Spring 2010

Co-delivery of cationic polymers and adenovirus in immunotherapy of prostate cancer

Jessica Beth Graham
University of Iowa

CO-DELIVERY OF CATIONIC POLYMERS AND ADENOVIRUS IN IMMUNOTHERAPY OF PROSTATE CANCER

by

Jessica Beth Graham

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Chemical and Biochemical Engineering
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Associate Professor Aliasger K. Salem

ABSTRACT

Prostate cancer is the most common non-skin cancer in America, and the most commonly diagnosed cancer among males. When metastatic, the disease can ultimately be incurable. Consequently, alternative strategies to current treatments are sought, especially in the area of immunotherapy. Vaccine immunotherapy using a specific antigen, such as prostate specific antigen (PSA) seeks to stimulate both the innate and adaptive immune system to destroy tumor cells in the body. PSA is an ideal target antigen given that it has a narrow distribution in tissues and is expressed in virtually all prostate cancer cases. An adenovirus encoding for PSA (Ad-PSA) can be used to deliver the genomic data encoding for PSA production and secretion to the target cell. This type of viral gene delivery system has already been shown to have the potential to stimulate anti-tumor activity.

To enhance this activity and increase transfection efficiency, we proposed the combination of a viral system with a non-viral system, in the form of a cationic polymer such as poly(ethyl)enimine (PEI) or chitosan. Cationic polymers will complex with the negatively charged adenovirus to form nanoparticles that can be used in gene delivery. Delivery in nanoparticle form can give enhanced uptake by the antigen-presenting cells necessary to initiate the targeted immune response. To further augment this response, previous research has shown that CpG sequences act as an adjuvant to enhance the efficacy of the Ad-PSA vaccines' tumor protection. CpG delivered in particulate form has also been shown to be more effective than delivery in solution. The objective of this proposal was to test the hypothesis that co-delivery of this targeted viral/non-viral gene delivery system will enhance tumor protection in a mouse model of prostate cancer.

Using the OVA model antigen system, we found that the adenovirus encoding OVA (AdOVA), coupled with the polymer PEI, enhanced tumor protection in vivo compared to AdOVA alone. To move towards our therapeutic model, these experiments were repeated using chitosan as the cationic polymer carrier, delivering AdOVA, and incorporating CpG into some particles. In this set of experiments, we found that AdOVA + CpG gave the best tumor protection in challenge studies. AdOVA + chitosan + CpG showed a decrease in protective levels and numbers of antigen-specific immune cells.

Further experiments focused on elucidating the mechanisms by which chitosan and CpG modulate the immune response. Using the therapeutic AdPSA model, chitosan was not found to enhance tumor protection or numbers of antigen-specific immune cells. Additional experiments found that this depression was not due to problems with viral infectivity or secretion due to chitosan complexation. A series of kinetics studies were performed which showed that peak levels of effector T cells were present 14 days later in AdPSA + CpG immunized mice than in AdPSA alone. This delayed effect may explain the increased levels of protection in AdPSA + CpG mice, and be useful in future vaccine design concerning the timing of peak response.

Abstract Approved: _____
Thesis Supervisor

_____
Title and Department

_____
Date

CO-DELIVERY OF CATIONIC POLYMERS AND ADENOVIRUS IN IMMUNOTHERAPY OF PROSTATE CANCER

by

Jessica Beth Graham

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Chemical and Biochemical
Engineering
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Associate Professor Aliasger K. Salem

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

PH.D. THESIS

_____

This is to certify that the Ph.D. thesis of

Jessica Beth Graham

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Chemical and Biochemical Engineering at the May 2010 graduation.

Thesis Committee: _____
Aliasger K. Salem, Thesis Supervisor

_____
David Lubaroff

_____
Allan Guymon

_____
Julie Jessop

_____
Jennifer Fiegel

To my grandparents

ACKNOWLEDGMENTS

I would first like to thank my thesis supervisor, Dr. Aliasger Salem, for his help in matching a project to my research interests, and guiding me through its development. Our regular meetings and discussions kept the project moving and evolving, and Dr. Salem was always receptive to questions and took the time to address any concerns. I would also like to thank Dr. David Lubaroff for assistance in bringing this collaborative project together. The groundwork that was completed in his lab was vital to the existence of this research, and he was very helpful in designing experimental methods and aiding my training in immunology. I would like to thank my committee members Dr. Jennifer Fiegel, Dr. Allan Guymon, and Dr. Julie Jessop for their direction and advice towards the development and completion of my research.

This research could not be undertaken without the help of all of the current and former members of the Salem Lab. Special thanks to Dr. Caitlin Lemke for all of her help with the project, and for instruction on flow cytometry analysis. Many long hours in the cell culture room (and even a half-marathon!) were passed more pleasantly with our discussions and chats. Trent Waugh from the Lubaroff Lab assisted with hybridoma growth and antibody production, and was always willing to help troubleshoot an assay. Former members Dr. Xueqing Zhang, Dr. Treniece Terry, Dr. Janjira Intra, and Megan Pearce aided in my early training in the lab and guided me in navigating graduate school. Current lab members Dr. Sean Geary, Aiman Abbas, Dahai Jiang, NaJung Kim, Yogita Krishnamachari, Vijaya Joshi, Dennison John, Sheetal D'mello, and Kristan Sorenson have been a pleasure to work with, and have been helpful and encouraging with my research work. I have no doubt that without these friendships formed with my colleagues, I would not have been able to work to the best of my abilities, and the ride surely would not have been as enjoyable.

I would also like to thank my family and friends for all of their love and support through the graduate school process. My parents have given me emotional, financial, and

material support, and has always done whatever they could to help me achieve my goals. Their willingness to jump in the car and drive to Iowa so that we could spend a holiday together while I had experiments going, is just one example of their endless support. Shane, Ashlie, and Kali have also made the trek to Iowa to visit us and our time together was always helpful to recharge and relax. The friends we have made in Iowa are too numerous to list, and we will miss them dearly. Many of us have made the journey through graduate and professional school together, and this support through the tough times was essential towards maintaining sanity and keeping a smile on my face.

Finally, I thank my husband Gavan for all of his love, support, and humor throughout my graduate studies. Your support of everything I do was instrumental in my development as a researcher, and your strength and beliefs in me the underlying reason to any of my success. You always know how to make me smile, whether with a funny story or a container of Ben and Jerry's (which is exactly what I need after a 14 hour day of pipetting!). I look forward to the future we are building together.

ABSTRACT

Prostate cancer is the most common non-skin cancer in America, and the most commonly diagnosed cancer among males. When metastatic, the disease can ultimately be incurable. Consequently, alternative strategies to current treatments are sought, especially in the area of immunotherapy. Vaccine immunotherapy using a specific antigen, such as prostate specific antigen (PSA) seeks to stimulate both the innate and adaptive immune system to destroy tumor cells in the body. PSA is an ideal target antigen given that it has a narrow distribution in tissues and is expressed in virtually all prostate cancer cases. An adenovirus encoding for PSA (Ad-PSA) can be used to deliver the genomic data encoding for PSA production and secretion to the target cell. This type of viral gene delivery system has already been shown to have the potential to stimulate anti-tumor activity.

To enhance this activity and increase transfection efficiency, we proposed the combination of a viral system with a non-viral system, in the form of a cationic polymer such as poly(ethyl)enimine (PEI) or chitosan. Cationic polymers complex with the negatively charged adenovirus to form nanoparticles that can be used in gene delivery. Delivery in nanoparticle form can give enhanced uptake by the antigen-presenting cells necessary to initiate the targeted immune response. To further augment this response, previous research has shown that CpG sequences act as an adjuvant to enhance the efficacy of the Ad-PSA vaccines' tumor protection. CpG delivered in particulate form has also been shown to be more effective than delivery in solution. The objective of this proposal was to test the hypothesis that co-delivery of this targeted viral/non-viral gene delivery system will enhance tumor protection in a mouse model of prostate cancer.

Using the OVA model antigen system, we found that the adenovirus encoding OVA (AdOVA), coupled with the polymer PEI, enhanced tumor protection in vivo compared to AdOVA alone. To move towards our therapeutic model, these experiments were repeated using chitosan as the cationic polymer carrier, delivering AdOVA, and

incorporating CpG into some particles. In this set of experiments, we found that AdOVA + CpG gave the best tumor protection in challenge studies. AdOVA + chitosan + CpG showed a decrease in protective levels and numbers of antigen-specific immune cells.

Further experiments focused on elucidating the mechanisms by which chitosan and CpG modulate the immune response. Using the therapeutic AdPSA model, chitosan was not found to enhance tumor protection or numbers of antigen-specific immune cells. Additional experiments found that this depression was not due to problems with viral infectivity or secretion due to chitosan complexation. A series of kinetics studies were performed which showed that peak levels of effector T cells were present 14 days later in AdPSA + CpG immunized mice than in AdPSA alone. This delayed effect may explain the increased levels of protection in AdPSA + CpG mice, and be useful in future vaccine design concerning the timing of peak response.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INRODUCTION AND BACKGROUND

Prostate Cancer

Prostate cancer is the most commonly diagnosed cancer among males in the United States, accounting for 33% of all cases, and is the second leading cause of death in this population. In 2005, over 232,000 men were diagnosed with prostate cancer, with nearly 30,000 deaths that year.[1] Early treatments for prostate cancer include surgery, radiation, chemotherapy, and hormone therapy. In beginning stages, prostate cancer tumors require testosterone to grow, so systemic treatments for hormonal blockade can initially limit further tumor growth. With time, the cancer progresses into a hormone refractory state, androgen independent cancer cells proliferate. Metastases are possible, and the tumor burden may ultimately become fatal.[2]

In the late 1980s, the development of the prostate specific antigen (PSA) blood test increased early detection rates in patients with prostate cancer. PSA is a single chain 34 kDa glycoprotein that is secreted from epithelial cells of the prostate gland. PSA levels are often elevated in men with cancer, and in 1986 the FDA approved the PSA test to monitor levels in patients. In 1994, the test was also approved as a prostate cancer screening tool. As a result, nearly 90% of all prostate cancers are diagnosed at an early stage. The treatment options for prostate cancer involve serious systemic side effects, due to surgery, chemotherapy, or radiation.[3] In the 1990s, many improvements in these methods were developed. Surgical removal of the prostate can be accomplished with less nerve damage than earlier methods, lessening the side effects in some patients. The use of brachytherapy, which implants small radioactive seeds in the prostate, has shown good results in some men with early-stage prostate cancer. Advances in chemotherapy drugs, such as docetaxel, have also improved the treatment of patients no longer responding to hormonal therapy.[1,3]

While these advances have improved the treatment of cancer over time, they are still not without side effects, and treatment is limited when men have reached a hormone-refractory state. The developing area of immunotherapy, in which the body's own immune system is activated to fight the cancer, is particularly attractive to the treatment of prostate cancer. Immunotherapy targets tumor-associated antigens (TAAs) that are specific to the cancer cells, sparing healthy tissue and organs.[4] Given that the target cells have a narrow tissue distribution, targeting PSAs can allow destruction of tumor cells without extensive systemic side effects.[5,6]

Several immunotherapeutic approaches have been investigated, including the use of viral vectors for gene transfer into cancer cells[7,8,9,10], dendritic cell therapy[4,6,11,12], and the Bacillus Calmette-Guérin **(**BCG) tuberculosis vaccine.[13,14] Vaccine immunotherapy is especially attractive in prostate cancer treatment, as it can be used to stimulate both innate and adaptive immunity for tumor destruction.[15,16,17] Prostate cancer is slow-growing and can be detected in early stages, so it is possible that vaccine immunotherapy could destroy and limit the tumor burden. Additionally, a therapeutic vaccine could build memory cells necessary to maintain an effective immune response over time. These therapeutic vaccines teach the immune system to recognize a protein, such as prostate specific antigen (PSA), which rises with prostate cancer cell growth. These gradually rising PSA levels remain under the level of detection by the body's immune system, and vaccine immunotherapy can be used to break tolerance.[18,19] In order for an antigen-specific, targeted immune response to occur, the target antigen must be presented in the appropriate pathway, to signal that the target cells are a threat and should be eliminated.[9,20] Vaccine immunotherapy can present the antigen in the pathway necessary to launch an effective immune response against PSA-secreting cells. It is anticipated that this antigen-specific immunization could eliminate both hormone-dependent and hormone-independent prostate cancer growth.

Immunotherapy for cancer

Before further discussion of the therapeutic goals of this research project, additional background information on the immune response is necessary. The utilization of the body's adaptive immunity is crucial in developing an immunotherapeutic vaccine. While many types of white blood cells make up the body's immune defenders, there are three important classes of effector cells involved in the immune response: NK cells, B cells, and T cells. Natural killer (NK) cells are a type of cytotoxic lymphocyte that kills tumor and virus-infected cells by perforating the cell membrane. B cells produce antibodies that recognize foreign antigens. The T cells are lymphocytes that are responsible for orchestrating the immune response. There are two types: CD4+, or helper T cells, and CD8+, also called cytotoxic T lymphocytes or CTLs. CD4+ T cells are necessary to activate other immune cells, including B cells to produce antibody response, and the CD8+ T cells. The CTLs directly target cells infected with a virus or particular antigen marker.[19,21](Figure 1)

The T cells only respond to antigen that is bound to the Major Histocompatibility Complex (MHC) class I or II on an antigen-presenting cells (APC). These cells, such as macrophages and dendritic cells, take up the antigen and present it on the cell surface as a marker. Presenting the antigen as a marker in this fashion directs effector cells to recognize this marker, and take action. Then, when CD4+ cells encounter an APC with the MHC class II antigen presentation, or CD8+ cells encounter an APC with the MHC class I antigen presentation, they will become activated to provide a response to the target antigen.[21,22] In this way, the PSA antigen can be used as target, and when the helper T cells encounter this antigen, they quickly expand and activate CTLs to attack the cancer cells presenting the PSA antigen. One method by which the PSA antigen can be processed and presented to macrophages and dendritic cells is by immunization with an adenovirus encoding for the production of PSA.[2,23,24,25] . The general scheme for antigen presentation and T cell expansion in a prostate cancer model is shown in Figure 2.

Gene Delivery for treatment of cancers

The treatment of prostate cancer by vaccine immunotherapy requires a vector to deliver the PSA gene, to cause production and presentation of the antigen for an anti-PSA immune response. Gene delivery aims to transfer functional genetic material to fight diseases caused by damaged or malfunctioning genes. Genes are delivered by vectors, which can be viral vectors, such as viruses or adenoviruses, and non-viral vectors, such as liposomes or polymer complexes. One potential goal of gene delivery is in the area of immunotherapy, as genes may be used to repair, stimulate, or enhance the immune system's response.[4,9,26] The body often will not recognize cancer cells as foreign since they are derived from the host, and therefore cancer can go undetected by the immune system. Gene delivery can be used to stimulate an immune response to a target cell or protein. This response within the patient is optimal towards an efficient and cost-effective immunization.

To date, research has shown both the benefits and drawbacks of viral vs. non-viral gene delivery. Viral vectors result in strong transfection efficiencies, as viruses are programmed to recognize certain cells and insert their DNA into them. However, their undesirable immunogenicity cannot be ignored.[27] After the death of a patient in a University of Pennsylvania clinical trial, the use of viral vectors was severely set back. Adenovirus-based vaccines are now being used with deletion of the replication portion of the viral gene, so that the virus can infect and produce the target antigen, but not multiply to cause these undesired systemic responses. Non-viral vectors are less immunogenic, but generally give lower transfection efficiencies than viral vectors.[28]

There are specific advantages and limitations limitations of each type of delivery vehicle, as will be discussed shortly. To exploit these advantages while overcoming any limitations, we hypothesize that a co-delivery of a non-viral polymer nanoparticle with the viral adenovirus gene delivery system will generate greater immune stimulation and tumor protection than either system would alone. Optimizing each part of the

nanocomplex will be key to the overall performance. This includes the polymer, such as poly(ethyl)enimine (PEI) or chitosan, the adenovirus vaccine with PSA antigen, and any adjuvants, such as CpG oligodeoxynucleotides.

Viral Gene Delivery

Adenovirus

Adenoviruses have successfully been used as viral delivery vehicles in cancer immunotherapy, and are capable of targeting of self antigens.[16] In theory it had been questionable whether differences in targeting to cancer and healthy cells would be possible, as both are derived from the host. However, it has been observed that some immunocompetent cancer patients show spontaneous tumor regression, while immunocompromised patients show a higher cancer rate.[7] This indicates that the immune system is able to recognize tumor cells and initiate an immune response to eliminate them. After introduction into the cell, the tumor associated antigen is processed by both the MHC I & II pathways, giving a CD8+ and CD4+ T cell response, both of which are necessary for tumor destruction. Adenovirus vaccines are therefore very attractive for effective cell processing and MHC expression, and in stimulating a cell-mediated immunity.[23,29]

One limitation with adenovirus vaccines is the fact that adenoviruses are quite prevalent and most people have neutralizing antibodies against the most common serotypes, such as Ad5. To overcome this prior immunity, increased vaccine dose or booster approaches might be employed.[7] Additionally, co-delivery of the vaccine with a polymeric carrier might help to protect the adenovirus by masking it to the immune system during delivery.[27,30] Several adenovirus vaccines have been used in animal studies and clinical trials, including an adenovirus/prostate-specific antigen vaccine in men with metastatic prostate cancer.[24,31]. Several adenovirus vaccines have been used

in animal studies and clinical trials, including an adenovirus/prostate-specific antigen vaccine in men with metastatic prostate cancer.[24,31]

Non-viral Gene Delivery

Cationic Polymer: PEI

The cationic polymer PEI comes in two forms: linear and branched. (Figure 3) The repeat unit of the polymer is two carbon atoms followed by one nitrogen atom. In the branched form, PEI has 1^o, 2^o, and 3^o amines that can be protonated, allowing PEI to serve as a buffer through a wide pH range.[26] The positive charge of the PEI gives effective binding to the negatively charged DNA, and this condensation protects the DNA from digestion as the complex travels through the cell. Once in the endosomal compartment, PEI might act as a buffer to induce osmotic swelling and cause release from the endosome, which is necessary to avoid degradation of the DNA when the endosome fuses with the lysosome.[32] Previous studies have found that PEI/DNA complexes with higher buffering capacities give better transfection efficiencies- uptake by the desired cell.[28]

PEI has drawn interest for gene delivery applications because of its endosomolytic activity and strong DNA compaction ability. However, cell viability has been shown to be adversely affected by exposure to PEI, possibly because of membrane permeabilization by PEI. Our group has previously observed that PEI has an LD50 value (value at which 50% of the cells die) of 25 μg/ml.[33] When bound to a ligand, the cytotoxic effects of PEI appear to be lessened. Lower molecular weight PEI has shown lower toxicity than higher molecular weight PEI, most likely because of aggregation of the larger molecules on the outer cell membrane, causing necrosis.[34]

Several groups have studied how PEI's structure and molecular weight affect transfection efficiencies in varying systems. The branched form of the molecule has

given greater success in transfection experiments and is usually the form of PEI used in these studies. It has been reported that the molecular weight of the PEI affects transfection efficiencies, with lower molecular weight PEI (25 kDa) giving better uptake than higher molecular weight PEI (800 kDa), possibly because of aggregation of higher molecular weight particles.[35] However, these findings are controversial, since additional studies found that PEI22, a linear molecule, had greater transfection efficiencies than the branched molecules PEI25 or PEI800 when applied to neuroblastoma and colon carcinoma cell lines *in vitro* in a salt buffer.[36] The choice of PEI structure for improved transfection efficiency will be influenced by the cell line under consideration.

Another method of characterizing the PEI complexes is through analysis of the ratio of the nitrogen atoms in PEI to the phosphate atoms in the compacted DNA, called the N/P ratio. In order for the DNA to condense, approximately 90% of its charged groups must be neutralized with the positive charge from the amine group in PEI, requiring a N/P ratio of at least 2-3. Complexes with a higher charge ratio give better transfection efficiency, but also show higher toxicity effects. It is hypothesized that the additional PEI present aids in the complex's release from the endosome; however, excess PEI may also cause membrane permeabilization.[37,38]

When PEI accepts protons in the endosomal compartment, the pH there is lowered, causing osmotic swelling and possibly allowing the endosome to burst, releasing the PEI before it reaches the degradative compartment of the lysosome.[32] The PEI complex can then travel to the cell nucleus for transgene expression. This hypothesis for how PEI escapes lysosomal degradation is often referred to as "the proton sponge effect".[28] Originally, the proton sponge effect was thought to take place in the lysosomes, before reaching the endosomal compartment. This mechanism was then disputed by a number of investigators, who argued there was no lysosomal involvement in freeing the PEI. Several groups have since found further proof for this mechanism occurring in the

endosomes rather than the lysosomes, and their data is consistent with these previous findings.[26,28,32,36]

Cationic Polymer: Chitosan

As PEI is synthetic and not biodegradable, its high transfection efficiency must be weighed against negative toxicity issues. Chitosan is an abundant natural cationic polysaccharide, derived from crustacean shells. Being non-toxic with good biocompatibility and DNA binding affinity, it is an attractive polymer for DNA delivery.[39,40,41,42] Additionally, it has a chemical structure similar to PEI, with free amino groups that can be protonated, aiding in endosomal escape. (Figure 3). The polymer consists of β1-4 linked glucosamine partly containing *N*-acetyl-glucosamine. The chitosan backbone has several amino groups, requiring a pH below 6 to be soluble. Not all of the amino groups are protonated at physiological pH. The N/P ratio can also be used to relate the number of amino groups in the chitosan to the number of phosphates in the DNA it is complexed with, as in PEI. [39] The safety of chitosan has been demonstrated in both animal and human studies. Currently, it is used in a variety of applications, including as a pharmaceutical excipient[43], as a weight loss supplement[44], and in wound-healing products.[42]

In vitro studies show that the linear chitosan cannot deliver as high levels of transfection as branched PEI, likely due to these structural differences. However, chitosan-DNA complexes formed are of similar sizes to PEI-DNA complexes at optimal charge ratios, all well below the 500nm mark that is necessary for endocytosis.[42] Toxicity studies show that chitosan LD_{50} levels are significantly higher than PEI, which is advantageous towards future clinical vaccine development. There are some differences observed in the gene expression kinetics between the two vectors. Chitosan particles give a slower onset of gene expression with levels increasing out to 6 days post-transfection. PEI particles reach high transfection levels within 24 hours.[26,28,36] The expression levels

are also dependent on cell line. PEI and chitosan particles eventually reach similar expression in the commonly transfected HEK-293 cell line, but chitosan transfection efficiencies are much lower in the highly differentiated HT-1080 (human sarcoma) and Caco-2 (human epithelial colorectal adenocarcinoma) cell lines.[42] Localization studies detect that PEI is found in the endosomal compartment at 24 hrs post-transfection, with the endosomal membranes appearing irregular and showing breaks. At 24 hours chitosan can be found in the endosomal compartments, but the membrane shows no irregularities. Not until 72 hours does the chitosan-containing endosomal compartment begin to rupture. As this is in line with the timing for gene expression that is seen within the two models, it is suggested that the ability to buffer the endosome, causing it to swell and burst, is what limits release and gene expression for PEI and chitosan.[45]

As with PEI, there are conflicting studies debating the overall effectiveness of chitosan as a gene delivery vehicle. Several studies have investigated the use of chitosan as a mucosal delivery agent, as it has shown muco-adhesive properties, allowing for transport through the epithelium in the gastrointestinal tract.[43,46] One study found that oral delivery of chitosan complexed with DNA encoding a peanut allergen could induce tolerance against peanut allergy when challenged.[47] Another study, using a subcutaneous model, showed that an aqueous chitosan solution could be used as an adjuvant to enhance both cell-mediated and humoral immune responses.[48]

Because of chitosan's proven safety and biocompatibility, it will also be investigated in our co-delivery system. We believe that some of the disadvantages in gene transfection, compared to PEI, might be overcome by combining the polymer with and adenovirus. Given that vaccine immunotherapy requires time for the immune system to be primed and upregulate responder cells, a slower gene expression time might not be an issue in this delivery system. As chitosan already has FDA approval, this also facilitates the process towards development of a therapeutic vaccine that can be used clinically.

Vaccine immunotherapy for the treatment of prostate cancer

Several clinical trials have been completed or are in progress for the treatment of cancer using vaccine immunotherapy. Provenge®, produced by the biotech company Dendreon, is an autologous cancer vaccine, produced from each individual patient's immune cells. Dendritic cells, which are responsible for presenting the antigen to other immune cells to launch a response, are removed from the patient and pulsed with a PSA-specific protein, prostatic acid phosphotase(PAP) and adjuvants to help upregulate the immune response, GM-CSF. The cells are then transferred back into the patient, having been altered to now appear "foreign" to the body, and to launch a response against cells expressing this "foreign" (PAP) protein that is found on most prostate cancer tumors.[49] Provenge® was shown in a Phase III clinical trial to extend survival to an average of 26 months, compared to 21.4 months for those who received only a placebo injection. Currently the drug is awaiting FDA approval, with company estimates of availability in mid-2011.

One disadvantage of autologous dendritic-cell based therapies is their labor intensive and costly production. Each patient requires his own production and supply of the drug. With vaccine immunotherapy, the goal is still to target cancer cells in an antigen-specific way, but solely within the patient's body. In this way, production costs are limited to the vaccine alone, and the patient is subject only to immunization, rather than multiple infusions. Because the vaccine is not patient-specific, the overall costs of production would be much lower, due to production in larger batches. Several vaccine immunotherapy trials are currently in progress in the U.S., including at the University of Iowa. The Lubaroff Lab has developed an adenovirus that has been transformed with the PSA gene (Ad5-PSA). The immunization has been shown to generate responses both *in vitro* and *in vivo* from anti-PSA immune cells. Additionally, tumor growth was suppressed *in vivo*, demonstrating a sustained response against PSA-secreting cancer

cells.[24,31] The vaccine demonstrated safety in a phase I clinical trial, and is currently being used in a phase II trial to monitor toxicity and levels of immune response in patients. At this point in the trial, no serious toxicities related to vaccination have been observed, and high anti-PSA T cell responses have been induced in a high percentage of patients.[23,24]

Adjuvants

An adjuvant is defined as any substance that enhances the immunogenicity of substances mixed with it[19]. In the case of our antigen-specific vaccine, the use of antigens can help enhance and ramp up the immune response. The use of a polymeric carrier such as PEI or chitosan can help present the antigen in particulate form, which would be more readily taken up by the antigen presenting cells such as macrophages and dendritic cells.[50] Additionally, studies have reported that chitosan itself can act as not only a polymer carrier, but an adjuvant, as it increases contact with the mucosal tract and can facilitate transport across the epithelium.[45,48,51,52]

Plasmid DNA contains small sequences of unmethylated CpG motifs. CpG sequences have a pathogen-associated molecular pattern, similar to a bacteria-like DNA pattern, and these similarities cause an upregulation of the immune response by activating more antigen presenting cells. This can cause an increase in the production of inflammatory cytokines that also set the immune cascade in action.[19] These sequences, through the Toll-like Receptor 9 (TLR-9) directly stimulate B cells and dendritic cells to promote the production of cytokines and Th1 cells, which stimulates and accelerates the immune response.[19] The adjuvant activity of CpG can result in 5-500 fold higher immune responses.[53] Recent and current studies have proven that CpG motifs are safe for patient trials and can greatly enhance the vaccine's potential immune response.[50,53,54,55] Additional studies in our lab have proven that CpG is more effective when delivered in particulate form that in solution.[50] Complexing the CpG with the

cationic polymer will allow delivery in the particulate form. *In vivo* studies show a decrease in tumor growth, and increased survival time, for mice that were vaccinated with the Ad5-PSA vaccine containing the stimulatory CpG motifs.[2,56]

Summary and Objectives

In summary, the goal of this research is to develop a therapeutic vaccine for the treatment of prostate cancer. Recent success in clinical trials with the Ad5-PSA vaccine demonstrates that an adenoviral vector can be used to deliver the PSA gene to launch an anti-PSA response. This targets only cells secreting PSA, sparing the healthy tissue. With this as the basis of our vaccine delivery system, our goal is to further augment the strength of the immune response, and the duration of protection. To do this, we aim to combine the advantages of the viral vector with the advantages of a class of non-viral vectors, the cationic polymers. Cationic polymers can condense negatively charged virus into nanoparticles through electrostatic interaction, requiring no chemical bonds and leaving the viral structure unharmed. In this way, we hope to capitalize on the advantages of delivery in particulate form that is possible with non-viral vectors, while retaining the viral machinery for PSA production that makes the adenoviral vector a successful gene delivery tool. Additionally, we will develop and test vaccines using the adjuvant CpG to augment the immune response. Combining CpG into the nanoparticle can allow all parts of the vaccine to be delivered to the same cell, boosting the strength of the overall immune response.

Both viral and non-viral vectors have their advantages, and have shown previous success in both the literature and clinical studies. Our goal is to develop a novel vaccine that retains the advantages of both types of delivery, allowing for an enhanced immune response compared to either vector used alone. In the experimental methods to follow, we first will characterize the nanoparticles *in vitro*, optimizing before moving to an *in vivo* model. Experiments in a mouse model of prostate cancer will measure and further

characterize the immune response through enumeration of antigen-specific T cells sets, measurement of CTL activity, and tumor protection studies. Additionally, we will examine how the immune response changes over time, and investigate how CpG can be used as an adjuvant to enhance the immune response. We hypothesize that co-delivery of a non-viral polymer nanoparticle with the viral adenovirus gene delivery system will generate greater immune stimulation and tumor protection than either system would alone, and provide valuable information towards improving vaccine immunotherapy of prostate cancer.

Figure 1: Schematics of immune response: T cell maturation. A T cell is activated when it encounters an APC, such as a dendritic cell or macrophage, which has encountered antigen and is expressing it on its surface in the context of an MHC molecule. This signals to the T cell that it is appropriate and necessary to mature into a specific T cell that is targeted against the antigen. Depending on the antigen presented, the T cell might mature into a CD4+ helper T cell, or a CD8+ cytotoxic T cell.

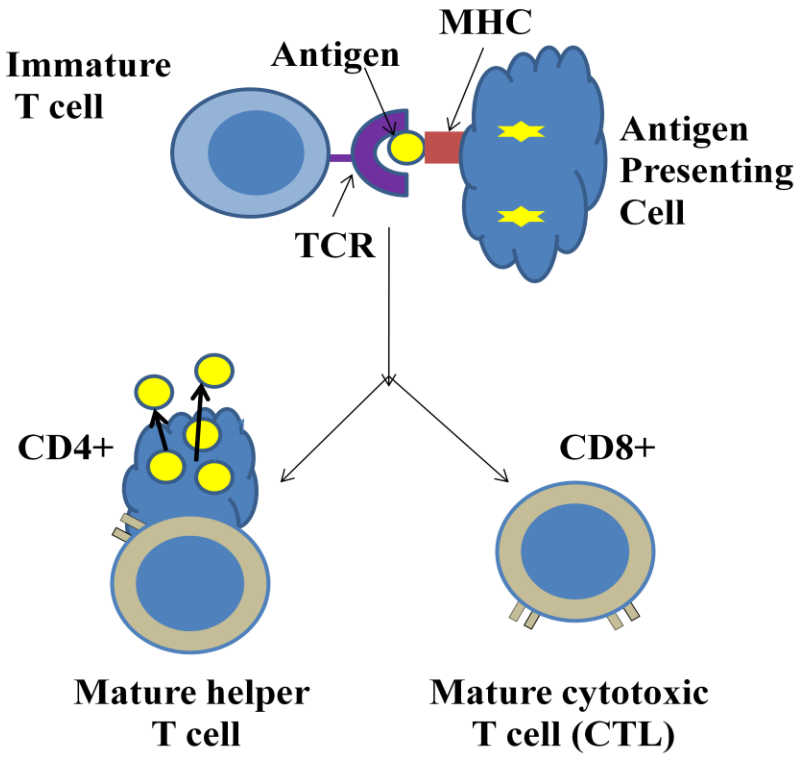

MHC
Antigen
Immature
T cell
Antigen
Presenting
Cell
TCR
CD4+
CD8+
Mature helper
T cell
Mature cytotoxic
T cell (CTL)

Figure 2: Activation of immune response via encoded viral vector. Adenovirus encoded with the PSA gene causes host cell PSA production, giving an antigen specific activation of immune cells that target PSA-producing tumor cells

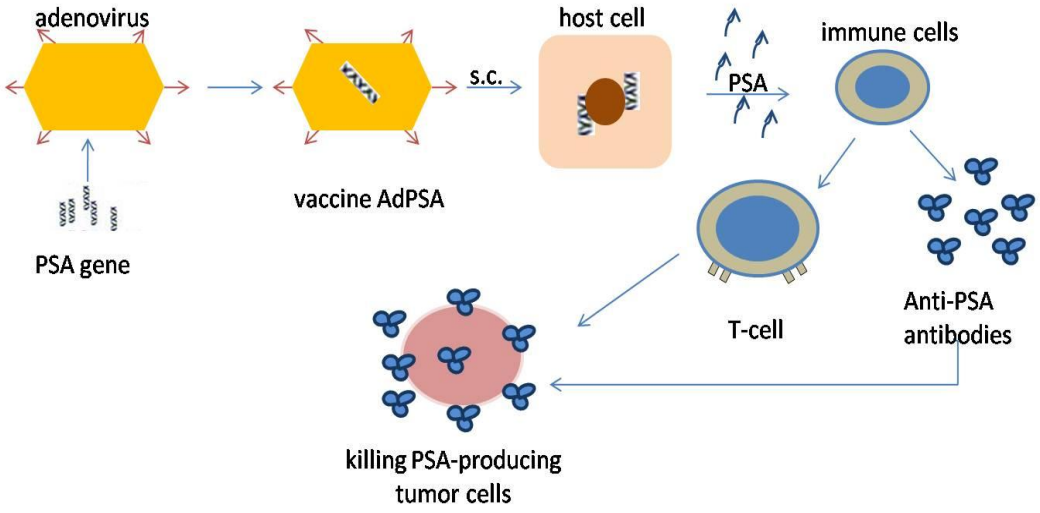
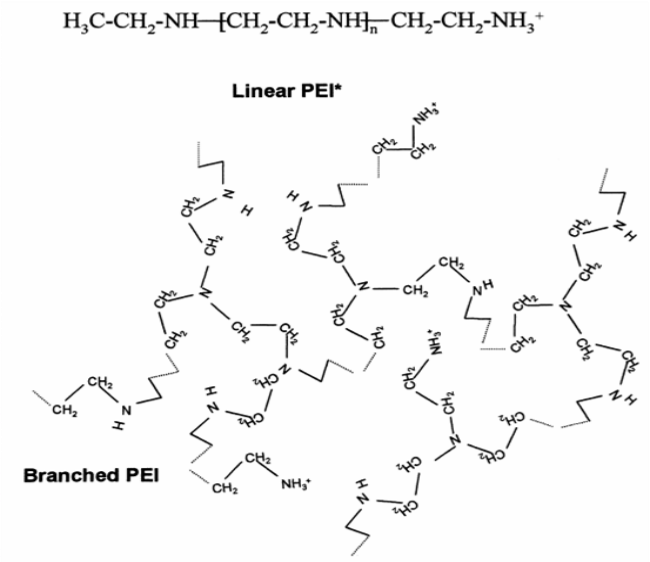
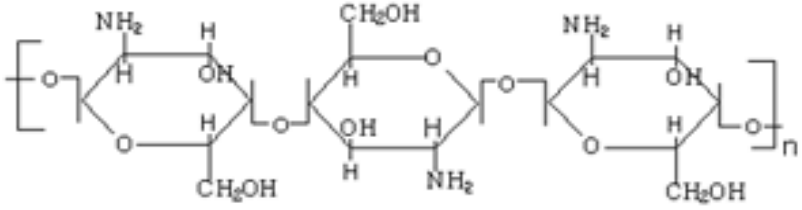

adenovirus
PSA gene
vaccine AdPSA
s.c.
host cell
PSA
immune cells
T-cell
Anti-PSA
antibodies
killing PSA-producing
tumor cells

Figure 3: Structure of cationic polymers PEI and chitosan



Chitosan



CHAPTER 2: MATERIALS AND METHODS

This chapter will cover the theory and details of the experimental methods used in this research in more detail than is presented in the individual chapters. This is done to introduce the assays and experiments in detail, and give background information explaining why these particular experiments are appropriate for data collection and analysis within the research goals. Additionally, figures and references are provided to illustrate and further clarify how each type of experiment was carried out.

Immunization

In order to test the ability of our nanoparticle vaccines to cause an immune response *in vivo*, we used a mouse model to test whether a response occurs. There are several immunizations routes that have been used in previous experimental and clinical models. Subcutaneous (s.c.) injection directs the vaccine between the epidermis and dermal layers; intraperitoneal(i.p.) vaccines deliver directly into the abdominal cavity, and intravenous injections (i.v.) deliver directly into the bloodstream.[19,22] Of these routes, subcutaneous is the method of choice for future translation into a clinical therapy. This is also the best method for eliciting an antigen-specific response, as the Langerhans' cells can take up the antigen and travel to the draining lymphoid organs. Here these cells present the antigen to effector T cells, eventually resulting in T-cell activation. Due to these reasons and success in previous studies using subcutaneous injection[31,56], we used this immunization route in our mouse model.

Animal Care and Handling

Before beginning any animal experiments, animal training and education courses were carried out through the University of Iowa's Animal Care and Use committee.[57] Instruction including proper handling and restraint of mice, which minimizes stress on the animal and prevent aggression or defensive responses to handling. For all cases

involving anesthesia, a ketamine/xylazine mixture was injected i.p. to deliver 87.5 mg/kg ketamine and 2.5 mg/kg xylazine in a 100 μl injection. This provides full anesthesia within a few minutes for approximately 20-30 minutes, with animals returning to full consciousness and mobility within 2 hours.[22] At the end of the studies, or to harvest spleens, mice were euthanized using CO_2 and death confirmed by cervical location.

Throughout the course of all experiments, mice were monitored daily by the University of Iowa Animal Care. During tumor challenge studies, mice were euthanized if at any point they appeared ill from tumor burden, or the tumor grew larger than 25 mm in any direction. Great care was taken to optimize formulations prior to immunization to minimize the number of animals necessary for each experiment while retaining significance.

Lymphocyte Isolation

In order to analyze the immune response, we needed to isolate the lymphocyte population to study their behavior and response to stimulation *in vitro*. Lymphocytes can be isolated from many lymphoid organs such as the spleen, bone-marrow, and lymph nodes. (Figure 4) On average, 60-100 million lymphocytes can be purified from the spleen, and because antigen specific populations can be quite small in percentage of total cells, we will process and further purify the splenocytes in order to best analyze the response.[22] For each experiment, the mouse was first euthanized with CO_2, and death confirmed by cervical dislocation. The spleen was removed, and the tissue ground through a 75μm cell strainer. The cell suspension was centrifuged at 1000 rpm for 8 minutes, and then the cell pellet was treated with ACK lysis buffer to lyse the red blood cells. ACK was prepared by mixing 8.29g NH4Cl, 1g KHCO3, and 37.2g Na2-EDTA in 1L deionized water.[22] The pH was then adjusted to 7.2-7.4 and the buffer stored at 4^oC. ACK is a hypotonic solution, and red blood cells lack Na^+ pumps, so the sodium buildup will cause the red blood cells to swell and burst.[19] After ACK treatment for 5 minutes,

fresh media was added and the cells were centrifuged again. The remaining cells are lymphocytes which can be counted and resuspended at the appropriate concentration for our assays and experiments.

Flow cytometry and FACS analysis

In order to identify and enumerate the antigen-specific T cell population, we chose to use flow cytometry to characterize these cell subsets. In a flow cytometry setup, laser light is focused on a narrow stream of the cell suspension which passes cells through the detector one at a time. The detectors that measure light scattering convert the light to electrical pulses. An analog to digital converter allows the events to be plotted on a graphical scale, for viewing information about the chemical and physical structure of the cell. Based on the forward and side scatter of the light from the cells, the cell volume and shape can be determined. Fluorescence Activated Cell Sorting (FACS) is a specialized type of flow cytometry that allows cells to be labeled with fluorochromes, and then from a mixed population, be separated into subpopulations based on the fluorescence and light scattering characteristics of the cell. In our studies, for example, the use of florescent-labeled antibodies to cell surface proteins for CD4 and CD8 can allow us to identify and enumerate these subsets of lymphocyte populations. (Figure 5)

Lymphocyte characterization

There are several methods to characterize the lymphocyte's frequency, specificity, and function. The goal of this research is to provide an effective antigen-specific immunization, which targets only cells expressing the antigen of interest. Because PSA has a narrow tissue distribution and is expressed by target cancer cells, this will target the killing of cancer cells while other cells of the body, which do not express PSA, are left alone. For this reason, we will focus on several assays that help enumerate antigen specific cell populations and measure their functionality. To test for specificity, cells can be identified by the presence of an antigen-specific receptor which is detected through

flow cytometry techniques, or by detecting cellular activation. To measure functionality, the degree to which cells respond to the antigen of interest can give a measure of the intensity of the immune response and the cells' ability to perform their intended function.

Intracellular Cytokine Staining (ICS)

Activated T cells secrete cytokines, which act specifically to cause changes in other cells of the immune system. For example, T cells can produce IFN-γ, which aids in further macrophage activation and an increase in antigen processing components.[19] This makes IFN-γ production a good measure for T cell activation, but, it can be difficult to measure as this cytokine is secreted out into the surrounding media. To allow a measure of cytokine levels directly associated with the cell that produced it, the intracellular cytokine staining (ICS) method has been developed. Cells can be treated with brefeldin-A, which inhibits transport out of the cell, allowing the protein to accumulate in the endoplasmic reticulum. The cells can then be fixed and permeabilized, which creates holes for antibodies to access these intracellular areas while cross-linking proteins to prevent loss. When these antibodies are fluorescently labeled, the individual cells which are producing IFN-γ can be detected and quantified. (Figure 6)

Tetramer staining

Within the past decade, the use of tetramer technology has allowed scientists to quantify antigen-specific T cells directly through the antigen-specific receptor. Previously, this was not possible, as T cells require both the antigen and appropriate MHC molecules to bind the antigen. Normally the interactions of the cells with the antigen were limited by this, and labeling the cells with the antigen alone was extremely difficult. By creating multimers, where four of the MHC-peptide complexes are bound to avidin or streptavidin by biotin, the strength of the interaction of the antigen complex and T cell is increased. (Figure 7) Additionally, fluorochromes can be added to the streptavidin to allow detection by flow cytometry of T cells which bind the MHC

tetramer complex. This allows for detection of cells that are specific for the antigen at interest. OVA is the ovalbumin protein, derived from chicken egg white, and is a model antigen used in many immunotherapy and gene delivery applications. At present time, the OVA tetramer complex is available and shows high specificity and avidity in previous studies. [19,58]

Cytotoxic T Lymphocytes (CTLs)

Beyond flow cytometry analysis to determine the presence of specific cell populations, assays to measure functionality can give insight to the strength of the immune response created, and the ability of cells to actually kill the target antigen-specific cell. For example, when an animal is immunized with AdPSA, T cells should become activated that kill cells that display PSA in the context of MHC I. To gauge the level of target cell destruction, a ^{51}Cr-release assay is used. Live cells will take up radioactively labeled sodium chromate, Na_2CrO_4, with very little spontaneous release. Upon cell lysis by the cytotoxic T cells the radioactive chromate be released into the supernatant, and can be measured in a gamma counter. (Figure 8) By comparing the levels of spontaneous release, when labeled cells are incubated in the absence of splenocytes, and maximum release, when labeled cells are incubated with Triton for complete lysis, a percentage of target cells lysed can be calculated. The strength of the immune response can then be compared amongst the immunization groups.

In vivo tumor challenge

Beyond measuring the functionality of the CTLs *in vitro,* measures of the *in vivo* response give the best representation of the level of protective immune responses that our immunization induces. To measure this response, mice were immunized with the vaccine formulations as previously described, and then challenged 14 days later with tumor cells expressing the specific antigen that the mice have been immunized against. Tumor outgrowth was then measured, with tumor volumes calculated as (length x width x depth

x (π/6)), as is used for *in vivo* measurements of ellipsoid tumors.[8] The survival of the mice was also monitored, and mice were sacrificed if the tumor dimensions became larger than 25mm in any direction. All animal experiments were carried out in accordance to the University of Iowa Animal Care and Use committee's guidelines and regulations.

In vivo depletion studies

In order to determine which subset of lymphocytes had the greatest impact on tumor protection, cells were depleted *in vivo*, followed by tumor challenge. Antibodies produced by hybridoma cells were purified by ammonium sulfate precipitation to be used in all depletion studies.[56] Optimization of the depletion regimen showed that 150 μg of antibody could deplete and maintain depletion levels for the course of the experiment. Antibodies used to deplete specific subsets were anti-CD8 (2.43), anti-CD4 (GK1.5), and anti-NK (PK-136). Mice were immunized on day -14, and then given depleting antibody on days -3, -2, and -1, prior to tumor challenge on day 0. Additionally, confirmation of depletion on day 0 by flow cytometry (CD4 and CD8) or NK assay (NK cells) was performed on one mouse per group. For the duration of tumor challenge, mice were injected with the antibodies twice weekly to maintain depletion.

Figure 4: The lymph nodes and spleen of the mouse. To obtain lymphocytes for analysis, mice were euthanized with CO_2 and death confirmed by cervical dislocation. The spleen was removed by making approximately a 1-in. incision on the left side of the body, midway along the abdominal cavity. Connecting tissue to the stomach is cut to allow removal of the spleen from the peritoneum. The spleen is transferred to a centrifuge tube containing complete media until it can be further processed.(Image)[22]

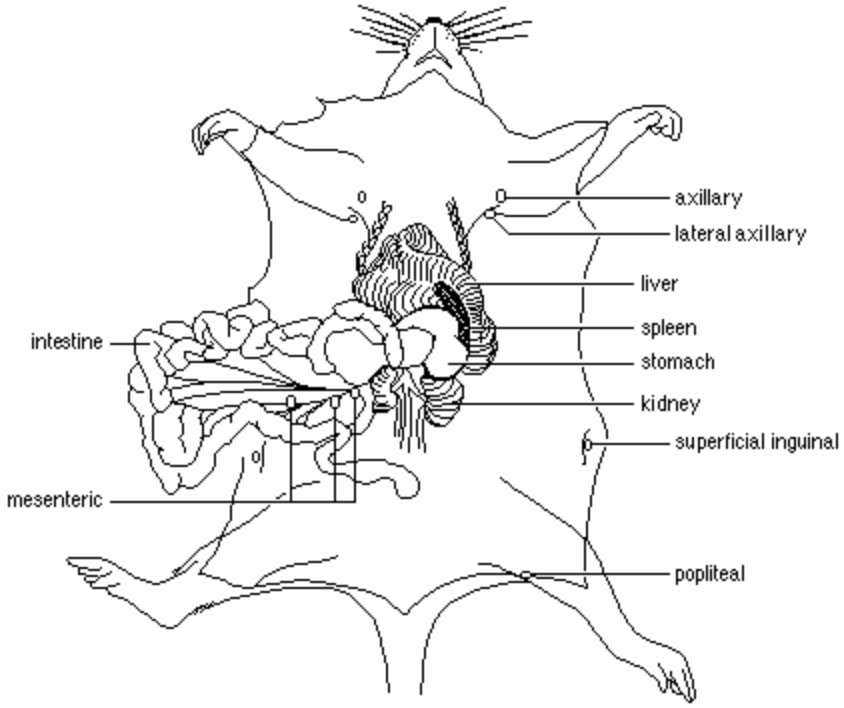

axillary
lateral axillary
liver
spleen
stomach
kidney
superficial inguinal
popliteal
intestine
mesenteric

Figure 5: FACS setup. Flow cytometry setup allowing cells to be sorted and quantified by their cell surface antigens. A mixture of cells can be labeled with fluorescent antibodies specific to different cell surface antigens. The labeled cells are forced through a nozzle into a single cell stream which passes through the laser beam. Laser beams delivering light at different wavelengths excite the fluorochromes and the emitted light is analyzed by computer. Additionally, the scattering of light allows identification of different cell populations, sorted by cell size and granularity. The cells that certain characteristics can be quantified and the level of expression can be measured, to compare effects of immunization on cell populations. [19]

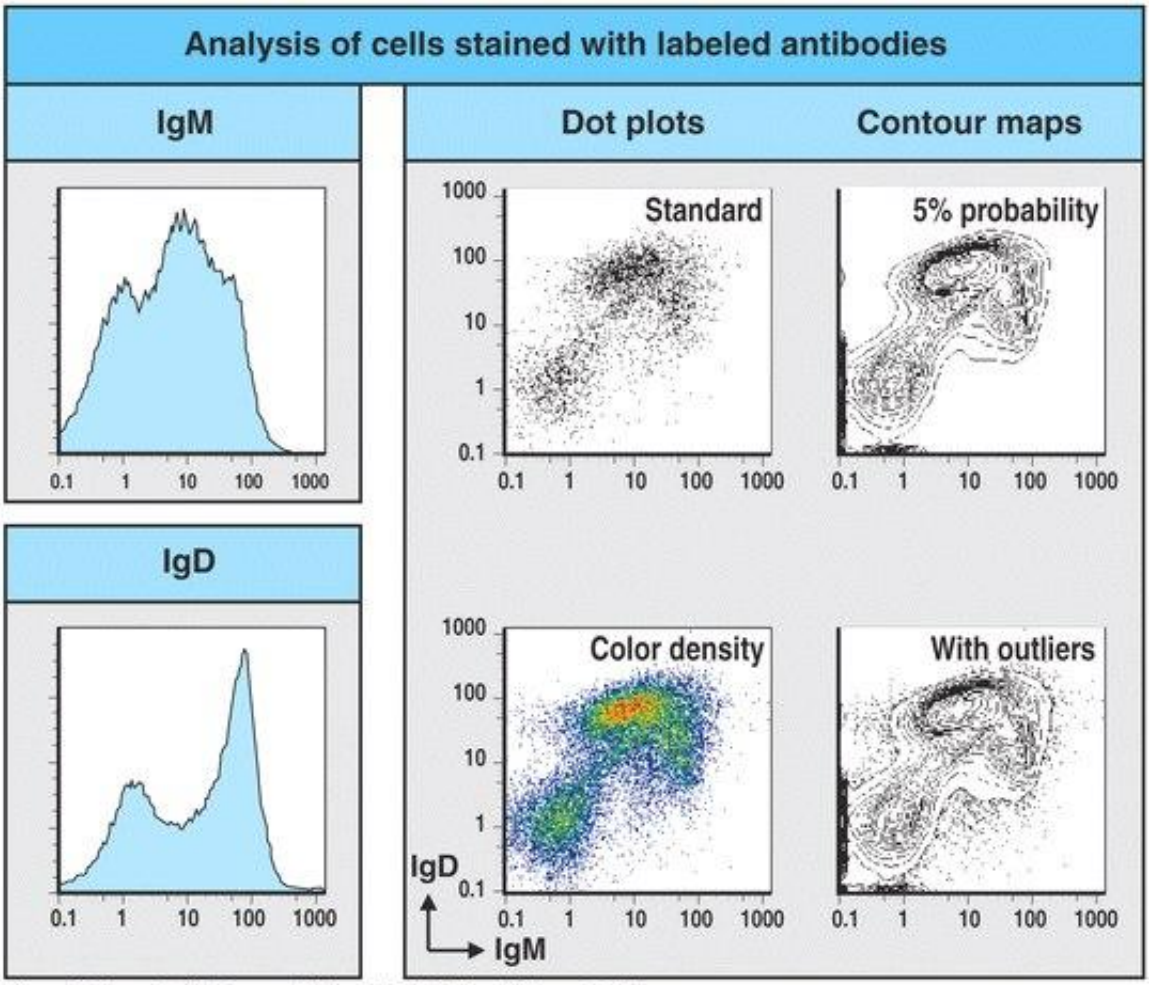


Figure A-25 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)

Figure 6: Intracellular Cytokine Staining. To quantify the number of T cells which are producing IFN-γ, the cells are treated with Golgi-stop to inhibit transport outside of the cell. The cells are fixed to cross-link proteins and prevent their loss after permeabilization. This enables fluorochromes-linked antibodies enter the cell and bind to the cytokines, allowing for quantification by flow cytometry. [19]

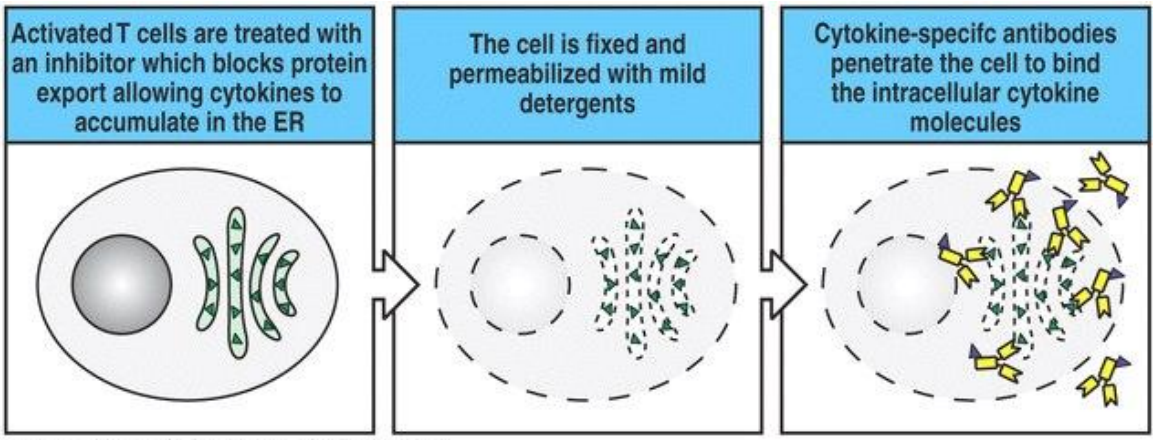


Figure A-30 Immunobiology, 6/e. (© Garland Science 2005)

Figure 7: Tetramer staining to enumerate antigen-specific cell populations. The tetramer, providing four opportunities for MHC: peptide to bind the cell receptor, gives a stronger interaction of the complex with the cell. The tetramer complex is also bound with fluorochrome to allow analysis by flow cytometry. The bottom panel shows T cells stained with CD3 and CD8, and tetramer for HLA-A2 molecules. The CD3+ cells were gated on, and the figure shows a cell population in the upper right quadrant that are the tetramer positive CD8+ cells.[19]

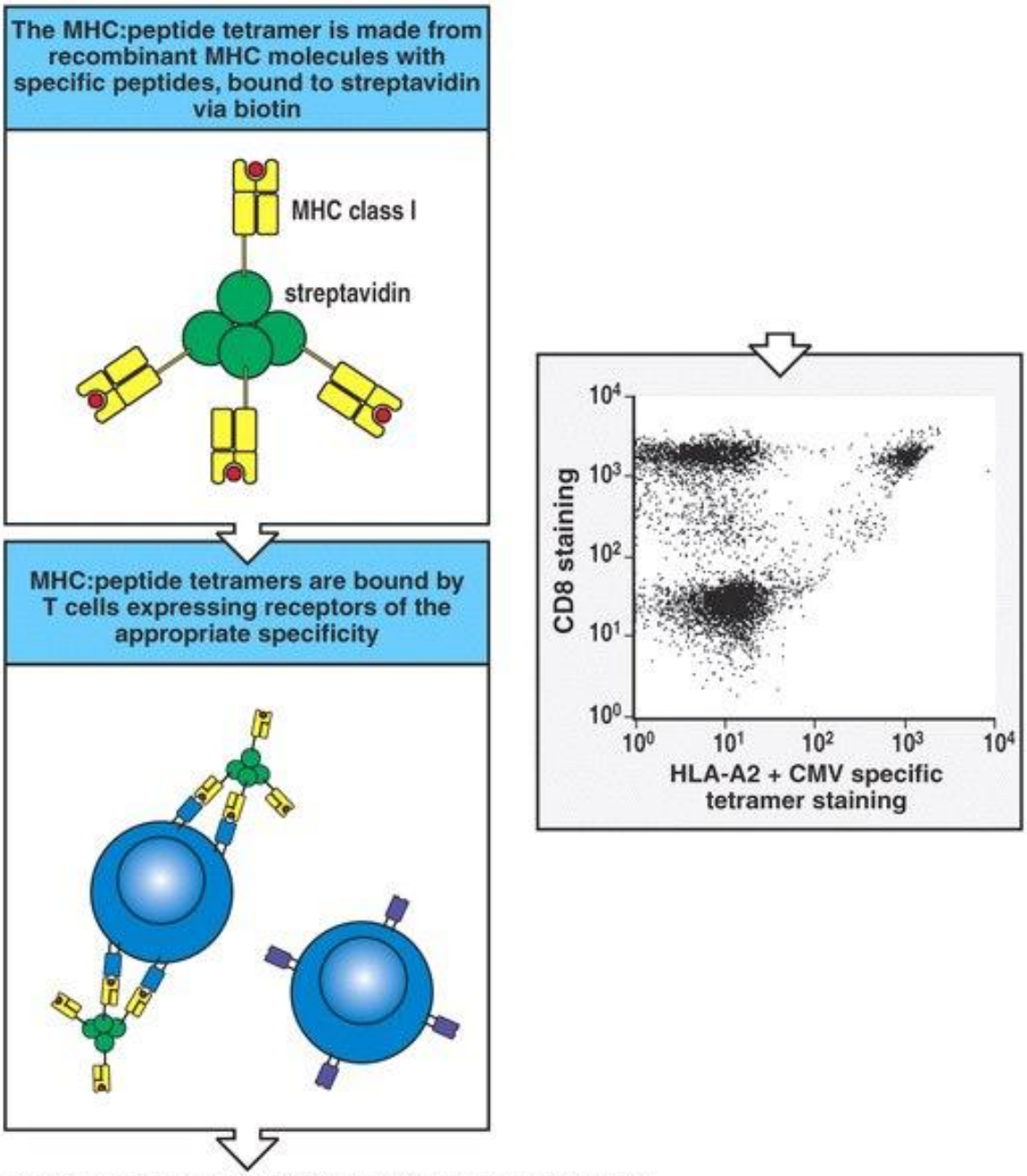


Figure A-32 Immunobiology, 6/e. (© Garland Science 2005)

Figure 8: CTL Assay. The target tumor cells are labeled with radioactive chromium, and washed to remove any excess radioactivity. Splenocytes from the varying immunization groups are then added to the labeled target cells and incubated for 4 hrs at 37 ^{o}C. As the target cells are lysed, the radioactive chromium will be released into the medium. The amount of chromium is quantified using a gamma counter, and can be compared to positive and negative controls to determine the percentage of target cells lysed by each immunization group.[19]

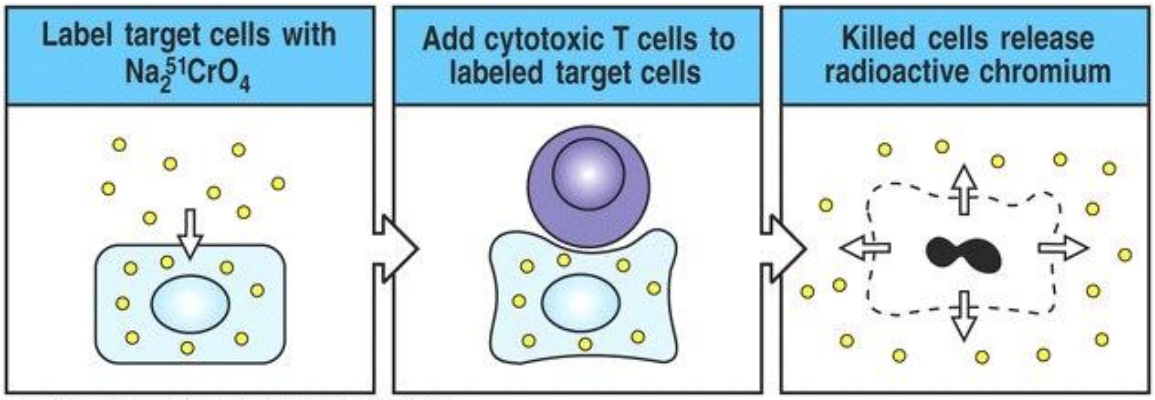


Figure A-38 Immunobiology, 6/e. (© Garland Science 2005)

CHAPTER 3: THE USE OF PEI IN A VIRAL/NON-VIRAL VACCINE SYSTEM

Introduction

Previous high efficiency with the use of adenoviral vectors has led to their continued use and research in the gene delivery and immunotherapy fields. Because they can infect a wide variety of cell types, this gives a greater chance of production of the encoded antigen of interest.[7,8,17] Previous studies have shown the ability of targeted anti-tumor vaccines to enhance CTL response and the activation of IFN-g secreting NK cells.[59] Based on this data, the Lubaroff lab has previously constructed a replication-deficient adenovirus of the Ad-5 serotype. One version, AdPSA, encodes for the therapeutic target prostate specific antigen, while another version, AdOVA, encodes for the model OVA antigen. *In vivo* experiments demonstrating tumor protection in mouse models using both AdOVA and AdPSA led to a phase I clinical trial of the AdPSA in men with metastatic hormone-refractory prostate cancer. The vaccine was shown to be safe at all administration levels, in aqueous form or delivered by Gelfoam matrix.

Based on these previous studies, we wanted to explore approaches to delivering the antigen-specific adenovirus in order to enhance the vaccine-induced immune response. The Gelfoam matrix that was previously used was one strategy to deliver the adenovirus in a time-release form, as the matrix degraded over time. This could allow the AdPSA to be released gradually rather than in one large burst. Another method to enhance the response is to increase the uptake of the virus particles, so that more cells can respond to the PSA antigen production. Forming viral/non-viral nanoparticles through the use of cationic polymers can present the adenovirus in particulate form, which increases the uptake by macrophages and dendritic cells.

PEI has been used in many gene delivery applications, and several investigators have explored its use for receptor-mediated targeted delivery or delivery of

adenoviruses[37]. Following uptake of the complex, the amino groups in PEI provide endosomal buffering leading to osmotic swelling and release, giving the adenoviral machinery the ability to initiate antigen expression. In these studies, we chose to use the AdOVA model in C57Bl/6 mice, together with the EG.7-OVA cell line. The OVA antigen gives us access to tetramer technology and other tools to further characterize and analyze the effectiveness of our immunization groups. Additionally, the model system has been well documented in previous studies and can provide a comparison to the relative effectiveness of these formulations.[56] The immunization groups used in this study were:

1) AdOVA
2) AdLacZ
3) AdOVA + PEI
4) AdLacZ + PEI
5) PEI

The virus titer used was 10^8 pfu for all experiments. To characterize the immune response, we used both phenotypic and functional *in vitro* assays from our mouse model. This is important to characterize changes in frequencies of cell populations, which is an indicator of expansion of cell subsets in response to immunization and antigen challenge. Functional assays tested that the lymphocytes can actually carry out an immune response: for example, lysis of OVA-expressing tumor cells by cytotoxic-T cells following AdOVA immunization. Working in the *in vivo* mouse model, we investigated the inhibition of tumor growth following immunization and a subsequent tumor challenge. Additionally, we used depletion of lymphocyte subsets *in vivo* during immunization and subsequent tumor challenge to determine the specific subsets of lymphocytes that provide tumor protection. This information can help shape further vaccine design and targeting strategies, and give further insight into the processing and mechanisms of the nanoparticles in order to induce an immune response.

Materials and Methods

Materials

Branched PEI (MW 25kDa) was purchased from Sigma-Aldrich (St. Louis, MO). A 0.01M stock solution was prepared as previously described and used for all formulations. The Ad-OVA vaccine was obtained from the University of Iowa Gene Vector Core, as was designed previously in the Lubaroff Group.

Nanoparticle vaccine preparation

Nanoparticles were formulated at various ratios of PEI nitrogen to DNA phosphate (N/P ratio), with the amount of PEI solution used based on a 50 μg/mouse DNA dose. 10^8 pfu viral vaccine was used. The solutions were vortexed for 20s and then left to incubate at room temperature for 30 min. The vaccines were injected subcutaneously into the right flank. For transfection studies, PEI-DNA nanoparticles were produced using VR1255 DNA, which encodes for luciferase. The VR1255 was amplified and purified in our lab as previously described.

Evaluation of luciferase expression in HEK293 cell line

Human Embryonic Kidney cells (HEK293) were purchased from the American Type Culture Collection(ATCC), and maintained in DMEM supplemented with 10% Fetal Bovine Serum(FBS), 1% sodium pyruvate, 1% HEPES, and 0.05 mg/ml gentamycin at 37 ^oC in a humidified 5% CO_2- containing atmosphere. Cells were seeded in 24-well plates at a density of 80,000 cells/well, 24 hours prior to transfection. 100 μl of the PEI-DNA nanoparticle solution at various N/P ratios were added to the cells, with each well receiving the constant DNA dose of 1 μg. The nanoparticle solutions were delivered in serum-free DMEM and allowed to incubate with the cells for 4 hrs at 37 ^oC.

The cells were then carefully washed with PBS and left to incubate in serum containing media for an additional 44 hours. Following this incubation, the cells were treated with 200 μl of lysis buffer (Promega, Madison, WI). The cell lysate was subjected to two freeze/thaw cycles, and then transferred into tubes for centrifugation at 13200 rpm for 5 minutes. 20 μl of the supernatant was mixed with 100 υl of the luciferase assay reagent (Promega, Madison, WI) and the RLU(relative light units) for each sample read on a luminometer (Lumat LB9507, EG & G Berthold, Bad Wildbad, Germany). The Micro BCA protein assay kit (Pierce, Rockford, IL) was used to normalize the RLU to protein concentration in the cell extracts. The luciferase activity could then be expressed as RLU/mg protein in the cell lystate. The transfection experiment was performed three times, with results reported as mean ± standard deviation.

Tumor cell lines and animals

EG.7-OVA cells and their parental line, the non-OVA expressing mouse lymphoma EL4, were used to measure *in vivo* tumor growth, and *in vitro* antigen specific CTL activity. Both cell lines were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% sodium pyruvate, 1% HEPES, and 0.05 mg/ml gentamycin in a humidified 5% CO_2- containing atmosphere. 6 to 8 week C57Bl/6 mice were purchased from the National Cancer Institute (Bethesda, MD) and were maintained in filtered cages before use.

Phenotypic assay: Tetramer staining

To enumerate the antigen-specific CD8+ T cells, tetramer staining using MHC I SIINFEKL tetramer (Beckman Coulter, Fullerton, CA) was performed. For each treatment group, a mouse was sacrificed and the spleen removed, and a spleen cell

suspension created. Red blood cells were lysed using ACK buffer, and the cell suspensions were filtered through a 70μm cell strainer. The splenocytes were counted and resuspended at 10^7 cells/ml. 100 μl of cells were plated per well in a 96 well plate, and blocked with 24G2 F_c receptor block for 15 minutes on ice. Cells were stained for 30 minutes with tetramer, then for 20 minutes for anti-CD8 FITC and anti-CD3 PE-Cy5. The cells were washed, fixed, and permeabilized with the Cytoperm/Cytofix kit (BD Biosciences, San Diego, CA) and resuspended in FACS buffer for flow cytometry analysis, collecting 10^5 events. The data was analyzed using FlowJo software (Tree Star, Stanford).

Intracellular Cytokine Staining

Splenocytes were processed as previously described, and plated at 10^6/well in a 96 well plate. Golgistop (BD Biosciences) was added to each well to inhibit IFN-γ secretion, wells were treated with peptide stimulation with SIINFEKL, and the plate incubated for 5 hrs at 37 ^oC. After blocking and staining with anti-CD8 FITC and anti-CD3 PE-Cy5 as described above, the cells were stained with anti-IFNγ PE, and flow cytometry performed.

In vitro cytotoxic assay

A ^{51}Cr release assay was used to measure OVA-specific lysis of target cells. In a 24 well plate, 1 x 10^7 cells/well were seeded for each immunization group, along with the cytokine IL-2(10 U/ml) and mitomycin C treated EG.7-OVA cells (2 x 10^5 cells/well) as stimulators. Following a 5 day co-culture at 37 ^oC, the effector splenocytes were harvested and separated from dead cells using a Ficol separation. Target EG.7-OVA cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hr, washed twice, and resuspended at 5 x 10^4/ml. The effector cells were diluted serially down a 96 well round bottom plate, and 100 μl targets added to each well, giving effector: target (E:T) ratios from 100:1 to 3.125:1. After a 4 hr incubation at 37 ^oC, the plate was centrifuged at 1000 rpm for 10

minutes, and 100 μl of the supernatant was taken from each well and counted in the COBRA II gamma counter (Packard Instrument Company, IL). The specific lysis was calculated using the formula:

(sample lysis-spontaneous lysis)/(maximum lysis-spontaneous lysis) *100

Tumor challenge study

In tumor challenge studies, the cells were resuspended in PBS at a concentration of 10^7 cells in 100 μL. The cells were injected subcutaneously into the right flank of the mouse. Tumor outgrowth was measured twice weekly, with tumor volume calculated as: [length x width x height x 0.5236] as described by *Shariat el al*.[8] Survival of the mouse treatment groups was also monitored. Mice were sacrificed for ethical reasons if they appeared ill from tumor burden or if measurements exceeded 25 mm in any direction. Each experimental group consisted of 4 mice and experiments were repeated 3 times. All animal experiments were conducted in accordance with the procedures outlined in the University of Iowa's Guidelines for Care and Use of Experimental Animals.

Antibody depletion regimen

To examine the effects of immune cell depletion on tumor growth *in vivo*, the mice were first immunized with AdOVA + PEI, as this formulation provided the best tumor protection in challenge studies. After immunization on day 0, the mice were then injected i.p on days 11, 12, and 13 with 100 μg of 2.43 (anti-CD8), GK1.5 (anti-CD4), PK136 (anti-NK), or both 2.43 and GK1.5, to deplete these lymphocyte subsets. For the rest of the study, the mice were injected twice weekly with 100 μg antibody to maintain cell depletion. All injection volumes were 100 μl. The antibodies were synthesized from hybridoma cell lines as previously described. On day 14, one mouse from each group

was sacrificed to confirm depletion by flow cytometry for CD4(91.4% depletion) and CD8 (98.6% depletion) cells, and the ^{51}Cr-release assay was performed for NK cells against NK-sensitive YAC-1 cells. All remaining groups of mice were challenged on day 14 with EG.7-OVA cells, and the tumor outgrowth monitored as previously described.

Results

Optimization of PEI nanoparticle formation

Previous studies in our lab have demonstrated that PEI gives high transfection in HEK-293 cells at varying N/P ratios.[60] For our studies, we wanted to optimize the amount of PEI used to complex the polymer, before proceeding with animal experiments. To test this, PEI and DNA or adenovirus at varying N/P ratios were mixed and allowed to complex through electrostatic interaction for 30 minutes at room temperature. All immunization groups formed particles in the 150-300 nm range (Figure 9), which is adequate for endocytosis by cells. Additionally, the formulations had positive zeta potentials, which will aid in attraction to the negatively charged cell membrane. (Table 1). In transfection studies, the complexes created at N/P = 5 had significantly lower protein expression than complexes at N/P = 10 (Figure 10). There was no significant difference between expression of N/P=10 and N/P= 20, with N/P 10 giving slightly higher expression, so N/P= 10 will be used for the duration of the studies. This minimizes the amount of polymer used in the formulation, which is especially important given that PEI can have toxicity concerns at higher doses.

PEI with adenovirus depresses the CD8+ OVA+ T cell population

To test the ability of the cationic polymer to act as an adjuvant in enhancing the immune response, we needed to fully characterize the *in vitro* and *in vivo* response, examining immunizations with and without PEI for comparison. To investigate the

modulation of the OVA-specific CD8+ T cell population in immunized mice, tetramer staining was performed 14 days post-immunization. Splenocytes were harvested and processed as previously described and then stained for CD8, CD3, and OVA tetramer. Figure 11 shows the representative gating on the CD3+ CD8+ population, and then FL1 vs. FL2 identifying cells double positive for FITC CD8 and PE tetramer. The LMOVA immunization, a listeria immunization encoding for OVA with a strong antigen-specific response, was used as a positive control. In our studies, we found that AdOVA + PEI significantly depresses the tetramer frequency compared to equivalent viral load in AdOVA alone ($p<0.01$). AdOVA + PEI showed similar CD8+ tetramer+ frequencies to the naïve and AdLacZ control mice. Given the many types of cell populations that may modulate the immune response, further study and experimentation into PEI's modulation of the immune response was necessary to see how immunization would affect other cell populations. Additionally, we wanted to further examine the changes in the immune response that may occur following *in vivo* immunization and tumor challenge using OVA-specific cells.

AdOVA + PEI effects on CD8+ IFN-γ levels

IFN-γ is a cytokine that plays a crucial role in anti-tumor immune responses. Measurement of any changes in IFN-γ secretion after immunization with the nanoparticle vaccines would allow us to see if the addition of PEI changes the IFN-γ+ levels. The cells were stained for CD8, CD3, and IFN-γ, treated with Golgistop to inhibit protein secretion, and incubated for 5 hrs at 37 ^{o}C with media, SIINFEKL peptide, or PMA/ionomycin. As with the tetramer staining, we gated on the CD8+ CD3+ cells, and then the CD8+ IFN-γ+ cell frequencies were compared between the groups. Overall, there are no significant differences in CD8+ IFN-γ+ frequencies between any of the groups, compared to naïve mice. (Figure 12) Due to variation within each group and the narrow range between positive and negative control values, significant differences

between the groups were not observed, as seen in 1-way ANOVA and Tukey's Multiple Comparison Post Test.

AdOVA + PEI CTL response is reduced compared to AdOVA

To further characterize the cell populations modulated by adenoviral and cationic polymer immunization, the CTL assay was performed 14 days after immunization. This assay is used to measure the ability of immunized mouse lymphocytes to mount a response against tumor cells expressing OVA. Cells were harvested and processed as previously described, and co-cultured for 5 days at 37^{o}C with stimulator EG.7-OVA cells. After the co-culture, fresh EG.7-OVA cells were labeled with radioactive chromium and plated at different ratios with the effector splenocytes recovered from the co-culture plate. The CTL results show that while AdOVA produces high specific lysis, all other groups do not, including AdOVA + PEI (Figure 13). Again, it was very interesting that the addition of a non-antigen specific component would appear to negate the effects of the adenovirus vaccine. Given that previous studies in the literature have shown that *in vitro* and *in vivo* activities do not necessarily mirror each other, completing these studies with examination of tumor challenge was the next step in further explanation and characterization of the immune response.[2,56,61]

AdOVA + PEI provides tumor protection in vivo

Mice were immunized and challenged 14 days later with 10^7 EG.7-OVA or EL4 cells, which were injected subcutaneously on the right flank. Tumor outgrowth was measured twice weekly, and mouse health and survival monitored. Mice were sacrificed if they appeared ill from tumor burden or if the tumor exceeded 25 mm in any direction. In this representative experiment, 75% of both the AdOVA and AdOVA + PEI mice remained tumor free, and had the lowest overall tumor growth (Figures 14 and 15). The mice immunized with AdLacZ formulations grew tumors quickly and exhibited no antigen-specific protection, with 100% of mice developing tumors. Interestingly, mice

immunized with PEI alone all developed tumors, but with slower growth and smaller size at the same time points as AdLacZ containing vaccines. We hypothesized that due to the inherent toxicity of PEI, a non-specific immune response may initially been launched which aided in suppressing tumor growth. In the EL4 tumor model, growth was rapid in all groups, and no antigen-specific protection was seen in any groups, as was expected since these cells do not express OVA.

CD8+ cells mainly suppress tumor growth, with contributions from NK cells

In hopes of elucidating which cell types contributed to tumor protection, we examined the effects of immune cell depletion on tumor growth *in vivo*. For these studies, mice were first immunized with AdOVA + PEI as previously described. Previous studies have shown that CD8+ cells mediate tumor protection with AdOVA vaccination, so immunization with AdOVA + PEI would allow us to see any changes that occur with the incorporation of PEI in the delivery system. After immunization on day 0, the mice were depleted of CD8+, CD4+, and NK cells, as described in the materials and methods. For the rest of the study, the mice were injected twice weekly with 100 μg antibody to maintain cell depletion. The mice were challenged on day 14 with EG.7-OVA cells, and the tumor outgrowth monitored as previously described. Figure 16 shows the tumor growth rates for individual mice, as well as the average tumor volumes and survival curves for each group. Tumor protection after immunization with AdOVA + PEI appeared to be mediated by CD8+ cells, as seen from the tumor growth rate in the absence of CD8+ T cells compared to the PBS group with no subset depletion. Additionally, there seems to be some contribution from NK cells, as tumor growth occurred more rapidly when these cells were depleted, but not as quickly as with CD8+ depletion.

Discussion

The goal of these experiments was to develop an effective antigen-specific vaccine, which would be measured through *in vitro* functionality tests, and *in vivo* tumor challenges. Based on previous success with the adenovirus encoded the model antigen OVA (the AdOVA vaccine), we wanted to formulate nanoparticles using PEI as the carrier vehicle. Our hypothesis was that increased delivery of the nanoparticles, and presentation of the adenovirus in particulate form, would give better uptake in macrophages and dendritic cells, leading to better antigen presentation and a more robust immune response. Based on transfection studies, the amount of PEI to be used per vaccine was found optimal at an N/P ratio of 10, based off of a 50 μg DNA dose per vaccine. N/P ratios above 10 did not show significantly higher transfection efficiency, and an N/P ratio of 10 did show statistically significant differences compared to N/P of 5. Because of concerns with toxicity of PEI, using the lowest possible amount of the polymer while still obtaining increased transfection efficiency is preferred, and so the N/P ratio of 10 was chosen for our studies. Particle size and zeta potential were measured to confirm that the nanoparticles were within the size and charge realm to be adequately delivered, able to interact with the cell membrane, and be taken up by antigen presenting cells.

As previously discussed, to determine how a viral/non-viral carrier in the form of adenovirus and PEI might modulate the immune response, a series of experiments was performed to compare AdOVA + PEI to previous work with AdOVA alone.[2,56] Interestingly, we found that AdOVA + PEI immunizations in all cases did not enhance OVA-specific CD8+ T cell numbers or function, as measured by IFN-γ secretion and CTL activity. We next considered whether the number of OVA-specific CD8+ T cells were affected, and employed tetramer staining to enumerate these cells. Again, we found that the addition of PEI to the AdOVA immunization decreases the number of antigen-specific T cells. One hypothesis for this change is that the delivery of adenovirus

complexed in nanoparticles, rather than in aqueous solution, changes the routing and processing of the adenovirus, thus reducing the availability or immunostimulatory capacity of the viral-encoded antigen. It is unclear at this time whether it is the change in antigen uptake, or a change in processing, that could be responsible for the decrease in *in vitro* activity of the cells.

In contrast to the *in vitro* assays of CD8+ T cell function, tumor challenge studies over a 6 week time period showed that AdOVA + PEI immunized mice were best protected from challenge, having the lowest average tumor volume of all immunization groups. Both AdOVA and AdOVA + PEI mice were 75% tumor free at the end of the experiment. Also of interest, while all PEI immunized mice developed tumors throughout the experimental time period, these mice had slower growing tumors, with overall smaller volumes than AdLacZ and AdLacZ + PEI. One main point of consideration in the modulation of PEI on tumor protection is whether PEI's inherent immunostimulatory activity can launch a general immune response that suppresses tumor growth regardless of antigen-specificity. Addressing these points, PEI's immunostimulatory activity has been noted in the literature[32,37,62], and is one reason why this polymer was chosen for development of a viral/non-viral adjuvant delivery system. PEI has been used in many *in vitro* gene delivery systems, but little information on clinical uses exists in the literature, due to systemic toxicity issues.[34,37] Several studies have suggested that a reason for PEI's toxicity is that its positive charges may cause more interaction at the negatively charged plasma membrane, leading to perforations, and cell death.[32,35] Activation of dendritic cells can be induced by these types of "danger signals", such as cell death or stress and damage to the tissues. Through this activation of cellular responses to necrotic cells, a general elevation in the immune response could cause suppression of tumor growth.[63]

The mechanism for PEI's modulation of the immune response is not completely understood, but our studies present several key findings toward the development of a

viral nanoparticles system. We show that *in vitro*, AdOVA + PEI depresses the antigen-specific T cell population and CTL response compared to AdOVA alone. Conversely, we find that with *in vivo* tumor challenge post AdOVA+ PEI vaccination, mice have the best tumor protection of all groups. This demonstrates that while the *in vitro* T cell activation is diminished, the overall goal of achieving tumor protection is not diminished *in vivo.* Antibody depletion studies show that while the response is mainly CD8 mediated, there is a contribution from NK cells for tumor protection as well. These differences from AdOVA vaccination might be due simply to the presence of PEI in the system, or different antigen processing from the nanoparticles. These studies demonstrate that formation of adenoviral nanoparticles, using cationic polymer as the complexing and delivery adjuvant are successful in providing *in vivo* tumor protection, and may activate additional lymphocyte subsets. With regards to AdOVA + PEI's tumor protection in spite of decreased antigen-specific T cell populations in vitro, we hypothesize that PEI's inherent immunostimulatory activity might be contributing to a general elevation in the immune response that is not antigen-specific. Additionally, further studies might investigate whether the use of PEI changed the kinetics of the immune response, as compared to the usual peak response seen 14 days post-immunization. Given these encouraging results, the next steps toward developing a clinically relevant nanoparticle immunization would involve investigation of cationic polymers similar to PEI, such as chitosan, which are already approved for human use.

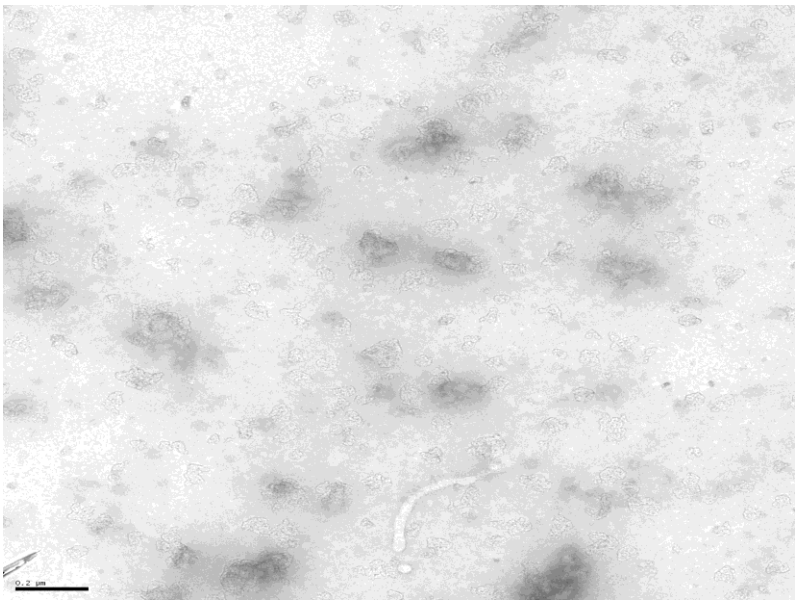


Figure 9: TEM image of PEI/DNA, N/P=10. Line corresponds to 500nm

Table 1: PEI particle size/zeta potential table

PEI: DNA	Particle Size	Zeta Potential
5	295.5± 23.8 nm	22.1± 2.5 mV
10	202.5± 18.9 nm	29.1± 8.9 mV
20	204.6± 15.6 nm	35.89 ± 8.4 mV

Note: Components for the nanoparticle formations were mixed by pipetting, and then vortexed for 30s. After 30 minutes incubation at room temperature, the particle size and zeta potential were measured using the Malvern Zetasizer. Measurements taken are from 3 formulations per group.

Figure 10: PEI- DNA transfection efficiency in HEK-293 cells. Luciferase activity is normalized to protein level and expressed as mean ± STD (n=3)

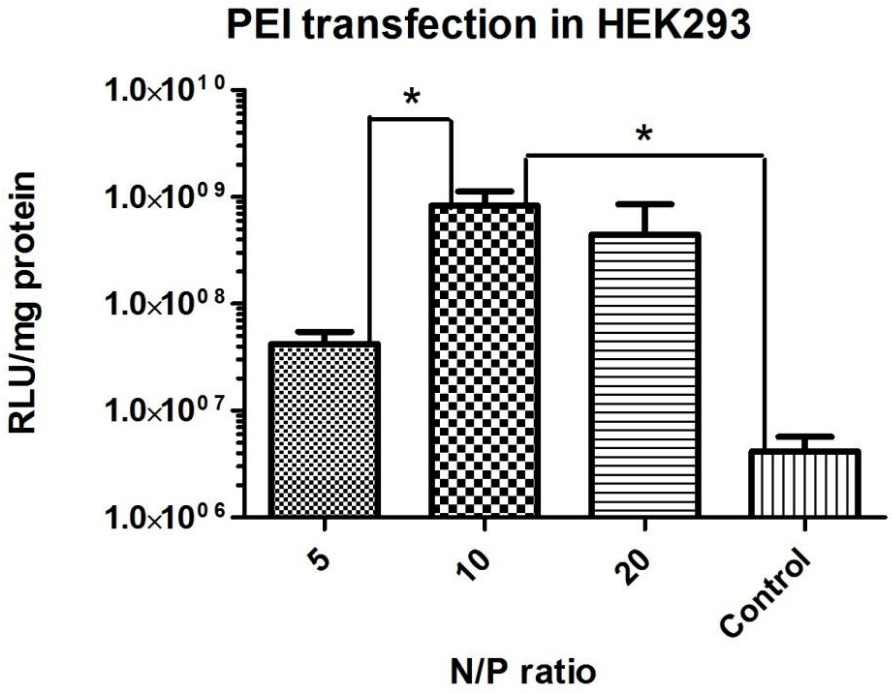
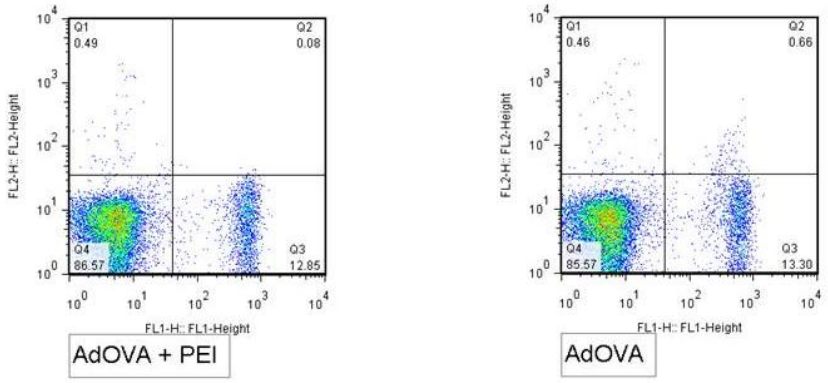

PEI transfection in HEK293
RLU/mg protein
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
*
*
5
10
20
Control
N/P ratio

Figure 11: Addition of PEI depresses OVA-tetramer population. To enumerate the antigen-specific CD8+ T cells, tetramer staining using MHC I SIINFEKL tetramer was performed. Cells suspensions of splenocytes were prepared as previously described. *A*, Flow cytometric acquisition of CD8 and tetramer stained splenocytes. *B*, Graphical presentation of the % tetramer+ CD8+ T cells for the immunization group.

A


Q1
0.49
Q2
0.08
Q4
86.57
Q3
12.85
FL2-H:: FL2-Height
FL1-H:: FL1-Height
AdOVA + PEI
Q1
0.46
Q2
0.66
Q4
85.57
Q3
13.30
FL2-H:: FL2-Height
FL1-H:: FL1-Height
AdOVA

B

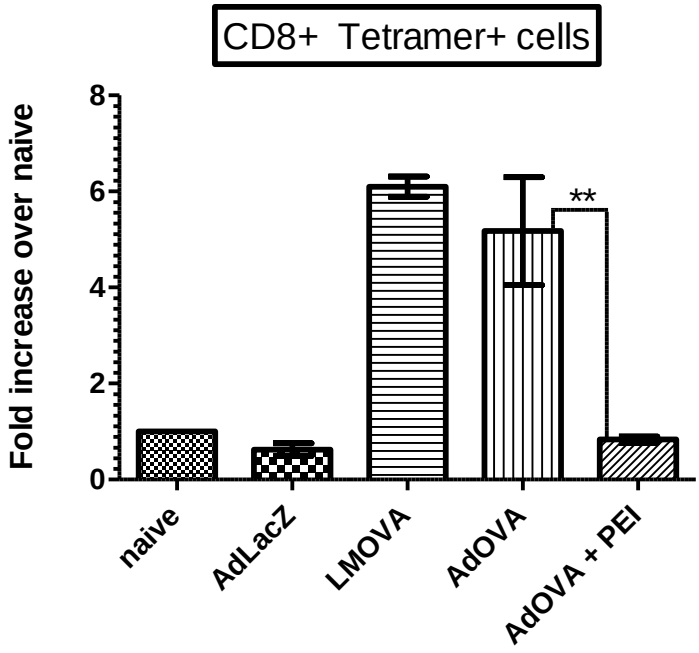
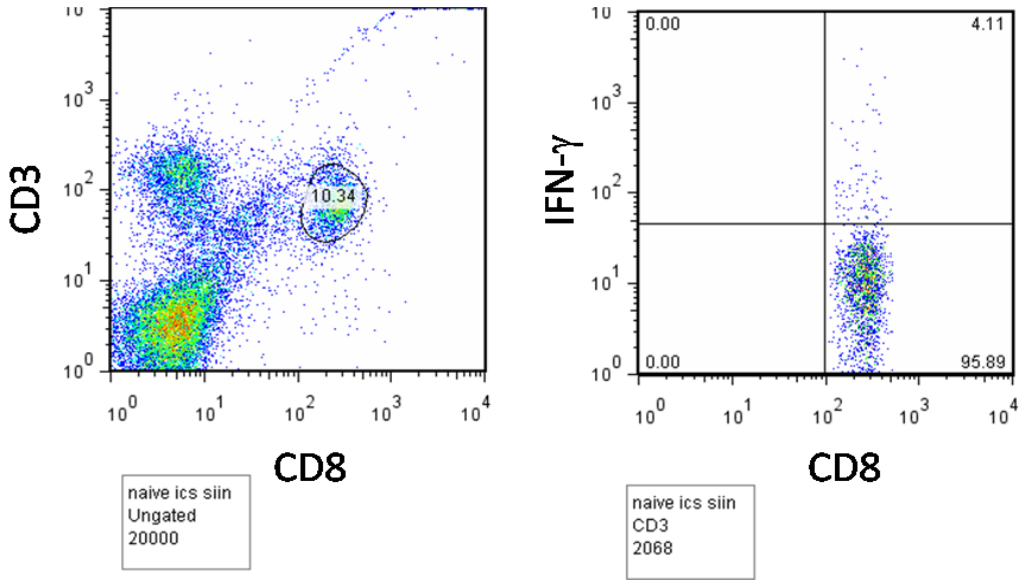

CD8+ Tetramer+ cells
Fold increase over naive
0
2
4
6
8
**
naive
AdLacZ
LMOVA
AdOVA
AdOVA + PEI

Figure 12: IFN-γ cytokine staining. Splenocytes were processed as previously described in Materials and Methods. A, Representative flow cytometric plot showing gating on CD8+ CD3+ T cells(left) and the CD8+ IFN-γ+T cells within this population(right). B, Fold induction over naïve control of CD8+ IFN-γ+ T cell frequency in response to OVA peptide restimulation**.**

A


CD3
CD8
10.34
naive ics siin
Ungated
20000
IFN-γ
CD8
0.00
4.11
0.00
95.89
naive ics siin
CD3
2068

B

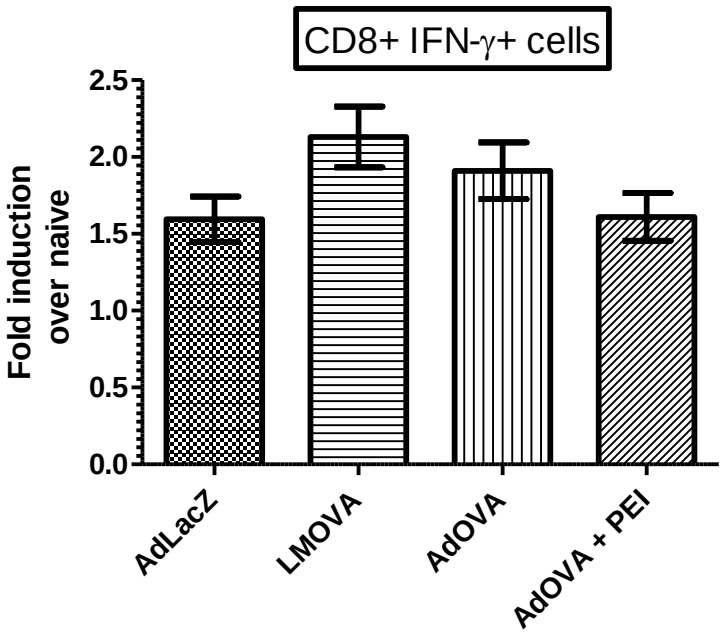
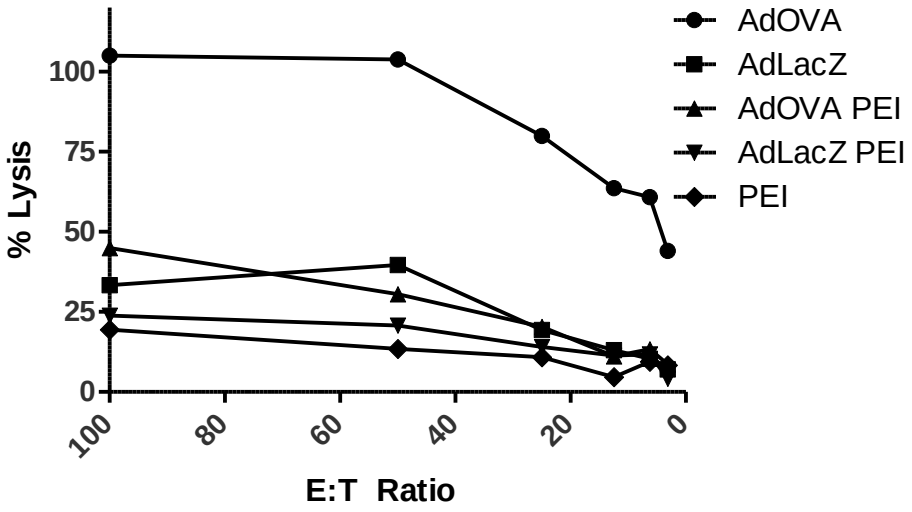

CD8+ IFN-γ+ cells
Fold induction over naive
2.5
2.0
1.5
1.0
0.5
0.0
AdLacZ
LMOVA
AdOVA
AdOVA + PEI

Figure 13: AdOVA + PEI depresses *in vitro* CTL activity. A ^{51}Cr release assay was used to measure OVA-specific lysis of target cells, as described in Materials and Methods. 2 mice were pooled for each experiment, and the experiment performed 3 times.


AdOVA
AdLacZ
AdOVA PEI
AdLacZ PEI
PEI
% Lysis
100
75
50
25
0
100
80
60
40
20
0
E:T Ratio

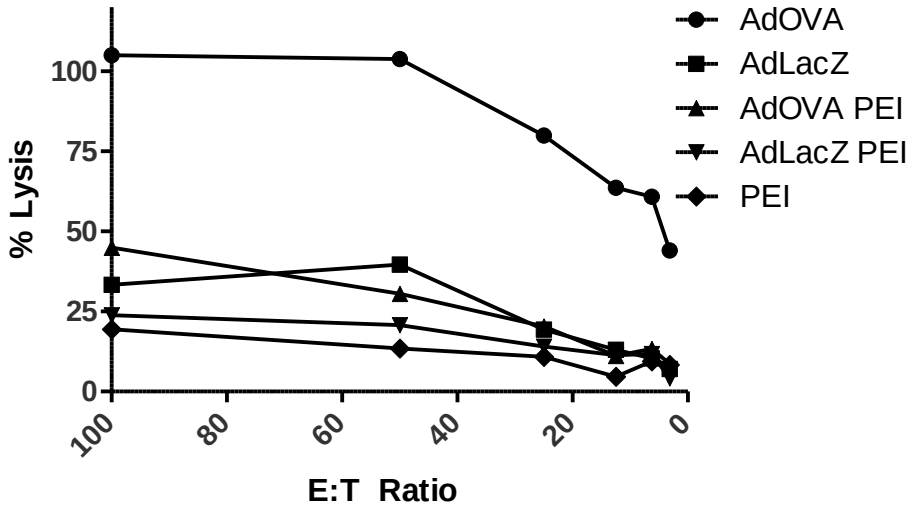
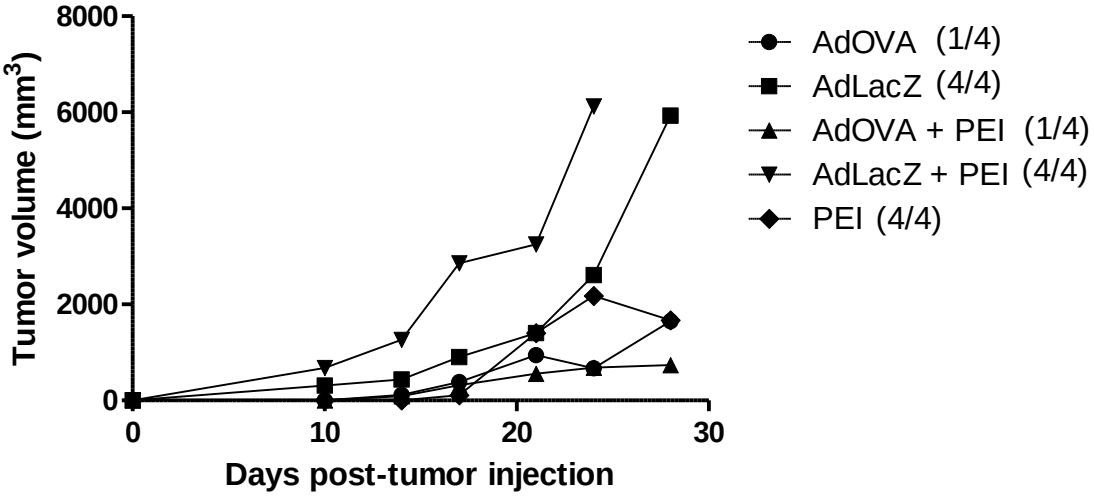

AdOVA
AdLacZ
AdOVA PEI
AdLacZ PEI
PEI
% Lysis
100
75
50
25
0
100
80
60
40
20
0
E:T Ratio

Figure 14: AdOVA + PEI enhances tumor protection *in vivo*. Mice were immunized as previously described and then challenged 14 days later with 10^7 EG.7-OVA or EL4 cells. Graph represents the average tumor volume at each time point for the groups, with error bars showing standard deviation. Number in parenthesis denotes number of mice bearing tumors by the end of the study. Note: due to sacrifice of mice when tumor burden becomes too great, the average volume may appear to decrease at later time points due to this loss. Comparison with survival curves at same time points clarifies these differences.

EG.7-OVA Tumor Challenge



EL4 Challenge

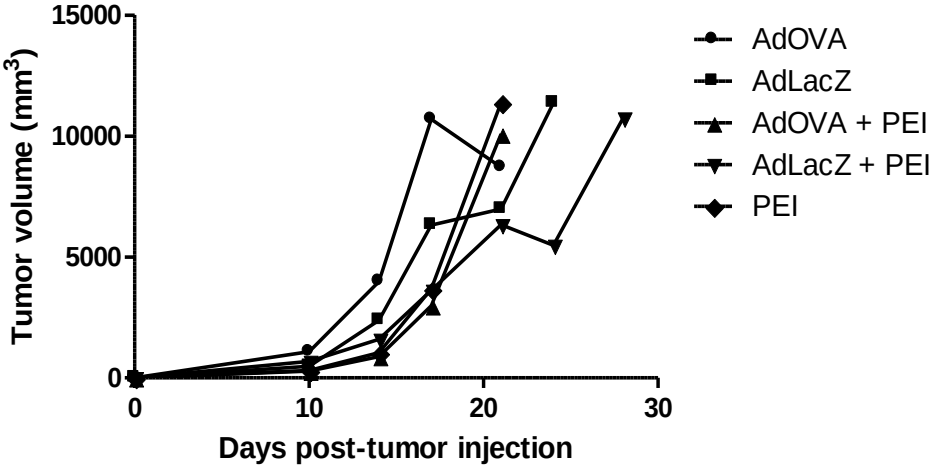
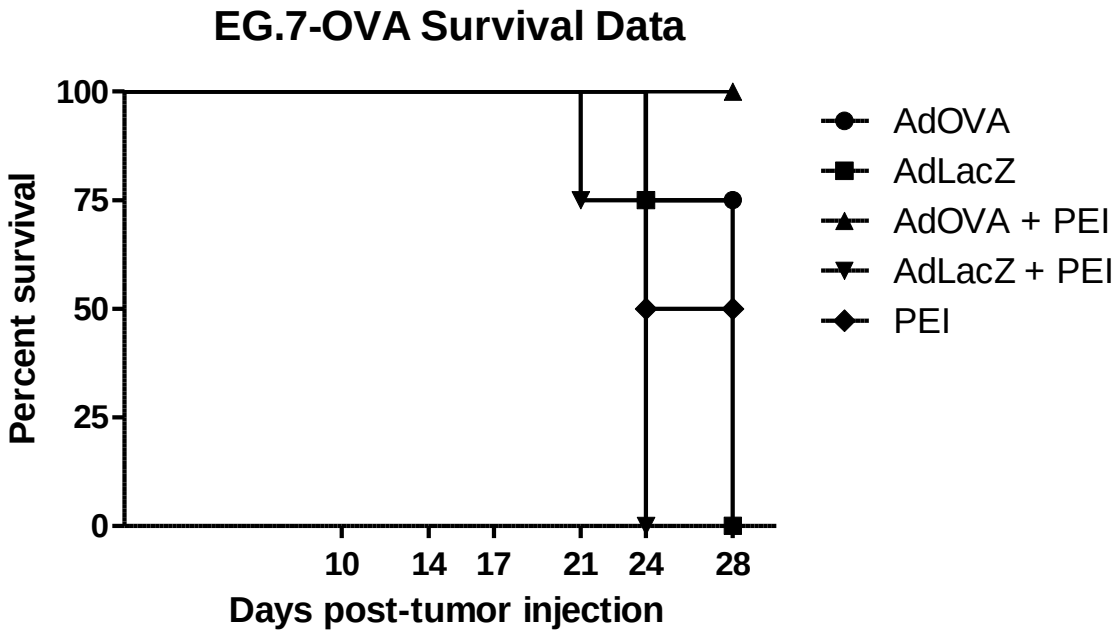


Figure 15: Survival Data: EG.7-OVA and EL4 tumor challenge. Survival data from mice in Figure 6. Mice were monitored twice weekly and sacrificed if tumor measurements exceeded 25 mm in any direction. The positive-control EL4 mice grew tumors quickly and had to be sacrificed at earlier time points, demonstrating no antigen-specific protection and the ability for tumor cells to grow in the C57Bl/6 mouse model.


EG.7-OVA Survival Data
Percent survival
100
75
50
25
0
10
14
17
21
24
28
Days post-tumor injection
AdOVA
AdLacZ
AdOVA + PEI
AdLacZ + PEI
PEI

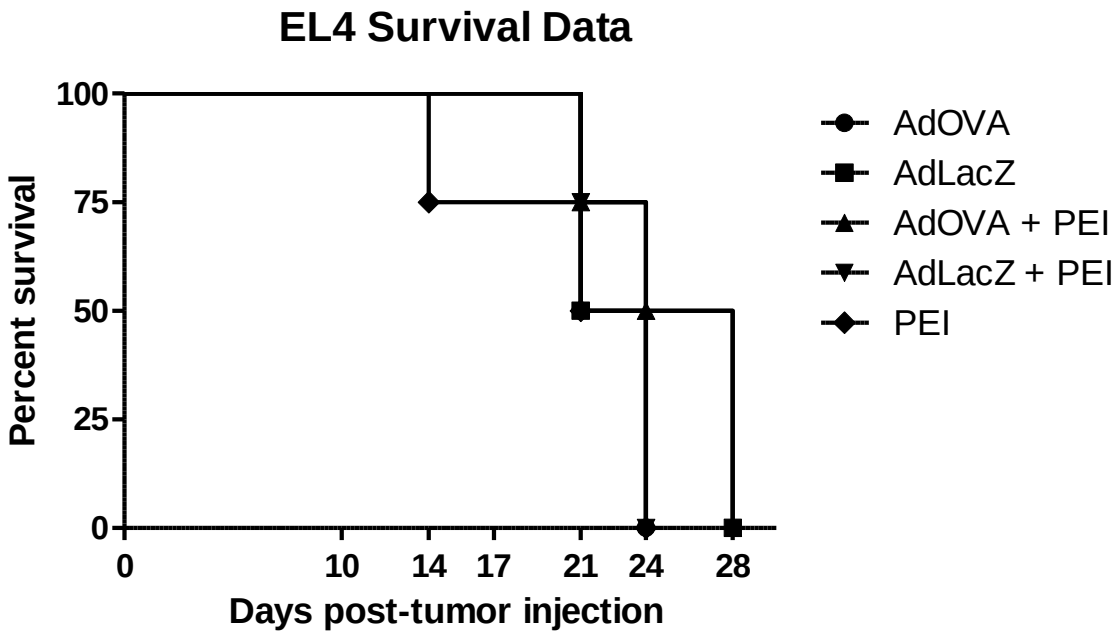

EL4 Survival Data
Percent survival
100
75
50
25
0
0
10
14
17
21
24
28
Days post-tumor injection
AdOVA
AdLacZ
AdOVA + PEI
AdLacZ + PEI
PEI

Figure 16: Antibody Depletion Studies. Mice were immunized with AdOVA + PEI on day 0, and then depleted for lymphocytes as previously described. On day 14 post-immunization, mice were challenged with 10^7 EG.7-OVA cells and tumor outgrowth and survival measured. *A*, Tumor growth rate of individual mice per group. *B*, Individual mice relative to all groups. *C*, Average tumor volume per time point for each depletion group. *D*, Survival curves for each depletion group.

A

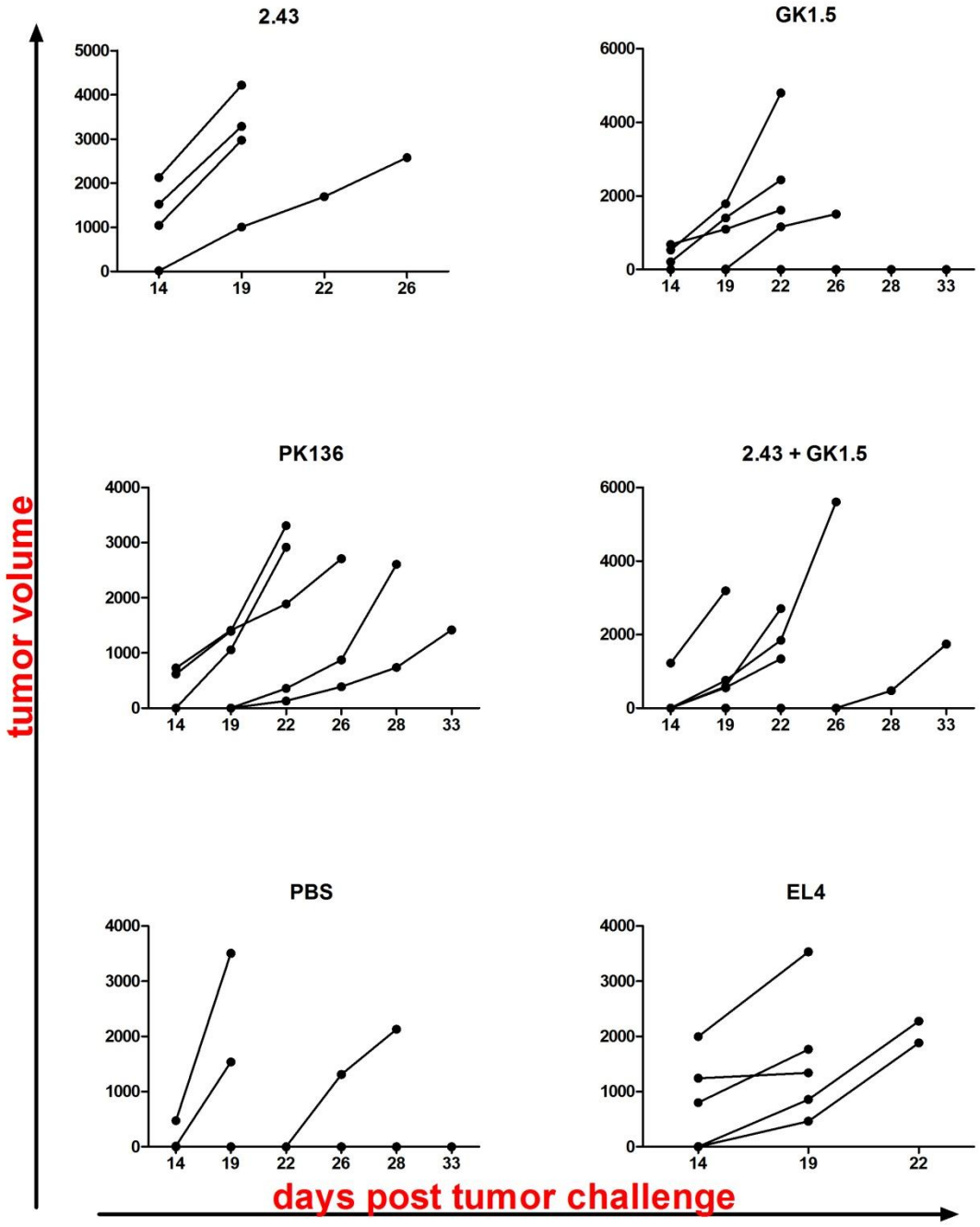
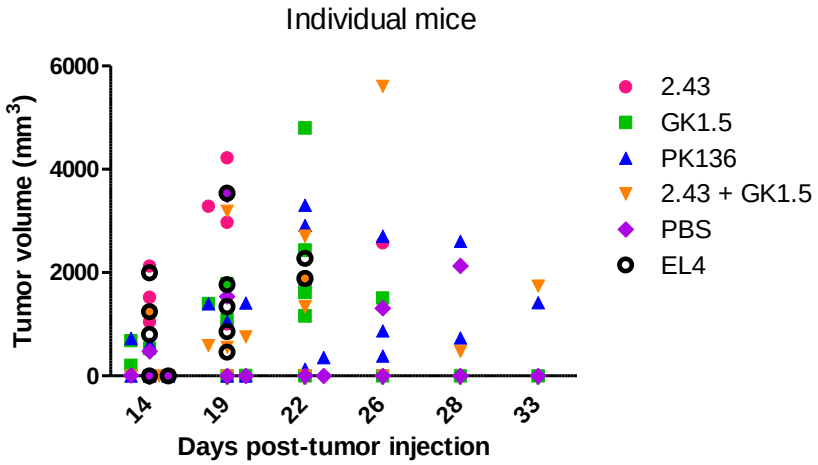
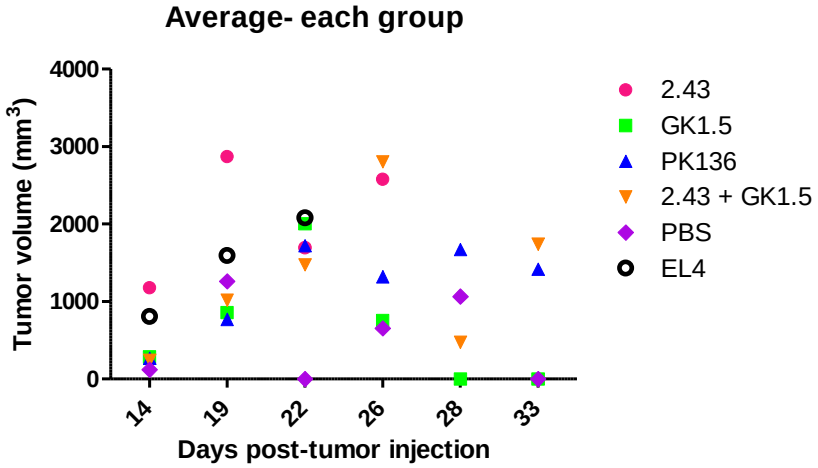

2.43
GK1.5
PK136
2.43 + GK1.5
PBS
EL4
tumor volume
days post tumor challenge

Figure 16 continued

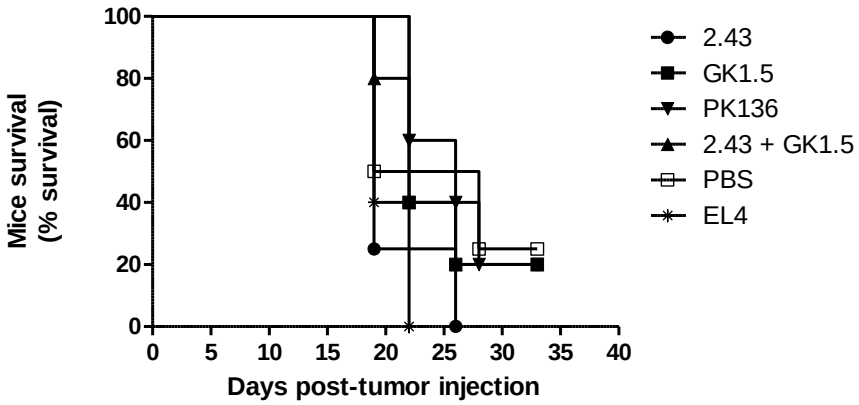
B



C



D



CHAPTER 4: ADOVA CO-DELIVERED WITH ADJUVANTS IN NANOPARTICLE FORM

Introduction

The combination of viral and non-viral vectors in the form of AdOVA + PEI showed an increase in tumor protection, though there was not an increase in CD8+ tetramer or CTL activity. This dichotomy between tumor protection and CD8+ activity has been previously demonstrated with the AdOVA model using CpG as adjuvant.[2] After demonstrating that adenoviruses can be complexed with the cationic polymer PEI to be delivered in nanoparticle form, we wanted to further test and consider more clinical applications of the vaccine formulations. While PEI is widely used for *in vitro* gene delivery studies, due to its complexation abilities and endosomal buffering capacity, issues with cell death due to toxicity is a concern for *in vivo* applications.[26,28,35] PEI is not FDA approved for use in humans and would cause a roadblock to therapeutic applications. For this reason, we chose to further test our viral/non-viral delivery system using the cationic polymer chitosan.

Chitosan is similar to PEI in that both have positively charged amino groups that can complex with negatively charged components such as proteins expressed on adenoviruses and DNA. It is biocompatible and FDA approved and is used in health supplements, wound healing agents, and as vaccine adjuvants for multiple routes of administration.[42,64] Additionally, chitosan has been shown to enhance both humoral and cell-mediated immune responses after subcutaneous vaccination.[48] For these reasons, we proceeded to optimize and test nanoparticle vaccine formulations using chitosan as the polymeric complexation adjuvant. As with the PEI vaccine formulations, we optimized the N/P ratio for enhanced transfection efficiency, while using the least amount of polymer necessary for effective protein expression.

Additionally, due to the dichotomy between tumor protection and CTL lysis that has been previously shown with CpG, as well as in our PEI studies, we wanted to expand our vaccine formulations to include CpG as an adjuvant. The combination of adenovirus, CpG, and chitosan gives three different approaches to modulating the immune response. With the adenoviral route of infection, there is antigen production, which will prime immune cells for an antigen-specific immune response. CpG as an adjuvant has been shown to boost the overall immune response, which is especially of interest when dealing with small subsets of antigen-specific T cells. Chitosan as a non-viral carrier serves to complex the adenovirus and CpG into nanoparticles, allowing delivery in particulate form, and assuring that adenovirus and CpG can reach the same cell. This can help further boost the response, as our lab has previously shown that CpG in particulate form is more efficacious than when it is delivered in aqueous solution.[50] Additionally, it has been reported to have intrinsic adjuvant activity, in that it can cause DC maturation.[54,55,61] The combination of these factors could enhance the strength of the immune response and levels of protection, as measured in both *in vitro* assays and *in vivo* tumor studies. To study the ability of AdOVA, chitosan, and CpG to initiate and enhance an antigen-specific immune response, we chose to look at 6 immunization groups:

1) AdOVA
2) AdOVA + CpG
3) AdOVA + chitosan
4) AdOVA + CpG + chitosan
5) AdLacZ + CpG + chitosan

We again assayed the induction of an antigen-specific immune response with tetramer and IFN-γ staining as well as an antigen-specific CTL assay and *in vivo* tumor challenge. These tests allowed us to both enumerate the T cell antigen-specific population, and assess the cells' ability to launch and function as effector cells and kill target tumor cells, thus preventing tumor growth in a mouse model of prostate cancer.

Materials and Methods

Materials

Medium molecular weight chitosan (~20kDa) was purchased from Sigma-Aldrich (St. Louis, MO). A 0.01M chitosan solution in 1% acetic acid was prepared as previously described, for the stock chitosan solution. The AdOVA vaccine was obtained from the University of Iowa Gene Vector Core, as was previously produced for the Lubaroff Group.[17]

Nanoparticle vaccine preparation

Nanoparticles were formulated at various ratios of chitosan's nitrogen to DNA phosphate (N/P ratio), with the amount of chitosan solution used based on a 50 μg/mouse DNA dose. 10^8 pfu viral vaccine was used for each immunization. The solutions were vortexed for 20s and the left to incubate at room temperature for 30 min. The vaccines were then injected subcutaneously. For transfection studies, chitosan-DNA nanoparticles were produced using VR1255 DNA, which encodes for luciferase. The VR1255 was amplified and purified in our lab as previously described.[60]

Evaluation of luciferase expression in HEK293 cell line

Human Embryonic Kidney cells (HEK293) were purchased from the American Type Culture Collection(ATCC), and maintained in DMEM supplemented with 10% Fetal Bovine Serum(FBS), 1% sodium pyruvate, 1% HEPES, and 0.05 mg/ml gentamycin at 37 ^oC in a humidified 5% CO_2- containing atmosphere. Cells were seeded in 24-well plates at a density of 80,000 cells/well, 24 hours prior to transfection. 100 μl of the chitosan-DNA nanoparticle solution at various N/P ratios were added to the cells, with each well receiving the constant DNA dose of 1 μg. The nanoparticle solutions

were delivered in serum-free DMEM and allowed to incubate with the cells for 4 hrs at 37 ^{o}C. The cells were then carefully washed with PBS and left to incubate in serum containing media for an additional 44 hours. Following this incubation, the cells were treated with 200 μl of lysis buffer (Promega, Madison, WI). The cell lysate was subjected to two freeze/thaw cycles, and then transferred into tubes for centrifugation at 13200 rpm for 5 minutes. 20 μl of the supernatant was mixed with 100 υl of the luciferase assay reagent (Promega, Madison, WI) and the RLU(relative light units) for each sample read on a luminometer (Lumat LB9507, EG & G Berthold, Bad Wildbad, Germany). The Micro BCA protein assay kit (Pierce, Rockford, IL) was used to normalize the RLU to protein concentration in the cell extracts. The luciferase activity could then be expressed as RLU/mg protein in the cell lystate. The transfection experiment was performed three times, with results reported as mean ± standard deviation.

Tumor cell lines and animals

EG.7-OVA cells and their parental line, the non-OVA expressing mouse lymphoma EL4, were used to measure *in vivo* tumor growth, and *in vitro* antigen specific CTL activity. Both cell lines were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% sodium pyruvate, 1% HEPES, and 0.05 mg/ml gentamycin in a humidified 5% CO_2- containing atmosphere. 6 to 8 week C57Bl/6 mice were purchased from the National Cancer Institute (Bethesda, MD) and were maintained in filtered cages before use.

Phenotypic assay: Tetramer staining

To enumerate antigen-specific CD8+ T cells, tetramer staining using MHC I SIINFEKL tetramer (Beckman Coulter, Fullerton, CA) was performed. For each treatment group, a mouse was sacrificed and the spleen removed, and a splenocyte suspension created. Red blood cells were lysed using ACK buffer, and the cell suspensions were filtered through a 70μm cell strainer. The splenocytes were counted and resuspended at 10^7 cells/ml. 100 μl of cells were plated per well in a 96 well plate, and blocked with 24G2 Fc receptor block for 15 minutes on ice. Cells were stained for 30 minutes with tetramer, then for 20 minutes for anti-CD8 FITC and anti-CD3 PE-Cy5. The cells were washed, fixed, and permeabilized with the Cytoperm/Cytofix kit (BD Biosciences, San Diego, CA) and resuspended in FACS buffer for flow cytometry analysis, collecting 1 x 10^5 events. The data was analyzed using FlowJo software (Tree Star, Stanford, CA).

Intracellular Cytokine Staining

Splenocytes were processed as previously described, and plated at 10^6/well in a 96 well plate. Golgistop (BD Biosciences) was added to each well to inhibit IFN-γ secretion, wells were treated with peptide stimulation with SIINFEKL, and the plate incubated for 5 hrs at 37 ^oC. After blocking and staining with anti-CD8 FITC and anti-CD3 PE-Cy5 as described above, the cells were stained with anti-IFNγ PE, and flow cytometry performed.

In vitro cytotoxic assay

A ^{51}Cr release assay was used to measure OVA-specific lysis of target cells. In a 24 well plate, 10^7 cells/well were seeded for each immunization group, along with the cytokine IL-2(10 U/ml) and mitomycin C treated EG.7-OVA cells (2 x 10^5 cells/well) as stimulators. Following a 5 day coculture at 37 ^oC, the effector splenocytes were harvested and separated from dead cells using a Ficoll separation. Target EG.7-OVA

cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hr, washed twice, and resuspended at 5 x 10^4 cells/ml. The effector cells were diluted serially down a 96 well round bottom plate, and 100 μl targets added to each well, giving effector: target (E:T) ratios from 100:1 to 3.125:1. After a 4 hr incubation at 37 ^oC, the plate was centrifuged at 1000 rpm for 10 minutes, and 100 μl of the supernatant was taken from each well and counted in the COBRA II gamma counter (Packard Instrument Company, IL). The specific lysis was calculated using the formula:

(sample lysis-spontaneous lysis)/(maximum lysis-spontaneous lysis) *100

Tumor challenge study

In tumor challenge studies, both E.G.7 and EL4 cells were used to challenge mice with antigen-specific, or non-specific, tumor cells. Following optimization of cell dose to ensure consistent tumor growth, the cells were resuspended in PBS at a concentration of 10^7 cells in 100 μL. The cells were injected subcutaneously into the right flank of the mouse. Tumor outgrowth was measured twice weekly, with tumor volume calculated as: [length x width x height x 0.5236] as described by *Shariat el al*.[8] Survival of the mouse treatment groups was also monitored. Mice were sacrificed for ethical reasons if they appeared ill from tumor burden or if measurements exceeded 25 mm in any direction. Each experimental group consisted of 4 mice and experiments were repeated 3 times. All animal experiments were conducted in accordance with the procedures outlined in the University of Iowa's Guidelines for Care and Use of Experimental Animals. Mice were monitored daily by the University of Iowa Animal Care Facilities, as well as by the investigators.

Results

Chitosan forms nanoparticles with AdOVA and is successfully transfected

To determine if chitosan could effectively form nanoparticles complexing AdOVA and CpG, components were mixed as previously described and allowed to complex through electrostatic interaction for 30 minutes at room temperature. All formulation groups formed particles in the 150-300 nm range, which is appropriate for endocytosis by cells. Additionally, the formulations had positive zeta potentials, which aids in attraction to the negatively charged cell membrane.[38](Table 2). To further test the ability of chitosan formulations to transfect cells and initiate protein expression, chitosan complexes mixed with DNA at varying N/P ratios were incubated with the permissive cell line HEK-293. The complexes created at N/P = 10 and 20 showed protein expression of 7-9 x 10^9 RLU/mg protein, compared to the control which was less than 10^6 RLU/mg protein. (Figure 17) There was no significant difference between expression of N/P=10 and N/P= 20. For this reason, an N/P of 10 was used for the duration of the studies. As described previously, the DNA dose per formulation remains 50 μg, which forms the basis for the N/P calculation. Table 1 shows results using chitosan at N/P=10.

Chitosan complexation with AdOVA does not promote antigen-specific T cell development

To investigate the modulation of the antigen-positive T cell population in immunized mice, tetramer staining was performed 14 days post-immunization. Splenocytes were harvested and processed as previously described and then stained for CD8, CD3, and OVA tetramer. Figure 18 shows the representative gating on the CD3+ CD8+ population, and then FL1 vs. FL2 identifying cells double positive for FITC CD8 and PE tetramer. Interestingly, we found that AdOVA + chitosan depresses the tetramer frequency, with levels equal to the control, AdLacZ + CpG + chitosan. (Figure 18) The

other vaccination groups which included OVA (AdOVA, AdOVA + CpG, and AdOVA + CpG + chitosan) all showed 6-10 fold increases over the control. Chitosan complexed with adenovirus appeared to negate the antigen-specific effects of AdOVA immunization.

Tetramer kinetics following tumor challenge

After observing the tetramer frequencies 14 days post-immunization, the mice were challenged with EG.7-OVA or EL4 tumor cells to determine how the frequencies would change after a specific or non-specific challenge. The mice that were challenged with the antigen-specific EG.7-OVA cells maintained the levels of CD8+ OVA tetramer + T cells that were seen at day 14 post immunization. The AdOVA + chitosan group's response was again depressed, compared to the other AdOVA formulations. (Figure 19) All groups challenged with parental EL4 cells showed a decrease in tetramer frequencies compared to day 14 post-immunization. Without a continuing antigen challenge, the frequencies have likely returned to memory levels.

Chitosan with AdOVA also depresses CD8+ IFN-γ levels

IFN-γ is an inflammatory cytokine that may promote immune responses and protect against tumor development. Upregulation of this cytokine has been observed for activation of the cytotoxic T cell response necessary for tumor rejection.[18] For these reasons, we wanted to measure the changes in IFN-γ secretion after immunization with our nanoparticle vaccines. As with the tetramer staining, first we gated on the CD8+ CD3+ cells, and then the CD8+ IFN-γ+ cells compared between the groups. Compared to the media background levels, no group showed large increases when stimulated with the OVA-specific peptide. It was also observed that all of the groups containing AdOVA + CpG had higher media background levels than those without. (Figure 20) Overall, the trends were the same as in tetramer staining, with AdOVA + chitosan depressing the CD8+ IFN-γ+ levels compared to AdOVA alone.

CpG and chitosan differentially impact CTL response

To examine the ability of immunization to stimulate a CD8 T cell response against tumor cells expressing OVA, the CTL assay was performed 14 days after immunization. Cells were harvested and processed as previously described. After the restimulation co-culture, EG.7-OVA cells were labeled with radioactive chromium and plated at different ratios with the effector splenocytes recovered from the coculture plate. The CTL results show that AdOVA + CpG produced the highest lysis, with an increase over AdOVA alone. (Figure 21) The opposite is true for the addition of chitosan, as the AdOVA + chitosan group exhibited the lowest specific lysis of the 4 immunization groups containing AdOVA. The AdLacZ group showed minimal background lysis at the varying E:T ratios.

Differential impact of chitosan and CpG is also present in tumor protection studies

The CTL assay demonstrated that splenocytes obtained from previously immunized mice could lyse target tumor cells *in vitro*, with the adjuvants CpG and chitosan having a differential impact on the modulation of the immune response. Next, we wanted to test the protection afforded by our immunization formulations in an *in vivo* tumor challenge. Mice were immunized and challenged 14 days later with 1 x 10^7 EG.7-OVA or EL4 cells, which were injected subcutaneously on the right flank. Tumor outgrowth was measured twice weekly, and mouse health and survival monitored. Mice were sacrificed if they appeared ill from tumor burden or if the tumor exceeded 25 mm in any direction. In this representative experiment, 75% of the AdOVA + CpG mice remained tumor free, and had the lowest overall tumor growth. (Figure 22) The AdOVA immunized mice initially had the same levels of tumor protection as the AdOVA + CpG mice, but beyond day 25 the tumor growth rate accelerated and by the end of the experiment 75% of mice had developed tumors. The AdOVA + chitosan mice had the

least tumor protection of all formulations including AdOVA, with all mice developing tumors and decreased survival time. In the EL4 tumor model, growth was rapid in all groups, and no antigen-specific protection was seen in any groups, as was expected since these cells do not express OVA.(Figure 23)

Discussion

In search of a therapeutic prostate cancer vaccine, we have considered both PEI and chitosan as cationic polymers that can enhance delivery and thereby increase the immune response initiated by our vaccines. PEI has been widely used as a gene delivery vehicle but toxicity issues remain a barrier to its use in human studies for clinical trials. Chitosan is FDA approved, making it a much better candidate for future therapeutic models. While continuing to use the model antigen OVA system, we characterized the cell types activated by the vaccine formulations, and tested the lymphocytes' ability to mount an effective immune response both *in vivo* and *in vitro*. Chitosan effectively condenses negatively charged adenovirus or CpG into nanoparticles that are of the appropriate size and charge to be endocytosed. Additionally, transfection experiments with chitosan complexing luciferase-encoding DNA showed high transfection at N/P ratios of 10 and 20, so the amount of chitosan used in all future experiments was based off of N/P=10 to minimize the amount of polymer per vaccine.

The use of adjuvants in vaccine formulations is well documented in both research [2,50,53] and in clinical practice[65,66]. A balance between harnessing the immunogenicity of the antigen, delivery vehicle, and adjuvant must be made in order to avoid unwanted inflammatory responses that may counteract the original objectives. Pattern recognition of CpG by TLR-9 can produce cytokine responses that may aid in tumor rejection. In previous studies, CpG was shown to enhance tumor protection when complexed with adenovirus, while not showing an increase in CTL lysis.[56] We wanted to

examine how chitosan would modulate the tumor protection and T cell response, when delivered with adenovirus, or with adenovirus and CpG.

In tumor challenge studies, initially both AdOVA and AdOVA + CpG suppressed tumor growth, at 3 weeks post-challenge (5 weeks post-immunization) (Figure 22). At this timepoint, the AdOVA group began to develop tumors, with 75% of mice bearing tumors by the completion of the experiment at day 60. The AdOVA + CpG immunized mice continued to suppress tumor growth and by day 60, 75% of the mice remained healthy and tumor free. Seeing this shift in protection approximately 3 weeks after tumor challenge, we believe that the CpG may cause a shift in the kinetics of tumor response, allowing longer lasting protection than the AdOVA alone. In these and previous studies, the CTL response has been analyzed 14 days post-immunization, as that is when the optimum immune response is found. After analyzing the tumor protection and survival curves, further experiments were planned to analyze the CTL response up to 6 weeks after immunization, in both challenged and naïve mice.

Chitosan delivered solely with AdOVA depressed the tetramer and CTL response, compared to AdOVA alone. Tumor protection was also reduced with this immunization group. The AdOVA + CpG+ chitosan group was intermediate in T cell activation and tumor protection, showing better results than AdOVA + chitosan, but less enhancement than AdOVA + CpG. The amount of virus and CpG present was consistent in all formulations, but the presence of chitosan appeared to depress the effects of the virus and CpG. Numerous studies have reported that chitosan may have adjuvant activity and be advantageous as a delivery agent in mucosal systems and as an oral gene delivery vehicle.[46,47,48,67] Studies have also shown that *in vitro*, chitosan may increase adenovirus infectivity of cells.[41] However, our *in vivo* experiments do not give an enhancement of the immune response that would be possible from increased cell uptake and processing. Because the tumor challenge studies also showed reduced tumor protection post-immunization, we hypothesized that chitosan may either be interfering with viral

infectivity in our model cell lines, or interfering with antigen production post-infectivity. This was investigated further in the Chapter 5 data.

Our viral/non-viral gene delivery system, in the form of antigen-encoding adenovirus and chitosan, is a novel system aimed at exploiting the benefits of each delivery vehicle. Adenoviruses are programmed to infect cells, making them superior to non-viral carriers in cell transfection studies. Non-viral vehicles such as the cationic polymers PEI and chitosan have the ability to complex negatively charged DNA and adenoviruses, delivering these components in particulate form. This is important to assure that all components and adjuvants are delivered to the same cells to maximize the synergistic effects of the gene delivery strategies. Additionally, delivery in particulate form makes the particles more likely to be taken up and processed by antigen presenting cells such macrophages and dendritic cells, which is necessary to initiate an immune response. Our rationale for testing chitosan was based on previous vaccination strategies that have successfully employed it.[46,47,48,68] However, our current results strongly indicate that complexation between chitosan and adenoviruses is not beneficial for initiating or enhancing antigen-specific CD8+ T cell responses necessary for tumor protection in the OVA antigen model system.

Our previous studies and other studies in the adenovirus + CpG model[2] have shown that the tumor response is mainly mediated by CD8 activity. Studies citing an enhancement of delivery with chitosan as delivery vehicle have focused on an upregulation of CD4 cells[48], or looked at alternate routes of administration rather than subcutaneous.[46,47,68] In our studies, we do not see an enhancement of CD8 activity with chitosan as adjuvant in a subcutaneous vaccine. This is of interest, as chitosan may not be an appropriate adjuvant for responses requiring activation through the CD8 pathway, when combined with subcutaneous delivery, as is necessary for clinical relevance. We believe this information is of value to the scientific community, as the area of immunotherapy and gene delivery continues to develop, and more exploration into these mechanisms is of great interest. To

further elucidate the effects of chitosan on the immune response, we wanted to continue to move from our model antigen system, OVA, into the therapeutic model, using PSA. This allows us to repeat many of the same experiments in a different mouse and tumor cell model, to determine if the results would be consistent in different systems. Additionally, we wanted to more closely examine the kinetics of tumor protection and CTL activity, and determine the mechanism by which chitosan may modulate the immune response.

Table 2: Chitosan particle size/zeta potential table

Formulation	Particle Size	Zeta Potential
Chitosan+ DNA	160.5± 16.2 nm	20.1± 3.4 mV
PEI + DNA	202.5± 18.9 nm	29.1± 8.9 mV
Chitosan + CpG	255.9± 14.5 nm	19.79 ± 2.24 mV
Adenovirus + CpG + chitosan	291.0±12.4 nm	18.49±5.45 mV

Note: Components for the nanoparticle formations were mixed by pipetting, and then vortexed for 30s. After 30 minutes incubation at room temperature, the particle size and zeta potential were measured using the Malvern Zetasizer. Measurements taken are from 3 formulations per group

Figure 17: Chitosan DNA transfection efficiency in HEK-293 cells. Results were normalized for the RLU to protein concentration in the cell extracts. The luciferase activity could then be expressed as RLU/mg protein in the cell lystate. The transfection experiment was performed three times, with results reported as mean ± standard deviation.

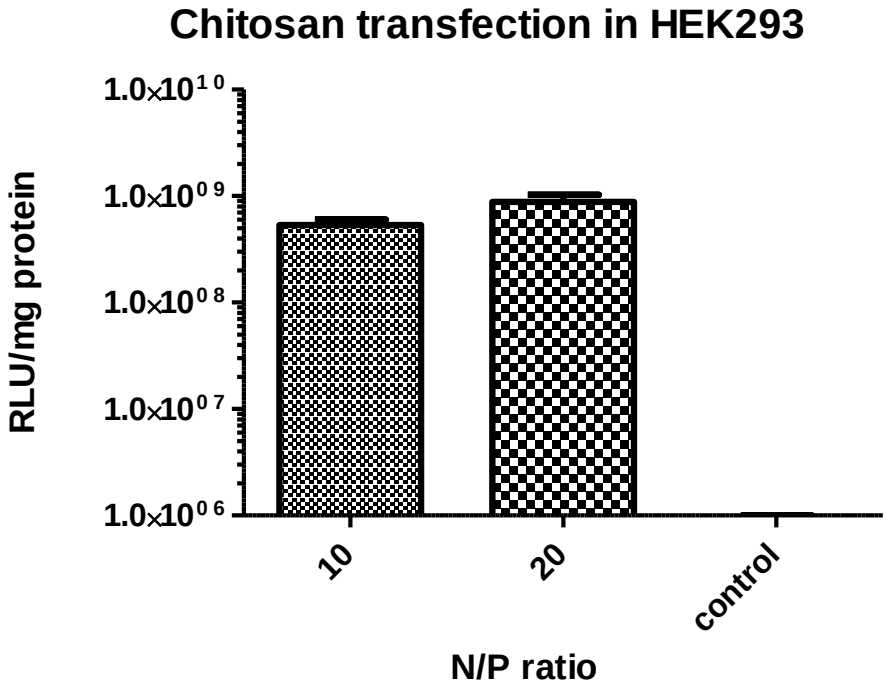
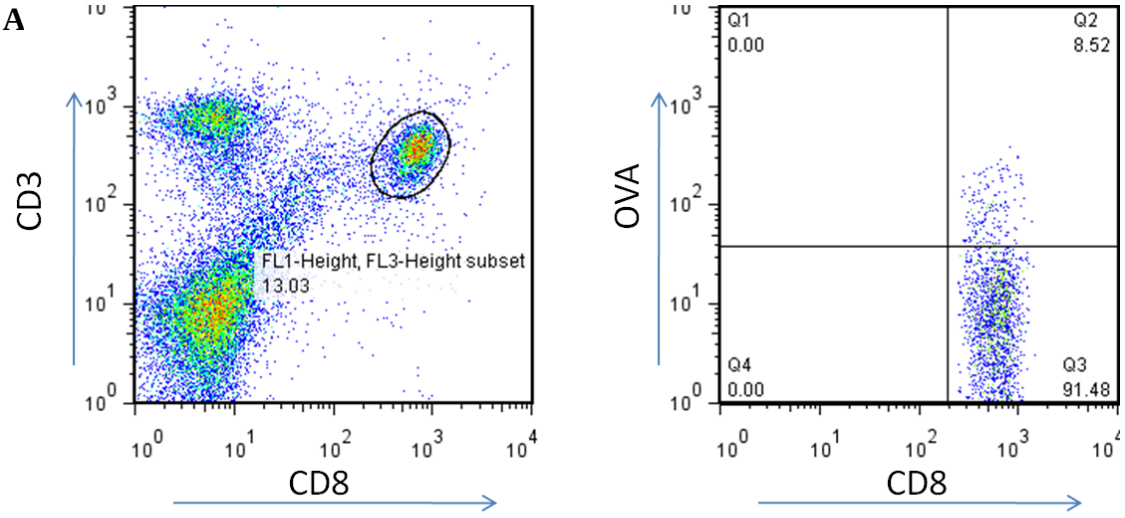

Chitosan transfection in HEK293
RLU/mg protein
1.0×10^10
1.0×10^09
1.0×10^08
1.0×10^07
1.0×10^06
10
20
control
N/P ratio

Figure 18: Chitosan complexed with AdOVA does not promote antigen-specific T cell development. To enumerate the antigen-specific CD8+ T cells, tetramer staining using MHC I SIINFEKL tetramer was performed. Cells suspensions of splenocytes were prepared as previously described. *A*, Flow cytometric acquisition of CD8 and tetramer stained splenocytes. *B*, Graphical presentation of the % tetramer+ CD8+ T cells for the immunization group.

A


CD3
CD8
FL1-Height, FL3-Height subset
13.03
OVA
CD8
Q1
0.00
Q2
8.52
Q4
0.00
Q3
91.48

B

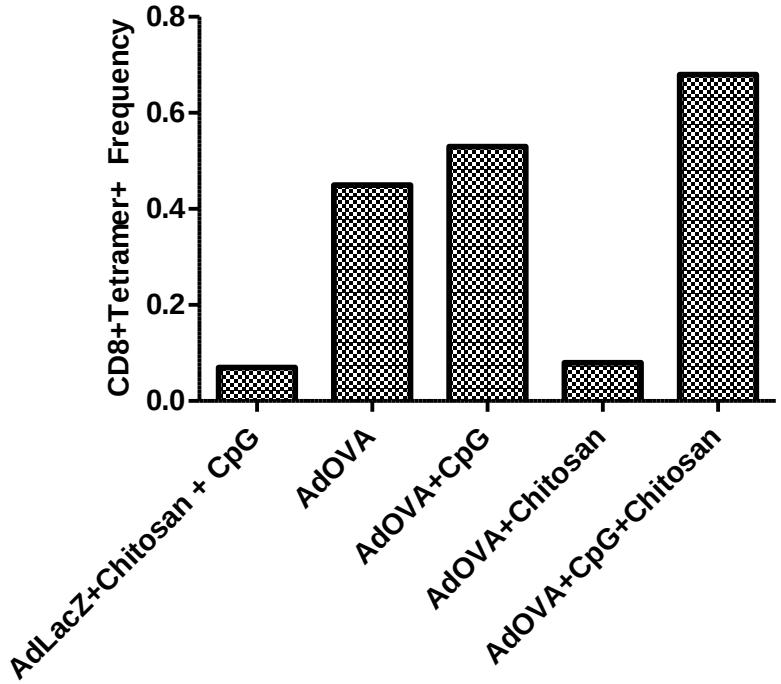

CD8+Tetramer+ Frequency
0.0
0.2
0.4
0.6
0.8
AdLacZ+Chitosan + CpG
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan

Figure 19: OVA Tetramer Kinetics. Mice were immunized with the AdOVA/chitosan formulations on day 0, and bled on day 14 to obtain the lymphocytes for staining. Also on day 14, the mice were challenged with either EG7 (OVA expressing) cells or EL4 control cells. On day 28, the tetramer staining was again repeated to measure fluctuations in the cell population after immunization, with and without challenge**.**

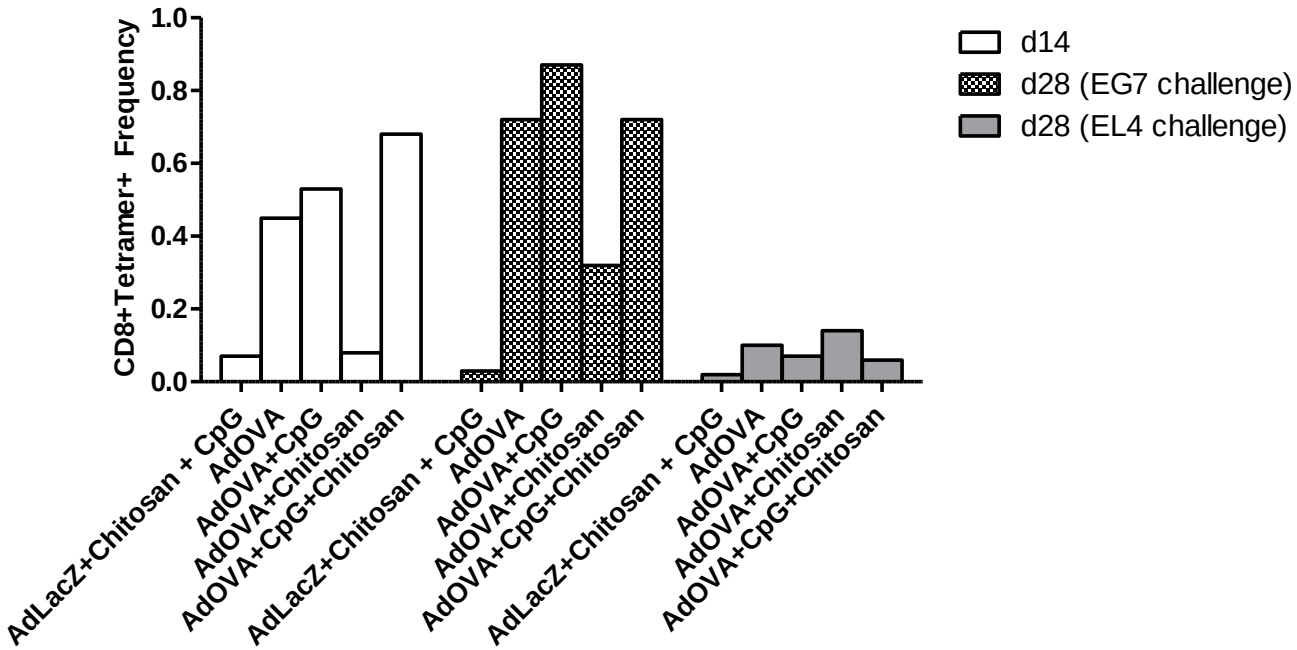
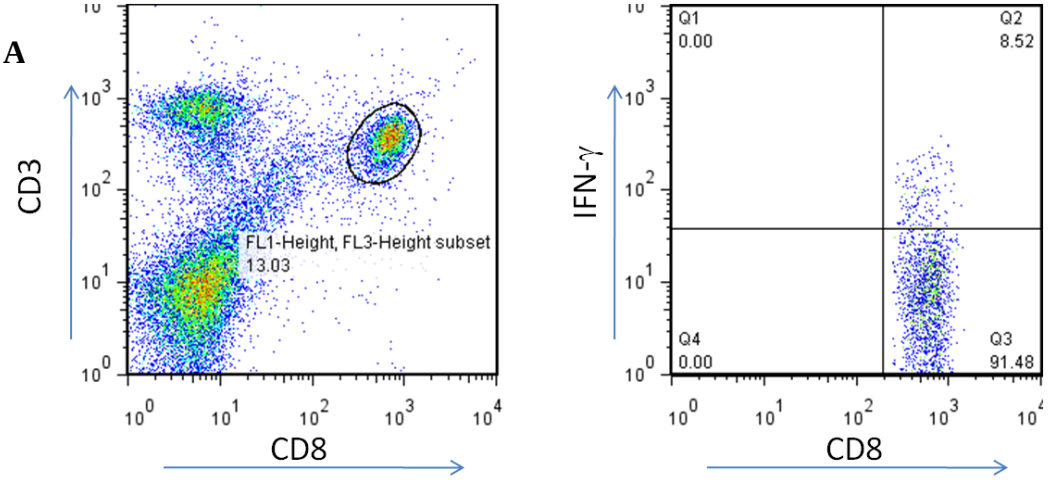

CD8+Tetramer+ Frequency
1.0
0.8
0.6
0.4
0.2
0.0
AdLacZ+Chitosan + CpG
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan
AdLacZ+Chitosan + CpG
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan
AdLacZ+Chitosan + CpG
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan
d14
d28 (EG7 challenge)
d28 (EL4 challenge)

Figure 20: CD8+ IFN-γ+ T cell frequency. Splenocytes were processed as previously described in Materials and Methods. *A*, Representative flow cytometric plot showing gating on CD8+ CD3+ T cells(left) and the CD8+ IFN-γ□ □ T cells within this population(right). *B*, Fold induction over naïve control of CD8+ IFN-γ+ T cell frequency in response to OVA peptide restimulation.

A


CD3
CD8
FL1-Height, FL3-Height subset
13.03
IFN-γ
CD8
Q1
0.00
Q2
8.52
Q4
0.00
Q3
91.48

B

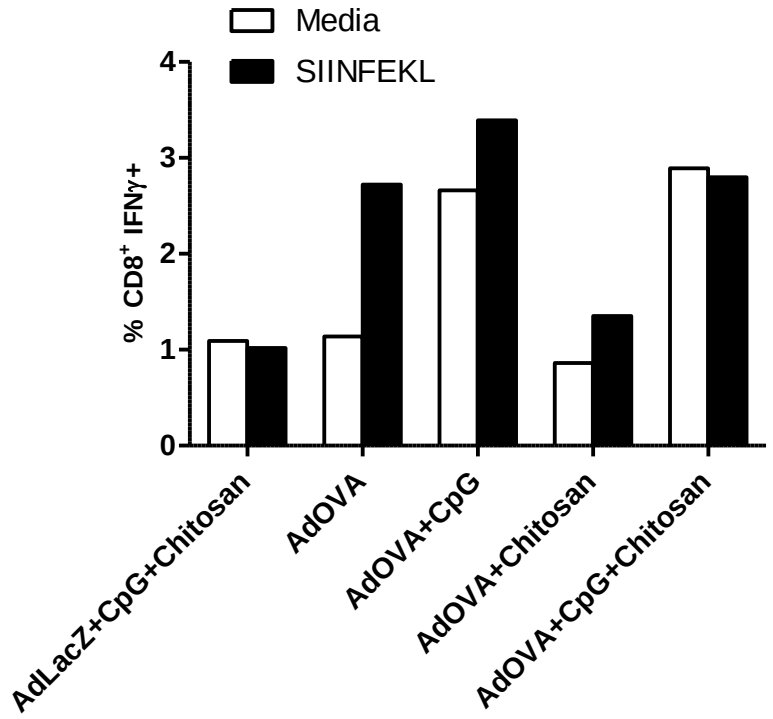

Media
SIINFEKL
% CD8+ IFNγ+
0
1
2
3
4
AdLacZ+CpG+Chitosan
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan

Figure 21: CpG and chitosan differentially impact the CTL response. A ^{51}Cr release assay was used to measure OVA-specific lysis of target cells, as described in Materials and Methods. 2 mice were pooled for each experiment, and the experiment performed 3 times.

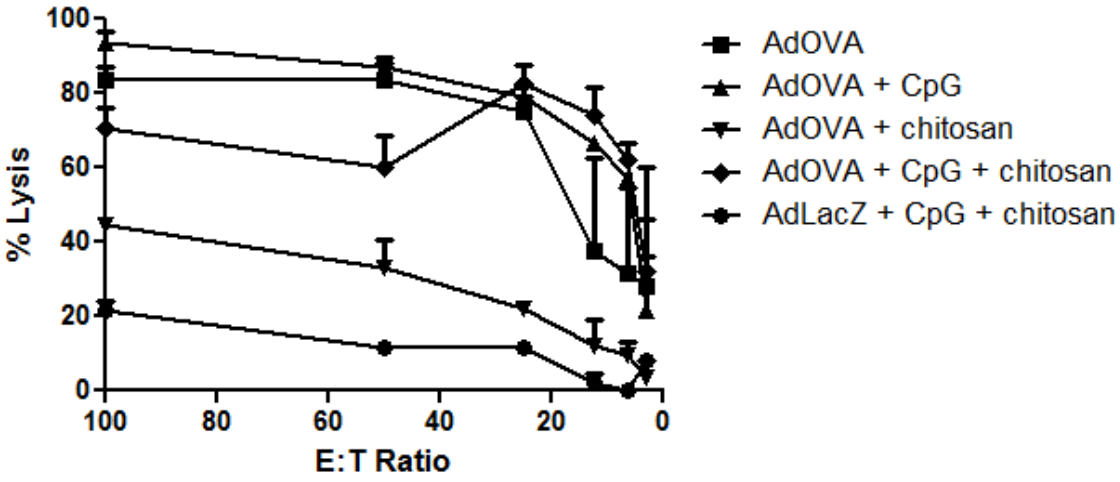

100
80
60
40
20
0
% Lysis
100
80
60
40
20
0
E:T Ratio
AdOVA
AdOVA + CpG
AdOVA + chitosan
AdOVA + CpG + chitosan
AdLacZ + CpG + chitosan

Figure 22: Differential impact of chitosan and CpG is also present in tumor protection studies. Mice were immunized as previously described and then challenged 14 days later with 10^7 EG.7-OVA or EL4 cells. Graph represents the average tumor volume at each time point for the groups, *A*, EG.7-OVA, and *B*, EL4. Error bars denote standard deviation. Number in parenthesis denotes number of mice bearing tumors by the end of the study. Note: due to sacrifice of mice when tumor burden becomes too great, the average volume may appear to decrease at later time points due to this loss. Comparison with survival curves at same time points clarifies these differences.

A

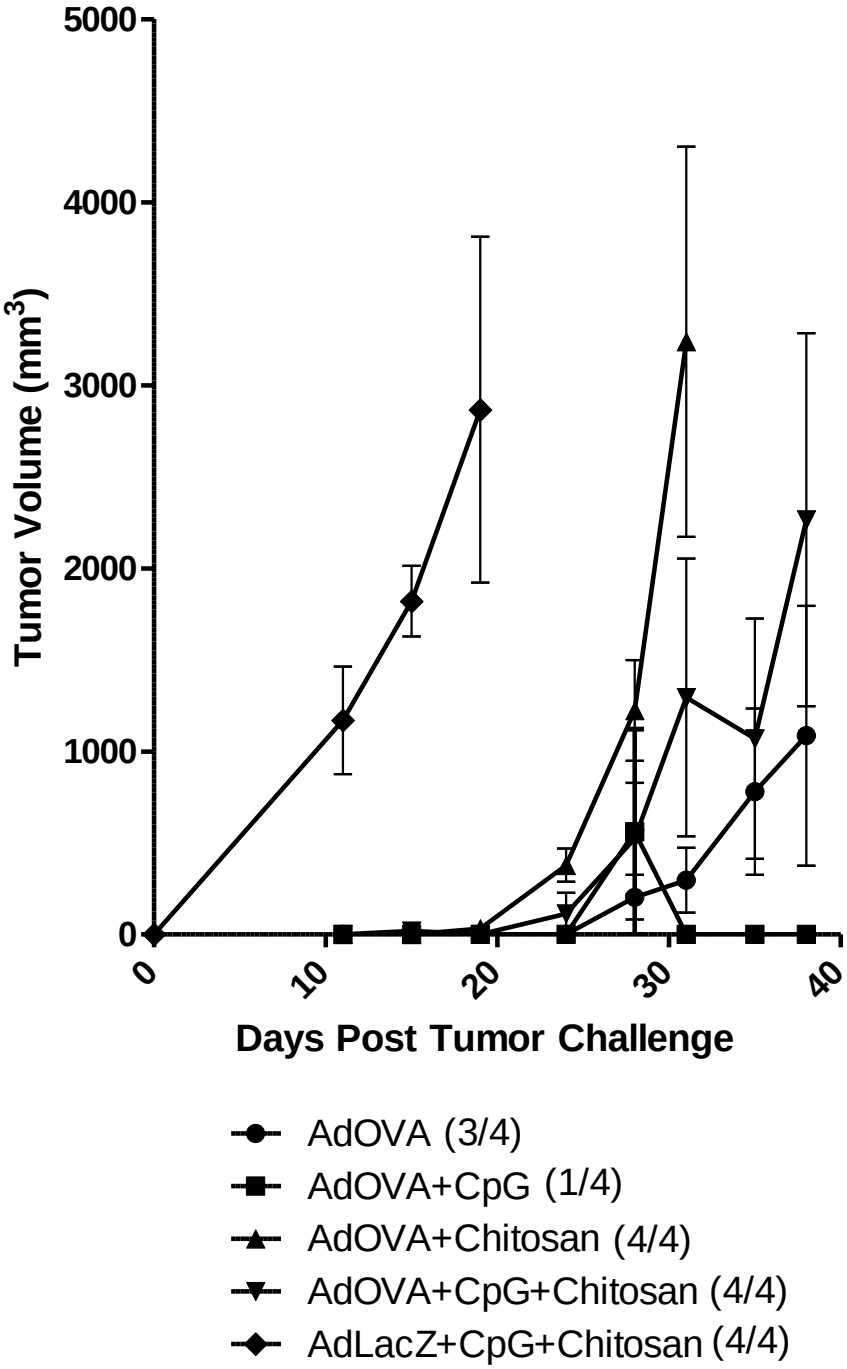

5000
4000
3000
2000
1000
0
Tumor Volume (mm3)
0
10
20
30
40
Days Post Tumor Challenge
AdOVA (3/4)
AdOVA+CpG (1/4)
AdOVA+Chitosan (4/4)
AdOVA+CpG+Chitosan (4/4)
AdLacZ+CpG+Chitosan (4/4)

Figure 22 continued

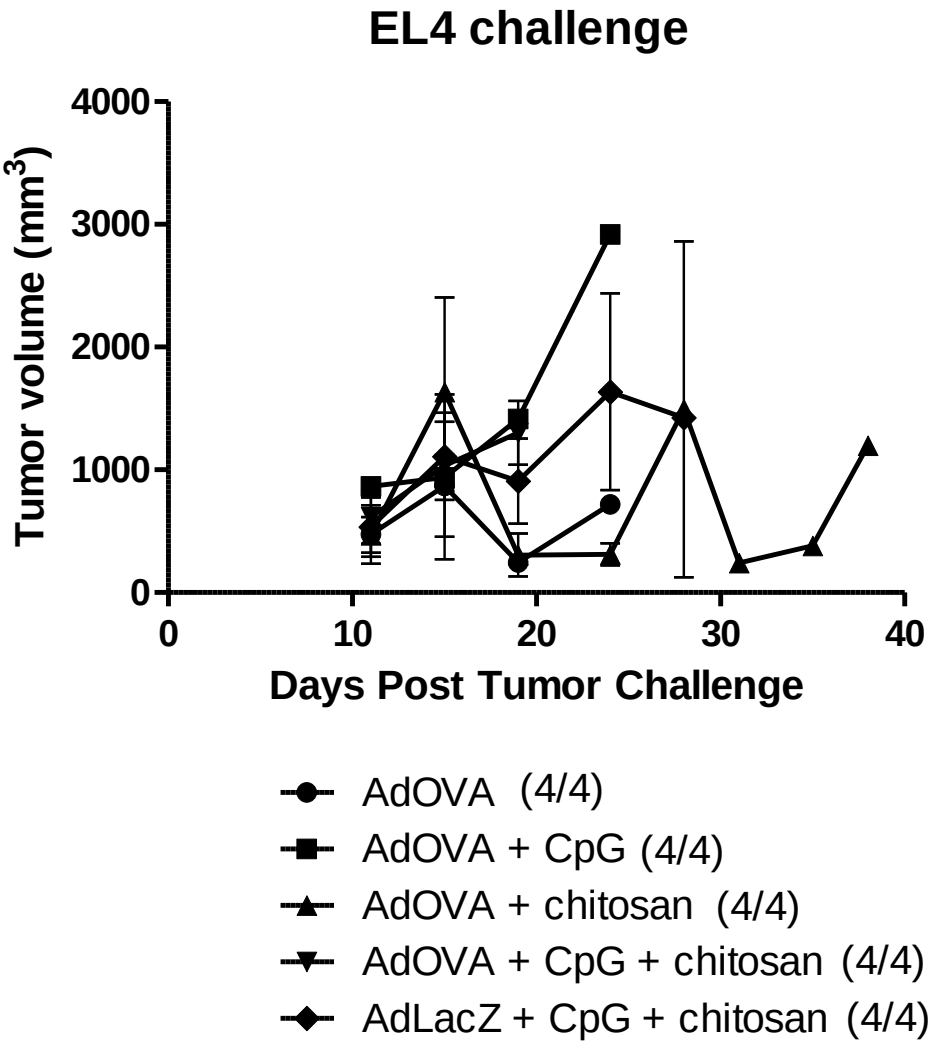
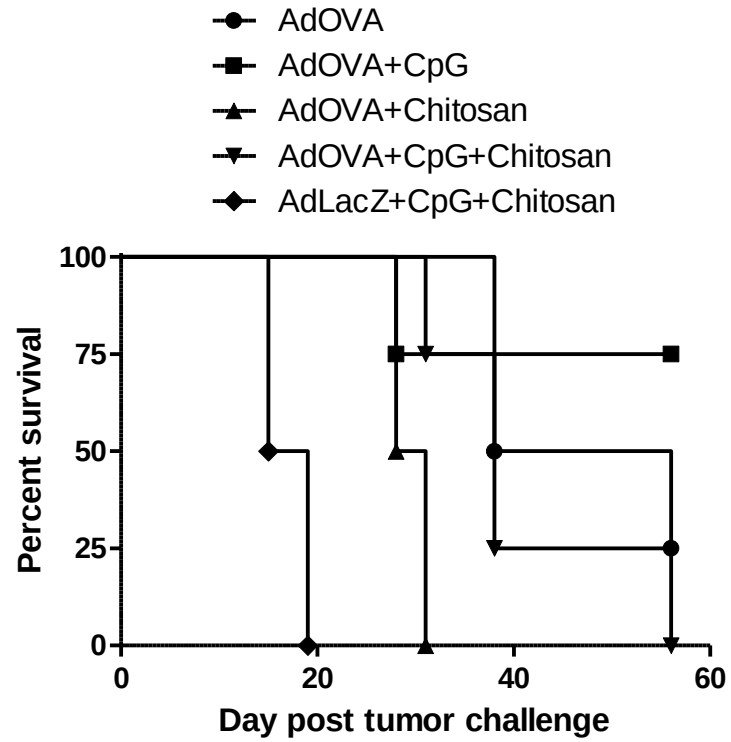
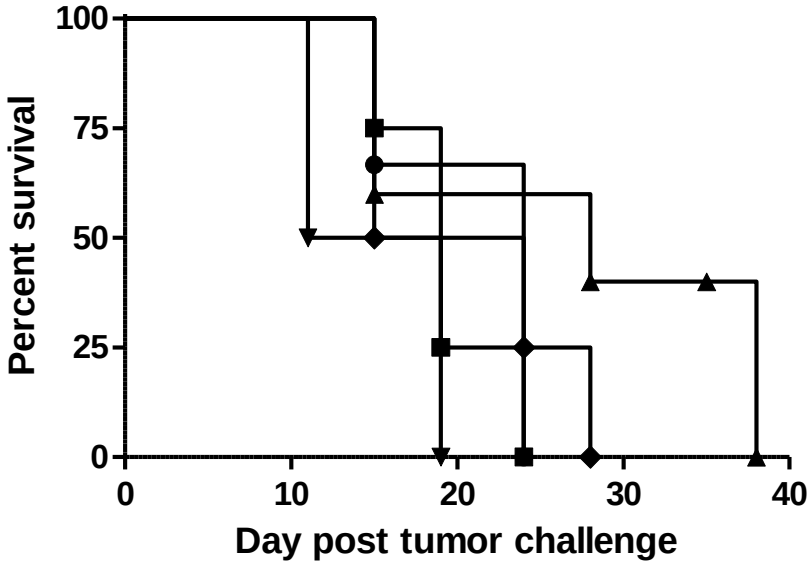


Figure 23: Survival Data: EG.7-OVA and EL4 tumor challenge. Survival data from mice in Figure 22. Mice were monitored twice weekly and sacrificed for ethical research if the tumor measurement exceeded 25 mm in any direction. *A*, EG.7-OVA survival. *B*, EL4 survival The remaining AdOVA + CpG EG.7-OVA mice were tumor free at the completion of the experiment(day 56).


AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chitosan
AdLacZ+CpG+Chitosan
Percent survival
100
75
50
25
0
0
20
40
60
Day post tumor challenge


Percent survival
100
75
50
25
0
0
10
20
30
40
Day post tumor challenge

CHAPTER 5: THERAPEUTIC NANOPARTICLE VACCINATIONS USING ADPSA IMMUNIZATIONS

Introduction

Based on the previous results using the AdOVA model with adjuvants CpG and chitosan, we have shown that the use of CpG enhances tumor protection *in vivo*, possibly by changing the kinetics of the antigen-specific immune response. But the exact mechanism for chitosan's modulation of the immune response is not yet understood. To further elucidate the effects of chitosan on the immune response, we wanted to continue to move from our model antigen system, OVA, into the therapeutic model, using PSA. This allows us to repeat many of the same experiments in a different mouse and tumor cell model, to determine if the results would be consistent in different systems. Additionally, we wanted to more closely examine the kinetics of tumor protection and CTL activity, and determine the mechanism by which chitosan may modulate the immune response. The PSA system is currently in use in clinical trials by the Lubaroff Lab[24,31], and has demonstrated safety with no major side effects in the Phase I study. Additionally, early results of the Phase II study indicate generation of anti-PSA T-cell responses in a large percentage of the vaccinated patients and stabilization or declines in serum PSA levels.

Keeping in mind the overall research objectives, our goal is to develop a therapeutic nanoparticle vaccine that enhances tumor protection in a mouse model of prostate cancer. In order to best optimize this vaccine and exploit the most positive attributes of each component, our goal is to further elucidate the mechanism of both chitosan and CpG to modulate the immune response. Previously, studies have focused on the time point 14 days after immunization, as that is when the maximal response occurs and when *in vivo* challenge is often performed. With this set of experiments, we have

chosen to analyze the CTL response throughout tumor challenge, to see if the kinetics of the immune response changes beyond the usual time point.

Additionally, given that chitosan appears to depress the immune response both *in vitro* and *in vivo*, we wanted to further examine the mechanisms behind this change. Because chitosan has been advantageous in other delivery vehicles[39,42,46,47,64], but no studies have looked at a subcutaneous, adenoviral-complex activating CD8 pathways, it is important to understand the limitations of chitosan usage in immunization strategies. Discovering how chitosan may modulate the immune response, and if interaction with the adenovirus causes changes in its processing or activation, can help us better understand how to develop an effective nanoparticle immunization. Two considerations that will be addressed are whether chitosan might interfere with PSA production or virus infectivity. If this is the case, then PSA will not be secreted to launch an antigen-specific immune response.

To test antigen production, we measured PSA secretion after AdPSA infection in a permissive cell line for all immunization formulas. To examine virus infectivity, the adenoviral vector which encodes GFP was used to infect cells and measure fluorescence in the cell population. We also wanted to test whether the way that chitosan was incorporated into the nanoparticle vaccine would change the activation capabilities, and resulting CTL response. To test this, chitosan was administered 24 hours before or after an injection of AdPSA + CpG, and results compared to AdPSA+CpG and AdPSA+CpG+chitosan, where the components are administered together in one immunization. Additionally, to test whether the order of nanoparticle formulation changed the ability to induce an immune response, we experimented with the order in which AdPSA, CpG, and chitosan were mixed prior to immunization and measurement of the CTL response.

Materials and Methods

Materials

Medium molecular weight chitosan (~20kDa) was purchased from Sigma-Aldrich (St. Louis, MO). A 0.01M chitosan solution in 1% acetic acid was prepared as previously described, for the stock chitosan solution. The Ad-PSA vaccine was obtained from the University of Iowa Gene Vector Core, as was designed previously in the Lubaroff Group.

Nanoparticle vaccine preparation

Nanoparticles were formulated at various ratios of chitosan's nitrogen to DNA phosphate (N/P ratio), with the amount of chitosan solution used based on a 50 μg/mouse DNA dose. 10^8 pfu viral vaccine was used. The solutions were vortexed for 20s and the left to incubate at room temperature for 30 min. The vaccines were injected subcutaneously in the right flank.

Tumor cell lines and animals

Mouse prostate cancer cells (RM11) were previously transfected with the full-length human PSA gene to produce two subclones. Clone E5 produces high levels of PSA and was used to measure the CTL activity, while clone E6 produces lower levels of PSA and was used in tumor challenge studies. The lymphoma cell line A20 was previously transfected with the human PSA gene to yield A4 cells, which were used in the cytotoxic assay as stimulator cells. A20 cells were used as control.

All cell lines were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% sodium pyruvate, 1% HEPES, and 0.05 mg/ml gentamycin in a humidified 5% CO_2- containing atmosphere. 6 to 8 week Balb/c mice were purchased from the National Cancer Institute (Bethesda, MD) and were maintained in filtered cages before use.

Intracellular Cytokine Staining

Splenocytes were processed as previously described, and plated at 10^6/well in a 96 well plate. Golgistop (BD Biosciences) was added to each well to inhibit IFN-γ secretion, wells were treated with peptide stimulation with PSA peptide or with PSA expressing A4 cells, and the plate incubated for 5 hrs at 37 ^oC. After blocking and staining with anti-CD8 FITC and anti-CD3 PE-Cy5 as described above, the cells were stained with anti-IFN-γ PE, and flow cytometry performed.

In vitro cytotoxic assay

A ^{51}Cr release assay was used to measure PSA-specific lysis of target cells. In a 24 well plate, 1 x 10^7 cells/well were seeded for each immunization group, along with the cytokine IL-2(10 U/ml) and mitomycin C treated E5 (2 x 10^5 cells/well) as stimulators. Following a 5 day co-culture at 37 ^oC, the effector splenocytes were harvested and separated from dead cells using a Ficol separation. Target EG.7-OVA cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 hr, washed twice, and resuspended at 5 x 10^4/ml. The effector cells were diluted serially down a 96 well round bottom plate, and 100 μl targets added to each well, giving effector: target (E:T) ratios from 100:1 to 3.125:1. After a 4 hr incubation at 37 oC, the plate was centrifuged at 1000 rpm for 10 minutes, and 100 μl of the supernatant was taken from each well and counted in the COBRA II gamma counter (Packard Instrument Company, IL). The antigen specific lysis was calculated using the formula:

(sample lysis-spontaneous lysis)/(maximum lysis-spontaneous lysis) *100

CTL kinetics study

6-8 week old male Balb/c mice were immunized as previously described, fourteen days prior to tumor challenge on day 0. On day 0, half the mice in each immunization group were injected with E6 cells resuspended in PBS at a concentration of 5 x 10^4 cells

in 100 μL. The remaining mice in each group remained unchallenged. On day 0, and on days 7, 14, 21, and 28, mice from each immunization group were sacrificed and spleens harvested to measure PSA-specific CTL lysis.

Chitosan timing studies

To test how the timing and co-delivery of chitosan changed the CTL response, mice were immunized with 3 different formulations:

1) AdPSA + CpG, followed by chitosan 24 hours later
2) Chitosan, followed by AdPSA + CpG 24 hours later
3) AdPSA + CpG + chitosan, in nanoparticle formulation

Two mice per group were immunized, subcutaneously in the right flank, and any subsequent injection 24 hours later was injected in the same location. Spleens were pooled and processed for CTL analysis as previously described. The CTL assay was performed on day 21, so results could be compared to previous studies where AdPSA + CpG gave high lysis at this time point.

Chitosan mixing formulations

To determine if the way chitosan is incorporated into the nanoparticle formulation effects the induction of immune response, the AdPSA/CpG/chitosan were mixed in different ways. The three components were incorporated as:

1) (AdPSA + CpG) mixed, followed by addition of chitosan 30 minutes later
2) (AdPSA + chitosan) mixed, followed by addition of CpG 30 minutes later
3) (chitosan + CpG) mixed, followed by addition of AdPSA 30 minutes later

In all cases, the first two components were mixed and vortexed for 20 seconds, and allowed to incubate together at room temperature before the third component was

added. Mice were then immunized with the formulations and the CTL response measured on day 21.

AdGFP infectivity study

To test whether there are any changes in infectivity with the various nanoparticle formulations, the AdGFP virus was used. One million cells were seeded in 6-well plates 6 hours prior to infectivity, and cells were infected at 36, 24, and 12 hours prior to analysis by flow cytometry. In all formulations, 10^8 AdGFP was used, corresponding to a moi of 100.

PSA secretion in HEK-293 cells

To test the ability of our nanoparticle formulations to induce PSA production, complexes were tested *in vitro* with the permissive HEK-293 cell line. The AdPSA virus is able to infect, but is lacking the E1 gene necessary for replication. Permissive cell lines, such as HEK-293, contain the E1 gene and therefore are permissive for the replication of AdPSA. One million cells in complete DMEM were seeded in a 6-well plate and infected with the immunization groups containing virus 10^8 pfu AdPSA, at a moi of 100:1. The plates were incubated at 37 ^oC, and after 36 hours, supernatants were collected and analyzed by immunoassay.

Results

CpG and chitosan have differential effects on IFN-γ production

Using the PSA model and PSA-expressing cells, we wanted to measure the changes in IFN-γ secretion after immunization with our nanoparticle vaccines. Traditionally, A4 cells which express PSA have been used in the 5 hr stimulation step. Additionally, we chose to test stimulation by PSA peptide, to see how peptide stimulation compares to A4 in this short time period. If stimulation by PSA peptide is possible, our

lab would like to collaborate with the NIH on a PSA-tetramer that would aid in further characterization in the PSA model. Figure 24 shows that our results mirror previous studies in the AdOVA model, where AdOVA + CpG shows increased IFN-γ frequencies, while all formulations with chitosan show decreased values, similar to control levels. Additionally, we found the frequencies to be very similar in both the PSA peptide and A4 stimulations, indicating that the PSA peptide is capable of stimulating cells *in vitro*.

CpG shifts kinetics of CTL response

Previously, we have measured the CTL response only 14 days post-immunization. To examine the kinetics of the immune response both with and without tumor challenge, 6-8 week old male Balb/c mice were immunized as previously described, fourteen days prior to tumor challenge on day 0. On day 0, half the mice in each immunization group were injected with E6 cells resuspended in PBS at a concentration of 5×10^4 cells in 100 μL. The remaining mice in each group remained unchallenged. On day 0, and on days 7, 14, 21, and 28, mice from each immunization group were sacrificed and spleens harvested to measure PSA-specific CTL lysis. Figure 25 shows that on day 0, only AdPSA shows high lysis, as was previously reported.[56] For the rest of the study, the unchallenged mice show no PSA-specific lysis, as was expected when no challenge occurred post-immunization. In the challenged mice, on day 7 AdPSA continued to be the only immunization group with high lysis. But by day 14, the AdPSA + CpG group now had high lysis as well. This trend continued in day 21, as AdPSA lysis started to decline. By day 28, all immunization group lysis had returned to background levels. These results shed light on why AdPSA + CpG provides *in vivo* tumor protection, though day 0 studies show a depression in CTL lysing ability. The immunizations with AdPSA + CpG see a shift in the kinetics of the immune response, with the highest responses on

days 14 and 21, 4 to 5 weeks post-immunizations. This delayed response overlaps with the periods of tumor protection in our tumor challenge studies. For the AdOVA + chitosan and AdOVA + CpG + chitosan groups, lysis remains low throughout the course of the study.

Chitosan timing studies

Following the CTL kinetics, we had a better understanding of how CpG can modulate and enhance the immune response, but were still lacking in an explanation of how chitosan may be acting. The CTL response at day 21 showed high lysis with AdPSA + CpG, but not with AdPSA+ chitosan or AdPSA + CpG + chitosan. (Figure 26) We wanted to test whether simply the presence of chitosan in the system during immune response was leading to the decrease, or if it is the complexation/delivery process that causes the change. To do this, mice were immunized either with AdPSA + CpG + chitosan, AdPSA + CpG, followed by chitosan 24 hours later, or with chitosan, followed by AdPSA + CpG 24 hours later. Our results show that when chitosan is injected either 24 hours before AdPSA + CpG is administered, a high CTL response occurs. AdPSA + CpG + chitosan together again show no specific lysis. These results demonstrate that chitosan administered separately from the virus does not decrease the overall immune response, but that some mechanism of the complexation or presentation process must be responsible for the changes.

Chitosan mixing formulations

To test whether the way in which chitosan is incorporated into the nanoparticle changes the immune response, mice were immunized with 3 different formulations of the AdPSA/CpG/chitosan components. These results were also compared to AdPSA alone and AdLacZ alone. The CTL results post-immunization show that of the 3 mixing formulations, (AdPSA + CpG) + chitosan yields the highest lysis. (Figure 27) This

response is not as strong as AdPSA alone. While the differences between the groups are not significant, the same trends were seen as the experiment was performed three times.

PSA secretion study

To test the ability of our nanoparticle formulations to induce PSA production, complexes were tested *in vitro* with the permissive HEK-293 cell line. The same formulations were tested as in the previous chitosan mixing formulations study, with the addition of AdPSA + CpG as well. After 36 hours the supernatants were measured by immunoassay, with no significant differences in PSA production between any of the groups. (Figure 28) AdPSA alone had similar PSA secretion levels to all groups containing chitosan, so no correlations could be made between chitosan in the immunization groups and lack of PSA production as causation for a depression of the immune response.

AdGFP infectivity study

To test whether there are any changes in infectivity with the various nanoparticle formulations, the AdGFP virus was used. One million cells were seeded in 6-well plates 6 hours prior to infectivity, and cells were infected at 36, 24, and 12 hours prior to analysis by flow cytometry. In all formulations, 10^8 AdGFP was used, corresponding to a moi of 100. Cell suspensions were analyzed by flow cytometry, where the level of florescence corresponds to total infectivity. There were no significant differences in infectivity levels between AdGFP, AdGFP + CpG, and AdGFP + chitosan, at each of the three timepoints. (Figure 29)

Frequency of Treg cells following immunization

Given that the immune response appears to be suppressed in formulations containing chitosan, we wanted to examine the levels of T regulatory(Treg) cells present after immunization. Tregs are responsible for maintaining immune homeostasis, giving a

balance between tolerance to self, and allowing a response when a foreign infection occurs.[19] When the desired balance fails, problems can occur. In the case that there too high of a response, the body's immune system can turn on itself, such as in autoimmune disorders. If a threat is not recognized as "foreign" enough, Treg cells might suppress immune responses that should occur to fight infection and disease.[19,69,70] One of chitosan's positive attributes is the fact that it is very biocompatible, but we are uncertain of any changes in Treg cell levels after immunization. Measuring Treg levels post-immunization will allow us to determine if these regulatory cells play any role in chitosan's modulation of the immune response.

To measure the levels of Tregs in immunized mice, the CD4+Foxp3+ cell populations were analyzed for each group. High expression of Foxp3 has been shown in CD4+CD25+ T regulatory cells, so it is a relevant marker for this cell population. Balb/c mice were immunized with AdPSA, AdPSA + CpG, AdPSA + chitosan, AdPSA + CpG + chitosan, AdLacZ, and AdLacZ + chitosan, and a naïve group was included. The same immunization groups were used in the C57Bl/6 model, with AdOVA as adenovirus. Results show that there is no significant difference in either model of Treg frequencies in immunized mice, compared to background levels present in naïve mice. (Figure 30)

Discussion

Moving from the model antigen OVA into the therapeutic antigen PSA, this set of experiments focused on further identification into the roles CpG and chitosan play in modulation of the immune response. In order to understand how to optimize our nanoparticle vaccine formulations, and how this research might translate into other cancer models, further study was necessary to elucidate the mechanisms of induction of the immune response. As was previously seen in the AdOVA model, the AdPSA formulations involving chitosan had a decrease in the level of CD8+IFN-γ+ T cell frequencies. Tetramer technology is not yet available in the PSA system, so frequencies

of CD8+PSA+ T cells could not be determined. However, the successful use of PSA peptide for stimulation *in vitro* with the ICS assay shows that development of a PSA tetramer may be possible, and our lab plans to pursue further studies with the NIH Tetramer Facility towards the development of a PSA-specific tetramer.

In previous studies, we had seen tumor protection from the AdOVA + CpG immunization group *in vivo*, but no corresponding CD8+ activation *in vitro*. To try and understand what changes might be occurring during tumor challenge, we chose to perform a CTL kinetics study from the day of challenge, day 0, through day 28. This would assess the lysing ability of mouse splenocytes as far out as six weeks past immunization. Our results show that while AdPSA shows high lysis from days 0-14 post challenge, before dropping off, AdPSA + CpG does not show CTL upregulation until days 14 and 21. This shift in the kinetics of the immune response gives insight as to why the AdPSA + CpG immunized mice show the best tumor protection, as CTL upregulation is shifted back from the tumor challenge. This delayed response might be useful in booster or multiple immunizations, where AdPSA initial induces a response and the AdPSA + CpG vaccine's delayed kinetics causes an extension of this response.

Throughout the kinetics study, all immunization groups containing chitosan remained at a low level of antigen-specific CTL lysis. We wanted to further probe into the mechanism of CTL's effects on the immune response. One aspect warranting further investigation was if chitosan was in some way interfering with the adenoviral infectivity. Without successful infection, there could be no PSA production, which could result in the depression of immune cell indicators we find *in vitro* and the lack of tumor protection *in vivo.* The infectivity experiments showed no significant difference in fluorescence levels between AdGFP, AdGFP + CpG, and AdGFP + chitosan at any of the three time points observed. The complexation of chitosan with the adenovirus did not cause a decrease in the level of infectivity.

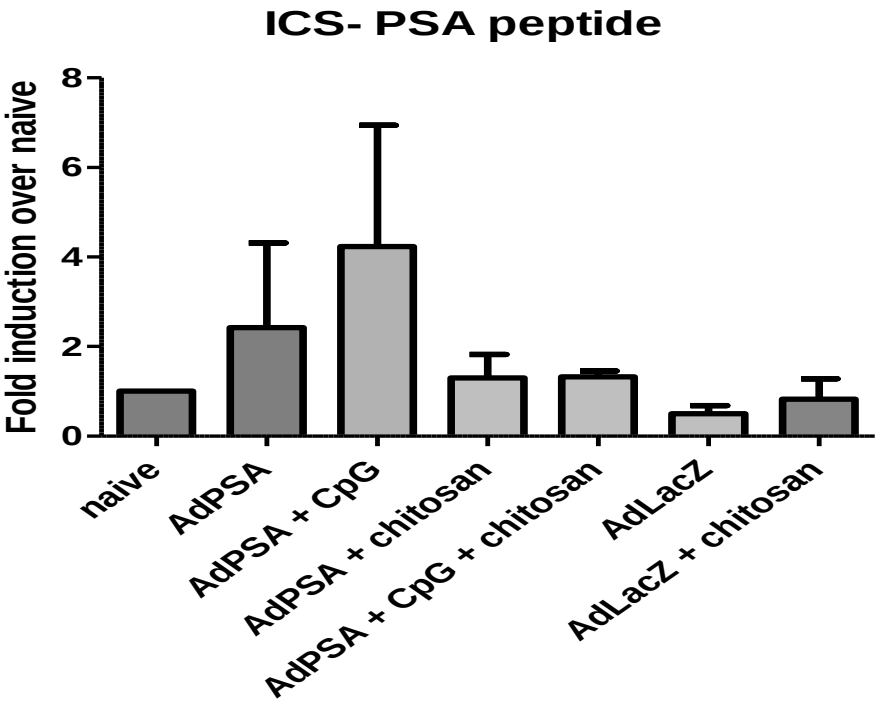
Next, we wanted to examine the virus's ability to induce PSA production in the cells. Even if infectivity occurs, if production was hampered, there would be no PSA increase to upregulate PSA-specific T cells, and we would not expect to see a robust immune response without this important step. To test this, the immunization groups were allowed to incubate with the permissive cell line HEK-293, which would allow for PSA production. After 36 hours, the supernatants were analyzed by immunoassay, and there were no significant differences found in PSA secretion between the groups. From the results of this experiment, we concluded that chitosan does not appear to be hampering the actual production of PSA after immunization.

After performing these additional studies to better understand the mechanism of chitosan's effects on the immune response, we have eliminated the most common causes of immune suppression as possible causes for suppressing AdPSA vaccine efficacy. These effects were not expected, given the previous success of chitosan-DNA vaccinations, and previous studies with the adenoviral vaccines. However, our system is novel, as it incorporates adenovirus with chitosan in a subcutaneous vaccine. It is possible that chitosan might be a helpful delivery vehicle in mucosal[46] or oral vaccines[47], for example, but not with adenovirus in subcutaneous administration. As was previously discussed, while CD8 and CD4 T cells mediate MHC class I and II immune responses, they are other subpopulations of T cells that can also mediate the immune response.

T regulatory cells (Tregs) are essential for preventing autoimmunity, by suppressing over-active immune responses against self, but they could pose a challenge to launching robust protective immune response. For example, previous studies have found that *in vivo* elimination of Tregs can increase the level of vaccine-mediated, tumor-specific T cell responses in humans.[69] Many tumor antigens can be recognized as self-antigens, which can lead to suppression of the immune response. We wondered if some intrinsic property of chitosan might be upregulating Treg activity, in this particular vaccination scheme. To investigate this, splenocytes were analyzed 14 days post-

immunization to enumerate Treg frequencies. In naïve mice, approximately 2% of total cells in the C57Bl/6 strain and approximately 3-5% in the BALB/c mouse strain are Treg cells.[69] Our results show that there is no significant difference between any of the immunization groups for Treg levels. All of the frequencies fall within the expected range for the AdOVA model in C57Bl/6 mice, and the PSA model in BALB/c mice. Given these results, the mechanism of chitosan's modulation of the immune response is still not fully understood. This may be one reason for the lack of previous research with chitosan and adenoviruses delivered subcutaneously cited in the literature. Many of the immunological tools and assays used in this research have only been recently developed, so time may lead to additional tools that can better examine other aspects of the immune results that we have not been able to explore at this time. Mechanisms of Treg activation and regulation are largely unknown at this time as well, so further research and examination of their modulation of the immune response, when exposed to chitosan, could lead to interesting results as well. Following the kinetics study, we were able to see a shift in AdPSA + CpG CTL activation that corresponds to increased tumor protection *in vivo*. This knowledge on how to best use CpG as adjuvant, and the way it can augment the immune response, can be very useful in future vaccine design. CpG can be used to enhance the antigen-specific response in this way and its incorporation into vaccine formulations has proven to provide increased tumor protection, through an enhanced immune response.

Figure 24: CD8+ IFN-γ+ T cell frequency. Splenocytes were processed as previously described, and plated at 106/well in a 96 well plate. Golgistop was added to each well to inhibit IFN-γ secretion, wells were treated with peptide stimulation with SIINFEKL or media as control, and the plate incubated for 5 hrs at 37 ^{o}C. After blocking and staining with anti-CD8 FITC and anti-CD3 PE-Cy5 as previously described, the cells were stained with anti-IFNγ PE, and analyzed by flow cytometry. The top panel shows the IFN-γ fold induction over naïve after stimulation by PSA peptide, and the bottom panel demonstrates stimulation by A4 cells.


ICS- PSA peptide
Fold induction over naive
0
2
4
6
8
naive
AdPSA
AdPSA + CpG
AdPSA + chitosan
AdPSA + CpG + chitosan
AdLacZ
AdLacZ + chitosan

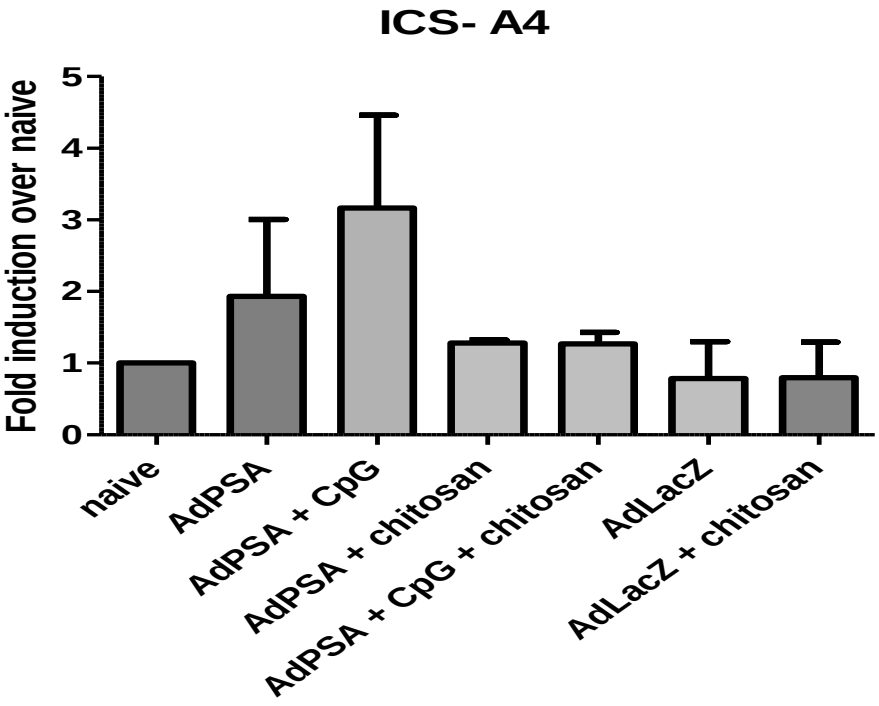

ICS- A4
Fold induction over naive
0
1
2
3
4
5
naive
AdPSA
AdPSA + CpG
AdPSA + chitosan
AdPSA + CpG + chitosan
AdLacZ
AdLacZ + chitosan

Figure 25: CTL activity of AdPSA/chitosan/CpG formulations. A ^{51}Cr release assay was used to measure PSA-specific lysis of target cells. 2 mice were pooled for each experiment. 14 days after immunization, corresponding to day 0 in our kinetics study, half the mice in each immunization group were challenged with E5 cells, and the rest of the mice remained unchallenged. The CTL assay was performed on days 0, 7, 14, 21, and 28.

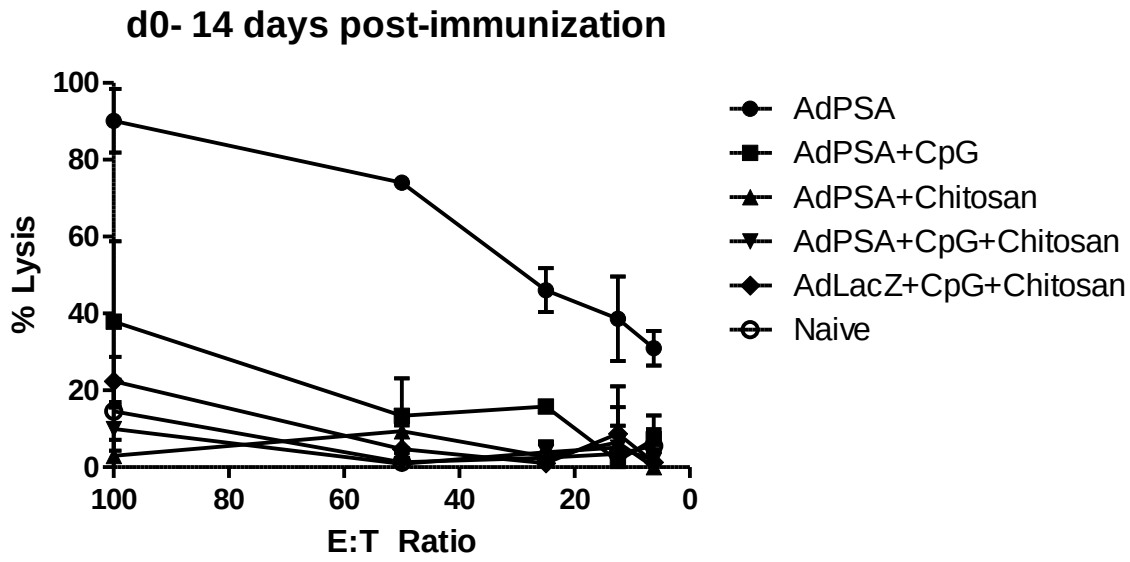

d0- 14 days post-immunization
% Lysis
100
80
60
40
20
0
100
80
60
40
20
0
E:T Ratio
AdPSA
AdPSA+CpG
AdPSA+Chitosan
AdPSA+CpG+Chitosan
AdLacZ+CpG+Chitosan
Naive

Figure 25 continued

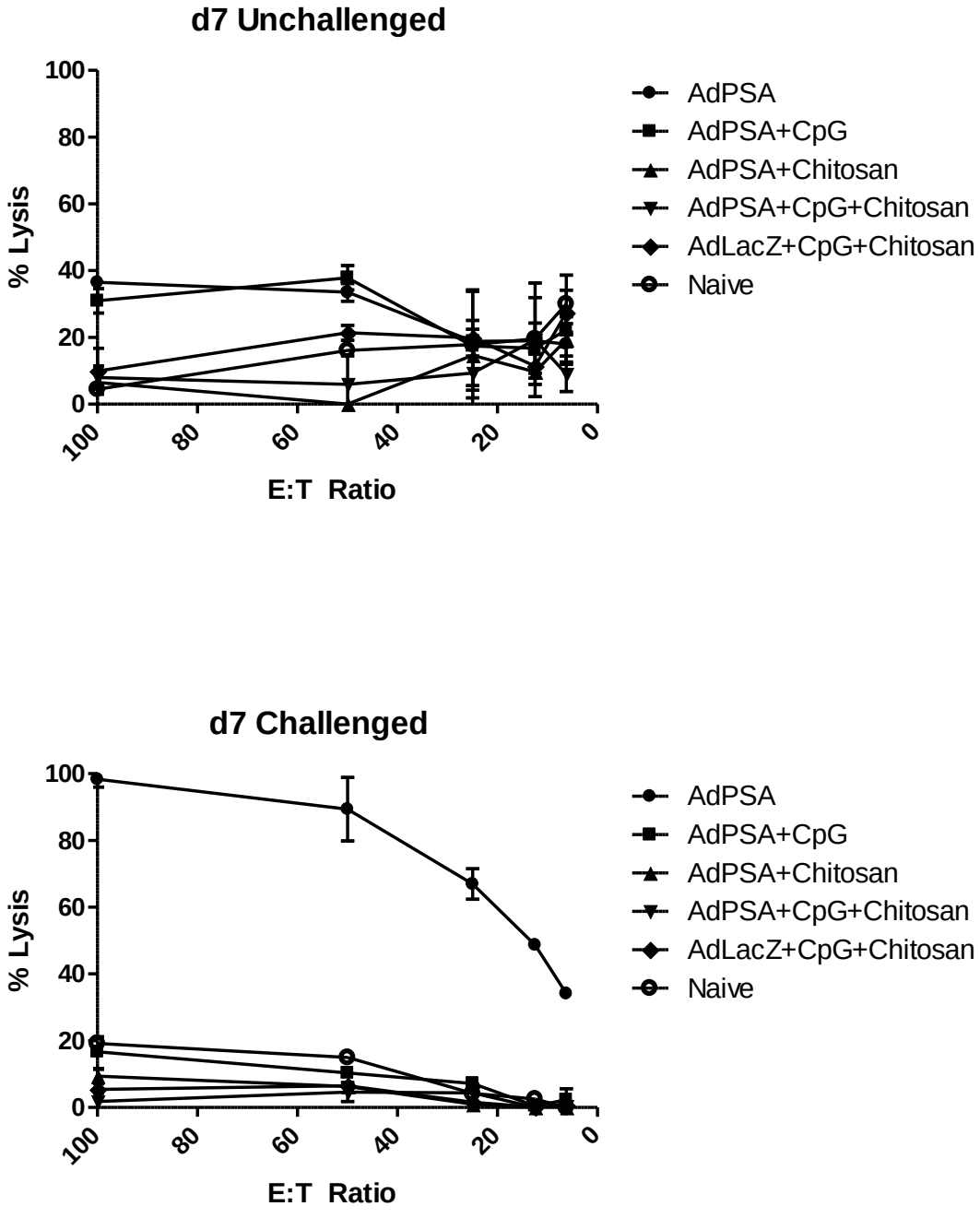
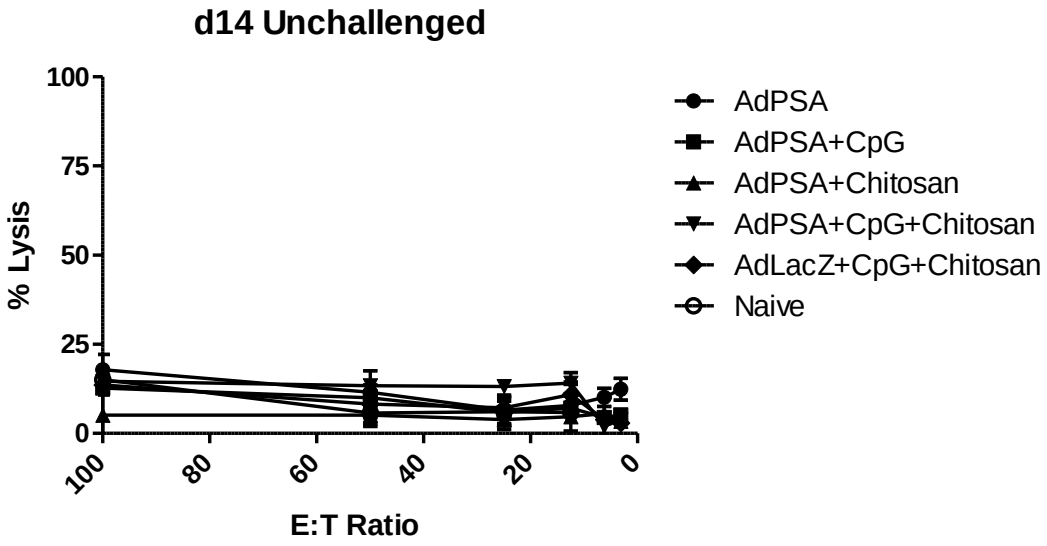


Figure 25 continued



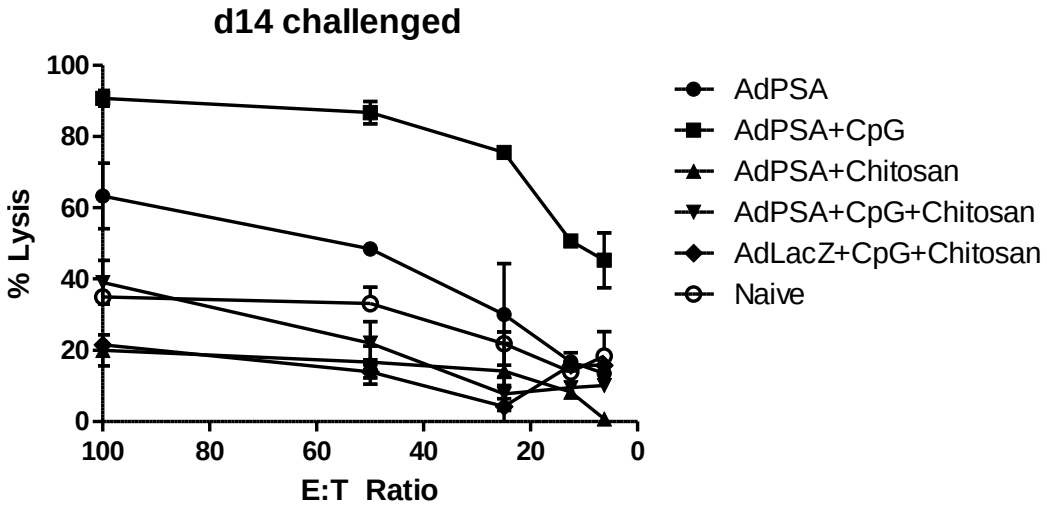
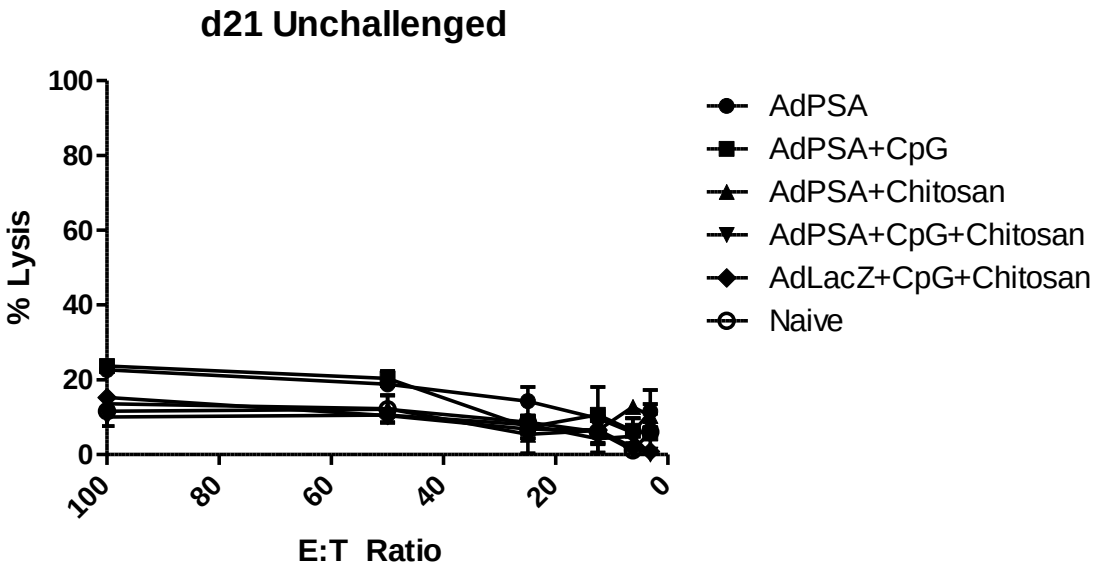


Figure 25 continued



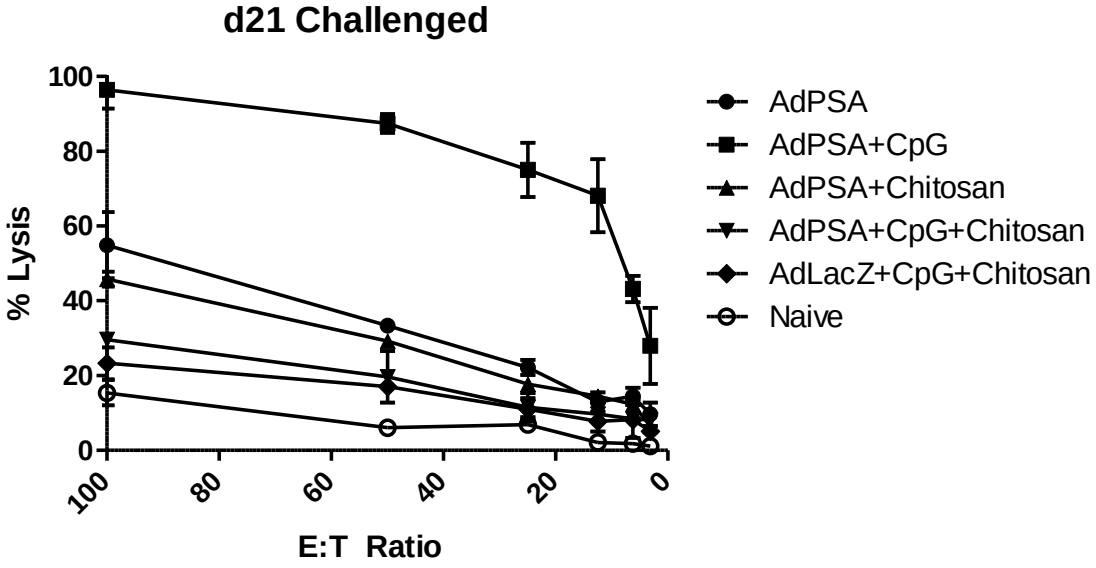


Figure 25 continued

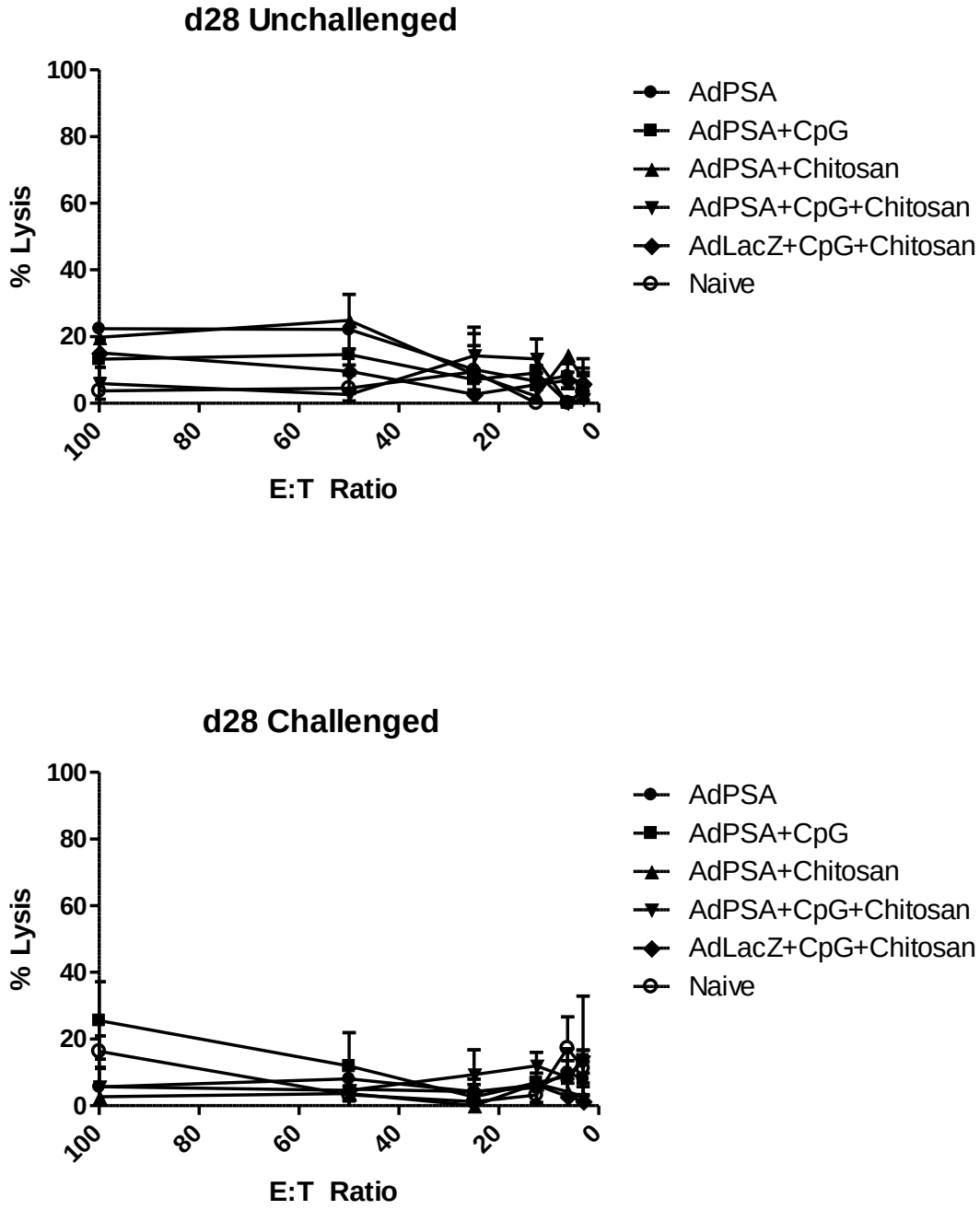


Figure 26: Chitosan timing studies. To test how the timing and co-delivery of chitosan changed the CTL response, mice were immunized with 3 different formulations: AdPSA + CpG, followed by chitosan 24 hours later, chitosan, followed by AdPSA + CpG 24 hours later, or AdPSA + CpG + chitosan, in nanoparticle formulation. Two mice per group were immunized, subcutaneously in the right flank, and any subsequent injection 24 hours later was injected in the same location. Spleens were pooled and processed for CTL analysis as previously described. The CTL assay was performed on day 21, so results could be compared to previous studies where AdPSA + CpG gave high lysis at this time point.

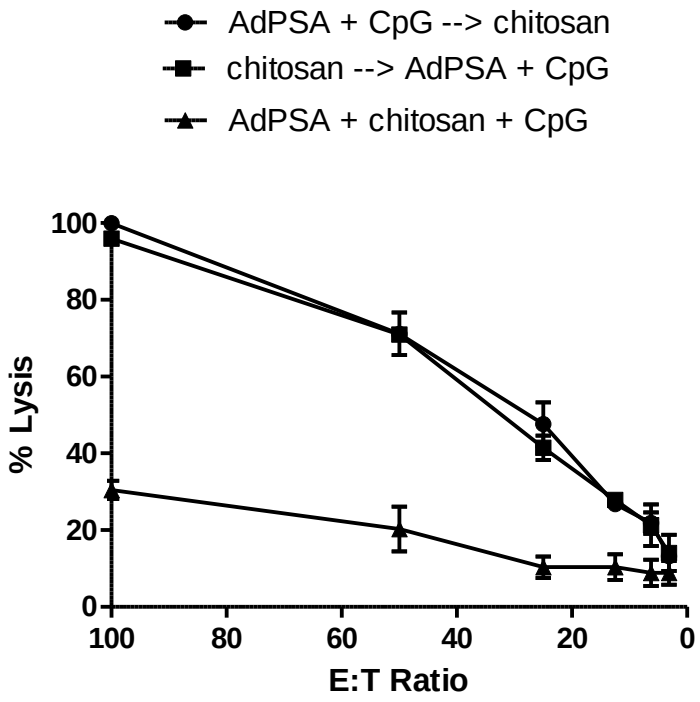

AdPSA + CpG --> chitosan
chitosan --> AdPSA + CpG
AdPSA + chitosan + CpG
% Lysis
100
80
60
40
20
0
100
80
60
40
20
0
E:T Ratio

Figure 27: Chitosan mixing formulations. To determine if the way chitosan is incorporated into the nanoparticle formulation effects the induction of immune response, the AdPSA/CpG/chitosan were mixed in different ways. The three components were incorporated as: (AdPSA + CpG) mixed, followed by addition of chitosan 30 minutes later, (AdPSA + chitosan) mixed, followed by addition of CpG 30 minutes later, or (chitosan + CpG) mixed, followed by addition of AdPSA 30 minutes later. In all cases, the first two components were mixed and vortexed for 20 seconds, and allowed to incubate together at room temperature before the third component was added. Mice were then immunized with the formulations and the CTL response measured on day 21.

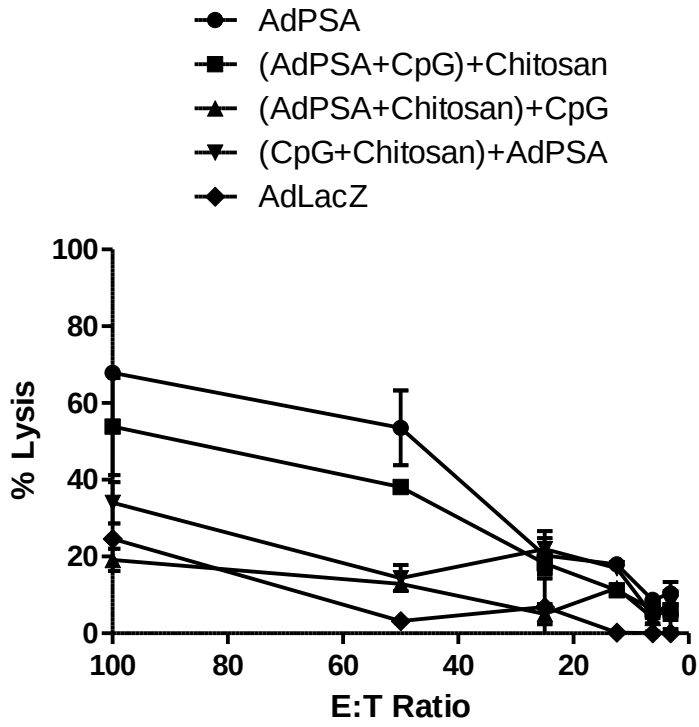

AdPSA
(AdPSA+CpG)+Chitosan
(AdPSA+Chitosan)+CpG
(CpG+Chitosan)+AdPSA
AdLacZ
% Lysis
100
80
60
40
20
0
100
80
60
40
20
0
E:T Ratio

Figure 28: AdGFP infectivity. To test whether there are any changes in infectivity with the various nanoparticle formulations, the AdGFP virus was used. One million cells were seeded in 6-well plates 6 hours prior to infectivity, and cells were infected at 36, 24, and 12 hours prior to analysis by flow cytometry. In all formulations, 10^8 AdGFP was used, corresponding to a moi of 100.

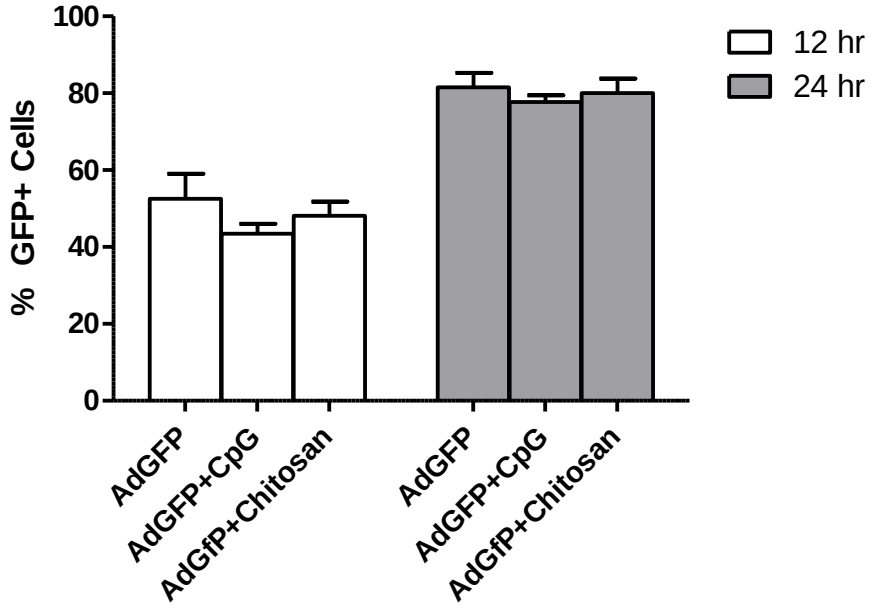

100
80
60
40
20
0
% GFP+ Cells
12 hr
24 hr
AdGFP
AdGFP+CpG
AdGfP+Chitosan
AdGFP
AdGFP+CpG
AdGfP+Chitosan

Figure 29: PSA secretion study. To test the ability of our nanoparticle formulations to induce PSA production, complexes were tested *in vitro* with the permissive HEK-293 cell line. One million cells in complete DMEM were seeded in a 6-well plate and infected with the immunization groups containing virus 10^8 pfu AdPSA, at a moi of 100:1. The plates were incubated at 37 ^{o}C, and after 36 hours, supernatants were collected and analyzed by immunoassay.

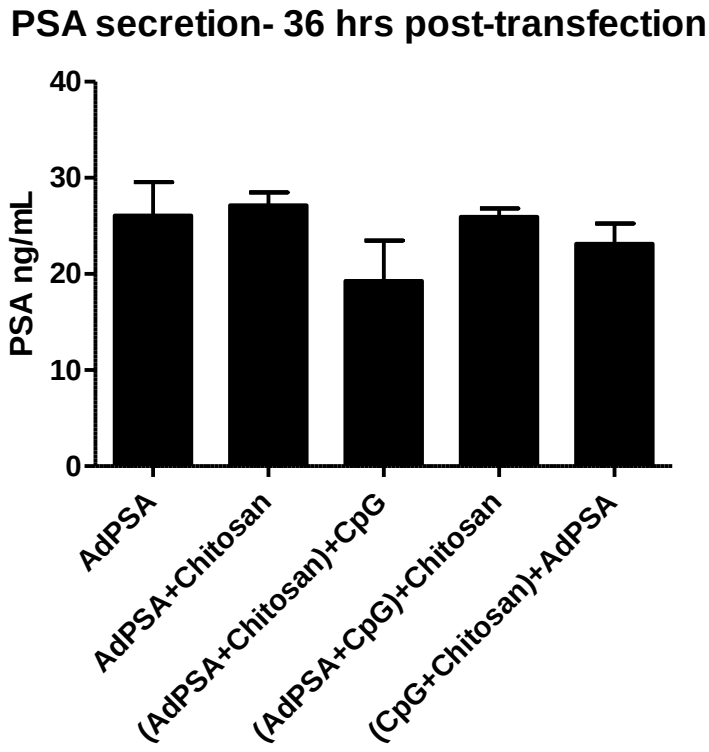
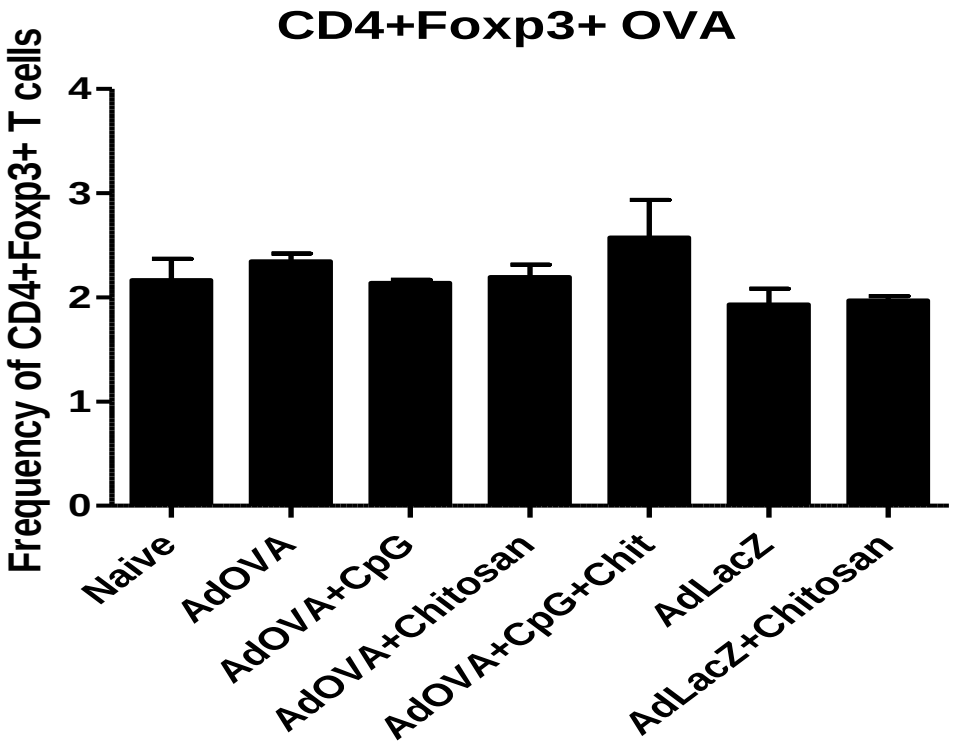
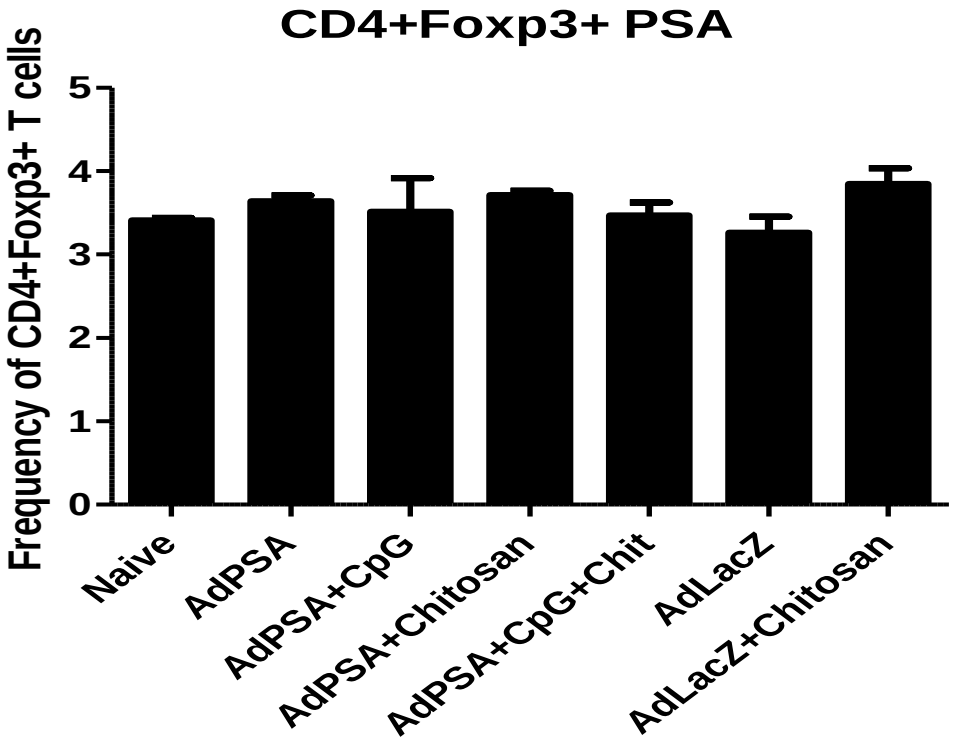

PSA secretion- 36 hrs post-transfection
PSA ng/mL
40
30
20
10
0
AdPSA
AdPSA+Chitosan
(AdPSA+Chitosan)+CpG
(AdPSA+CpG)+Chitosan
(CpG+Chitosan)+AdPSA

Figure 30: Analysis of T regulatory cell levels, post-immunization. Mice were immunized 14 days prior to experiment and spleens harvested and processed as for other flow cytometry experiments. AdOVA immunizations were used for the C57Bl/6 model, and ADPSA in the BALB/c model. The cells were stained with CD4+ FITC and CD3+ PE-Cy5, and blocked with F_c block. After washing and fixing, the cells were then stained with Foxp3+ PE, and analyzed by flow cytometry.


CD4+Foxp3+ OVA
Frequency of CD4+Foxp3+ T cells
0
1
2
3
4
Naive
AdOVA
AdOVA+CpG
AdOVA+Chitosan
AdOVA+CpG+Chit
AdLacZ
AdLacZ+Chitosan


CD4+Foxp3+ PSA
Frequency of CD4+Foxp3+ T cells
0
1
2
3
4
5
Naive
AdPSA
AdPSA+CpG
AdPSA+Chitosan
AdPSA+CpG+Chit
AdLacZ
AdLacZ+Chitosan

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The goal of this research has been to improve upon an existing adenovirus-based vaccine for the treatment of prostate cancer, using adjuvants such as cationic polymers and CpG to enhance delivery and uptake, while boosting the strength of the immune response. The Ad5-PSA vaccine demonstrates that an adenoviral vector can be used to deliver the PSA gene to launch an anti-PSA response, as was shown first *in vivo*, and currently in clinical trials.[23,24,31] This delivery scheme utilizes the body's own immune system to target only cells secreting PSA, sparing the healthy tissue. With this as the basis of our vaccine delivery system, our goal is to further augment the strength of the immune response, and the duration of protection. To do this, we aimed to combine the advantages of the viral vector with the advantages of a class of non-viral vectors, the cationic polymers. Cationic polymers can condense negatively charged virus into nanoparticles through electrostatic interaction, requiring no chemical bonds and leaving the viral structure unharmed.[28,34,36,42] With these methods, we hoped to capitalize on the advantages of delivery in particulate form that is possible with non-viral vectors, while retaining the viral machinery for PSA production that makes the adenoviral vector a successful gene delivery tool. Combining CpG into the nanoparticle as an adjuvant can allow all parts of the vaccine to be delivered to the same cell, boosting the strength of the overall immune response.[50]

In chapter 3, we examined the use of PEI as the non-viral delivery vehicle in our nanoparticle system. Optimization of transfection efficiency showed that a N/P ratio of 10 gave significant increases in expression compared to lower N/P ratios, while there were no significant advantages to using higher N/P ratios than 10. Given toxicity issues with PEI *in vitro* and *in vivo*, we chose to proceed with this ratio to minimize total polymer use without sacrificing efficacy. Interestingly, we found that while AdOVA + PEI depressed the CD8+ OVA+ T cell and CTL responses, the formulation provided the

best tumor protection in *in vivo* challenge studies. Antibody depletion studies showed that tumor growth was mainly mediated by CD8 cells, with some contribution from NK cells as well. PEI has been cited in the literature to have inherent immunostimulatory effects, though the exact mechanism for this is not yet understood.[62] We hypothesized that this activity, whether due to structure or PEI's mechanism of release from the endosome, could contribute to an overall immune response, which though unspecific to antigen, aids in overall suppression.

PEI's inherent immunostimulatory may actually aid in a non-specific immune response due to cell death that is triggered in the presence of danger signals. In this case, dendritic cells can be primed against specific antigens to activate CTLs.[18,20,71,72,73,74]. Additionally, toxicity effects due to compexation by PEI may cause some cells that take up the complexes to become necrotic or apoptotic. As DCs scavenge these dead or dying cells, that may be able to present the encoded antigen, such as OVA or PSA, to launch a response that is now antigen-specific against tumor cells expressing these proteins. Future studies in this area might include an examination of general cellularity after immunization, to enumerate the DCs after immunization with the adenovirus, with or without PEI. Flow cytometry studies to determine if there is an upregulation of activation receptors would also show whether the DCs had become activated following immunization.

After demonstrating that adenoviruses can be complexed with the cationic polymer PEI to be delivered in nanoparticle form, we wanted to further test and consider more clinical applications of the vaccine formulations. Next, we developed formulations using chitosan, as it has already gained FDA approval and has been used in clinical applications. [46,47,68] Using a similar polymeric carrier that had shown less toxicity, we hoped to show a safer carrier and immunoadjuvant for our vaccination scheme. In our studies, we did not see an enhancement of CD8 activity with chitosan as a delivery adjuvant in a subcutaneous vaccine. This is of interest, as chitosan may not be an

appropriate adjuvant for responses requiring activation through the CD8 pathway, when combined with subcutaneous delivery, as is necessary for clinical relevance. To further elucidate the effects of chitosan on the immune response, and to determine the mechanism by which CpG is providing tumor protection, further experiments were conducted with the therapeutic model, using PSA.

A CTL kinetics study was conducted to determine how the response might change during the course of a tumor challenge. Our results show that while AdPSA shows high lysis from days 0-14 post challenge, before dropping off, AdPSA + CpG does not show CTL upregulation until days 14 and 21. This shift in the kinetics of the immune response gives insight as to why the AdPSA + CpG immunized mice show the best tumor protection, and this delayed response might be useful in booster or multiple immunizations. AdPSA can induce an initial response, while the AdPSA + CpG vaccine's delayed kinetics causes an extension of this response. When chitosan was delivered separately from the adenovirus and CpG, the CTL lysis response was equivalent to the AdPSA + CpG immunization. To better understand the mechanism of chitosan's effects on the immune response, we tested the formulations' ability to infect cells, secrete PSA, and modulate the Treg population. These experiments showed no significant differences between groups containing chitosan, and formulations without the polymer, so we are still unsure of the means by which chitosan depresses the *in vitro* and *in vivo* effects of the adenovirus. These effects were not expected, given the previous success of chitosan-DNA vaccinations, and previous studies with the adenoviral vaccines. Our novel system incorporates adenovirus with chitosan in a subcutaneous vaccine, with adenovirus primarily activating CD8 cells. The combination of this activation pathway and administration route may not be compatible for enhanced activation in this particular cancer model. However, our experiments have lead to a better understanding on how CpG can be used as an adjuvant, and the way it can augment the immune response. Additionally, though chitosan did not prove efficacious with our adenovirus and

administration route, further investigation into different treatment systems, and different pathways, might be better evaluated following the results of these experiments.Additional studies have reported that the use of CpG enhances the strength of an adenovirus-based response, whether through a multi-layer effect, or possibly by changing the kinetics of the immune response. [53,54,55,75,76] Our results have allowed us to better understand the changes in kinetics of the immune response using CpG as an adjuvant, and this will be very useful in future vaccine design. As CpG also activates dendritic and B cells, further studies to measure upregulation and activation of these cells types could give a better understanding of how various types of immune cells respond to immunization using CpG. A similar kinetics study to that which we performed on the CTLs would give data on how the levels of these additional immune cells fluctuate during the course of immunization and challenge.

This might give a clearer picture of the mechanism to chitosan's modulation of the immune response. Our investigation into issues of viral infectivity or PSA secretion proved that chitosan did not inhibit the viral machinery in either of these cases. This eliminates the most common causes of immune suppression that would reduce the AdPSA + chitosan vaccine efficacy. Again, an examination of dendritic cells might show how their role in antigen presentation might account for the decreased immune response.[15,16,77] In our studies for infectivity and secretion, HEK-293 cells were cultured *in vitro*, and were the only cells present in the assay. A study of dendritic cell activation might demonstrate whether the cells are capable of being infected and upregulating receptors necessary for antigen presentation and the activation of an immune response. Following this experiment, the dendritic cells which had been exposed to the target antigen could then be incubated with CTLs or other immune cells to examine whether antigen presentation and activation successfully occurs.

Further study into the kinetics of the Treg population would be recommended to examine whether these regulatory cells are upregulated at a later time point, similar to the

changes in kinetics seen with CpG. Examing these levels with and without tumor challenge would also show whether Tregs are upregulated at different time points, and if a challenge changes the routing of these cells. Additionally, depletion of Tregs in an *in vivo* tumor model would allow the kinetics of the response to be measured throughout the course of the study.

In developing a vaccine with enhanced tumor protection, a combination of AdPSA, followed by AdPSA + CpG as booster, could be used to maintain tumor protection and high CTL lysis for longer time periods than AdPSA alone. Additionally, the idea of a sustained release vector might be useful for this vaccination scheme. Previously, work with the Gelfoam matrix showed that lower viral doses could be used to obtain the same level of immune response.[25] The use of pluronics has been examined for future experiments in this vaccination system to enhance and strengthen the immune response. Pluronics are polyoxyethylene(A) and polyoxypropylene(B) triblock copolymers (ABA) that are currently FDA approved for use in sustained-release gel depots. This polymer is unique in that it undergoes reverse thermal gelation, becoming a liquid at 4^{o}C, and a gel at 37 ^{o}C.[46,78,79] This could allow a vaccine formulation to be mixed in an aqueous solution, and then injected where it would form a gel depot at body temperature. Using pluronics as a delivery vehicle, increased production of PSA could lead to a greater number of APCs taking up antigen, presenting it to T cells, and thereby increasing the overall immune response. Future work might examine whether a combination of an intial AdPSA immunization, coupled with an AdPSA + CpG or AdPSA + pluronics formulation, could be used to obtain both a strong initial response, and maintain protection for longer time periods.

While chitosan is a negative modulator of the immune response in our immunization scheme, several recent studies have consider the use of both chitosan and PEI in the same vector, to overcome high toxicity while still maintaining adequate levels of transfection efficiency and cellular activation[80,81,82,83] Specifically, these studies have

examine the importance of the N/P ratio on reducing polymer level to reduce toxicity, as well as whether PEI's endosomolytic activity is retained by further conjugation to chitosan or other vaccine components. Given the complex interactions of the cells of the immune system and the cascade of events necessary to launch an effect immune response, continued research into effect immunization strategies will aid in the optimization of a delivery system which provides effective levels or protection while minimizing unwanted immune reactions.

REFERENCES

1 PCF. *Prostate Cancer Foundation*, <www.prostatecancerfoundation.org> (2007).

2 Lubaroff, D. M. *et al.* Decreased cytotoxic T cell activity generated by co-administration of PSA vaccine and CpG ODN is associated with increased tumor protection in a mouse model of prostate cancer. *Vaccine* **24**, 6155-6162 (2006).

3 Institute, N. C. *Cancer Advances in Focus: Prostate Cancer* <www.cancer.gov/cancertopics/cancer-advances-in-focus/prostate> (2009).

4 Schlom, J., Gulley, J. L. & Arlen, P. M. Paradigm Shifts in Cancer Vaccine Therapy. *Experimental Biology and Medicine* **233**, 522-534 (2008).

5 Rhodes, D. R., Barrette, T. R., Rubin, M. A., Ghosh, D. & Chinnaiyan, A. M. Meta-Analysis of Microarrays: Interstudy Validation of Gene Expression Profiles Reveals Pathway Dysregulation in Prostate Cancer. *Cancer Res* **62**, 4427-4433 (2002).

6 Arlen, P. M., Mohebtash, M., Madan, R. A. & Gulley, J. L. Promising novel immunotherapies and combinations for prostate cancer. *Future Oncology* **5**, 187-196 (2009).

7 P Gallo, S. D., B Cipriani and P Monaci. Adenovirus as vehicle for anticancer genetic immunotherapy. *Gene Therapy* **12**, S84-S91 (2005).

8 Shariat, S. F. *et al.* Adenovirus-mediated Transfer of Inducible Caspases: A Novel "Death Switch" Gene Therapeutic Approach to Prostate Cancer. *Cancer Res* **61**, 2562-2571 (2001).

9 Bramson, J. L. & Wan, Y.-H. The efficacy of genetic vaccination is dependent upon the nature of the vector system and antigen. *Expert Opinion on Biological Therapy* **2**, 75-85 (2002).

10 Garen, Z. H. a. A. Intratumoral injection of adenoviral vectors encoding tumor-targeted immunoconjugates for cancer immunotherapy. *PNAS* **97**, 9221-9225 (2000).

11 Murphy, G., Tjoa, B., Ragde, H., Kenny, G. & Boynton, A. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. *The Prostate* **29**, 371-380 (1996).

12 Small, E. J. *et al.* Immunotherapy of Hormone-Refractory Prostate Cancer With Antigen-Loaded Dendritic Cells. *J Clin Oncol* **18**, 3894-3903 (2000).

13 Hrouda, Baban, Dunsmuir, Kirby & Dalgleish. Immunotherapy of advanced prostate cancer: a phase I/II trial using Mycobacterium vaccae (SRL172). *British Journal of Urology* **82**, 568-573 (1998).

14 Guinan, P. D., John, T., Baumgartner, G., Sundar, B. & Ablin, R. J. Adjuvant immunotherapy (BCG) in Stage D prostate cancer. *American Journal of Clinical Oncology* **5**, 65&hyhen;68 (1982).

[15] Xia, D. J., Moyana, T. & Xiang, J. Combinational adenovirus-mediated gene therapy and dendritic cell vaccine in combating well-established tumors. *Cell Research* **16**, 241-259 (2006).

[16] Xiang, J., Chen, Y. & Moyana, T. Combinational immunotherapy for established tumors with engineered tumor vaccines and adenovirus-mediated gene transfer. *Cancer Gene Therapy* **7**, 1023-1033 (2000).

[17] Elzey, B. D., Siemens, D. R., Ratliff, T. L. & Lubaroff, D. M. Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors. *International Journal of Cancer* **94**, 842-849 (2001).

[18] Kowalczyk, D. W. *et al.* Vaccine-induced CD8(+) T cells eliminate tumors by a two-staged attack. *Cancer Gene Therapy* **10**, 870-878 (2003).

[19] Janeway, C. *Immunobiology*. 5 edn, (Garland Science, 2005).

[20] Brossart, P., Goldrath, A. W., Butz, E. A., Martin, S. & Bevan, M. J. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. *Journal of Immunology* **158**, 3270-3276 (1997).

[21] Kuby, J. *Immunology*. 3rd edn, (W.H. Freeman & Company, 1997).

[22] 2007. *Current Protocols in Immunology*.

[23] D. M. Lubaroff, D. A. V., R. D. Williams, F. N. Joudi, M. C. Smith, P. S. Zehr, J. Eastman, K. Griffith, T. M. Madsen, K. Johnson. A phase II trial of an adenovirus/PSA vaccine for prostate cancer. *J Clin Oncol* **27**, suppl; abstr 3065 (2009).

[24] Lubaroff, D. M. *et al.* Adenovirus PSA (Ad5-PSA) gene therapy for prostate cancer: A phase I clinical trial. *Journal of Urology* **169**, 395-395 (2003).

[25] Siemens, D. R. *et al.* Cutting Edge: Restoration of the Ability to Generate CTL in Mice Immune to Adenovirus by Delivery of Virus in a Collagen-Based Matrix. *J Immunol* **166**, 731-735 (2001).

[26] Neu, M., Fischer, D. & Kissel, T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J Gene Med* **7**, 992 (2005).

[27] Stefaan C. De Smedt , J. D. a. W. E. H. Cationic Polymer Based Gene Delivery Systems **17**, 113-126 (2000).

[28] Lungwitz, U., Breunig, M., Blunk, T. & Gopferich, A. Polyethylenimine-based non-viral gene delivery systems. *European Journal of Pharmaceutics and Biopharmaceutics* **60**, 247 (2005).

[29] Essand, M. Gene therapy and immunotherapy of prostate cancer: Adenoviral-based strategies. *Acta Oncologica* **44**, 610 - 627 (2005).

[30] Wong, S. Y., Pelet, J. M. & Putnam, D. Polymer systems for gene delivery-past, present, and future. *Progress in Polymer Science* **32**, 799-837 (2007).

[31] Lubaroff, D. M. *et al.* Clinical protocol: Phase I study of an adenovirus/prostate-specific antigen vaccine in men with metastatic prostate cancer. *Human Gene Therapy* **17**, 220-229 (2006).

[32] Godbey, W. T., Wu, K. K. & Mikos, A. G. Poly(ethylenimine) and its role in gene delivery. *Journal of Controlled Release* **60**, 149 (1999).

[33] Salem, A. K., Searson, P. C. & Leong, K. W. Multifunctional nanorods for gene delivery. *Nature Materials* **2**, 668-671 (2003).

[34] Kircheis, R. *et al.* Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Therapy* **4**, 409 (1997).

[35] Godbey, W. T., Barry, M. A. & Saggau, P. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J Biomed Mater Res* **51**, 321 (2000).

[36] Wightman, L. *et al.* Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med* **3**, 362 (2001).

[37] Diebold, S. S. *et al.* Efficient Gene Delivery into Human Dendritic Cells by Adenovirus Polyethylenimine and Mannose Polyethylenimine Transfection. *Human Gene Therapy* **10**, 775 (1999).

[38] K.W. Leong, H.-Q. M., V.L. Truong-Leb, K. Roya, S.M. Walsha, J.T. August. DNA-polycation nanospheres as non-viral gene delivery vehicles. *Journal of Controlled Release* **53**, 183-193 (1998).

[39] Borchard, G. Chitosans for gene delivery. *Advanced Drug Delivery Reviews* **52**, 145-150 (2001).

[40] Heo, S. H. *et al.* Characterization and preparation of low molecular weight water soluble chitosan nanoparticle modified with cell targeting ligand for efficient gene delivery. *Polymer-Korea* **31**, 454-459 (2007).

[41] Kawamata, Y. *et al.* Receptor-independent augmentation of adenovirus-mediated gene transfer with chitosan in vitro. *Biomaterials* **23**, 4573-4579 (2002).

[42] M Koping-Hoggard, I. T., H Guan, K Edwards, M Nilsson, KM Varum & Artursson, A. P. Chitosan as a nonviral gene delivery system. Structure–property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Therapy* **8**, 1108-1121 (2001).

[43] Singla, A. K. & Chawla, M. Chitosan: some pharmaceutical and biological aspects-an update. *Journal of Pharmacy and Pharmacology* **53**, 1047-1067 (2001).

[44] Mhurchu, C. N., Dunshea-Mooij, C., Bennett, D. & Rodgers, A. Effect of chitosan on weight loss in overweight and obese individuals: a systematic review of randomized controlled trials. *Obesity Reviews* **6**, 35-42 (2005).

[45] Ghendon, Y. *et al.* Evaluation of Properties of Chitosan as an Adjuvant for Inactivated Influenza Vaccines Administered Parenterally. *Journal of Medical Virology* **81**, 494-506 (2009).

[46] Julie Westerink, M. A. *et al.* ProJuvant(TM) (Pluronic F127®/chitosan) enhances the immune response to intranasally administered tetanus toxoid. *Vaccine* **20**, 711-723 (2001).

[47] Roy, K., Mao, H.-Q., Huang, S. K. & Leong, K. W. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med* **5**, 387-391 (1999).

[48] Zaharoff, D. A., Rogers, C. J., Hance, K. W., Schlom, J. & Greiner, J. W. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. *Vaccine* **25**, 2085-2094 (2007).

[49] Burch, P. A. *et al.* Immunotherapy (APC8015, Provenge®) targeting prostatic acid phosphatase can induce durable remission of metastatic androgen-independent prostate cancer: A phase 2 trial. *The Prostate* **60**, 197-204 (2004).

[50] Zhang, X.-Q. *et al.* Potent Antigen-specific Immune Responses Stimulated by Codelivery of CpG ODN and Antigens in Degradable Microparticles. *Journal of Immunotherapy* **30** (2007).

[51] Ghendon, Y., Markushin, S., Krivtsov, G. & Akopova, I. Chitosan as an adjuvant for parenterally administered inactivated influenza vaccines. *Archives of Virology* **153**, 831-837 (2008).

[52] Zaharoff, D. A., Rogers, C. J., Hance, K. W., Schlom, J. & Greiner, J. W. Chitosan solution enhances the immunoadjuvant properties of GM-CSF. *Vaccine* **25**, 8673-8686 (2007).

[53] Kipps, R. R. a. T. J. CpG Oligodeoxynucleotides Enhance the Capacity of Adenovirus-mediated CD154 Gene Transfer to Generate Effective B-Cell Lymphoma Vaccines. *[CANCER RESEARCH* **63**, 4128-4135 (2003).

[54] Baines, J. & Celis, E. Immune-mediated Tumor Regression Induced by CpG-containing Oligodeoxynucleotides. *Clinical Cancer Research* **9**, 2693-2700 (2003).

[55] Ballas, Z. K. *et al.* Divergent Therapeutic and Immunologic Effects of Oligodeoxynucleotides with Distinct CpG Motifs. *J Immunol* **167**, 4878-4886 (2001).

[56] Karan, D., Krieg, A. M. & Lubaroff, D. M. Paradoxical enhancement of CD8 T cell-dependent anti-tumor protection despite reduced CD8 T cell responses with addition of a TLR9 agonist to a tumor vaccine. *International Journal of Cancer* **121**, 1520-1528 (2007).

[57] *University of Iowa Office of Animal Resources*, <http://research.uiowa.edu/animal/> (2009).

[58] Skinner, P. J., Daniels, M. A., Schmidt, C. S., Jameson, S. C. & Haase, A. T. Cutting Edge: In Situ Tetramer Staining of Antigen-Specific T Cells in Tissues. *J Immunol* **165**, 613-617 (2000).

[59] Karimi, K. *et al.* Enhanced Antitumor Immunity Elicited by Dendritic Cell Vaccines Is a Result of Their Ability to Engage Both CTL and IFN[gamma]-producing NK Cells. *Mol Ther* **16**, 411-418 (2007).

[60] Intra, J. & Salem, A. K. Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo. *Journal of Controlled Release* **130**, 129-138 (2008).

[61] Lubaroff, D. M. & Karan, D. CpG oligonucleotide as an adjuvant for the treatment of prostate cancer. *Advanced Drug Delivery Reviews* **61**, 268-274, (2009).

[62] Regnstrom, K. *et al.* PEI - a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and FasL-mediated cell death in mice. *Gene Ther* **10**, 1575-1583 (2003).

[63] Gallucci, S. & Matzinger, P. Danger signals: SOS to the immune system. *Current Opinion in Immunology* **13**, 114-119 (2001).

[64] Mao, H. Q. *et al.* Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *Journal of Controlled Release* **70**, 399 (2001).

[65] Pashine, A., Valiante, N. M. & Ulmer, J. B. Targeting the innate immune response with improved vaccine adjuvants. *Nat Med* (2005).

[66] O'Hagan, D. T. & Valiante, N. M. Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov* **2**, 727-735 (2003).

[67] Nishimura, K. *et al.* Immunological activity of chitin and its derivatives. *Vaccine* **2**, 93-99 (1984).

[68] Toshinori Sato, T. I., Yoshio Okahata. In vitro gene delivery mediated by chitosan. Effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. *Biomaterials* **22**, 2075-2080 (2001).

[69] Dannull, J. *et al.* Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *The Journal of Clinical Investigation* **115**, 3623-3633 (2005).

[70] Nizar, S. *et al.* T-regulatory cell modulation: the future of cancer immunotherapy. *Br J Cancer* **100**, 1697-1703 (2009).

[71] Blohm, U., Potthoff, D., van der Kogel, A. J. & Pircher, A. Solid tumors "melt" from the inside after successful CD8 T cell attack. *European Journal of Immunology* **36**, 468-477 (2006).

[72] Chen, Z. *et al.* Efficient antitumor immunity derived from maturation of dendritic cells that had phagocytosed apoptotic/necrotic tumor cells. *International Journal of Cancer* **93**, 539-548 (2001).

[73] Rovere, P. *et al.* Cutting edge: Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *Journal of Immunology* **161**, 4467-4471 (1998).

[74] Spiotto, M. T., Rowley, D. A. & Schreiber, H. Bystander elimination of antigen loss variants in established tumors. *Nature Medicine* **10**, 294-298 (2004).

[75] Perkins, S. D., Williams, A. J., O'Brien, L. M., Laws, T. R. & Phillpotts, R. J. CpG Used as an Adjuvant for an Adenovirus-Based Venezuelan Equine Encephalitis Virus Vaccine Increases the Immune Response to the Vector, But Not to the Transgene Product. *Viral Immunology* **21**, 451-457 (2008).

[76] Rieger, R. & Kipps, T. J. CpG oligodeoxynucleotides enhance the capacity of adenovirus-mediated CD154 gene transfer to generate effective B-cell lymphoma vaccines. *Cancer Research* **63**, 4128-4135 (2003).

[77] Sorensen, M. R., Holst, P. J., Pircher, H., Christensen, J. P. & Thomsen, A. R. Vaccination with an adenoviral vector encoding the tumor antigen directly linked to invariant chain induces potent CD4(+) T-cell-independent CD8(+) T-cell-mediated tumor control. *European Journal of Immunology* **39**, 2725-2736 (2009).

[78] Coeshott, C. M. *et al.* Pluronic® F127-based systemic vaccine delivery systems. *Vaccine* **22**, 2396-2405 (2004).

[79] Alonso, M. J., Gupta, R. K., Min, C., Siber, G. R. & Langer, R. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine* **12**, 299-306 (1994).

[80] Gao, J. Q. *et al.* Gene-carried chitosan-linked-PEI induced high gene transfection efficiency with low toxicity and significant tumor-suppressive activity. *International Journal of Pharmaceutics* **387**, 286-294 (2009).

[81] Kim, T. H., Kim, S. I., Akaike, T. & Cho, C. S. Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. *Journal of Controlled Release* **105**, 354-366 (2005).

[82] Zhao, Q. Q. *et al.* N/P Ratio Significantly Influences the Transfection Efficiency and Cytotoxicity of a Polyethylenimine/Chitosan/DNA Complex. *Biological & Pharmaceutical Bulletin* **32**, 706-710 (2009).

[83] Zhou, X. F. *et al.* Controlled release of PEI/DNA complexes from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine. *Journal of Controlled Release* **121**, 200-207 (2007).