltration. On day 5, the subcutaneous nodule formed by PEG modified MSRs was noticeably larger than that formed by unmodified, PEG-RGD MSRs and PEG-RDG MSRs (Fig2.13 A). Analysis of the cell content revealed that nearly 10 times more cells infiltrated the PEG MSR scaffold than the unmodified MSR scaffold. Although both PEG-RGD MSR and PEG-RDG MSR scaffolds also contained significantly more cells than the unmodified MSR scaffold, nearly 4 times fewer cells were found than in the PEG MSR scaffold (Fig2.13 B). No significant difference was observed between PEG-RGD modified and PEG-RDG modified scaffolds.
The types of immune cells that infiltrated the scaffolds were next analyzed. Specifically, DCs, macrophages and myeloid cells/neutrophils were quantified. DC and macrophage infiltration has been shown to be important for eliciting a potent adaptive immune response, and myeloid cells/neutrophils are among the first responders to a foreign material at sites of inflammation. Myeloid DCs (Fig2.14 A) were determined as CD11c⁺CD11b⁻ cells, myeloid cells/neutrophils (Fig2.14 B) were determined as Ly6GhiLy6cmid cells, and macrophages (Fig2.14 C) were determined as CD11b⁻F4/80⁺ cells.

Figure 2.13. Effect of surface modified MSRs on cell recruitment in vivo. (A) Images of subcutaneous nodules formed by the unmodified and surface modified MSR scaffolds after 5 days. Scale bar = 5 mm. (B) Total cell count in the explanted MSR scaffolds. Data are shown as mean and s.d. with n = 4. * indicates p < 0.05. ns indicates no significance.
Figure 2.14. Primary flow cytometry plots showing gating strategies used to identify (A) CD11c and CD11b double positive DCs, (B) Ly6G\textsuperscript{high} and Ly6c\textsuperscript{mid} myeloid cells/neutrophils and (C) F4/80 and CD11b double positive macrophages. Plots on the left shows the isotype control staining and plots on the right shows example of cells positive for the gate.
Interestingly, PEG scaffolds contained a significantly lower percentage of DCs than unmodified scaffolds (<1% of total infiltrating cells), and PEG-RGD scaffolds and PEG-RDG scaffolds contained more DCs than PEG scaffolds, but lower numbers than unmodified scaffolds (Fig2.15 A). Nevertheless, the total number of DCs was not significantly different among the scaffold conditions (Fig2.15 B). This trend was also observed for activated DCs and macrophages in the scaffolds (Fig2.15 C-D). Myeloid cells/neutrophils made up more than 50% of immune cells in all scaffold conditions at this time point. In contrast to what was observed with DCs and macrophages, PEG scaffolds had a significantly higher percentage and total number of myeloid cell/neutrophils compared to unmodified, PEG-RGD modified and PEG-RDG modified scaffolds (Fig2.15 E-F and F).
In addition to immune cell infiltration, the development of a fibrous capsule around the scaffold was also analyzed. Scaffolds were explanted, and the weight of the fibrous tissue around the scaffold was measured. The fibrous capsule around the PEG

Figure 2.15. Effect of surface modified MSRs on immune cell recruitment in vivo. (A) Flow cytometry analysis of CD11c and CD11b expression in cells isolated from the scaffolds. Cells double positive for CD11c and CD11b were quantified. (B) Total number of CD11c and CD11b double positive DCs in the scaffolds. (C) Flow cytometry analysis of CD11c and CD86 expression in cells isolated from the scaffolds. (D) Flow cytometry analysis of CD11b and F4/80 expression in cells isolated from the scaffolds. (E) Flow cytometry analysis of Ly6c and Ly6G expression in cells isolated from the scaffolds. Cells expressing high levels of Ly6G and medium levels of Ly6c were quantified. (F) Total number of cells expressing high levels of Ly5g and medium levels of Ly6c in the scaffolds. Data is expressed as mean and s.d with n = 4. * indicates p < 0.05. ns indicates no significance.
scaffold was significantly heavier and noticeably thicker than that around unmodified and PEG-RGD scaffolds (Fig2.16 A). The fibrous capsule around the PEG scaffold also contained significantly higher IL-1β levels than unmodified and PEG-RGD scaffold fibrous capsules (Fig2.16 B). Analysis of a panel of inflammation related cytokines revealed that IL-1β was one of the highest secreted inflammatory cytokines in unmodified scaffolds (Fig2.16 C).

Figure 2.16. Scaffold tissue analysis. (A) Weight of fibrous capsule around the scaffolds. (B) ELISA analysis of IL-1β concentration in the fibrous capsule. (C) Bioplex analysis of 21 inflammation related cytokines in unmodified MSR scaffolds explanted and digested after day 7. Data is expressed as mean and s.d with n = 4. Data are shown as mean and s.d. with n = 4. * indicates p < 0.05. ns indicates no significance.
The role of the Nlrp3 inflammasome on immune cell infiltration \textit{in vivo} was subsequently examined. Unmodified and modified MSRs were injected subcutaneously into the flanks of wild type B16 mice and Nlrp3\textsuperscript{−/−} mice. Scaffolds in Nlrp3\textsuperscript{−/−} mice showed significantly reduced total cell infiltration from all surface modifications as compared to wild type mice (Fig\textbf{2.17 A}). However, similar to the previous observations in wild type mice, PEG scaffolds in Nlrp3\textsuperscript{−/−} mice exhibited significantly increased cell infiltration as compared to the other surface chemistries. In both wild type and Nlrp3\textsuperscript{−/−} mice, the primary cells that infiltrated the scaffolds were again myeloid cells/neutrophils, and there was no significant difference between wild type and Nlrp3\textsuperscript{−/−} mice (Fig\textbf{2.17 B}). Interestingly, both unmodified and PEG modified scaffolds contained increased percentage of DCs when placed in Nlrp3\textsuperscript{−/−} mice compared to wild type mice (Fig\textbf{2.17 C}). However, unmodified scaffolds in Nlrp3\textsuperscript{−/−} mice contained fewer activated DCs as compared to scaffolds in wild type mice, suggesting most of the DCs infiltrating the scaffold in Nlrp3\textsuperscript{−/−} mice may exhibit an immature phenotype (Fig\textbf{2.17 D}). Finally, and consistent with the previous observations, in both wild type and Nlrp3\textsuperscript{−/−} mice, PEG modified scaffolds had a reduced DC population and were enriched in the myeloid cell/neutrophil population.
Figure 2.17. Involvement of the Nlrp3 inflammasome in immune cell infiltration into unmodified and surface modified MSR scaffolds in vivo. (A) Total cell counts in the MSR scaffolds. (b) Flow cytometry analysis of Ly6c and Ly6g expression in cells from scaffolds injected in Nlrp3⁻/⁻ and WT mice. Cells expressing high levels of Ly6g and medium levels of Ly6c were quantified. (c) Flow cytometry analysis of CD11c and CD11b expression in cells from scaffolds injected in Nlrp3⁻/⁻ and WT mice. Cells double positive for CD11c and CD11b were quantified. (d) Flow cytometry analysis of CD86 expression. Results expressed as percent of CD86+ cells within the CD11c⁺ population. Data are shown as mean and sd with n = 4. * indicates p < 0.05. ns indicates no significance.

Collectively, these data suggest that modifying the surface chemistry on the MPS/MSRs could have profound effects on their inherent immune response. Functionalizing the MPS/MSRs with reactive groups such as amine, thiol, chloro and
phophonate groups did not increase the particle’s immunogenicity. Unexpectedly, modifying the surface with PEG significantly enhanced the particle’s immunogenicity. However, the dominant immune cell population recruited to PEG-MSRs is the myeloid cell/neutrophil phenotype. It is unclear, so far, what benefit myeloid cells will bring to the activity of a cancer vaccine. Therefore, going forward, unmodified MPS/MSRs will be used.

2.4 Discussion

The results from this chapter demonstrated that MSRs are capable of forming, in situ, macroporous structures that provide a 3D microenvironment for housing and modulating large numbers of immune cells in vivo. Although there has been past work to assemble DNA, peptides, and nanoparticles, the resulting structures were limited to nanostructures for drug delivery or microstructures for in vitro cell culture. In this study the interparticle macropores formed by mesoporous silica microparticles could hold host cells. This approach eliminates the need for ex vivo construction of a predefined 3D scaffold and brings the assembly process in vivo. Although the current microparticles create random pore structures, designing molecular interactions between microparticles could allow self-assembly into a pre-designed, macroporous structure in vivo. In addition to cell recruitment, the structure of the MSRs could alter various aspects of cellular phenotype, including cytoskeletal architecture, and differentiation and proliferation.

The number of cells within MSR scaffolds is strikingly higher than the number previously reported to be recruited to preformed macroporous polymer scaffolds.
Generating interparticle macropores using high-aspect-ratio MSRs appears to be crucial to cell number, and the ability of resident cells to reorganize the particles may generate space for additional cell infiltration. Cell infiltration into blank MSRs is likely a result of the innate sensing of the silica material. Although MSRs used in this system are larger than cells, and are unlikely to be internalized intact, it is possible that smaller particles are produced during their degradation in vivo over time and internalized by cells, resulting in NALP3 activation.

The surface of the MSRs can be readily functionalized and modified. Surface chemistry was shown to impact how the innate immune system sensed and responded to the MSRs. PEG modified MSRs displayed substantially higher inflammatory responses compared to unmodified, PEG-RGD modified and PEG-RDG modified MSRs. PEG MSRs stimulated increased production of the pro-inflammatory cytokine IL-1β, up-regulation of DC maturation markers in vitro, and increased immune cell infiltration into the scaffolds in vivo. PEG is typically considered a “stealth” polymer and, by extension, has been shown to decrease serum protein adsorption and reduce host response. However, recent evidence has suggested that treatment with some PEGylated drugs can result in immune responses against the PEG, which could limit the drug efficacy. For example, clinical studies of PEG-uricase and PEG-asparaginase show substantial increase of serum anti-PEG antibodies that were associated with decreased drug efficacy. Likewise, a number of studies have also suggested that PEG containing materials can elicit pro-inflammatory responses under specific contexts. For instance, human monocytes seeded on PEG diacrylate hydrogels secreted more IL-1β, TNF-α and GM-CSF compared to those seeded on...
tissue culture plastic. More recently, a series of studies show that implantation of PEG hydrogels in vivo resulted in a more robust inflammatory response, characterized by a thicker fibrous capsule around the hydrogel, compared to PEG-RGD hydrogels. Murine macrophages seeded on PEG hydrogels showed higher expression of IL-1β and TNF-α and PEG hydrogels adsorbed more serum proteins related to acute inflammation than PEG-RGD hydrogels.

Several reasons for the immunogenicity from PEGylation have been proposed, including the direct interaction between PEG itself and serum proteins and through the immunogenicity of the anchoring material or drug. It has been shown that PEG hydrogels can activate serum complement proteins, leading to subsequent immune cell recognition. Monodispersed and highly concentrated PEG polymers ranging from 2K to 11K, PEG5000 stabilized carbon-nanotubes and lipid-PLGA-PEG nanoparticles were shown to generate complement activation when incubated with human serum. Though the exact mechanism by which PEG may activate complement proteins remains unclear, the effect likely depends on surface PEG modification density. For example, 10% PEG-PLA nanocapsules increased complement activation over PLA nanocapsules, but 30% PEG-PLA decreased the effect. In this study, relatively short PEG polymers (PEG12 and PEG9 for PEG MSR and PEG-RGD/RDG MSR, respectively) were used. Since the MSRs have large surface area to volume ratio, it is likely that PEG covered only a fraction of the total surface and increasing PEG coverage may alter the inflammatory response. PEG was anchored onto the MSRs through stable amide bonds. Mesoporous silica nanoparticles have been shown to be stable in serum in vitro over a period of 60 days, and PEGylation was shown to
decrease the biodegradation rate of silicon-based materials\textsuperscript{58}. However, the
degradation of the MSRs could be significantly accelerated in vivo due to the presence
of proteases and reactive oxygen species in the inflammatory microenvironment, and
the local concentration of the degradation products may contribute to the
immunogenicity. Finally, the immunogenicity of PEG may depend on the
immunogenicity of the anchoring material. For example, immunization with PEG-OVA
given in Freund’s complete adjuvant resulted in increased anti-PEG serum antibody,
but not after immunization with PEG-OVA alone\textsuperscript{59}. Since the MSRs are inherently
inflammatory, as shown by increased activation markers and pro-inflammatory
cytokine secretion in BMDCs, it is possible that the immunogenicity of the MSRs
amplified the immune response towards PEG. Overall, future studies are required to
investigate the effect of PEG surface chain density, length and conformation.

PEG-RGD modified and PEG-RDG modified MSRs showed decreased
inflammatory responses compared to PEG MSRs. The effect was not specific to the
integrin-binding domain, as no difference was observed between the behaviors of
PEG-RGD and PEG-RDG MSRs in vitro and in vivo. In this context, it is more likely that
incorporating the small peptide to PEG hindered the interaction between the PEG
chain, or its terminal group, and components in the serum, reducing immune cell
recognition. A number of studies have shown that terminal groups, such as hydroxyl
groups, can selectively activate proteins in the complement pathway\textsuperscript{60-62}. The present
study utilized PEG polymers carrying a methoxy group, which has been suggested to
limit complement activity in some contexts\textsuperscript{54, 63}. However, the effect of the terminal
group in this system on complement protein activation and serum protein adsorption needs to be evaluated.

The Nlrp3 inflammasome was found to be necessary for IL-1β production after stimulation with the MSRs in vitro, and significantly impacted the degree of immune cell trafficking in vivo. Previous works showed that uptake of amorphous and crystalline silica particulates by macrophages can induce intracellular membrane damage and the production of reactive oxygen species\textsuperscript{64, 65}. DAMPs released by this process are sensed by the Nlrp3 inflammasome, leading to enhanced IL-1β and IL-18 secretion. The MSRs likely acted through a pathway similar to the previously studied amorphous silica particulates in vitro, but the strength and duration of pathway activation remains to be elucidated. Abolishing Nlrp3 also significantly reduced total immune cell infiltration in vivo. Myeloid cells/neutrophils have been shown to exhibit high expression of Nlrp3, but there was no reduction in the percentage of myeloid cells/neutrophils in scaffolds from Nlrp3\textsuperscript{-/-} mice\textsuperscript{66, 67}. Rather, scaffolds in Nlrp3\textsuperscript{-/-} mice enriched for CD11c\textsuperscript{+}CD11b\textsuperscript{+} myeloid DCs, and these DCs exhibited a more naïve phenotype in the unmodified scaffolds.

PEG modification of MSRs could potentially make the resulting scaffold a stronger adjuvant. The PEG MSRs could increase the response to the vaccine by acting directly on infiltrated immune cells through NLRs and TLRs. On day 5 and 7 post injection, PEG MSRs were highly enriched in CD11b\textsuperscript{+}Gr.1\textsuperscript{+} cells but exhibited a reduced percentage of APCs. It is possible that APCs infiltrated the scaffold at an earlier time point, were activated upon interacting with PEG MSRs and emigrated from the scaffold, as it has been demonstrated that activated APCs up-regulate CCR7,
leading them to home to draining lymph nodes. While biomaterial design has traditionally avoided immune-activation to minimize rejection, adjuvant properties of the scaffold could be exploited and may act synergistically with other adjuvants such as CpG or poly(I:C) to induce potent adaptive immune responses\textsuperscript{68,69}. For instance, aluminum salt adjuvants were shown to activate immune cells through Nlrlp3 by a mechanism of lysosomal disruption. However, as CD11b\textsuperscript{+}Gr.1\textsuperscript{+} myeloid cells may inhibit DC and NK cell function and mediate T cell suppression\textsuperscript{70-72}, it will be important to investigate the functional interaction between CD11b\textsuperscript{+}Gr.1\textsuperscript{+} myeloid cells, DCs and macrophages in the scaffolds to better understand their functional roles.

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2.6 References


Chapter 3: Recruitment and activation of host DCs to generate antigen specific adaptive immunity

Adapted from:


* Equal contribution

3.1 Introduction

Active cancer vaccination strategies can induce anti-tumor immunity either priming new immune responses, or expanding existing host immune responses against various tumor antigens. The strength of vaccination relies on inducing potent antigen specific adaptive immunity (T and B cell responses) that efficiently clears tumor cells and establishes long-term immunological memory. Dendritic cells (DCs) are a type of professional antigen presenting cells (APCs) that function as sentinels that constantly move around the periphery and sample foreign proteins before relocating to secondary lymphoid organs to prime adaptive immunity. Because of their superior capability to uptake, process and present antigens compare to other APCs, they are a potent regulator of T and B cell immunity. DCs are derived from the hematopoietic stem and progenitor cells (HSPCs) in the bone marrow and are differentiated two main categories: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Conventional DCs include myeloid lineage DCs (CD11c+CD11b+) and lymphoid lineage DCs (CD11c+CD8α). cDCs are specialized in antigen presentation and initiating primary T-cell responses. In particular, CD8+ DCs are characterized by their ability to cross-
present extracellular antigens onto the MHC-I molecule and stimulate CTLs.\textsuperscript{5-7}. On the other hand, pDCs do not typically stimulate T cells into cycles but rather produces large amounts of type I interferon upon stimulation\textsuperscript{8}. After stimulation, subsets of DCs are mobilized to the draining LNs (dLNs), where they either directly stimulate T cells, or transfer the acquired antigens to other LN resident DCs for further processing and presentation. In the latter case, this inter-DC antigen transfer function can drastically amplify the antigen presentation signal across a large network of LN resident DCs\textsuperscript{9,10}. This concerted effort by a broad set of DCs result in a robust adaptive response.

In order to construct a cancer vaccine in situ, large numbers of host DCs must be actively recruited to the site of vaccination. Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is a potent regulator of DC generation and proliferation\textsuperscript{11}. Additionally, GM-CSF preferentially generates myeloid lineage DCs, characterized as CD11c\textsuperscript{+}CD11b\textsuperscript{+}CD8a\textsuperscript{−} DCs, as opposed to lymphoid-lineage (CD11c\textsuperscript{−}CD11b\textsuperscript{low}CD8a\textsuperscript{+}) or plasmacytoid (CD11c\textsuperscript{−}CD11b\textsuperscript{−}B220\textsuperscript{+}) DCs\textsuperscript{12-14}. It has been demonstrated that another cytokine, Flt3-ligand (FLt3L), preferentially expand plasmacytoid DCs\textsuperscript{15,16}. However, previous work from our group showed that a cancer vaccine delivering either GM-CSF or Flt3L to recruit host DCs generated comparable levels of tumor protection.

The role of GM-CSF in tumor immunology remains controversial\textsuperscript{17}. On one hand, it has been shown that GM-CSF can be secreted by various tumors and promote immune suppressive activities that enable tumor escape\textsuperscript{18-20}. Increased level of GM-CSF level in the serum correlates with poor prognosis\textsuperscript{21}. On the other hand, there is solid evidence that GM-CSF can mediate protective anti-tumor immunity by stimulating mature DC infiltration into the tumor. Clinical trials using irradiated GM-CSF secreting
autologous tumor vaccines induced potent and diverse protective anti-tumor responses mediated by CD8 T cells, T\textsubscript{H}1 and T\textsubscript{H}2 CD4 T cells, B cells and NK cells\textsuperscript{22-24}. These contradicting roles of GM-CSF suggest that other factors present in the context of GM-CSF likely contribute to skewing its tolerogenic or pro-inflammatory roles.

DCs can be activated through stimulating Pattern Recognition Receptors (PPRs), which recognize various Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These PAMPs and DAMPs include structures common to bacteria, viruses, particulates and cellular damage\textsuperscript{25-27}. Toll-Like Receptors (TLRs) are among the most commonly studied PPRs in DCs. Collectively, TLRs are capable of recognizing extracellular and intracellular components conserved among microorganisms\textsuperscript{28}. Unmethylated CpG oligonucleotide (CpG-ODN) is an agonist for the Toll-Like Receptor 9 (TLR-9) in DCs\textsuperscript{29}. Preclinical studies and clinical trials have indicated that CpG is a promising vaccine adjuvant. TLR-9 activation enhanced antigen specific B cell and T cell responses by promoting Type 1 cytokine secretion, up-regulation of maturation markers and antigen cross presentation\textsuperscript{30-32}.

Here, we hypothesize that GM-CSF and CpG can be incorporated into and released from the mesopores in the MSR scaffold (described in Chapter 2) to recruit and activate host DCs and prime antigen specific adaptive immunity. In this chapter, we will explore the ability of the MSR scaffold to induce antigen specific responses using a model protein ovalbumin (OVA).
3.2 Materials and Methods

**In vitro release study**

To examine the in vitro release of CpG-ODN, 100 µg of CpG-ODN (Oligofactory) was loaded onto 5 mg of MSRs at room temperature for 8 h under vigorous shaking, and MSRs were subsequently lyophilized and redispersed in 1% BSA. Release media was collected periodically, and the concentration of CpG-ODN was measured with the OliGreen ssDNA Assay (Invitrogen) according to manufacturer’s protocol. Similarly, to examine the in vitro release of ovalbumin, 300 µg of ovalbumin (Sigma Aldrich) was loaded onto 5 mg of MSRs, lyophilized and re-dispersed in PBS. Release media was collected periodically, and the ovalbumin concentration was determined using a micro-BCA Protein Assay (Pierce). All release studies were done in 37°C under moderate shaking.

**Confocal analysis**

Animals were injected with MSRs containing with OVA conjugated with Alexa Fluoro 488 (AF488). At various time points, the scaffolds were explanted, and fixed in neutral buffered formalin at 4 °C overnight. Scaffolds were then embedded in OCT. Frozen sections of the scaffolds were stained with rhodamine-phalloidin (Biotium) and DAPI (Life Technologies) and visualized using a Zeiss LSM 710 confocal microscope.

**Preparation of MSR vaccine and immunization**

5 mg of MSRs were loaded with 1 µg of GM-CSF, 100 µg of CpG-ODN and OVA (50, 150, or 300 µg) for 12 h at room temperature under vigorous shaking. The particles
were then lyophilized, resuspended in cold PBS (150 μl) and injected subcutaneously into the flank of female C57Bl/6J mice.

**Analysis of DC recruitment to MSR scaffolds and their emigration to lymph nodes**

APC-conjugated CD11c (eBioscience 17-0114), FITC-conjugated CD11b (eBioscience 11-0112) stains were conducted for DC and leukocyte recruitment analysis, and APC-conjugated CD11c, FITC-conjugated MHC II (eBioscience 11-5332), and PE-conjugated CD86 (eBioscience 12-0862) stains were conducted for DC maturation analysis. Cells were gated according to positive FITC, APC and PE using isotype controls, and the percentages of cells staining positive for each surface antigen were recorded. To track DC emigration to lymph nodes (LNs), OVA labeled with Alexa Fluor 647 was loaded in MSRs and injected subcutaneously. The inguinal lymph nodes were harvested at 7 days post injection. Cell suspensions from LNs were prepared by mechanical disruption and pressing of the tissue through 40 μm cell strainers, and examined for CD11c+ AF647+ cell numbers by flow cytometry. All flow cytometry antibodies were diluted according to the manufacturer’s suggestions.

**In vivo cytokine analysis**

Tissue samples between 1 mm and 3 mm from the scaffold were extracted from the animals and digested using the Tissue Protein Extraction (T-Per) Reagent (Pierce), and homogenized via brief sonication. Cell debris was pelleted with centrifugation. The supernatant was analyzed using Bio-Plex Pro™ mouse cytokine 23-plex immunoassay
(Bio-Rad M60009RDPD) and ELISA (R&D systems DY415), according to the manufacturer's instructions.

**Detection of SIINFEKL presenting DCs in the draining lymph node (dLN)**

To analyze OVA257-264 (SIINFEKL) peptide (Anaspec) presenting DCs, dLNs were isolated and digested on day 7 post vaccination. dLN cells were enumerated and stained with APC conjugated anti-mouse CD11c (eBioscience 17-0114) and PE conjugated anti-mouse SIINFEKL peptide bound to H-2Kb (eBioscience 12-5743) for 15 minutes on ice. Cells were washed and assessed by flow cytometry.

**Detection of germinal center formation**

To analyze GC formation, dLNs were isolated on day 7 post vaccination. Cells were enumerated, stained with FITC conjugated anti-mouse B220 (eBioscience 11-0452) and APC conjugated anti-mouse GL7 (eBioscience 51-5902) for 15 minutes on ice. Cells were washed and assessed by flow cytometry.

**Detection of OVA-specific humoral responses**

Animals were vaccinated with bolus 300 μg OVA (bolus OVA), bolus vaccine containing 300 μg OVA, 100 μg CpG-ODN and 1 μg GM-CSF (bolus vaccine), 5 mg MSR containing 300 μg OVA (MSR + OVA), and MSR vaccine containing 5 mg MSRs, 300 μg OVA, 100 μg CpG-ODN and 1 μg GM-CSF (MSR vaccine). Blood sera were collected once every 14 days. Sera were then analyzed for IgG1 and IgG2a antibodies against ovalbumin using ELISA (Biolegend 406603 and Biolegend 407104, respectively). High
affinity plates were coated using OVA (Invivogen vac-pova) and anti-OVA titers were defined as the lowest serum dilution at which the ELISA OD reading was equal to OD value 0.365. In the booster experiments, animals were vaccinated on day 0 with bolus OVA, bolus vaccine, Imject Alum (Pierce) loaded with 300 μg OVA (Alum + OVA), MSR + OVA, and MSR vaccine, and re-vaccinated on day 30 with the same formulations. Imject Alum was loaded with OVA according to the manufacturer's protocol.

**CD4+ T cell cytokine secretion assay**

The spleens of the animals were isolated and digested at day 10 post vaccination. CD4+ T cells were magnetically sorted from each spleen (Miltenyi Biotec). The T cells were then co-cultured with LPS (100 ng/ml)-primed DCs pulsed with 1 μM OVA323-339 peptide (Invivogen) for 24 hours in round bottom 96 well plates. CD4+ T cells and DCs were co-cultured at the ratio of 2 to 1 (T to DC). Media was collected after 24 hours and analyzed for IL-4 and IFNγ using ELISA (eBioscience 88-7711).

**OVA-specific CD4+ T cell expansion and follicular helper T-cell analysis**

Recipient Thy 1.1+ mice were vaccinated subcutaneously with 5 mg MSRs containing 300 μg OVA, 5 mg MSRs containing 300 μg lysozyme, or MSR vaccine containing 5 mg MSRs, 300 μg OVA, 100 μg CpG-ODN and 1 μg GM-CSF. On day 3, 2 ×10^7 splenocytes were isolated from donor OT-II (Thy 1.2+) mice, labeled with CFSE and adoptively transferred i.v. into the recipient vaccinated mice. On day 7, recipient mice were sacrificed, and dLN and spleens were isolated. Cells were analyzed for Thy1.2+ CD4+ (eBiosciences 17-0902, eBioscience 25-0041, respectively) T cell expansion, and
Thy1.2\(^+\) CD4\(^+\) CXCR5\(^+\) (eBiosciences 12-1851) follicular helper T-cells using flow cytometry.

**OVA-specific CD8\(^+\) T cell expansion analysis**

Recipient mice were vaccinated subcutaneously with 5 mg MSRs containing 5 mg MSRs, 300 \(\mu\)g OVA, 100 \(\mu\)g CpG-ODN and 1 \(\mu\)g GM-CSF. On day 3, \(2 \times 10^7\) splenocytes were isolated from donor OT-I mice, labeled with CFSE and adoptively transferred i.v. into the recipient vaccinated mice. Seven days following transfer, recipient mice were sacrificed, and dLN and spleens were isolated and analyzed for CD3\(^+\) CD8\(^+\) (eBioscience 25-0031, eBioscience 17-0081, respectively) T cell expansion.

**Prophylactic tumor study**

Animals were immunized with 5 mg MSRs containing 150 \(\mu\)g OVA (MSR + OVA), MSR vaccine containing 5 mg MSRs, 150 \(\mu\)g or 50 \(\mu\)g OVA, 100 \(\mu\)g CpG-ODN and 1 \(\mu\)g GM-CSF (MSR vaccine), and bolus vaccine containing 150 \(\mu\)g OVA, 100 \(\mu\)g CpG-ODN and 1 \(\mu\)g GM-CSF (Bolus vaccine). After 10 days, animals were challenged with a subcutaneous injection of \(1 \times 10^6\) EG7.OVA lymphoma cells (ATCC) in the back of the neck. Tumor growth was monitored by measuring the tumor length, width and height. Animals were euthanized for humane reasons when tumors grew to 20 mm in longest diameter.
Animal protocol

All animal studies were performed in accordance with NIH guidelines, under approval of Harvard University’s Institutional Animal Care and Use Committee.

Statistical analysis

All values in the present study were expressed as mean ± S.D unless indicated otherwise. Sample sizes were calculated, using InStat software, to allow the statistical significance of differences of 50% or greater to be determined. The specific sample size required depended on the experiment. Statistical analysis was performed using GraphPad Prism and Microsoft Excel. Sample variance was tested using the F test. For samples with equal variance, the significance between the groups was analyzed by a, two-tailed, student’s t test. For samples with unequal variance, a two tailed Welch’s t-test was performed. In all cases, a P value of less than 0.05 was considered significant.

3.3 Results

Incorporation and sustained release of GM-CSF

To recruit host DCs, we sought to incorporate a DC chemoattractant GM-CSF into the mesopores of the MPS/MSRs. GM-CSF has a hydrodynamic radius of about 2nm \(^{33}\), and in theory, can be loaded into the mesopores of the MSRs. The ability of GM-CSF to be loaded into the aligned mesopores of the MSRs and released from the particles in a sustained manner was next examined. 1ug of GM-CSF was loaded into 1mg of MSRs for 8 hours at room temperature and subsequently lyophilized. The
release of GM-CSF, measured with radioactive factor, showed a typical burst of release followed by a sustained release of 66% of the total loaded GM-CSF by day 40 (Fig3.1 A), while the release measured by ELISA shows nearly first-order release with lower level of bioactive GM-CSF released (Fig3.2 B). Previous reports have shown that ELISA is a reliable indicator of GM-CSF bioactivity. These data suggest that though GM-CSF was released from the MPS/MSRs in a sustained the manner, the bioactivity of the released GM-CSF was significantly impaired.

![Fig3.1 A](image1)

**Radio labeled GM-CSF release**

![Fig3.1 B](image2)

**GM-CSF release detection by ELISA**

**Figure 3.1. GM-CSF release profile in vitro.** (A) Cumulative GM-CSF release from MSR measured using radioactive GM-CSF. (B) Cumulative GM-CSF release from MSR measured using ELISA. Data are shown as mean and sd with n = 3.

It has been previously reported that lyophilization in the absence of carrier proteins or surfactants may damage the 3D conformation of proteins, damaging their bioactivity. We investigated whether the low cumulative release of GM-CSF from the MSRs was due to the lyophilization of GM-CSF after loading into the MSR mesopores.
1ug of GM-CSF was loaded into 1mg of MSRs for 8 hours at room temperature. GM-CSF loaded MSRs were either used directly for the release study or lyophilized before the release study. Bioactive GM-CSF release, measured using ELISA, showed that lyophilization led to loss of GM-CSF activity (Fig3.1 B) and direct loading (without lyophilization) completely preserved GM-CSF activity (Fig3.2). In the following studies, GM-CSF was loaded 1 hour before vaccination and was not lyophilized. The bioactivity of GM-CSF after lyophilization can likely be maintained by the addition of surfactants and other carrier proteins, but these possibilities are not investigated here.

![Figure 3.2. GM-CSF release kinetics in vitro.](image)

**Figure 3.2. GM-CSF release kinetics in vitro.** Cumulative GM-CSF release from MSR measured using ELISA. Data are shown as mean and sd with n = 3

**Recruitment of host dendritic cells *in vivo* with GM-CSF releasing MPS/MSRs**

We hypothesized that the recruitment of immature DCs could be enhanced by GM-CSF release. MSRs (5 mg) loaded with 0, 500, 1000, or 3000 ng of GM-CSF were injected into subcutaneous tissue, and retrieved after 7 days to analyze the DC
population. CD11c⁺ CD11b⁺, a widely accepted marker for conventional myeloid lineage DCs was used to determine DC numbers. In addition, the expression levels of the major histocompatibility complex, MHC II, were used to assess DC maturation. Dose dependent increases in CD11c⁺ CD11b⁺ (Fig3.3 A), and CD11c⁺ MHC II⁺ (Fig3.3 B) DCs were found in the scaffolds with GM-CSF release.

The numbers of total cells and DCs were still maintained higher in vaccine scaffolds than in blank scaffold at day 14 (Fig3.4). Henceforward, 1ug GM-CSF was used in vaccination studies.

Figure 3.3. Recruitment of host DCs. (A) Numbers of CD11c⁺ CD11b⁺ and (B) CD11c⁺ MHC II⁺ DCs recruited to MSR-scaffold loaded with different amounts of GM-CSF (n=4). Data are shown as mean and sd with n = 4. * indicates p < 0.05. ns indicates no significance.
The ability of MSRs to provide cues to modulate the phenotype of DCs was then studied. The unmethylated cytosine-phosphate-guanine oligonucleotide (CpG-ODN) sequence, a potent agonist for TLR-9, was used in this study, as previous studies have shown that vaccines containing CpG-ODN can elicit strong CD8⁺ killer T cell mediated immune responses. In vitro, a burst of CpG-ODN release was followed by a sustained release at a much lower rate (Fig3.5 A). To examine whether the incorporated CpG was bioactive, BMDCs were stimulated with either MSR alone or MSR+CpG for 18 hours. BMDCs stimulated with MSR+CpG secreted significant level
of IL-12 compared to MSRs alone, suggesting that CpG maintained its bioactivity after incorporation (Fig3.5 B).

To evaluate whether co-loading of CpG-ODN with GM-CSF could activate DCs in vivo, the expression of CD86 and MHC II in DCs retrieved from MSR releasing solely GM-CSF (MSR-GM), and MSR releasing both GM-CSF and CpG-ODN (MSR-GM-CpG) at day 3 post injection was analyzed. MSR-GM-CpG yielded 1.3- and 2.5-fold increases in the number of recruited DCs expressing CD86⁺ and MHC II⁺, as compared to MSR-GM (Fig. 3.6). As the level of endotoxin in the MSRs was below the detection limit of the endotoxin assay, the activation of DCs was not derived from undesired contamination of MSRs.

Figure 3.5. CpG release from MSRs . (A) Cumulative CpG-ODN release from MSRs as detected using the OliGreen ssDNA detection dye. (B) ELISA analysis of IL-12 secretion from BMDCs stimulated with 5ug MSRs alone or 5ug MSRs loaded with 18mM CpG-ODN. In (B), data are shown as mean and sd with n = 3. ** indicates p < 0.01
MPS/MSR scaffold presents protein antigens in a sustained manner

We next verified that MSRs can be loaded with protein antigens, and present them in a sustained manner. In vitro, OVA was released relatively quickly, with ~45% released within 5 days (Fig3.7 A). Similarly, a mixture of protein lysate derived from B16F10 melanoma tumors can be incorporated into the MSRs and released slowly over time (Fig3.7 B). These release studies suggest that the MPS/MSR scaffold can likely incorporate a range of protein antigens of a variety of molecular weights and net charge.

Figure 3.6. Host DC activation. Levels of CD86 and MHC-II of recruited CD11c⁺ DCs with injection of MSRs loaded with GM-CSF, or both GM-CSF and CpG, respectively. Left: primary flow cytometry plot. Right: quantification of CD86⁺ population or MHC-II⁺ population within the CD11c⁺ population (n=4). Values represent mean and s.d. * represents p<0.05.
To investigate in vivo release of OVA from MSRs, MSRs were loaded with Alexafluor 647-labeled OVA (MSR-OVA*), and the duration of OVA* presence at the vaccine site was compared to the injection of bolus OVA*, using near infrared fluorescent imaging (Fig3.8). The fluorescence at the site of bolus OVA* injection decreased to 10% of the initial level by day 1 post injection, while the MSR-OVA* condition maintained 60% of the initial OVA* level at this time point. At day 10 post injection, the OVA remaining at the vaccination site was still 10.6-fold higher in MSR-OVA* than in the bolus OVA* condition, suggesting that MSRs are a good candidate for sustained release of antigenic proteins in microenvironments housing antigen presenting cells.

**Figure 3.7. Antigen release from MSRs.** (A) Cumulative *in vitro* release of OVA from MSRs determined using BCA. (B) Cumulative *in vitro* release of B16F10 tumor lysate from MSRs determined using BCA. N=3. Values represent mean and s.d.
Figure 3.8. In vivo OVA release kinetics. Representative NIR fluorescent images of mice injected with bolus OVA labeled with Alexafluor-647 (OVA*) or MSRs loaded with same amount of OVA* (top), and relative OVA* remaining in injection sites as a function of time, as based on fluorescent imaging (bottom) (n=4). Values represent mean and s.d.

MSR vaccine modulates DCs to exert systemic effects

MSR-based scaffolds (5 mg MSRs) containing OVA, GM-CSF, and CpG-ODN were then examined for their ability to function as vaccines. Cell recruitment to vaccine scaffolds, compared with blank MSR scaffolds, at different time points was analyzed.
The total number of cells recruited to both systems increased over time, but the vaccine MSR scaffold held 1.8- and 6.5 fold higher numbers of cells at day 5 and 7, respectively, than blank MSR scaffold (Fig3.9 A). CD11c⁺ DCs increased over time, and ~4-6-fold higher numbers were observed than in blank MSR scaffolds (Fig3.9 B).

**Figure 3.9. Cell recruitment kinetics to MSR vaccine site.** Total number of recruited host cells (A) and CD11c⁺ DCs (B) in blank or vaccine MSR scaffolds (5 mg), respectively (n=4). Values represent mean and s.d. *represents p<0.05.

The level of GM-CSF in the tissue between 1 mm and 3 mm from the injection site was also analyzed, to confirm GM-CSF release in vivo (Fig3.10). Higher GM-CSF levels were maintained for over a week, compared to delivery of blank MSR particles.
The composition of immune cell infiltrate at the MSR vaccine site was analyzed. Immediately after vaccination, on day 1, few immune cells had infiltrated into either the blank MSR scaffold or the MSR vaccine scaffold, consistent with our previous findings (Fig 3.9A). On day 3 and day 5, the vaccine site was enriched with CD11c⁺ DCs (Fig 3.11A), Gr.1⁺ myeloid cells (Fig 3.11B) and NK1.1⁺ NK cells (Fig 3.11C) compared to the blank MSR scaffold. Myeloid cells were the predominant cell type infiltrating into both the blank and the vaccine scaffold (>50%), DCs accounted for ~20% of the cells infiltrating into the scaffolds, and NK cells were a relatively insignificant population.

Figure 3.10. GM-CSF level in MSR vaccine tissue. *In vivo* GM-CSF levels in tissue around the injection sites of blank or vaccine MSR scaffolds measured using Bioplex (n=4).

![Graph showing GM-CSF levels over time](image)

**Note:** The graph shows the levels of GM-CSF over time (days) with error bars indicating variability. The blue line represents the blank scaffold, and the red line represents the vaccine scaffold. Significant differences are indicated by asterisks.
The functional role of the myeloid cells was unclear. It is possible that they differentiate into myeloid-lineage DCs and participate in antigen presentation.

The ability of recruited host DCs to traffic to dLNs and prime T and B cells

The ability of assembled MSRs to allow recruited DCs to process antigen, subsequently traffic to the draining lymph nodes (dLN), and to interact with other immune cells, was then analyzed. Mice were initially immunized with MSR containing only OVA* or both OVA* and GM-CSF. Seven days after injection, the cells in dLN were extracted and analyzed. Delivery of the antigen alone in the MSR scaffold resulted in a slight increase in the number of AF647+ CD11c+ DCs in the dLN, while with the addition of GM-CSF, the number was drastically increased (Fig3.12 A). Including CpG with the GM-CSF and OVA* in the MSR scaffold further increased the number of CD11c+ CD86+ activated DCs in the dLN, compared to MSR releasing only GM-CSF and antigen (Fig3.12 B).
To test the ability of recruited DCs to process antigen at the site of vaccination and traffic to the dLN, MSRs were loaded with 300 µg of OVA, and the presence of DCs in the dLN presenting OVA257-264 peptide (SIINFEKL) on the major histocompatibility complex I (MHC I) was analyzed 7 days after injection. The MSR vaccine was capable of generating a significant number of SIINFEKL-MHC-I⁺ CD11c⁺ DCs in the dLN (Fig3.13).
To test the ability of the MSR-primed DCs to interact with other immune cells in the dLN, mice were again immunized and the dLNs were harvested and analyzed for formation of a germinal center (GC) where immature B cells with antigenic information undergo affinity maturation and somatic hypermutation to generate specific antibody producing plasma B cells. MSR with OVA alone, and the full MSR vaccine both elicited strong GC formation (Fig3.14 A) in an OVA dose-dependent manner (Fig3.14 B), indicating that the antigen-presenting DCs exert downstream effects on the B cells in the dLN. Taken together, these data suggest that the MSR vaccine is able to recruit DCs, program them with danger signal while loading them with antigen, and enhance their trafficking to the dLN to present processed antigen to other immune cells.

Figure 3.13. Antigen presenting DCs in dLN after vaccination. Numbers of DCs presenting SIINFEKL-MHCI in dLNs at day 7 post immunization (n=4). Values represent mean and s.d. *represents p<0.05.
Additionally, we also analyzed the change in spleen size after vaccination. There is significant increase in spleen size, determined by the number of splenocytes, after 5 days post vaccination (Fig 3.15). Strikingly, by day 7, spleens from mice that received the full MSR vaccine was ~4 times larger than mice that received only the blank MSR scaffold. It is likely that the spleen enlargement is induced by the rapid release of CpG from the vaccine; from our observations, mice that received a bolus CpG injection or a bolus vaccine also had significantly enlarged spleen on day 7. Further observations showed that the spleen enlargement was not persistent as they were reduced to normal size after ~14-20 days. The mice did not show abnormal signs of distress at all times after vaccination.

Figure 3.14. Germinal center in dLN after vaccination. (A) Numbers of B220$^+$ GL7$^+$ germinal center B cells at day 7 post immunization in untreated mice or mice treated with MSR containing various cargoes. (B) Impact of OVA dose on number of germinal center B cells (right) (n=4). Values represent mean and s.d. *represents p<0.05.
Finally, the ability of the MSR vaccine to induce antigen specific adaptive immune responses was studied. A single immunization with MSRs loaded with OVA, GM-CSF and CpG elicited strong and durable titers of sera anti-OVA IgG2a (Fig3.16 left) and IgG1 (Fig3.16 right), indicative of T₉₁ and T₉₂ responses, respectively. OVA specific antibody levels are measured using ELISA. ELISA plates coated with a control protein, BSA, showed no detectable titer. Notably, immunization with MSRs loaded with only OVA elicited a strong antibody response as well, but the response was primarily skewed towards a T₉₂ response. By contrast, vaccination with equivalent amounts of GM-CSF, CpG-ODN and OVA as delivered by the MSR, but in bolus form instead, only elicited a moderate and T₉₁ skewed response that soon decreased after
100 days. Vaccination with bolus OVA alone led to minimal antibody generation, as expected.

To further characterize the T\textsubscript{H}1 and T\textsubscript{H}2 responses elicited by MSRs loaded with OVA (MSR + OVA) and the MSR vaccine, we analyzed the production of IFN\textgamma, a key T\textsubscript{H}1 cytokine, and IL-4, a key T\textsubscript{H}2 cytokine, after co-culture of the spleen CD4\textsuperscript{+} T cells (isolated at day 10 post vaccination) with OVA\textsubscript{323-339} peptide-pulsed BMDCs (Fig3.17). MSR+OVA and the full MSR vaccine showed equally high production of IFN\textgamma, indicating that the MSR scaffold is capable of driving a strong immune-stimulatory response against OVA. However, the MSR+OVA condition resulted in significantly higher IL-4 production as compared to the MSR vaccine. Taken together, CD4\textsuperscript{+} T cells primed by the MSR+OVA had a lower IFN\textgamma to IL-4 ratio, which could explain the observation that the MSR vaccine leads to high levels of both IgG2a and IgG1 serum.

**Figure 3.16. Anti-OVA antibody titers after vaccination.** ELISA analysis of sera OVA-specific IgG\textsubscript{2a} (left) or IgG\textsubscript{1} (right) after immunization with bolus OVA, soluble components of the vaccine (bolus vaccine), MSRs loaded with OVA, or MSR vaccine, respectively (n=5). Values represent mean and s.d. *represents p<0.05 between vaccine and bolus groups.
antibodies, whereas the MSR+OVA formulation was only able to elicit a strong IgG1 serum antibody.

To compare the MSR vaccine with a conventional adjuvant vaccine using a prime followed by boost strategy, animals were vaccinated with bolus OVA, bolus vaccine formulation (GM-CSF + CpG + OVA), Imject Alum with OVA, MSR with OVA, and the MSR vaccine formulation on day 0, and boosted with the same formulations on day 30. We collected serum biweekly or weekly and measured serum IgG2a (Fig3.18 left) and IgG1 (Fig3.18 right) antibody against OVA. MSR vaccine resulted in the highest IgG1 and IgG2a titers as compared with all other groups after primary vaccination. Boosting further increased antibody production in all conditions. Boosting increased the anti-OVA IgG2a level resulting from the bolus vaccine formulation to a similar level as that induced by the first injection of the MSR vaccine. This suggests

Figure 3.17. Cytokine secretion from peptide stimulated T cells after vaccination. The level of (a) IFNγ and (b) IL-4 in co-culture of CD4+ T cells retrieved from vaccinated mice and peptide-pulsed BMDCs at 1:2 DC:T ratio for 24 hours (n=4). Values represent mean and s.d. *represents p<0.05.
that the MSR vaccine is a potent platform to induce high serum antibody titers and has potential as a single-shot vaccine technology.

**Figure 3.18. Anti-OVA antibody titers using a prime-boost vaccine strategy.** ELISA analysis of sera OVA-specific (c) IgG2a and (d) IgG1 after immunization with bolus OVA, bolus vaccine formulation (GM-CSF, CpG, OVA), Imject Alum with OVA, MSR with OVA, and the MSR vaccine formulation on day 0, and boosted with the same formulations for each condition on day 30 (n=5). Values represent mean and s.d. *represents p<0.05 between vaccine and bolus groups.

Strong humoral responses are highly dependent on the action of CD4+ T follicular helper (T_{FH}) cells. Therefore, we investigated whether the MSR vaccine induced the differentiation of antigen specific TFH cells. Following vaccination with MSRs containing OVA, MSRs containing lysozyme as a negative control, and the full MSR vaccine, 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) stained Thy1.2+ splenocytes from OT-II mice were adoptively transferred into Thy 1.1+ C57Bl/6 mice (**Fig3.19 A**). The CD4+ T cells in OT-II mice have T cell receptors that specifically recognize a sequence on the OVA protein. Four days after immunization, the dLN and spleens were harvested, and the transferred Thy 1.2+ cells were analyzed. Mice immunized with the full MSR vaccine or MSR loaded with OVA showed significant
proliferation of Thy1.2+ CD4+ T cells (Fig3.19 B), and generated a significant CD4+ CXCR5+ T helper cell clonal expansion (Fig3.19 C) and T_{FH} differentiation, whereas mice vaccinated with lysozyme as antigen did not. Together, these data suggest the MSRs have the ability to incorporate and release a variety of factors to induce both T_{H1} and T_{H2} responses.
To further investigate if the MSR vaccine can enhance CD8+ cytotoxic T lymphocyte (CTL) immune responses, C57Bl/6 mice were immunized with the full MSR.
vaccine. Seven days after immunization, the strength of the CD8+ T cell response was probed by analyzing the frequency of SIINFEKL-tetramer+ CD8+ T cells and intracellular IFNγ+ CD8+ T cells in the spleen. High numbers of SIINFEKL-tetramer+ CD8+ T cells (Fig3.20 A) and IFNγ secreting CD8+ T cells (Fig3.20 B) were found in the spleens of vaccinated mice.

![Figure 3.20. Antigen specific CD8 T cell response after vaccination. Number of tetramer+ CD8+ T cells (g) and IFN-γ+ CD8+ T cells (h) in spleen 7 days after vaccination with blank MSR (Blank) or complete MSR vaccine (Vaccine) (n=4). Values represent mean and s.d. *represents p<0.05.](image)

To evaluate antigen-specific CD8+ T cell expansion in vivo, CFSE-stained splenocytes isolated from OT-I transgenic mouse were adoptively transferred into
naïve mice, and mice immunized with the MSR-vaccine. CD8+ CTL T cells in OT-I mice have a transgenic T cell receptor designed to recognize SIINFEKL-MHC I complex expressed on antigen presenting cells (Fig3.21 A). Seven days after adoptive transfer of CFSE-stained OT-I T cells, the cells in LNs and spleen were retrieved from the recipient mice and CFSE fluorescence was analyzed by flow cytometry. The intensity of the CFSE fluorescence was measured as an indicator of T-cell proliferation, revealing that OT-I T cells proliferated substantially both in LNs and spleen of vaccinated mice (Fig3.21 B). These data indicate immunization with the MSR-vaccine induces antigen specific cellular responses that result in T cell proliferation in response to the antigenic information.
To demonstrate one possible application of this approach, we investigated its effect in generating prophylactic tumor protection. Mice were immunized with MSR vaccines or controls, and subsequently challenged with a subcutaneous injection of EG7.OVA lymphoma cells. MSR vaccines containing CpG-ODN, GM-CSF and OVA were found to delay the onset of tumor growth compared to bolus delivery of the same
immune stimulatory agents, a mimic of conventional vaccination (Fig. 3.22 A). MSRs containing OVA (MSR + OVA) suppressed tumor growth in the early stage, but differences over the control condition were lost with time. A bolus vaccine (GM + CpG + OVA 150 μg) resulted in tumor initiation at a similar time point (day 7) as naïve mice, but delayed tumor growth significantly. Tumor growth was much more delayed upon injection of MSR vaccines loaded with 50 μg or 150 μg of OVA. Tumor growth was first observed on day 21, and tumor volume was significantly less than the other conditions at all-time points. The corresponding survival rate also supports the potential utility of the current MSR strategy for prophylactic cancer vaccines (Fig. 3.22 B).
Figure 3.22. Prophylactic tumor study using the EG.7 model. Prophylactic cancer vaccine study using injectable MSRs. (A) EG.7-OVA tumor volume and (k) survival rate after subcutaneous injection of various vaccine formulations 10 days before tumor inoculation (n=10). In (B), the tumor volumes were compared on day 21, 23 and 25, following the onset of tumor growth in the vaccine group. N=10. Values represent as mean ± SEM. *, **, and *** represents p<0.05, p<0.01, and p<0.001, respectively.
3.4 Discussion

To modulate the recruited host immune cells, sustained release of a small inflammatory molecule (GM-CSF), an ssDNA TLR agonist (CpG-ODN), and a model antigen protein (OVA) was achieved by exploiting the high surface area and pore volume of the MSRs. This approach prolongs presentation of these molecules in the surrounding tissue, allowing one to modulate DC enrichment, activation and antigen processing, and subsequent homing to lymph nodes. The current release profile is based on diffusion of loaded molecules from the mesopores, but the release rate can be further actively controlled\textsuperscript{38,39}.

We observe significant enrichment of myeloid DCs in GM-CSF releasing scaffolds, which is likely a combined effect of peripheral DC migration and in situ differentiation of DCs from precursors. Prolonged presentation of antigens and adjuvants to immune cells, which is believed to be crucial to enable long-term stimulation of DCs in order to break immune tolerance\textsuperscript{40}, has been demonstrated to yield potent immune responses in past studies\textsuperscript{41,42}. MSRs loaded with OVA, GM-CSF, and CpG-ODN were here found to enhance systemic IgG2a and IgG1 serum antibody levels and CTL responses, compared to bolus vaccine formulations. The induction of these strong humoral and cellular immune responses is likely a result of the high number of DCs, their sustained and prolonged activation and priming, and their subsequent interactions with CD4 and CD8 T cells in the lymph node.

The ability to tune the immune response is one of the advantages of the MSR system. In this study, CpG was used to activate the DCs to secrete T\textsubscript{h}1 cytokines and prime CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells. The boost of IgG2a antibody response and anti-tumor
effect with the MSR vaccine are likely the manifestation of a T\textsubscript{H}1 response. Sustained delivery of antigens and adjuvants from the MSRs likely contributes to, though is not solely responsible for, generating high serum antibody titers. Previous reports using polymer nano- or microparticles to release antigens and adjuvants\textsuperscript{43,44} have led to moderate antibody titers, and required a secondary boost. In contrast, with MSRs, a single prime injection was sufficient to induce antibody titers over 2 orders of magnitude higher than the bolus control, and comparable to a prime-boost using bolus vaccination. Although Alum loaded with OVA and CpG induces high IgG1 serum antibody titer, it induces lower IgG2a serum titer than bolus formulations composed of OVA and CpG, suggesting it is not effective at inducing a T\textsubscript{H}1 skewed immune response\textsuperscript{45}. In contrast, the MSR vaccine leads to high levels of both IgG2a and IgG1 serum antibodies. These results indicate that cell enrichment along with sustained release of antigens and adjuvants plays a key role in driving the potent immune response. The immunoactive nodule generated by the MSR system can likely generate cell-cell and cell-material interactions that are crucial to the development of potent CTL and antibody responses.

The biodegradation and safety of the injected MSRs should be considered for potential medical applications. Mesoporous silica is composed of an amorphous silica which degrades in the body over time\textsuperscript{46,47}. Synthetic amorphous silica is generally recognized as safe by the FDA, and is used in cosmetics and as a food additive. The current administration route (subcutaneous injection) and quantity (5 mg) of MSR used in these studies resulted in a significant biodegradation in 28 days, and did not cause severe local inflammation, side effects, or animal mortality, indicative of the
biocompatibility of this technology. This is obviously in contrast with another type of fibrous silica, asbestos, which is a crystalline material that does not degrade in the body, and thus acts as a carcinogen. The in vivo degradation and clearance of the MSRs in the context of a full vaccine should be carefully examined in the future.

This injectable scaffold may provide a useful platform for prophylactic and therapeutic vaccination. The success of the HPV vaccine in cervical cancer has created interest in additional prophylactic opportunities for other types of cancer where individuals at high risk (e.g., breast cancer) can be identified in advance, and tumor antigens common to many patients’ or patient specific antigens are being actively identified. The prophylactic tumor vaccine study using MSRs clearly demonstrates that this injectable scaffold system has potential to suppress the growth of tumors. However, the major value of this new technology would likely be as a therapeutic cancer vaccine, and additional studies are required to test its activity in that setting. In addition, the striking serum antibody response induced by this approach is likely relevant to the treatment of viral diseases that are resistant to conventional treatment. The injection site reactions may not be acceptable in situations where an effective immune response can be generated with conventional vaccine technologies, nor in situations where the disease does not present a significant danger to the patient. However, in diseases that are both life-threatening and resistant to conventional therapy, we do not believe an injection site reaction would be a significant impediment to vaccine use.
3.5 Acknowledgements

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Chapter 4: Mesoporous silica scaffold vaccine for enhancing single-shot humoral responses against peptide antigens

4.1 Introduction

Immunotherapies have shown significant clinical success and preclinical promise. They are rapidly becoming a standard approach for cancer and chronic infectious disease treatment, and immunocontraceptive vaccination\(^1,2\). Vaccines can induce strong protective and therapeutic responses against many forms of live and attenuated virus, toxoids, irradiated autologous tumor cells and protein subunits\(^3\). However, most effective vaccines require several boosts after the primary immunization. These dosing regimens are costly, increase hospital/clinic visitation for patients and are especially challenging in regions where limited healthcare access poses a major logistical barrier to disease treatment and management\(^3,4\). An effective single-shot vaccine could overcome these limitations. Most approaches for single-shot vaccines in clinical testing to date have used depots of antigens that were subsequently released in a controlled manner for months after immunization\(^3,5-7\). However, passive diffusion of the antigen and adjuvant to the lymph nodes alone is likely unable to maximally and rapidly stimulate immune cells and provide life-long benefit. Therefore, achieving a robust and lasting immune response after a single injection of a vaccine remains an important challenge.

Peptide vaccines have many potential advantages but generating potent responses is challenging. Peptide vaccines can induce responses against defined
antigen epitopes while avoiding adverse off target effects. Peptides can be easily synthesized, up-scaled and have good safety and stability\(^8\). They can also be readily presented by antigen presenting cells (APCs) to induce subsequent effector T cell and humoral responses. Furthermore, antibodies raised against peptides have biological activity and high epitope specificity. For instance, a synthetic peptide vaccine against subtypes of influenza using Keyhole limpet hemocyanin (KLH) as a carrier protein generated anti-hemagglutinin antibodies and neutralizing activity\(^9\). However, synthetic peptide vaccines generally cannot confer long-lived, therapeutic benefit. This limitation is likely caused by a combination of 1) short peptide half-life due to rapid systemic clearance, 2) weak immunogenicity due to lack of a co-stimulatory signal, and 3) absence of B-cell receptor (BCR) cross-linking due to lack of multivalency\(^10\)-\(^12\).

One attractive target for vaccination is gonadotropin-releasing hormone (GnRH), a decapeptide hypothalamic hormone that controls male spermatogenesis, and female estrogen and follicular development. GnRH determines both fertility and sexual behavior and is highly conserved among mammals\(^13\). GnRH activity has also been implicated in the progression of several types of cancer including prostate, breast and ovarian\(^14\)-\(^16\). Blocking GnRH activity using androgen deprivation therapies (ADTs) and gene therapy has shown pre-clinical promise, but such therapies have strong toxic side effects and lack long term potency\(^17,18\). Active immunizations against GnRH using various carrier proteins such as KLH, ovalbumin (OVA) and diphtheria toxoid (DT) have been shown to decrease sexual organ function, reduce testosterone levels in patients with advanced prostate cancer and reduce reproductive capability in feral animals as an humane alternative to surgical desexing\(^17,19,20\). However, these vaccines require
multiple immunizations and the effects are variable and not potent enough to produce long-term immune-castration.

Here we propose a single injection vaccine platform using a mesoporous silica micro-rod (MSR) vaccine system to generate high antibody titers against synthetic peptide antigens. In the previous chapters, we demonstrated that MSR particles could spontaneously assemble into a 3D scaffold after subcutaneous injection; the macropores formed by random stacking allowed for cell infiltration and active cell-scaffold interactions. The controlled release of GM-CSF and CpG from the MSR vaccine modulated host APC infiltration into the vaccine and, consequently, continuously programmed host APCs. Continuous antigen uptake and processing by APCs and persistent toll-like receptor (TLR) priming have been shown to amplify antigen specific humoral immunity. Therefore, we hypothesized that a single injection of the MSR vaccine could generate robust and long-lasting humoral immunity against small peptides, and enhance the response compared to traditional vaccine approaches. We focus on GnRH as the antigen, but also explore this concept with a Her2 epitope within the Trastuzumab-binding domain.

4.2 Materials and Methods

Peptides and proteins.

All peptides used in this study were synthesized at least 95% purity from Peptide 2.0. Peptide sequences are as follows: CGnRH (CEHWSYGLRPG), scrambled CGnRH peptide (CRSYGPLHEWG), MVPGnRH (KLLSLIKGIVHRLEGVEGPSLEHWSYGLRPG), MVPHer2 (KLLSLIKGIVHRLEGVEGPSLIWKFPDEEGACQPL). Endotoxin free
ovalbumin was purchased from Invivogen (vac-pova), and maleimide activated KLH was purchased from ThermoFisher Scientific (77605).

**MSR synthesis and MSR vaccine formulation.**

MSRs were fabricated as described previously with minor modifications. Briefly, 4g of P123 surfactant (average Mn ~5800, Sigma-Aldrich) were dissolved in 150 g of 1.6 M HCl solution and stirred with 8.6 g of tetraethyl orthosilicate (TEOS, 98%, Sigma-Aldrich) at 40 °C for 20 h, followed by aging at 100 °C for 24 hours. TEOS was extracted in 1% EtOH in HCl at 80 °C for 18 hours. To prepare the MSR vaccines, 4mg of the MSR were adsorbed with 100ug murine class B CpG-ODN (sequence TCCATGACGTTCCTGACGTT, IDT) and 100ug or 30ug of the antigen (peptide or peptide-carrier protein conjugate) for 8 hours at room temperature under shaking, and subsequently lyophilized. Separately, 1mg of the MSRs were loaded with murine GM-CSF (Peprotech) for 1 hour at 37 °C under shaking. The MSRs were combined and resuspended in cold PBS prior to immunization.

**Peptide protein conjugation.**

To conjugate CGnRH to ovalbumin (OVA), OVA was first reacted with 30 molar excess of sulfo-SMCC (ThermoFisher Scientific) in PBS for 1 hour at room temperature. Subsequently, the maleimide-OVA was desalted to remove the excess sulfo-SMCC. Separately, CGnRH or CHer2 was reduced using 2 molar excess of TCEP (ThermoFisher Scientific) for 1 hour at room temperature. 100ug of Maleimide-OVA was
then reacted with 100ug the CGnRH peptide in PBS for 12 hours at room temperature under shaking.

**Immunization.**

Unless otherwise noted, all in vivo studies were carried out using female C57BL/6J mice (Jackson Laboratories) between 6 to 10 weeks old at the beginning of the experiment. MSR vaccines were resuspended in 150ul of cold PBS and injected, via an 18G needle, subcutaneously in the intrascapular region with the mouse under brief isoflurane anesthesia. We have demonstrated that the MSR vaccine can be injected using 23G needles, but we used 18G needles in these studies to be consistent with our previous studies. All animal studies were performed in accordance with NIH guidelines, under approval of Harvard University’s Institutional Animal Care and Use Committee.

**Sera titer analysis using ELISA.**

Peripheral blood was collected periodically after immunization. Sera samples were analyzed for IgG1 (BD Biosciences), IgG2a (BD Biosciences) or total IgG (Biolegend) levels using ELISA. Briefly, ELISA plates were coated overnight in 4 °C with 30ug/ml of GnRH or 10ug/ml Her2 peptides in PBS. ELISA was performed according to established procedures, and anti-GnRH or anti-Her2 titers were defined as the lowest serum dilution at which the ELISA OD reading was equal to OD value 0.2.
In vitro MVPGnRH release.

100ug of MVPGnRH was loaded onto 2mg of MSRs in PBS for 8 hours at room temperature. Separately, 100ug of MVPGnRH was loaded onto Alhydrogel Alum (Invivogen) according to the manufacturer’s protocols. The MSRs and Alum were lyophilized, and then reconstituted in PBS. Supernatant containing released MVPGnRH was collected periodically. The release of MVPGnRH was quantified using Lavapep (GelSciences) according to the manufacturer’s protocols.

Germinal center characterization.

To analyze GC formation, dLNs were isolated on days 7, 14, 25 and 50 after immunization. Cells were enumerated, and stained with anti-mouse B220 (eBioscience), anti-mouse GL7 (eBioscience) and Rhodamine-PNA (Vector) for 15 minutes on ice. Cells were washed and assessed using flow cytometry (BD Fortessa or BD LSR-II).

Lymph node histology.

dLNs were fixed for 1 hour with 4% paraformaldehyde (Thermofisher Scientific), embedded in Tissue-tek OCT (VWR) and cryo-sectioned using a Leica CM1950 Cryostat. Various sections from one LN were stained with anti-B220 (eBioscience) and anti-GL7 (eBioscience) and visualized using confocal (Zeiss LSM 710).

Cell isolation from MSR scaffolds explanted from animals.

Scaffolds were excised on day 1, 3, 5, 7 or 15 after immunization. The tissues were processed through mechanical disruption and suspended in PBS. The resulting cell
suspension was then filtered through a 40 μm cell strainer to isolate the cells from the larger sized MSRs. The cells and small remaining MSR particles were pelleted, washed with cold PBS, and counted (Beckman-Coulter). The portion of cells in the mixture of cells and small silica particles was accessed in SSC and FSC gating in flow cytometry (BD LSRII or BD Fortessa). Based on the counts from Coulter counter and the percentage of cells determined from FACS gating, the number of live cells in the MSR scaffolds could be calculated.

Analysis of DC recruitment to MSR scaffolds.

APC-conjugated CD11c (eBioscience), FITC-conjugated CD11b (eBioscience) stains were conducted for DC and leukocyte recruitment analysis, and APC-conjugated CD11c and PE-conjugated CD86 stains were conducted for DC maturation analysis. Cells were stained with 7-AAD and fluorophore conjugated antibody for 15 minutes on ice, washed thoroughly and analyzed using flow cytometry (BD LSRII or BD Fortessa). Cells were first gated according to the viability channel, live cells were then gated according to positive FITC, APC and PE using isotype controls, and the percentages of cells staining positive for each surface antigen were recorded.


Four weeks after immunization, peripheral blood was collected. SK-BR-3 cells or CT26 cells were incubated in sera diluted to 1:100 for 1 hour on ice. Cells were thoroughly washed and stained with PE-conjugated anti-mouse pan-IgG secondary antibody
(Jackson ImmunoResearch) for 20 minutes on ice, washed extensively and analyzed using flow cytometry.

**Mass spectrometry.**
GnRH-OVA conjugate was synthesized as described above and desalted. OVA and the conjugates were analyzed using Agilent 6460 Triple Quadrupole Mass Spectrometer equipped with Agilent 1290 uHPLC.

**Statistical analysis.**
All values in the present study were expressed as mean ± S.D. Statistical analysis was performed using GraphPad Prism and Microsoft Excel. For serum antibody analysis, significance was determined using unpaired Mann–Whitney tests. For all other analyses comparing multiple groups, two-way ANOVA tests were performed. For all statistical tests, p-values < 0.05 considered significant.

### 4.3 Results

**Single injection of MSR vaccine with GnRH peptide conjugated to OVA (GnRH-OVA).**

To evaluate whether a CD4+ helper T cell epitope was necessary to generate an anti-GnRH response, we incorporated either free GnRH peptide or GnRH peptide conjugated to the carrier protein ovalbumin (OVA) into the MSR vaccine. MSR vaccines were formulated to contain 1μg GM-CSF and 100μg CpG-ODN, as previously described, and either 100μg free GnRH or 300μg GnRH conjugated to OVA (GnRH-OVA) (Fig4.1).
GnRH-OVA conjugation was confirmed using mass spectrometry (Figure S1). The loading efficiencies of free GnRH and GnRH-OVA onto the MSRs were 91 \pm 2\% and 85 \pm 1\%, respectively.
Vaccines were injected into the subcutaneous tissue of mice, and antibody titers were subsequently measured using ELISA. Strikingly, a single immunization with the MSR vaccine containing GnRH-OVA elicited strong titers of anti-GnRH IgG1 (Fig4.3 A) and IgG2a (Fig4.3 B) serum antibody for over 12 months. In comparison, immunization with MSRs loaded with unconjugated GnRH did not elicit detectable anti-GnRH titer.

Figure 4.2. Characterization of GnRH-OVA conjugate by Mass Spectrometry.
Characteristic ESI-TOF profile of (A) Ovalbumin (OVA) and (B) GnRH peptide conjugated to OVA. GnRH-OVA conjugate show characteristic mass increase of 1519 to 1536 Da. The theoretical added mass of one GnRH moiety (1304.44 Da) plus one SMCC crosslinker (219.09) is 1523.53 Da.
To confirm the serum anti-GnRH antibody recognized the GnRH peptide sequence specifically, serum from animals vaccinated with the MSR GnRH-OVA vaccine were tested using an ELISA coated with GnRH peptide or a scrambled peptide (CRSYGPLHEWG) (Fig 4.4 A). The serum antibody recognized the GnRH peptide in a dilution-dependent manner but did not recognize the scrambled peptide. Finally, to confirm that the anti-GnRH antibody response was specific to the MSR GnRH-OVA vaccine, we immunized mice with MSR vaccines containing OVA. Serum from animals vaccinated with the MSR OVA vaccine did not elicit any detectable anti-GnRH IgG1 (Fig 4.4 B) or IgG2a (Fig 4.4 C) titers.

Figure 4.3. Serum anti-GnRH antibody titer after immunization. ELISA analysis of sera GnRH-specific IgG1 (C) or IgG2a (D) after immunization with MSR vaccines loaded with free GnRH or GnRH-OVA (mean and s.d., n = 4) * indicates p < 0.05.
We next investigated whether the MSR vaccine can elicit higher antibody titers compared to traditional bolus immunizations. Mice were again immunized with a...
single injection of the MSR vaccine containing 1ug GM-CSF, 100ug CpG-ODN and 300ug GnRH-OVA, or a single injection of a bolus vaccine containing 1ug GM-CSF, 100ug CpG-ODN and 100ug GnRH-OVA on day 0 (no MSR). The MSR vaccine induced significantly higher anti-GnRH IgG1 (Fig4.5 A) and IgG2a (Fig4.5 B) antibody titers compared to the bolus vaccine. The effect was also durable, lasting for at least 100 days post immunization. Impressively, the MSR vaccine induced rapid onset of antibody production. On day 14, the titer elicited by the MSR vaccine was 35 fold and 80 fold higher than that elicited by the bolus vaccine for IgG2a and IgG1 subtypes, respectively. KLH is the most commonly used carrier protein immunogen to induce antibody production, and is highly immunogenic due to its large molecular weight and complex chemical structure. Using KLH as the carrier protein in place of OVA, the MSR vaccine again significantly enhanced the total serum anti-GnRH IgG response compared to a bolus KLH-GnRH vaccine (Fig4.5 C). At the peak of the response, the titer elicited by the MSR vaccine was nearly 10 fold higher than that elicited by the bolus vaccine.
Temporal impact of MSR vaccine on immune response.

To begin to determine if the duration of the MSR scaffold following injection impacted the anti-GnRH antibody titers, the kinetics of cell recruitment by the MSR GnRH-OVA vaccines was first quantified. The MSR vaccine has been engineered to recruit and activate host immune cells by incorporating and releasing GM-CSF and CpG-ODN (27). Consistent with work described in Chapter 3, host immune cells were shown to infiltrate the MSR scaffold in a time dependent manner, peaking at day 7 (Fig 4.6 A). CD11c⁺CD11b⁺ DCs were detectable at day 1 post immunization, their percentage peaked at day 3 and dropped sharply at day 5, followed by a secondary increase from day 7 to day 14 (Fig 4.6 B). Recruited DCs were activated by the
released CpG-ODN, as indicated by a higher percentage of CD11c⁺CD86⁺ DCs on day 3 after immunization (Fig 4.6 C). The percentage of activated DCs dropped from day 3 to day 5, likely due to their homing to the draining lymph node (dLN). The percentage of activated DCs in the scaffold remained low from day 5 to day 14.

![Figure 4.6. Cell infiltration into the MSR GnRH-OVA vaccine.](image)

The size of the MSR vaccine scaffold also varied over 100 days. The scaffold nodule was apparent 24 hours post immunization, and initially increased over time, with its size peaking at day 7. After day 7, the scaffold volume reduced and the scaffold was not visible after day 40 (Fig4.7 A). On day 50, skin around the immunized area was excised, and no visible MSR scaffold material was observed. Additionally, fluorescent microscopy of the MSR AF488-OVA (MSR-OVA*) vaccine on days 7, 15 and 30 revealed a reduction in MSR in the vaccine site (Fig4.7 B). Together, these data suggest the MSR GnRH-OVA vaccine induces rapid DC infiltration, activation and
exfiltration, and serves as a site of sustained antigen presentation that may program immune cells for extended periods.

**Figure 4.7. Kinetics of MSR GnRH-OVA vaccine.** (A) MSR vaccine scaffold vaccine volume over time. (mean and SD, n=4), **** indicates p <0.0001 (B) Confocal images of MSR-OVA+ vaccine scaffold explanted on day 7, 15 and 30. Scale bar: 500µm

To investigate the relationship between the infiltrated immune cells and the anti-GnRH titer, the MSR vaccines were explanted at days 1, 3, 5, 7 and 15 after immunization (**Fig4.8 A**). Vaccination durations of less than 5 days resulted in no significant overall anti-GnRH IgG1 (**Fig4.8 B**) or IgG2a (**Fig4.8 C**) titers. Vaccines that were explanted on day 5 led to detectable amounts of IgG1 titer on day 14 and 20, but the response was transient and no titers were detected from this condition after 20 days. In contrast, a vaccination duration of 7 days or greater led to prolonged anti-GnRH IgG1 (**Fig4.8 D**) and IgG2a (**Fig4.8 E**) titers. Vaccines that were explanted on day 7 resulted in a significantly slower onset of titer production compared to vaccines that were explanted on day 15 and those that were not explanted. There was no difference in titer levels between vaccines that were explanted on day 15 and those
that were not explanted. Together, these data suggest that vaccine duration of 15 days is necessary to generate both rapid onset and durable high anti-GnRH titers.

Germinal Center (GC) activity after immunization.

A strong humoral response depends on activated B cells forming GCs in secondary lymphoid organs, where they further undergo somatic hypermutation and

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**Figure 4.8. The effect of MSR vaccine duration on anti-GnRH antibody production.**

(A) Schematics of MSR vaccine regimen. Vaccine duration is varied from 1, 3, 5, 7 and 15 days, or indefinitely. (B+C) ELISA analysis of sera GnRH-specific IgG1 (B) or IgG2a (C) antibody from mice immunized with the MSR vaccine for a duration of 1 (Day 1), 3 (Day 3), 5 days (Day 5) or indefinitely (Vax). (D+E). ELISA analysis of sera GnRH-specific IgG1 (D) or IgG2a (E) antibody from mice immunized with the MSR vaccine for a duration of 7 (Day 7), 15 days (Day 15) or indefinitely (Vax). (mean and s.d., n=4) a indicates p < 0.05 between Vax and Day 1, b between Vax and Day 3, c between Vax and Day 7 and * between Vax and all other groups.
isotype switching, and eventually differentiate into plasma cells that can rapidly produce antibodies upon secondary encounter with the antigen\(^ {21}\). Therefore, we investigated whether a single injection of the MSR GnRH-OVA vaccine can stimulate enhanced GC formation compared to the conventional bolus vaccination. The MSR GnRH-OVA vaccine induced a prolonged immune response, as indicated by the increased total cell number in the dLN from 7 days to 25 days after immunization (Fig4.9). In contrast, although the bolus GnRH-OVA vaccine enhanced dLN cell number on day 7, the response rapidly dissipated.

![Figure 4.9. dLN cellularity kinetics after vaccination with MSR GnRH vaccine. Total number of cells in the dLN of untreated mice (Naïve) or after immunizing with the MSR vaccine (vax) and bolus formulations of the vaccine (Bolus). N=4. * indicates p <0.05 , ** p<0.01 and ns, no significance between Bolus and Vax](image)

Next, the percentage and total cell number of GC B cells in the dLN were quantified using the markers B220\(^ +\)GL7\(^ +\) (Fig4.10 A) and B220\(^ +\)GL7\(^ +\)PNA\(^ +\) (Fig4.10 B) using flow cytometry. By day 7 after immunization, both the MSR vaccine and the
bolus vaccine led to an enhanced GC response. Notably, the enhanced GC response in the MSR vaccinated animals persisted until after day 25. The number of GC B cells in the MSR vaccinated mice was approximately 10 fold higher than those in the bolus vaccine treated mice on days 14 and 25. In contrast, the GC response in the bolus vaccinated animals sharply dissipated after 7 days.

Figure 4.10. Germinal center kinetics after vaccination with the MSR GnRH vaccine. (A) Percentage of GC B cells in the dLN. GCs are determined as cells double positive for B220^+GL7^+. (B) Percentage of GC B cells in the dLN. GCs are determined as cells triple positive for B220^+GL7^+PNA^+. Data represent mean and s.d., n=4. a indicates p < 0.05 between Naïve and Bolus group, b between Naïve and Vax, * indicates p <0.05 , ** p<0.01 and ns, no significance between Bolus and Vax

Primary flow cytometry gating plots for B cells and GC B cells are shown in Fig 4.11
Furthermore, these findings were further supported by immunofluorescence staining of GC B cells in the dLNs over time (Fig4.12).
MSR vaccine using a tandem peptide as antigen

Vaccines that generate antibody responses using carrier proteins may require cumbersome chemical conjugation processes, depending on the complexity of the peptide, and elicit off-target antibody responses against other T and B cell epitopes on the carrier protein itself. Therefore, we investigated whether the MSR vaccine could induce anti-GnRH humoral responses using only a single peptide. A peptide construct (MVPGnRH) was designed that incorporates a promiscuous T helper cell epitope derived from measles virus pneumonia (MVP), a flexible linker, and the GnRH epitope (Fig4.13 A). Mice were immunized with a single injection of the MSR vaccine containing 1ug GM-CSF, 100ug CpG-ODN and 100ug MVPGnRH, or a single injection of a bolus vaccine containing the same actives (no MSR) on day 0. Titers were analyzed against only the GnRH peptide portion of the MVPGnRH peptide construct. The bolus vaccine only induced a moderate anti-GnRH response, likely due to the
weak immunogen property of peptide antigens. In comparison, a single injection of the MSR vaccine elicited titers that were over 1.5 orders of magnitude higher than that from the bolus vaccine (Fig4.13 B).

To start to understand whether the antibody response generated by the MSR MVP-GnRH vaccine can induce reproductive organ destruction, we euthanized the animals from the previous study (Fig 4.13) on day 160 and removed the ovaries. Mice that received the MPS vaccine resulted in visually smaller ovaries (Fig4.14 A) compared to those that received the bolus vaccine. The ovaries from mice immunized with the MPS vaccine had a statistically significant decrease in weight (Fig4.14 B).
MSR vaccine generates anti-Her2/neu antibody that reacted with the Trastuzumab-binding domain on human Her2 protein.

Finally, we assessed whether the MSR vaccine could induce humoral response against other types of small peptides. Human epidermal growth factor receptor 2 (Her2/neu) is highly expressed in 20–30% of invasive breast carcinomas and is associated with increased metastatic potential. Trastuzumab (Herceptin) is a humanized monoclonal antibody (mAb) against Her2 that has been shown to inhibit tumor growth by inhibiting Her2 signaling and inducing antibody dependent cell mediated cytotoxicity (ADCC) against the tumor. Peptide epitopes that closely mimic the native structure of the pocket-like trastuzumab-binding domain of Her-2 have been previously demonstrated. Here, we designed a peptide construct (MVPHer2) that incorporates a promiscuous T helper cell epitope derived from measles virus pneumonia (MVP), a flexible linker, and a short, linear peptide within the trastuzumab-binding domain.
binding domain of Her-2 (Fig.4.15 A). Mice were immunized with a single injection of the MSR vaccine containing 1ug GM-CSF, 100ug CpG and 150ug MVPHer2 or a bolus vaccine formulation. A single immunization with the MSR Her2 vaccine was able to generate strong anti-Her2 IgG1 and IgG2a antibodies compared to the bolus vaccine (Fig4.15 B-C).

**Figure 4.15. Serum anti-Her2/neu titer after vaccination with MSR MVPHer2 vaccine.** (A) Her2 peptide sequence. (B-C) ELISA analysis of sera Her2-specific IgG1 (B) or IgG2a (C) 23 days after immunization with MSR vaccines loaded with MVP-Her2 (Vax), the soluble components of the vaccine (Bolus) or in absence of treatment (Naïve). N=5. Data represent mean and s.d. * indicates p < 0.05, ** p<0.01

Furthermore, the reactivity of the induced antibody response against the native Her2 protein structure was analyzed, as it is essential for antibodies raised against a synthetic peptide to recognize the native protein to be a useful vaccine candidate. Whole serum of vaccinated mice was incubated with human Her2+ cells (SK-BR-3) or a Her2- control cell line (CT26), and antibodies from MSR vaccinated mice were capable of recognizing the native protein (Fig4.16 A-B). In contrast, antibodies from bolus-vaccinated mice did not show significant cell binding compared to the naïve serum. Serum antibodies from naïve, bolus and MSR vaccinated groups did not show binding to the negative control cell line.
4.4 Discussion

The findings of this chapter demonstrate that a single injection of the MSR vaccine elicits potent and durable serum antibody titers against small peptides. Both peptide-carrier protein constructs (e.g. GnRH-OVA and GnRH-KLH) and tandem peptide constructs (e.g. MVPGnRH and MVPHer2) demonstrated that the MSR vaccine induced more potent and durable humoral responses as compared to traditional
approaches. Notably, the MSR GnRH-OVA vaccine generated high anti-GnRH titers even 12 months after vaccination. Although carrier proteins can significantly increase immune responses against a peptide, this approach has several limitations. First, chemical coupling of specific residues on the carrier protein and the peptide can be difficult to control, and may lead to variability in vaccine response\(^2^2\). Second, because the carrier protein contains multiple antigenic epitopes, there is likely skewed clonal dominance of carrier-specific B cell responses\(^2^3\). For these reasons, tandem peptide constructs containing a single CD4 T-helper epitope and the target peptide were also explored. The MSR MVPGnRH vaccine produced 10-20 fold higher antibody titers compared to the bolus formulations, and MSR vaccination led to faster antibody production and overall higher response compared to a conventional Alum adjuvanted vaccine. Incorporating the antigen into the MSR scaffold likely resulted in multivalent display of the antigen on the MSR pores and surface, which may facilitate BCR crosslinking and enhance B cell activation for stronger humoral responses. Collectively, these data suggest that the MSR vaccine can promote continuous production of anti-GnRH antibodies. Future studies will examine the functional capabilities of the antibodies to reduce effector functions of reproductive organs and induce immune-castration, as well as subsequent effects on, for example, controlling feral animal reproduction or prostate tumor growth.

The MSR vaccine generated antibodies against a portion of the Her2 epitope that exhibited immunoreactivity to the native Her2 structure on a tumor cell surface. While Trastuzumab can inhibit tumor growth, mAb therapy lacks long-term efficacy due to the limited half-life time of the immunoglobulins, and repeated administration of the
mAb results in many side effects\textsuperscript{24-26}. It has been demonstrated that Trastuzumab binds to the portion of the Her2 extracellular domain spanning between residues 563-626 that contains three loops\textsuperscript{27}. Previous studies identified a number of peptides that mimic portions of the Her2 domain at the Her2/Trastuzumab interface\textsuperscript{28-30}. Repeated vaccination using these peptides led to antibodies that could recognize Her2/neu displayed on a cell surface and induced Her2 internalization in a similar manner as Trastuzumab\textsuperscript{31}. Here we chose a short and linear peptide Her2\textsubscript{613-626} to evaluate the robustness of the MSR vaccine. Other studies using the same peptide construct showed that multiple vaccinations were required to raise high titers\textsuperscript{30}. In comparison, a single injection of the MSR vaccine was able to induce antibody titers up to 2 orders of magnitude higher than the bolus vaccine. In particular, the antibodies recognized Her2 displayed on human breast tumor cells, whereas the antibodies produced by the bolus vaccine did not show any significant binding. Although linear peptides are highly flexible and inexpensive to synthesize, they can adopt a variety of conformations in buffer and only a subset of these conformations are ultimately responsible for antibody reactivity. Therefore, future studies should explore various cyclic peptides mimicking the Her2 loop to confer antibody specificity, and evaluate the anti-tumor efficacy. Overall, these data suggest that the MSR vaccine can likely promote continuous in situ antibody production, generate focused and robust humoral anti-tumor immunity and form long-term immunological memory.

The relationship between MSR vaccine duration and the kinetics and maintenance of the humoral response suggests the importance of prolonged DC programming in the MSR vaccine. The DC profile in the MSR vaccine showed two
distinct waves; the first one peaked on day 3 and rapidly diminished by day 5, and the second initiated after day 7. The first wave of DCs was likely due to recruitment mediated by release of GM-CSF. The residence of these DCs in the vaccine was transient, likely due to activation by the released CpG-ODN and homing to the dLN. The second wave of DCs was possibly a result of cytokines and chemokines secreted by the first wave of DCs and other immune cells. Interestingly, the first wave of DCs exhibited an activated phenotype, while the DCs in the second wave were mostly immature. Explanting the MSR vaccine between day 1 and day 5, before the first wave of DCs could be accumulated and subsequently home to the dLNs, resulted in no significant humoral response. When the vaccine was explanted after day 7, the vaccine effectively generated and maintained potent humoral responses over 100 days. Previous studies have showed that prolonged durations of vaccination (> 16 days) using engineered biomaterial scaffold vaccines significantly augmented persistent CTL responses locally and slowed tumor progression\textsuperscript{32}. Here, we showed that vaccine duration was also important in humoral responses.

The enhanced humoral responses generated by the MSR vaccine correlated strongly with persistent GC activity. After encountering an antigen, activated B cells form GCs where they switch their immunoglobulin constant region from IgM to IgG, IgA or IgE, and undergo somatic hypermutation in the variable region to produce antibodies with high specificity and avidity\textsuperscript{33}. A number of biomaterial-based vaccine systems have been shown to stimulate persistent GC activity. For instance, poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing multiple TLR ligands induced active GCs for \textasciitilde45 days, and multilamellar lipid vesicle based LN-targeting
nanoparticles showed superior GC formation compared to bolus formulations on day 14\textsuperscript{34-36}. However, these strategies required multiple injections to achieve this effect. In contrast, only a single injection of the MSR vaccine induced persistent GC activity for over 30 days. Specialized B cells that emerge from the GC secrete high affinity antibodies and can also differentiate into long-lived memory cells, which are necessary to generate life-long humoral response against various forms of antigens\textsuperscript{21}. Therefore, it will be important to characterize the avidity of the antibodies and the profile of memory B cell formation after immunizing with the MSR vaccine. The duration of the GC activity after immunization with the MSR vaccine also correlated with the amount of time the MSR vaccine remained visible at the injection site. Future studies could explore how varying MSR vaccine degradation kinetics impacts GC activity. In addition, the kinetics of antigen presentation after vaccination with the MPS system could likely be further optimized, as recent studies have demonstrated a dramatic impact of the antigen presentation profile on the immune response\textsuperscript{37,38}. The role of persistent antigen presentation by different subtypes of APCs is also an important topic for further study.

The findings of these studies suggest the MSR vaccine may have high utility as a single-injection platform to generate potent humoral responses. The MSR vaccine can generate potent anti-GnRH titers with a single vaccination, which may be a humane method to control the stray and feral animal population via immune-castration. The MSR vaccine may also provide a facile platform to induce humoral responses against new tumor associated and neoantigen peptides with a single vaccination to elicit antibody-dependent, cell-mediated cytotoxicity (ADCC) or prevent receptor-mediated tumor growth (e.g. GnRH and Her2/neu). Future studies will evaluate the
MSR vaccine to generate prophylactic and therapeutic responses. Additionally, the MSR vaccine could have significant applications in other areas, such as chronic bacteria or viral infection, and neurodegenerative diseases.

4.5 Acknowledgements

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4.6 References


Chapter 5: A facile approach to enhance antigen response for personalized cancer vaccination

5.1 Introduction

An effective vaccine platform to target multiple tumor specific peptide antigens to elicit a broad immune response could increase the likelihood of a robust response against at least some of the epitopes while decreasing the likelihood of tumor escape from immune-surveillance\textsuperscript{1,2}. Early work on the identification of tumor specific or associated antigens showed that tumor cells acquire protein-altering mutations during its progression that can be recognized by cytotoxic T lymphocytes (CTLs)\textsuperscript{1}. Cloning and sequencing of these reactive CTLs produced sequences of a number of tumor specific peptides such as melanoma-associated antigen 1 (MAGEA-1)\textsuperscript{3}. Recent advances in bioinformatics and genomics made it possible to rapidly identify and predict mutant proteins expressed exclusively in cancer cells (“neoantigens”)\textsuperscript{4-9}. These neoantigens serve as excellent vaccination candidates as they can be highly immunogenic and have strong MHC-I/II binding affinities. Additionally, checkpoint blockade therapies have been shown to facilitate the expansion of neoantigen specific preexisting T cells in melanoma patients, but the response rate depends on a preexisting immunity\textsuperscript{10,11}. Therefore, active vaccination against multiple neoantigens could augment the clinical success rate of checkpoint blockade therapies and prevent antigen escape.
Generating potent anti-tumor responses using neoantigen peptides has remained a challenge due to their rapid clearance and low immunogenicity, which limits optimal presentation by antigen presenting cells (APCs) to initiate a strong T cell response. Multiple macro- and nano-engineering strategies have been designed to overcome these challenges. However, many approaches require chemical modification or physical emulsification of the peptides, potentially altering their presentation capacity. Moreover, since neoantigen vaccines typically require several peptides, modification of individual peptides is cumbersome for clinical translation and likely results in high batch-to-batch variability during manufacturing.

We propose a facile strategy to enhance antigen immunogenicity using polyethylenimine (PEI) based on a mesoporous silica micro-rod (MSR) vaccine described in the previous chapters. The MSR vaccine can be easily injected using standard needles, was shown to effectively concentrate and activate large populations of host APCs, and induced more potent humoral responses and prophylactic tumor protection compared to traditional vaccine formulations. To augment its therapeutic efficacy, the surface of the MSR scaffold could potentially be modified to induce strong responses to tumor specific peptide antigens. PEI is a cationic polymer that has been widely used as a gene transduction agent. A number of recent studies have shown that PEI-based complexes can stimulate pro-inflammatory cytokine production, and induce potent humoral response when complexed with glycoproteins. Here, we explore the application of PEI to co-present antigen in a layered adsorption manner in the MSR vaccine to enhance T cell responses and drive therapeutic tumor control.
5.2 Materials and Methods

Materials, Cell line and peptides.

Branched 60K PEI (Sigma 181978), Branched 2K PEI (Polysciences 06089), Linear 25K PEI (Polysciences 23966) and Linear 2K PEI (Polysciences 24313) were diluted to 5.5mg/ml in dH2O and stored away from direct light. Endotoxin free ovalbumin was used throughout the study (Invivogen, vac-pova). All antibodies were used according to the manufacturer’s protocols. SIINFEKL- and E749-57- tetramers were obtained from the Emory NIH Tetramer Core Facility. TC-1 cells were generated in the laboratory of T-C. Wu (Johns Hopkins University, Baltimore, USA) and maintained in RPMI supplemented with 10% FBS, 1% penicillin–streptomycin and 50ug/ml G418. B16F10 and CT26 cells (ATCC) were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin according to protocols from ATCC. All peptides used in this study were synthesized at least 95% purity from Peptide 2.0.
Peptide information:

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<td>CT26-M20</td>
<td>PLLPFYPPDEAEIIGLELNSSALPPTM</td>
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<tr>
<td>CT26-M90</td>
<td>LHSGQNHKLKEMASVLEARACCAAGQS</td>
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<td>GP70</td>
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MSR and MSR-PEI vaccine formulation.

To formulate the MSR PEI-antigen vaccines, 2mg of the MSR were adsorbed with 100μg murine class B CpG-ODN, 2mg of the MSR were adsorbed with various doses of linear or branched polyethylenine at 37°C for 15 minutes (MSR-PEI particles). Subsequently, various doses of antigen were adsorbed onto MSR-PEI particles for 1-3 hours at 37 °C or overnight at RT under shaking. Separately, 1mg of the MSRs were loaded with murine GM-CSF (Peprotech) for 1 hour at 37°C under shaking. The MSR components (5mg total) were combined and resuspended in cold PBS prior to immunization. To formulate the MSR PEI-CpG vaccines, 2mg of the MSR were adsorbed with various doses of antigens for 6 hours at RT, and 2mg of the MSR were adsorbed with various doses of linear or branched polyethylenine at 37 °C for 15
minutes (MSR-PEI particles). Subsequently, 100ug murine class B CpG-ODN was adsorbed onto MSR-PEI particles for 1 hour at 37 °C or overnight at RT under shaking. Separately, 1mg of the MSRs were loaded with murine GM-CSF (Peprotech) for 1 hour at 37 °C under shaking. The MSR components (5mg total) were combined and resuspended in cold PBS prior to immunization. To prepare the MSR vaccines, 4mg of the MSR were adsorbed with 100ug murine class B CpG-ODN (sequence TCCATGACGTTCCTGACGTT, IDT) and the antigen for 6 hours at room temperature under shaking, and subsequently lyophilized. Separately, 1mg of the MSRs were loaded with murine GM-CSF (Peprotech) for 1 hour at 37 °C under shaking. The MSR components were combined and resuspended in cold PBS prior to immunization.

**Incorporation efficiency assays.**

To determine the incorporation efficiency of PEI onto the MSRs, PEI at the indicated doses was adsorbed onto MSR for 15 min at 37 °C. Subsequently, the MSRs were centrifuged at 2000g for 10 min. Supernatant was collected and the concentration of PEI was measured using Fluoraldehyde (Sigma Aldrich). To determine the incorporation efficiency of peptides onto the MSRs, 50ug of the indicated peptides was adsorbed onto 2mg of MSRs or MSR-PEI overnight. The MSRs were centrifuged at 2000g for 10 min. Supernatant was collected and the concentration of each peptide was quantified using micro-BCA (ThermoScientific).
BMDC differentiation, cytokine production and antigen presentation assays.

Bone marrow cells were isolated from female C57Bl/6J mice (Jackson Laboratories) and cultured in RPMI based media (Lonza) supplemented with 10% heat inactivated FBS (Sigma-Aldrich), 1% penicillin/streptomycin, 50µM β-mercaptoethanol and 20 ng/ml GM-CSF (Peprotech). To assess the uptake kinetics of PEI by BMDCs, 0.5x10^6/ml immature BMDCs were plated onto non-tissue culture treated 12-well dishes and stimulated with 7ug of rhodamine labeled L25K PEI for various time lengths (0h, 2h, 6h, 24h, 72h). BMDCs were harvested, resuspended in FACS buffer and the rhodamine signal was analyzed using flow cytometry (BDBiosciences). To assess the BMDCs activity after PEI stimulation, 0.5x10^6/ml immature BMDCs were plated onto non-tissue culture treated 12-well dishes and stimulated with various doses of L25K or B60K PEI or MSR-PEI for 24 hours. BMDCs were harvested, resuspend in FACS buffer and stained using 7-AAD (eBioscience), anti-CD11c, anti-CD86 and anti-MHCII for 15 minutes on ice. BMDCs were then analyzed using flow cytometry. Separately, supernatant was collected and analyzed for IL-6, TNFα and IFNγ using ELISA according to the manufacturer’s protocols (eBioscience). To assess BMDC cross presentation, 0.5x10^6/ml immature BMDCs were pulsed with 20ug ovalbumin (Invivogen) and either 5ug or 10ug B60K PEI for 48 hours. BMDCs were then harvested and stained using 7-AAD (MF), anti-CD11c and anti-SIINFEKL/H-2K^b antibody, and analyzed using flow cytometry (LSRFortessa, BD).
**Immunization.**

Unless otherwise noted, all in vivo studies were carried out using female C57BL/6J mice (Jackson Laboratories) between 6 to 10 weeks old at the beginning of the experiment. MSR vaccines were resuspended in 150ul of cold PBS and injected, via an 18G needle, subcutaneously in the intrascapular region with the mouse under brief isoflurane anesthesia. All animal studies were performed in accordance with NIH guidelines, under approval of Harvard University’s Institutional Animal Care and Use Committee.

**Therapeutic tumor studies.**

Cells were used at passage number <5 and passaged at least twice prior to inoculation. In the subcutaneous therapeutic TC-1 model, female C57BL/6 mice were injected with a single cell suspension of $2 \times 10^5$ TC-1 cells in 100ul cold PBS on the back of the neck on day 0. In the subcutaneous therapeutic B16F10 model, female C57BL/6 mice were injected with a single cell suspension of $1 \times 10^5$ B16F10 cells in 100ul cold PBS on the back of the neck on day 0. Mice were immunized on indicated days, and tumor area was monitored using a caliper: longest surface length (a) and width (b), and the tumor size was expressed as area (a×b). Mice were euthanized when the largest side reached 2cm or according to IACUC standards. In the therapeutic B16F10 or CT26 lung metastasis model, mice were injected intravenously with $2 \times 10^5$ B16F10 or CT26 cells in 100ul cold PBS on day 0. Lungs were excised on day 16 and fixed in Fekete’s solution. Lung metastasis nodules were then manually enumerated. For anti-CTLA4 therapies,
100μg per mouse of anti-mouse CTLA-4 antibodies (BioXcell, clone: 9D9) were administered intraperitoneally on days 1, 3, 6 and 9 after tumor inoculation.

**Scaffold explant and analysis.**
Scaffold tissues were surgically removed from the animals. They were then processed through mechanical disruption and digested for 30 min. at 37 °C in 250 U/ml Collagenase IV in RPMI. The resulting cell suspension was then filtered through a 40μm cell strainer to isolate the cells from the larger sized MSRs. The cells and small remaining MSR particles were pelleted, washed with cold PBS, and counted manually using a hemacytometer. The single cell suspension was stained with 7-AAD, anti-mouse CD11c, CD86, CCR7 and SIINFEKL/H-2Kb monoclonal antibodies for 15 min. on ice. The cells were then washed and analyzed using flow cytometry (LSRFortessa, BD).

**Lymph node analysis.**
Vaccine draining lymph nodes were surgically removed from the animals. They were then processed through mechanical disruption and digested for 30 min. at 37 °C in RPMI containing 0.5mg/ml Collagenase 4 and 0.1 mg/ml DNAse. Cells were then filtered through a 40μm cell and washed in cold PBS. Single cell suspensions were enumerated (Beckman-Coulter) and stained with an alexa-fluor 780 dead exclusion dye (eBioscience), anti-mouse CD11c, CD11b, CD86, MHC-II and F4/80 for 15 min. on ice. DQ-OVA was visualized in the FITC channel. The cells were then washed and analyzed using flow cytometry (LSRFortessa, BD).
Peptide restimulation and tetramer analysis of circulating PBMCs.

80μl of blood was collected into heparin-coated tubes (BD). Red blood cells were removed using 1ml RBC lysis buffer (BioLegend) for 1 min. at RT. To perform tetramer analysis, blood cells were incubated with 7μg/ml peptide-MHC tetramers conjugated with alexa fluor 647 for 30 min. at 37 °C. Subsequently, cells were collected and stained with 7-AAD, anti-mouse CD3e and CD8a for 15 min. on ice. Cells were then washed and analyzed using flow cytometry. To perform peptide restimulation, blood cells were washed and pulsed with 2μg/ml of various MHC-I restricted peptides for 1.5 hours at 37 °C. Subsequently, cytokine secretion was stopped with golgi plug (MF) and cells were cultured for 4 hours at 37 °C. Cells were collected and stained with Alexa-Fluor 780 dead exclusion dye, anti-mouse CD3e, CD8a and CD4 for 15 min. on ice before fixing and permeabilizing (eBioscience 88-8824-00). Cells were then stained with anti-mouse IFNγ for 30 min. in 4 °C and analyzed using flow cytometry (LSRFortessa, BD).

Tumor infiltrating lymphocyte (TIL) analysis.

Tumors were explanted and cut into small pieces in 5ml of RPMI. The samples were then digested in 15ml RPMI supplemented with 2% FCS, 50U/ml Collagenase IV (Invitrogen), 100μg/ml Hyaluronidase and 20U/ml DNase (Roche) for 2 hours at 37 °C using the gentleMACS™ Dissociators (Miltenyi Biotech). Suspensions were filtered through a 40μM strainer and washed 3x with PBS. Lymphocyte population was enriched through gradient centrifugation at 50g for 5 minutes and repeated 3 times. Supernatant was collected and stained with a Zombie-UV dead exclusion dye, anti-
mouse CD45, CD3e, CD4, CD8a, CD62L and CD44 for 15 min. on ice before fixing and permeablizing (eBioscience 00-5523-00). Cells were then stained with anti-mouse IFNγ, TNFα and Granzyme B for 30 min. in 4 °C and analyzed using flow cytometry (LSRFortessa, BD).

**Statistical analysis.**

All values in the present study were expressed as mean ± S.D unless otherwise indicated in the figure legends. Statistical analysis was performed using GraphPad Prism and Microsoft Excel. Sample variance was tested using the F test. For samples with equal variance, the significance between the groups was analyzed by a, two-tailed, student’s t test. For samples with unequal variance, a two-tailed Welch’s t-test was performed. In all cases, a P value of less than 0.05 was considered significant.

**5.3 Results**

**Modification of MSRs with PEI**

MSRs are modified with PEI by simply mixing with a PEI solution; subsequently, an antigen pool was directly adsorbed onto PEI modified MSRs (PEI-MSRs) in 3 hours at 37 °C (**Fig5.1**).
PEI was adsorbed into the MSRs at high efficiency (Fig5.2 A). The incorporation capacity of both 60K branched PEI (B60K) and 25K linear PEI (L25K) was ~20ug PEI/mg MSR. The high positive charge content of the PEI polymers likely enabled their rapid adsorption into the MSRs, as over 90% of B60K and L25K PEI polymers was adsorbed to the MSR surface after 1 minute of mixing with the MSRs, and over 95% of both PEI polymers was adsorbed after 15 minutes (Fig5.2 B).
Furthermore, zeta potential measurements of PEI-MSRs confirmed that the positive charge content increased after PEI incorporation, in a PEI dose dependent manner (Fig5.3).

Figure 5.2. PEI incorporation efficiency. (A) Incorporation efficiency of various doses of soluble B60K and L25K PEI into MSRs. (B) Incorporation kinetics of soluble B60K and L25K PEI into MSRs. N=3. Data represent mean and s.d.
The incorporation efficiency of six neoantigen peptides of the same molecular weight but different net charges at neutral pH was tested. MSRs and PEI-MSRs showed high incorporation efficiency for net positive and neutral peptides. The PEI-MSRs enhanced the incorporation of net negative peptides in a manner likely dependent on the peptide’s hydrophilicity (Fig5.4).

Figure 5.3. MSR-PEI surface charge. Zeta potential of MSR-PEI particles using various doses of soluble B60K and L25K PEI. N=3. Data represent mean and s.d.
The effect of PEI on bone marrow derived dendritic cells (BMDCs) was next examined, as previous studies have demonstrated an underlying adjuvant effect of PEI\textsuperscript{19-21}. BMDCs take up PEI in a time dependent manner, reaching their uptake capacity at 24 hours (Fig5.5).

Figure 5.4. Peptide incorporation efficiency into MSR-PEI. Incorporation efficiency of neoantigen peptides (sorted according to the net charge at neutral pH) onto bare MSR or MSR-PEI particles using B60K PEI. N=3. Data represent mean and s.d. ** indicates p<0.01
After 24 hours of stimulation by soluble B60K or L25K PEI, BMDCs exhibited a significant increase in CD86 and MHC-II expression (Fig5.6 A), and TNFα (Fig5.6 B) and IL-6 (Fig5.6 C), but not IFNγ (Fig5.6 D) production in a PEI dose dependent manner.

**Figure 5.5. BMDC uptake kinetics of PEI.** Flow cytometry analysis of rhodamine⁺ murine BMDCs after stimulating with soluble Rhodamine-PEI for 0, 2, 6, 24 and 72 hours.
Figure 5.6 BMDC stimulation by soluble PEI. (A) Flow cytometry analysis of CD86 and MHC-II expression on murine BMDCs after 18 hours of stimulation with 1ug or 7ug of soluble PEI or PBS. ELISA analysis of (B) TNFα, (C) IL-6 and (D) IFNγ concentration in murine BMDC supernatant after 18 hours of stimulation with 1ug or 7ug of soluble B60K and L25K PEI or PBS. Data depicts mean +/- sd, n=4, * indicates p < 0.05. ** indicates p < 0.01, *** indicates p < 0.001
Similarly, BMDCs stimulated by PEI-MSR particles also showed significantly increased CD86 expression (Fig5.7 A) and TNFα production (Fig5.7 B).

![Figure 5.7](image)

**Figure 5.7. BMDC stimulation by MSR-PEI.** (A) Flow cytometry analysis of CD86 expression on murine BMDCs after 18 hours of stimulation with MSR particles (MSR) or PEI-MSR particles containing 0.5ug or 2ug of B60K or L25K PEI. (B) ELISA analysis of TNF-a concentration in murine BMDC supernatant after 18 hours of stimulation with MSR particles (MSR) or PEI-MSR particles containing 0.5ug or 2ug of B60K or L25K PEI. Data depicts mean +/- sd, n=3-4, *** indicates p < 0.001 against the MSR control.

The impact of PEI on antigen cross presentation was next examined by pulsing BMDCs with the whole ovalbumin protein (OVA) either alone or together with B60K PEI for 2 days. B60K PEI led to ~10-20 fold increase in antigen cross-presenting DCs compared to OVA alone (Fig5.8). Together, these data suggest that PEI can be efficiently incorporated into MSRs in a facile manner, a diverse range of neoantigen
peptides can be subsequently incorporated using simple adsorption, and PEI and MSR-PEI can enhance DC activation and cross-presentation.

![Graph showing SIINFEKL-H2Kb+ CD11c+ (%)]

**Figure 5.8. BMDC cross presentation after stimulation with PEI.** Flow cytometry analysis of SIINFEKL presenting murine BMDCs after stimulation with PBS, OVA and OVA with 5ug or 10ug of soluble B60K PEI. Data depicts mean +/- sd, n=3-4, * indicates p < 0.05. **** indicates p < 0.0001.

Effect of MSR-PEI vaccine on host DC recruitment, activation and LN trafficking

The ability of an MSR-PEI vaccine to enhance DC activation in vivo was then analyzed by comparing MSR incorporating 1ug GM-CSF, 100ug Cpg and 100ug OVA as previously described to the MSR-PEI vaccine incorporating these agents (**Fig 5.9**).
Mice were immunized with the MSR vaccine (V) or the MSR-PEI vaccine (VP) subcutaneously in the flank. On day 3, the vaccine scaffolds were removed and analyzed for infiltrating cell content. The total number of cells recruited to the vaccine was comparable between MSR and MSR-PEI vaccine immunized mice (Fig5.10 A). However, the MSR-PEI vaccines showed a significant enrichment of CD11c⁺CD86⁺ activated DCs (Fig5.10 B), CD11c⁺CCR7⁺ LN homing DCs (Fig5.10 C) and CD11c⁺SIINFEKL-H2Kb⁺ antigen cross-presenting DCs (Fig5.10 D).

Figure 5.9. Schematics of the MSR vaccine (V) and MSR-PEI vaccine (VP).
To evaluate the impact of the MSR-PEI vaccine on DC trafficking to the vaccine draining lymph node (dLN), the dLN was analyzed on days 3 and 5 post immunization.

Figure 5.10. In vivo DC profile in MSR-PEI Vaccine. (A) Total cell number at the vaccine site explanted on day 3 post immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI. Total number of (B) CD11c⁺ CD86⁺ activated DCs, (C) CD11c⁺ CCR7⁺ LN homing DCs and (D) SIINFEKL presenting DC recruited to the vaccine site on day 3 post immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI. Data depicts mean +/- sd, n=4, * indicates p < 0.05. ** indicates p < 0.01, *** indicates p < 0.001, ns indicates no significance.
The total dLN cellularity between MSR and MSR-PEI vaccine immunized mice was similar on day 3, likely due to insufficient time for the recruited DCs to traffic to the dLN. On day 5, the dLN in MSR vaccine immunized animals showed a significant increase in cellularity compared to naïve animals. Strikingly, the MSR-PEI vaccine further increased dLN cellularity (Fig5.11 A). Additionally, the MSR-PEI vaccine elicited a strong enrichment in antigen presenting DCs (CD11c⁺DQ-OVA⁺) (Fig5.11 B) and activated DCs (CD11c⁺CD86⁺ and CD11c⁺MHC-II⁺) (Fig5.11 C).
Figure 5.11. DC profile in dLN after vaccination with MSR-PEI vaccine. (A) Total number of cells in the vaccine draining lymph node (dLN) on days 3 and 5 after immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI or left unimmunized (N). Total numbers of (B) antigen+ DC and (C) CD11c+ CD86+ or CD11c+ MHC-II+ activated DCs in the dLN on days 3 and day 5 post immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI or left unimmunized (N). Data depicts mean +/- sd, n=4, * indicates p < 0.05. ** indicates p < 0.01, *** indicates p < 0.001.
The macrophage populations were not affected on day 5 post vaccination (Fig5.12). Collectively, these data demonstrate that the MSR-PEI vaccine enhanced host DC activation, antigen presentation and trafficking to secondary lymphoid organs.'

Effect of MSR-PEI vaccine on CD8 T cell response in vivo

The ability of the MSR-PEI vaccine to induce CD8 killer T cell (CTL) responses in vivo was next examined. Animals were immunized with the MSR vaccine (V) or the MSR-PEI vaccine (VP), and circulating blood PBMCs 7 days after immunization were analyzed. Mice immunized with the MSR-PEI vaccine generated ~2-fold higher
circulating IFNγ+ (Fig5.13 A) and SIINFEKL-tetramer+ (Fig5.13 B) CTLs compared to the MSR vaccine.

**Figure 5.13.** Antigen specific CD8 T cell response. (A) Percentage of IFNγ+ CD3+CD8+ T cells isolated from peripheral blood on day 7 after immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI, or left unimmunized (N), and stimulated with SIINFEKL (primary FACS plots on the left, quantifications from the FACS plots on the right). (B) Percentage of SIINFEKL-tetramer+ CD3+CD8+ T cells isolated from peripheral blood on day 7 after immunization with the MSR vaccine (V), the MSR-PEI vaccine (VP) using B60K PEI or left unimmunized (N). Data depicts mean +/- sd, n=4, * indicates p < 0.05.

Furthermore, we analyzed the scaffold content on day 10 post vaccination and found that the CD8+ effector T cells outnumbered Foxp3+ CD4+ regulatory T cells by ~15 fold at the MSR-PEI vaccine site, almost 3 times higher than this ratio at the MSR vaccine site (Fig5.14).
The effect of PEI dose, molecular weight and physical structure in the MSR-PEI vaccine on CTL responses was also evaluated. Increasing the PEI dose above 40μg/vaccine led to a significantly decreased CTL response (Fig5.15 A). Similarly to the MSR-PEI vaccine using B60K PEI, the MSR-PEI vaccine formulation using L25K PEI also showed an enhanced CTL response compared to the MSR vaccine (Fig5.15 B). No significant differences were observed when varying PEI structure and molecular weight in the MSR-PEI vaccine (Fig5.15 C).

**Figure 5.14. Scaffold Effector to Regulatory T cell ratio.** Ratio of CD8⁺ effector T cells (T_{eff}) to Foxp3⁺CD4⁺ regulatory T cells (T_{reg}) at the MSR vaccine site on day 11 after immunization with the MSR vaccine (V) or the MSR-PEI vaccine (VP) using B60K PEI. Data depicts mean +/- sd, n=4, **indicates p < 0.01.
Finally, while maintaining a constant dose of GM-CSF (1ug), CpG (100ug), OVA (100ug) and PEI (B60K, 10ug), two types of MSR-PEI vaccines were tested: 1. A VPC vaccine that combined CpG adsorbed onto PEI-MSRs and OVA and GM-CSF adsorbed onto another set of naked MSRs, or 2. A VPO vaccine that combined OVA adsorbed onto PEI-MSRs with CpG and GM-CSF adsorbed onto a set of naked MSRs (Fig5.16 A). Interestingly, VPO, not VPC, immunized animals showed a statistically significant increase in the CTL response compared to MSR vaccine immunized animals.
To exclude the possibility that a higher dose of PEI in the VPC vaccines may improve the response, two increasing doses of PEI in the VPC vaccine were evaluated and the CTL response decreased with increasing dose of PEI (Fig 5.16 C).

Figure 5.16. Effect of PEI association on antigen specific CD8 T cell response. (A) Schematics of the MSR PEI-OVA associating vaccine (V PO) and the MSR PEI-CpG associating vaccine (V PC). (B) Percentage of IFNγ+ CD3+CD8+ T cells isolated from peripheral blood at day 7 after immunization with the MSR vaccine (V), the MSR PEI-CpG associating vaccine (VPC) using B60K PEI, and the MSR PEI-OVA associating vaccine (VPO) using B60K PEI, or left unimmunized (N) and stimulated with SIINFEKL. (C) Percentage of IFNγ+ CD3+CD8+ T cells after stimulating with SIINFEKL in peripheral blood on day 7 after immunization with MSRs delivering only GM-CSF (GM), the MSR vaccine (V), MSR PEI-CpG associating vaccines containing 20ug (VPC 20) or 60ug (VPC 60) of B60K PEI. Data depicts mean +/- sd, n=4, * indicates p < 0.05

Anti-tumor effect using a single HPV peptide

To test whether the MSR-PEI vaccine may serve as an effective platform to induce anti-tumor immunity against tumor-specific peptides, a synthetic long peptide derived from the E7 oncoprotein of human papilloma virus (HPV) was used as the antigen. Beyond cervical cancer, HPV is associated with 30-60% of oropharyngeal, vaginal and head-and-neck cancers22,23. Consistent with our findings using the model OVA antigen, the MSR-PEI vaccine (VP) induced ~2-3 fold higher E7 specific IFNγ+
(Fig5.17 A) and E7_{49-57}^\text{tetramer} (Fig5.17 B) circulating CTL cell response compared to the MSR vaccine (V).

**Figure 5.17. E7 specific CD8 T cell response.** (A) Percentage of IFNγ⁺ CD3⁺CD8⁺ circulating T cells after immunization with the MSR E7 vaccine (V), the MSR-PEI E7 vaccine (VP) using 5μg or 20μg of B60K PEI, or left unimmunized (N), and stimulated with E7_{49-57}. (B) Percentage of RAHYINVTF tetramer⁺ CD3⁺CD8⁺ T cells in peripheral blood on day 7 after immunization with the MSR E7 vaccine (V), the MSR-PEI E7 vaccine (VP) using 5μg or 20μg of B60K PEI, or left unimmunized (N). Data depicts mean +/- sd, n=4, * indicates p < 0.05.

The MSR-PEI vaccine also showed significantly better response compared to a traditionally formulated bolus vaccine containing the same dose of GM-CSF (1μg), CpG (100μg) and the E7 peptide (50μg) (Fig5.18).
Additionally, mice immunized with the MSR-PEI, but not the MSR vaccine, showed a significantly elevated blood TNFα level (Fig 5.19).

**Figure 5.18. E7 specific CD8 T cell response.** Percentage of IFNγ⁺ CD3⁺CD8⁺ T cells isolated from peripheral blood on day 8 after stimulating with RAHYNIVTF; mice were immunized with a bolus vaccine (B; 1μg GM-CSF, 50μg E7, 100μg CpG), or the MSR-PEI vaccine (VP; 5mg MSR, 1μg GM-CSF, 100μg CpG, 50μg E7) associated with either 5μg or 20μg of L25K PEI. Data depicts mean +/- sd, n=4, * indicates p < 0.05
To test whether the MSR-PEI vaccine confers enhanced therapeutic benefit against established tumors, mice were inoculated with E7 expressing TC-1 carcinoma subcutaneously and treated with a single injection of the MSR or MSR-PEI after 8 days, at which point the tumor area reached $\sim$25 mm$^2$. The MSR-PEI vaccine triggered rapid and complete regression, whereas the MSR vaccine induced only partial regression in the majority of the animals (Fig5.20 A). Importantly, subsets of mice treated with the MSR-PEI vaccine had large tumors ($\sim$1 cm x 1 cm) before completely regressing (Fig5.20 B).

Figure 5.19. Serum cytokine level. ELISA analysis of (c) TNF-a level in serum 24 hours post vaccination with the MSR E7 vaccine (V), the MSR-PEI (B60K) E7 vaccine (VP), or left unimmunized (N). Data depicts mean +/- sd, n=4, * indicates p < 0.05, ns indicates not significant.
Figure 5.20. TC-1 tumor growth. (A) Tumor growth of mice bearing established E7 expressing TC-1 tumors (inoculated with $2 \times 10^5$ TC-1 cells and allowed to develop for 8 days) and treated with the MSR vaccine (V) or the MSR-PEI vaccine (VP) using L25K PEI, or left untreated (N). (B) Representative photos of one mouse treated with the MSR-PEI vaccine. Data depicts mean +/- sem, n=9, *** indicates $p < 0.001$
The MSR-PEI vaccine also enhanced overall survival by ~2 fold compared to the MSR vaccine, as ~80% of animals treated with the MSR-PEI vaccine survived long term beyond 150 days (Fig5.21).

![Cumulative survival in mice inoculated with the TC-1 tumor.](image)

**Figure 5.21. Cumulative survival in mice inoculated with the TC-1 tumor.** Tumor growth of mice bearing established E7 expressing TC-1 tumors (inoculated with $2 \times 10^5$ TC-1 cells and allowed to develop for 8 days) and treated with the MSR vaccine (V) or the MSR-PEI vaccine (VP) using L25K PEI, or left untreated (N). n=9, *** indicates $p < 0.05$

In a separate experiment, the MSR-PEI vaccine was evaluated against a traditionally formulated bolus vaccine (Fig5.22 A). Consistent with previously reported findings, the bolus vaccine was partially effective and induced complete regression in only ~20% of the animals$^{10,24}$. In comparison, the MSR-PEI vaccine induced faster regression and ~80% of treated animals again showed complete regression (Fig5.22 B)
To further demonstrate that a direct association between the antigen and PEI in the vaccine is important, animals bearing established subcutaneous TC-1 tumors were treated with a MSR-PEI vaccine in which the GM-CSF and CpG are adsorbed onto bare MSRs and the E7 peptide was adsorbed onto PEI-MSRs (PEI-E7 Vax), and a MSR-PEI vaccine in which the GM-CSF and E7 are adsorbed onto bare MSRs and the CpG was adsorbed onto PEI-MSRs (PEI-CpG Vax). Mice treated with the PEI-E7 Vax showed superior tumor regression compared to those treated with the PEI-CpG Vax.

Figure 5.22. TC-1 tumor growth. (A) Tumor growth and (B) overall survival in mice bearing established E7 expressing TC-1 tumors (inoculated with 2x10^5 TC-1 cells and allowed to develop for 9 days) and treated with a bolus vaccine (Bolus Vax; 1ug GM-CSF, 50ug E7, 100ug CpG), or the MSR-PEI vaccine (VP; 5mg MSR, 1ug GM-CSF, 100ug CpG, 50ug E7) associated with either 5ug or 20ug of L25K PEI. Data depicts mean +/- sem and n= 6 for N, 8 for V and VP *** indicates p<0.001 between VP 5ug PEI and Bolus Vax, and ### indicates p<0.001 between VP 20ug PEI and Bolus Vax.

To further demonstrate that a direct association between the antigen and PEI in the vaccine is important, animals bearing established subcutaneous TC-1 tumors were treated with a MSR-PEI vaccine in which the GM-CSF and CpG are adsorbed onto bare MSRs and the E7 peptide was adsorbed onto PEI-MSRs (PEI-E7 Vax), and a MSR-PEI vaccine in which the GM-CSF and E7 are adsorbed onto bare MSRs and the CpG was adsorbed onto PEI-MSRs (PEI-CpG Vax). Mice treated with the PEI-E7 Vax showed superior tumor regression compared to those treated with the PEI-CpG Vax.
(Fig5.23), indicating that co-presentation of the antigens and PEI in the vaccine is likely a crucial component to generating anti-tumor immunity.

Finally, since effective cancer vaccines can also generate long-term immunological memory to prevent tumor recurrence, the animals that survived were re-challenged by inoculation with the same E7 expressing TC-1 carcinoma after 6 months. Impressively, 100% of the mice that were treated with either the MSR vaccine or the MSR-PEI vaccine and survived the first tumor inoculation were completely tumor free after the re-challenge (Fig5.24), indicating that both the MSR and the MSR-PEI vaccines induced potent long-term immunological memory.

**Figure 5.23. Effect of PEI association on therapeutic benefit.** Tumor growth in mice bearing established E7 expressing TC-1 tumors (inoculated with 2x10^5 TC-1 cells and allowed to develop for 8 days) and treated with the MSR PEI-CpG associating vaccine (PEI-CpG Vax) or the MSR PEI-E7 associating vaccine (PEI-E7 Vax), or left untreated (N). Data depicts mean +/- sem, n=5, *** indicates p < 0.001 between PEI-CpG Vax and PEI-E7 Vax.
Anti-tumor effect using libraries of neoantigen peptides

Finally, the MSR-PEI vaccine’s ability to serve as a facile and effective platform to elicit responses against multiple neoantigens and induce tumor control was tested. The MSR-PEI vaccine was evaluated in the highly aggressive and immune-suppressive B16F10 and CT26 models using combinations of recently identified neoantigen peptides\textsuperscript{7,25}. As the tumor microenvironment needs to be enriched with effector tumor infiltrating lymphocytes (TILs) to achieve tumor clearance\textsuperscript{8}, we first analyzed whether the MSR-PEI vaccines can improve TIL infiltration into the tumor. We inoculated the animals with B16F10 melanoma cells, treated them 5 days post inoculation with the MSR vaccine or the MSR-PEI vaccine containing 50ug of the B16-M27 and B16-M30 melanoma neoantigen peptides. On day 15, we measured the tumor sizes and analyzed TIL infiltration. The TIL population was identified as CD3\textsuperscript{+} CD45\textsuperscript{+} T-
lymphocytes that was CD62L\textsuperscript{low} (Fig 5.25 A). Primary flow cytometry plots for CD44\textsuperscript{+}IFN\textsuperscript{γ} and CD44\textsuperscript{+}TNF\textsuperscript{α} are shown in Fig 5.25 B.
At this time point, tumors in the untreated animals have developed to the maximum allowable size, and the tumors in the MSR-PEI vaccine treated animals were significantly smaller (Fig 5.26 A). Impressively, tumors in animals treated with the MSR-PEI vaccine showed ~3 fold higher enrichment in the effector IFNγ+ (Fig 5.26 B), TNFα+ (Fig 5.26 C) population in the CD4+ T lymphocyte compartment. In contrast, the MSR vaccine did not generate enhanced TIL response compared to untreated animals.

**Figure 5.25. Primary gating strategy of TIL analysis.** (A) Singlet cells were selected from the cell population and dead cells were then excluded. CD3+CD45+ T cells were selected from the live cell population. Subsequently, CD62L low effector T cells were selected from the T cell population. (B) CD44+TNFα+, CD44+IFNγ+, and CD44+Granzyme B+ cells were analyzed from the effector T cell population.
Figure 5.26. TIL infiltration after therapeutic vaccination. (A) Tumor size after 15 days in mice bearing established B16F10 tumors (inoculated with $1 \times 10^5$ B16F10 cells and allowed to develop for 5 days) and treated with the MSR vaccine (V) or the MSR-PEI vaccine (VP) using L25K PEI, or left untreated (N). (B) Percentage of CD44$^+$TNF$\alpha^+$ cells within CD4$^+$ or CD8$^+$ T lymphocytes. (C) Percentage of CD44$^+$ IFNy$^+$ cells within CD4$^+$ or CD8$^+$ T lymphocytes. Data depicts mean +/- sd and n=3-5. * indicates p < 0.05. ** indicates p < 0.01.
We then evaluated the therapeutic effect of the MSR-PEI vaccine. First, B16F10 melanoma cells were injected intravenously into C57BL/6 animals. After 24 hours, mice were treated with the MSR-PEI vaccine containing a combination of B16-M27 and B16-M30 peptides. After 16 days, lungs were removed and tumor metastases were counted. Over 200 melanoma metastases developed in the untreated animals, and the MSR-PEI vaccine efficiently cleared these metastases (Fig5.27 A). This finding was corroborated using another tumor cell line. CT26 colorectal cancer cells were injected intravenously into Balb/C animals. After 3 days, the animals were treated with a combination of CT26 neoantigens (CT26-M03, -M19, -M20, -M90, gp70). Lungs were removed after 16 days and tumor metastases were counted. Again, the MSR-PEI vaccine significantly reduced the formation of metastases nodules (Fig5.27 B).
Figure 5.27. Therapeutic lung metastasis studies. (A) Number of lung metastasis formed on day 16 post inoculation in mice that received IV inoculation of B16F10 melanoma cells ($2 \times 10^5$ cells injected and allowed to develop for 1 day) and treated with the MSR-PEI vaccine (VP) using L25K PEI and 50ug of M27 and M30 neoantigens, or left untreated (N). Primary representative photographs of excised lungs are shown in the figure. (B) Number of lung metastasis formed after 16 days in mice that received IV inoculation of CT26 colorectal carcinomas ($2 \times 10^5$ cells and allowed to develop for 3 days) and treated with the MSR-PEI vaccine (VP) using L25K PEI and 50ug of M03, M19, M20, M90 and gp70 neoantigens or left untreated (N). Primary representative photographs of excised lungs are shown in the figure. Data depicts mean +/- sd and n=6. ** indicates p < 0.01, **** indicates p < 0.0001.
Next, we evaluated the MSR-PEI vaccine using a subcutaneous B16F10 model. A standard prime and boost of the MSR-PEI vaccine improved B16F10 tumor growth control (Fig. 5.28A) and temporary regression after the boost in a subset of the animals (Fig. 5.28B).

**Figure 5.28. Therapeutic subcutaneous B16F10 tumor study.** (A) Tumor growth in mice bearing established B16F10 tumors (inoculated with $1 \times 10^5$ cells and allowed to develop for 3 days) and treated with two injections of the MSR-PEI vaccine (VP) using L25K PEI and 50ug of the M27 and M30 neoantigens on days 3 and 13, or left untreated (N). (B) Tumor volume change between days 13 and 17 after tumor inoculation. In (A) data depicts individual tumor growth and n=8. In (B), data depicts mean +/- sd and n=8. *** indicates p<0.001.
Finally, checkpoint blockade therapies have shown tremendous promise in various types of cancer, but the response rate depends on a preexisting immunity\textsuperscript{26}. In this aggressive subcutaneous B16 model, anti-CTLA4 therapy alone did not confer significant tumor growth control compared to untreated controls. However, one injection of the MSR-PEI vaccine in combination with anti-CTLA4 treatment significantly augmented the response from anti-CTLA4 treatment (Fig. 5.29).

![Graph](image-url)

**Figure 5.26. Combination therapy with anti-CTLA4 in B16F10 subcutaneous tumors.** Tumor growth of mice bearing established B16F10 tumors (inoculated with 1x10\(^5\) cells) and treated with anti-CTLA4 antibody (a-CTLA4, 100ug on days 3, 6, 9 and 12), anti-CTLA4 antibody in combination with the MSR-PEI vaccine (VP+a-CTLA4) using L25K PEI and 50ug of the M27, M30, M47 and M48 neoantigens on days 5, or left untreated (N). Data depicts individual tumor growth and n=8. Area under the curve was analyzed and ** indicates p < 0.01 compared to a-CTLA4 alone.

**5.4 Discussion**

The results from this chapter demonstrated a rapid and facile method to enhance peptide antigen immunogenicity to promote robust therapeutic anti-tumor immunity. PEI is a stable and easily manufactured synthetic cationic polymer that has
been widely used as a gene delivery agent. Plasmid DNA condensed with PEI showed dramatically enhanced uptake by cells and gene expression compared to naked DNA\textsuperscript{27,28}. This non-specific adsorptive endocytosis is likely a result of the electrostatic interaction between the cationic surface of the PEI-DNA condensate and the negatively charged cell membrane\textsuperscript{29-31}. Additionally, PEI offers advantage over other cationic polymers as a non-viral transfection vector because of its intrinsic endosomolytic activity. Here we instead describe a method to co-present PEI with peptide antigens in a layered adsorption fashion to enhance antigen immunogenicity. PEI can be incorporated into the MSRs in a rapid and facile manner. Near-complete incorporation of PEI into the MSRs can be achieved in 60 seconds by simple mixing. Peptides with various molecular weights and physical properties can be adsorbed onto PEI modified MSRs in less than three hours. The simplicity of this approach is expected to significantly reduce clinical burdens associated with manufacturing and vaccine variability.

Modifying the MSR vaccine with PEI drastically increased its anti-tumor therapeutic efficacy. We showed that the MSR-PEI vaccine using both branched and linear forms of PEI enhanced antigen specific CTL response and therapeutic tumor control compared to the MSR vaccine. It is likely that the enhanced anti-tumor responses were mediated by the enhanced CTL response. However, recent works have shown that vaccination with CD4\textsuperscript{+} immunogenic mutations (MHC-II restricted neoantigens), rather than with CD8\textsuperscript{+} mutations (MHC-I restricted neoantigens), conferred strong anti-tumor responses\textsuperscript{7}. CD4\textsuperscript{+} T cells play a crucial role in modulating the intratumoral microenvironment by mediating IFN\gamma production, stimulating CD8\textsuperscript{+} T
cell expansion and recruiting other killer cells such as NK cells. Therefore, it will be important to understand the effect of PEI on driving CD4 T cell responses, especially in the context of neoantigens.

In this system, PEI likely functions as a DC adjuvant for peptide and protein antigens. PEI induced DC maturation and pro-inflammatory cytokine production. Among the tested cytokines, IL-6 and TNFα was markedly increased, but not INFγ. Additionally, DCs stimulated with PEI showed markedly better antigen cross presentation capacity, which has been shown to be important in generating CTL responses. Consistent with these in vitro results, the MSR-PEI vaccine site and the dLNs were enriched in activated and antigen-presenting DCs compared to the MSR vaccine in vivo. 24 hours after vaccination, mice treated with the MSR-PEI vaccine also showed increased blood serum TNFα level. Interestingly, mice treated with the MSR vaccine or the MSR-PEI vaccine showed enhanced but comparable levels of blood IFNγ level. One possible explanation for this is that both vaccines contained the same amount of CpG-ODN, which has been shown to be a potent interferon inducer. A number of studies have provided evidence that PEI (mostly in condensed forms) can nonspecifically activate multiple TLR pathways, namely, TLR-4 and TLR-5. It is likely that PEI and CpG stimulate distinct TLR signaling in DCs and provide synergy. Future work will provide more in-depth mechanistic understanding of how PEI (in both condensed and adsorbed forms) is taken up by DCs and how it interacts with various TLR and NLRs. Although a degree of toxicity has been reported in studies using PEI as a transfection agent, the dose range used in this chapter was significantly lower in
comparison\textsuperscript{36}. Additionally, at the doses used here, we did not observe significant cell death \textit{in vitro or in vivo}.

The MSR-PEI vaccine induced impressive therapeutic anti-tumor immunity demonstrated using three independent tumor models. A number of peptide-, protein-, and viral-based cancer vaccines targeting the E6 and E7 domain in HPV\textsuperscript{+} tumors have been tested in preclinical and clinical settings, but they have failed to induce complete regression of established tumors. Here, we showed that most of the animals treated with the MSR-PEI vaccine resulted in complete regression of large established tumors, and the therapeutic regression accomplished with just one immunization of the MSR-PEI vaccine was stronger than multiple treatments of conventionally adjuvanted E7 vaccines and novel mRNA and nanoparticle-based technologies\textsuperscript{10,37-39}. Unlike the HPV\textsuperscript{+} TC-1 tumor model, the B16F10 and CT26 tumors are highly aggressive, immune suppressive and have low lymphocyte infiltration. When immunized with a pool of B16F10 or CT26 neoantigens, the MSR-PEI vaccine eradicated established lung metastases, induced therapeutic tumor growth control and synergized with anti-CTLA4 therapy. Notably, these therapeutic responses achieved with a single immunization of the MSR-PEI vaccine were comparable to repeated immunizations of RNA-based neoantigen vaccinations\textsuperscript{7}. Mechanistically, it was shown that the MSR-PEI vaccine dramatically enhanced lymphocyte infiltration into B16 tumors. Strikingly, CD4\textsuperscript{+} T lymphocytes, rather than CD8\textsuperscript{+} T lymphocytes, in the MSR-PEI treated tumors showed improved capacity to secret TNF-a and IFN-y upon antigen restimulation. These data provide further evidence that it is important to explore the role of CD4 T lymphocytes in vaccine induced anti-tumor responses. Finally, since tumor somatic mutation is well
correlated with the response rate to checkpoint blockade therapies, it will be interesting to explore whether the MSR-PEI vaccine is capable of expanding the spectrum of tumor specific mutation and augment epitope spreading.

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5.6 References


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Chapter 6: Conclusions, implications and future directions

6.1 Conclusions

In this thesis, a novel cancer vaccine platform was described. Long, rod-like mesoporous silica rods (MSRs) were designed to spontaneously assemble after injection, and individual particles stack on top in a random, low-packing density manner, to form inter-particle macropores in between that allow for cell infiltration. The material surface was highly amenable to modification, and chemical modification of the surface was shown to control the innate immune response to the material. Immune-modulating cues with different chemical properties, such as GM-CSF, CpG, Poly(I:C) and MPLA, were successfully incorporated into the mesopores or adsorbed onto the material surface, and released in a sustained manner. Likewise, tumor antigens in the forms of whole lysate, proteins and peptides with varying properties, were successfully incorporated into the MSR vaccine at high efficiency. Importantly, the modularity of this approach allows us to construct highly individualized vaccines by simply mixing and matching the individual cue-loaded particles. Finally, the vaccine platform was evaluated using three independent tumor models, and was shown to elicit potent therapeutic tumor growth control and regression, and synergy with checkpoint blockade therapies.

In chapter 2, we demonstrated that high-aspect-ratio, mesoporous silica rods (MSRs) injected with a needle spontaneously assemble in vivo to form macroporous structures that provide a 3D cellular microenvironment for host immune cells. High
aspect ratio MSRs were shown to recruit more immune cells than their low aspect ratio counterparts. Both the mesopores within individual particles and the interparticle space formed by the spontaneously stacking were important to recruit high numbers of immune cells. Additionally, it was demonstrated that modulating surface chemistry on the MSR scaffold significantly impacted its inherent inflammatory response in vitro and in vivo. PEG MSRs enhanced pro-inflammatory cytokine production in vitro and immune cell, especially CD11b+Gr.1+ myeloid cell, infiltration into the scaffold in vivo. Conversely, PEG-RGD and PEG-RDG MSRs displayed decreased inflammatory effects as compared to PEG MSRs, but the effect was not specific to the integrin-binding domain RGD. Finally, the Nlrp3 inflammasome was necessary for MSR stimulated IL-1b production and played a key role in regulating immune cell infiltration and cell phenotype in the MSR scaffolds. Overall, the findings from this chapter demonstrate a novel 3D scaffold material concept and that changing the surface chemistry on a scaffold could alter its immune cell infiltration profile and may aid the design of new materials-based vaccines for a variety of applications.

In chapter 3, a recruitment factor, GM-CSF, and a TLR9 agonist, CpG-ODN, were incorporated into the MSR scaffold developed in chapter 2. In mice, substantial numbers of DCs are recruited to the pores between the scaffold rods in response to GM-CSF. The recruitment of DCs and their subsequent homing to lymph nodes can be modulated by sustained release of CpG from the scaffold. Using a model antigen ovalbumin, it was demonstrated that injection of an MSR-based vaccine formulation enhances systemic T_{H}1 and T_{H}2 serum antibody and cytotoxic T cell levels compared to bolus controls. These findings suggest that injectable MSRs may serve as a
multifunctional vaccine platform to modulate host immune cell function and provoke adaptive immune responses.

In chapter 4, we explored whether a single injection of a mesoporous silica micro-rod (MSR) vaccine containing synthetic peptides can effectively generate potent and durable antigen specific humoral immunity, as continuous antigen uptake and processing by APCs and persistent toll-like receptor (TLR) priming have been shown to amplify antigen specific humoral immunity. It was shown that a single injection of the MSR vaccine against a gonadotropin-releasing hormone (GnRH) decapeptide elicited highly potent anti-GnRH response that lasted for over 12 months. The MSR vaccine generated higher titers than bolus vaccine and alhydrogel alum vaccine formulations. Moreover, a MSR vaccine directed against a Her2/neu peptide within the Trastuzumab binding domain showed immunoreactivity to native Her2 protein on tumor cell surface. Mechanistically, we found that the MSR vaccine induced persistent germinal center (GC) B cell activity for over 30 days after a single injection, and at least 7 days of immunostimulation by the vaccine was required to generate an effective humoral response. This work implies that the MSR vaccine represents a promising technology for synthetic peptide vaccines to bypass the need for multiple immunizations and enhance long-term production of antibodies against endogenous antigens in the context of reproductive biology, cancer, and chronic infection.

In chapter 5, we report a simple approach using polyethyleinemine (PEI) in a mesoporous silica micro-rod (MSR) vaccine to enhance antigen immunogenicity. Existing strategies to enhance peptide immunogenicity for cancer vaccination generally require direct peptide alteration, which beyond practical issues may impact peptide
presentation and result in vaccine variability. We showed that the MSR vaccine can be modified with PEI in a simple and rapid manner. The MSR-PEI vaccine significantly enhanced host dendritic cell activation and T cell response over the existing MSR vaccine and bolus vaccine formulations. Impressively, a single injection of the MSR-PEI vaccine using an E7 peptide completely eradicated large established TC-1 tumors in ~80% of mice and generated long-term memory. When immunized with a pool of B16F10 or CT26 neoantigens, the MSR-PEI vaccine eradicated established lung metastases, induced therapeutic tumor growth control and synergized with anti-CTLA4 therapy. Our findings using three independent tumor models suggest that the MSR-PEI vaccine approach may serve as a facile and powerful multi-antigen platform to enable robust personalized cancer vaccination.

Overall, the potency, flexibility, and simplicity of the MSR vaccine platform overcome many of the current barriers to unlocking the potential of personalized vaccines for cancer therapy. The potency of this approach can effectively drive immune responses against libraries of cancer specific mutations and synergize with other immunotherapies. The flexibility and modularity of this strategy are expected to drastically simplify the construction of personalized cancer vaccines. The simplicity of this approach is highly amenable to clinical translation as the vaccine is assembled in less than three hours by simple mixing of all components and can be stored in lyophilized form before or after antigens are added.
6.2 Implications

This work has advanced the fields of biomaterials and immunotherapy in three ways.

First, while most current macroporous scaffolds are fabricated ex vivo prior to implanting into a patient, this work described a new methodology that, instead, brings the scaffold assembly process directly in vivo via a simple injection. This approach dramatically simplifies the process of introducing macroporous scaffolds into a patient, and likely broadens their applications. Moreover, this thesis describes a modular approach to constructing biomaterial scaffolds. Individual rods could be modified with a library of bioactives, lyophilized for long-term storage, and mixed-and-matched prior to use to construct designer scaffolds. This approach enables us to easily manipulate the targeting, presentation and delivery of individual bioactives in the scaffold.

Second, the vaccine platform takes advantage of the inherent inflammatory properties of the biomaterial to form a highly rich immune milieu in vivo, and drives broad and potent innate and adaptive responses. Creating a synthetic space rich in various types of innate and adaptive immune cells opens up many possibilities to broadly interrogate the immune system. This thesis focused on manipulating DCs once they are recruited, but it is also possible to program neutrophils for pathogen clearance, T cells for specific cytokine production, etc.

Lastly, this thesis describes a facile approach to enhancing antigen immunogenicity. Current approaches to enhancing peptide immunogenicity generally require chemical or physical modification of the peptide, which, beyond practical challenges, could alter the bioactivity of the peptides. Personalized vaccines typically
require at least several peptide antigens. Modifying these antigens individually prior to incorporating into a vaccine could result in low yield, high variability and low efficacy. This thesis described novel methods to increase the immunogenicity of multiple peptides by simple adsorption of the antigens to modified material surfaces. This simple method led to potent anti-tumor immune responses using a pool of tumor specific peptides. Since the modified materials could be stored in lyophilized forms before or after antigen incorporation, this approach is also highly amenable to translation.

6.3 Future directions

This thesis describes a powerful biomaterial platform to modulate host cells in vivo. The strength of this platform lies with its ability to induce a strong immune response, its flexibility and its modularity. A variety of biological actives with different chemical and physical properties can be incorporated with high efficiency into the material via a simple mixing procedure. After incorporation, scaffolds containing combinations of the bioactive could be easily constructed by mixing individual particles. The power of this platform was demonstrated here in the context of a cancer vaccine, but many questions still remain and the potential of this material platform can be explored in many contexts. In this section, I describe only four examples, but many possibilities exist, such as using the platform to probe fundamental questions related to immunology, to deliver mRNA constructs to recruited DCs, to program the tumor microenvironment as an endogenous vaccine, and to recruit host innate and adaptive cells to aid tissue regeneration.
Other than mDCs, other types of innate and adaptive immune cells were recruited to the MPS/MSR vaccine site. In fact, the majority of the recruited cells have a myeloid/neutrophil phenotype, defined as CD11b+CD11c-Ly6GhighLy6Cmid. This population was present in blank MSR scaffolds, and was enriched in the presence of GM-CSF. In blank MSR scaffolds, this population was enriched after PEG modification of the scaffold. The functional role of this population in the scaffold has not been explored. Studies have speculated that monocytes can differentiate into DC-like cells with antigen presentation capabilities under inflammatory conditions\textsuperscript{1,2}. It would be interesting to understand the origin and function of these myeloid cells in the vaccine. For instance, do they eventually differentiate into APCs and contribute to driving an adaptive immune response? Alternatively, do they exhibit a tolerogenic phenotype (similar to MDSCs in the tumor\textsuperscript{3}) and suppress the immune activity in the vaccine? If so, does local depletion of this population, thereby creating a more “DC-pure” device, enhance the efficacy of the vaccine?

The MSR vaccine described in this thesis was shown to generate high antibody titers. The breadth of the antibody response has yet to be characterized in depth. From superficial analysis from this thesis and works in progress, the generated antibodies have broad IgG isotypes (we detected IgG1, IgG2a, IgG2b, IgG2c and IgM). The Fc portion on the IgGs can trigger a number of effector functions such as neutralization, opsonization and macrophage phagocytosis and antibody dependent cell-mediated cytotoxicity (ADCC)\textsuperscript{4}. In particular, ADCC has been strongly implicated in effective clearance of tumor cells by NK cells and macrophages\textsuperscript{5-8}. It will be informative to analyze the IgGs generated by the vaccine to understand their FcR engagement affinity.
and their effector functions. Another interesting area to pursue in the future is to engineer a biomaterial construct that is capable of preferentially stimulating isotype switching. For example, in the context of tumor immunotherapy, it would be exciting to engineer a vaccine construct that stimulated purely mouse IgG2 antibody production.

The kinetics of antigen release from the MSR platform can be tuned by altering particle size. So far, the antigens are presented and released from the MSR vaccine in a sustained manner. It has been speculated that the persistent Germinal Center and B cell response generated by the MSR vaccine is a result of an automatic “prime-boost” strategy through sustained antigen delivery. However, in a classical prime-boost strategy, the boost occurs after the immune response from the prime has subsided. The boost then reawakens the immune system once it has had some rest. Moreover, recent data suggest that vaccination regimens that slowly increasing in antigen dose induces a stronger T and B cell response compared to regimens with repeated injections of the same dose\textsuperscript{9,10}. It would be interesting to explore altered antigen release kinetics by simply tuning the size of the MSR particles. In theory, antigens loaded into particles between 20nm and 100nm will be efficiently drained to dLNs immediately after injection (within hours)\textsuperscript{11}, whereas antigens loaded into 100um particles will require DC recruitment to be taken up and transported to dLNs (days). By tuning the ratio of nano and micro particles in the material construct, it may be possible to achieve an optimal “prime-boost” kinetics to generate high affinity and avidity T and B cell responses.

The MSR scaffold can be modified with other materials to induce controlled tumor mutation to enhance responsiveness to checkpoint blockade therapies. Recent
studies show that somatic mutation load in the tumor correlates with responsiveness to checkpoint blockade therapies. Multiple strategies, such as radiotherapy, are being assessed to increase mutagenic tumor death and response to checkpoint blockade therapies\textsuperscript{12-14}. However, these approaches generally lack controlled cell targeting. One potential idea to test is the intratumoral co-delivery of radiation and checkpoint blockade inhibitors. Radiation sensitive materials and immune checkpoint blockade inhibitors can be into the MSRs, and injected directly into the tumor. It will also be possible to introduce more sophisticated tumor-targeting motifs for more localized radiation delivery. This approach could potentially provide a large dose in a localized manner, allow us to better understand the interplay between immunotherapy and radiation therapy, and reduce systemic toxicity.
6.4 References


Appendices: Preliminary Studies for Future Work and Detailed Protocol for MSR synthesis

Appendix A. Delivery of an antigen-adjuvant conjugate from the MSR vaccine

A.1: Introduction
Antigen-adjuvant conjugates have shown effective stimulation of cross presentation of exogenous antigens and CD8 T cell priming\(^1\). Here, we propose that delivery of an antigen-adjuvant conjugate from the mesoporous silica (MPS) vaccine scaffold will increase the immunogenicity and CD8 T cell response towards the antigen as compared to delivering the antigen and adjuvant as separate entities.

We covalently conjugated an antigen to a TLR adjuvant through maleimides (sulphhydryl-sulphhydryl), carbodiimide (amine-carboxylic acid) and photo-click (norbornene-thiol) linkers. We demonstrated success of conjugation and in vivo T cell response using a model antigen Ovalbumin (OVA) and its CD8 epitope SIINFEKL. However, broadly, the antigen could comprise of a) a protein or peptide against which an immune response needs to be elicited or b) a lysate of a cell associated with tumor. We are also currently exploring conjugation with other TLR agonists such as MPLA and Poly (I:C).
A.2: Materials and Methods

CpG-OVA conjugation.

OVA protein at 5 mg/ml was reacted with 50 molar excess of sulfo-SMCC NHS (Pierce) in pH 7.5 PBS for 2 hours to functionalize primary amines on the protein with maleimide. After purification via 7K desalting column (Pierce), the modified protein was added to a solution of reduced thiol-CpG (IDT) containing 1 free thiol per CpG molecule and reacted on shaker for 12 hours at room temperature. Excess CpG was removed using a 30K spin filter column (Millipore).

SDS PAGE

The CpG-OVA conjugate was denatured at 95°C for 10 minutes and subsequently chilled on ice. The conjugate was not reduced. To perform gel electrophoresis, 10ug of the conjugate was separated using a 10-20% Tricine gel (Invitrogen). Proteins were stained using 0.1% Coomassie (Thermo Fisher) and destained using 40% methanol and 10% glacial acetic acid.

Peptide restimulation and tetramer analysis of circulating PBMCs.

80ul of blood was collected into heparin coated tubes (BD). Red blood cells were removed using 1ml RBC lysis buffer (BioLegend) for 1 min. at RT. To perform tetramer analysis, blood cells were incubated with 7ug/ml peptide-MHC tetramers conjugated with alexa fluor 647 for 30 min. at 37 °C. Subsequently, cells were collected and stained with 7-AAD, anti-mouse CD3e and CD8a for 15 min. on ice. Cells were then washed
and analyzed using flow cytometry. To perform peptide restimulation, blood cells were washed and pulsed with 2ug/ml of various MHC-I restricted peptides for 1.5 hours at 37 °C. Subsequently, cytokine secretion was stopped with golgi plug (MF) and cells were cultured for 4 hours at 37 °C. Cells were collected and stained with Alexa-Fluor 780 dead exclusion dye, anti-mouse CD3e, CD8a and CD4 for 15 min. on ice before fixing and permeablizing (eBioscience 88-8824-00). Cells were then stained with anti-mouse IFNγ for 30 min. in 4 °C and analyzed using flow cytometry (LSRFortessa, BD).

**BMDC stimulation assay.**

Detailed protocol described in Chapter 3

**Release assay.**

Detailed protocol described in Chapter 2

**Prophylactic tumor study.**

Female C57BL/6 mice were vaccinated subcutaneously in the flank with the MSR vaccine containing 1ug GM-CSF, 100ug CpG conjugated to 100ug OVA. On day 11, mice were inoculated with 0.3x10⁶ B16-OVA cells subcutaneously behind the neck. Animal survival was monitored and mice were euthanized when the largest side reached 2cm or according to IACUC standards.
A.3: Results

CpG-OVA conjugation was confirmed using gel electrophoresis. OVA was visualized using Coomassie staining and conjugation was determined according to the increase of the protein's molecular weight. On average, each OVA protein contains 1 CpG molecule (FigA.1).

![Figure A.1 SDS-PAGE of the CpG-OVA conjugate](image)

To evaluate the ability of the CpG-OVA conjugate to be presented by DCs, mouse BMDCs were stimulated with the conjugate overnight, and the surface expression of SIINFEKL-MHC-I was analyzed. BMDCs stimulated with the CpG-OVA conjugate showed enhanced cross-presentation (FigA.2). In comparison, BMDCs stimulated with the unconjugated CpG+OVA only elicited a moderate increase.
The release kinetics of the CpG-OVA conjugate was subsequently examined. The CpG-OVA conjugate was loaded into the mesoporous silica (MPS) scaffold and was released in a sustained manner followed by a burst release (FigA.3).

The ability of the MSR vaccine containing the CpG-OVA conjugate was then analyzed. C57bl/6J mice were immunized with MSR scaffold containing 1μg GM-CSF.
and 100ug OVA, 1ug GM-CSF, 100 ug CpG and 100ug OVA (MPS vaccine) or 1ug GM-CSF and 100ug OVA conjugated to 100ug CpG (MPS conjugate vaccine). After 7 days, peripheral blood was analyzed for SIINFEKL CD8\(^+\) T cells. MPS conjugate vaccine increased the presence of SIINFEKL specific CD8\(^+\) T cells by 2 fold compared with the MPS vaccine (FigA.4).

![Figure A.4. Peripheral CD8 T cell response after vaccination.](image)

Figure A.4. Peripheral CD8 T cell response after vaccination. Percentage of SIINFEKL tetramer\(^+\) CD3\(^+\)CD8\(^+\) T cells isolated from peripheral blood on day 7 after immunization with the MSR+GM-CSF+OVA, the MSR vaccine (MPS vaccine), the MSR conjugate vaccine (MPS conjugate vaccine), or left unimmunized (Naive). Data depicts mean +/- sd, n=4, * indicates p < 0.05.

Finally, a preliminary study evaluating the ability of the MSR conjugate vaccine was conducted. C57bl/6J mice were immunized with MPS scaffold containing 1ug GM-CSF and 100ug OVA conjugated to 100ug CpG (MPS conjugate vaccine. After 11 days, mice were inoculated with 3x10\(^5\) B16 melanoma cells transfected with the OVA vector (B16-OVA) and tumor growth was monitored. The MPS conjugate vaccine resulted in 80% prophylactic tumor protection whereas unvaccinated naïve mice succumbed to tumor within 20 days (FigA.5).
Figure A.4. Overall survival in mice inoculated with B16-OVA tumors. Percentage of survival in mice vaccinated with the MSR conjugate vaccine (MPS conjugate vaccine) and subsequently inoculated with B16-OVA cells. Data depicts mean +/- sd, n=5.
A.4: References

Appendix B. MSR synthesis

Materials:
Pluronic P123: Sigma-Aldrich 435465
Tetraethyl orthosilicate (TEOS): Sigma-Aldrich 131903

Procedure:
1. Dissolve 4 g of pluronic 123 (p123) in 130 mL of H2O and 20 mL of 37% HCl
2. Stir to dissolve at room temperature
3. Once completely dissolved, increase temperature to 40 °C and set speed to 600rpm
4. Add 9.2ml of TEOS quickly using a 10ml pipet tip
5. Stir for 20 hours
6. Raise temperature to 100 °C and stir for 24 hours. (Note: do not completely cover the flask)
7. Let the flask cool down
8. Filter the particles and air dry at 60 °C
9. Extract the p123 using 500 ml concentrated ethanol and 5 ml concentrated HCl
10. Stir overnight at 60-80 °C (Note: the beaker or flask should be covered with vinyl wrap to prevent evaporation)