Immunological Benefits of a Novel polycaprolactone-polyorthoester-based Therapeutic Vaccine in a Mouse Model of Glioma

A Thesis

SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Karen Lynn Grinnen

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

John R. Ohlfest, PhD (*deceased*)

Timothy Wiedmann, PhD

August 2014

ABSTRACT

Cancer immunotherapy has led to significant improvement in the survival of patients with previously untreatable malignancies. The use of therapeutic vaccines is a promising form of immunotherapy, but their efficacy remains ambiguous. Much of the difficulty in identifying the optimal formulation and delivery is related to the complicated nature of the immune response, where it is uncertain which aspects would be most effective in destroying cancer cells. In this thesis, a novel polymeric delivery system, involving poly (caprolactone)-co-poly (ortho ester) [PCL-POE], was used to deliver tumor antigens and adjuvants in a controlled manner. We hypothesized that persistent release of tumor antigens from the biodegradable polymer would result in an increase in the number and persistence of anti-tumor lymphocytes in the effector state. To test this hypothesis, vaccines were administered to mice and the time dependent immunological response was evaluated. The polymeric delivery system resulted in an *in vitro* release profile characterized by a burst release of both antigen and adjuvant followed, in both cases, by a much slower phase of release. We also observed that the slow release provided by the PCL-POE polymer stimulated prolonged maturation of dendritic cells, activation and persistence of anti-OVA antibodies and antigen-specific T cells following a single vaccination. The vaccine system was also tested in a mouse model of glioblastoma multiforme (GBM). We observed a significant, potentially translatable increase in overall survival.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Brain Tumors

Incidence, types, and prognosis

A glioma is the general term used for a primary brain tumor. It can arise from any of three types of glial cells of the central nervous system, which include astrocytes, oligodendrocytes, and ependymal cells. Of special interest are the astrocytes that make up the supportive tissue of the brain. Tumors stemming from astrocytes are called astrocytomas and are graded according to criteria established by the World Health Organization (WHO) criteria. As an initial division, tumors are graded as being either benign or malignant. For malignant tumors, grade I (low grade) astrocytomas are more localized, have a slower growth rate, and carry a better prognosis. They are also most common in children. Astrocytomas in grades II through IV are progressively more severe as the tumor is much more aggressive and invasive. The highest grade (IV) astrocytomas may be referred to as a Grade IV Glioblastoma or, most commonly, Glioblastoma Multiforme (GBM). GBM is the most common primary brain malignancy in adults, and carries an extremely poor prognosis. An estimated 18,000 people die each year in the United States from GBM.[1]

Current treatment of brain tumors

Historically, the standard of care for GBM was surgical resection of the tumor followed by brain irradiation. With this approach, the median survival time following diagnosis was less than 12 months. In 2005, Stupp, et al[2] published the results from a phase III clinical trial that added temozolomide, an alkylating chemotherapeutic agent, to the combination of surgery and radiation. With this combination regimen, the median survival of patients with GBM was extended to approximately 15-18 months post-diagnosis. As such, this approach became the new standard of care and is presently used for comparison of new treatments. Given the mortality rate,

considerable effort has been devoted to increasing survival times in patients; however, progress has remained elusive.

The dismal prognosis of gliomas with implementing standard of care treatment has driven the search for innovative methods for curing patients with GMB. One promising avenue is immunotherapy. In the following sections, the current state of immunotherapy of GBM is addressed. For background, the salient features of the immune system are presented including the humoral and cell mediated response. Particular attention is given to the impact of the presence of persistent antigens on the magnitude and kinetics of the immune response, the role of a controlled-release vaccine in providing persistent antigens, and the effect of the magnitude and kinetics of the immune response on the survival of the glioma mouse model.

Fundamentals of the immune system

Antibody (humoral) immune response

B Cells

Immunity and the immune response rely on many cells and specialized entities in a complex interactive manner. The adaptive immune response is of particular interest, and therefore the general discussion begins with the humoral immune response. This arm of the immune system is comprised of B cells and antibodies, which is distinct from the cell mediated arm, albeit these two arms are interactive.

All naïve B cells express on their surface a B cell receptor (BCR), which is a membrane bound antibody. Unlike T lymphocyte receptors, BCRs recognized and bind antigens that are often portions of a three dimensional (3D) structure of large macromolecules, including many proteins and polysaccharides. Like T cells, B cells are present in secondary lymphoid organs to bind the specific antigen and thereby form an antibody-antigen cognate. Naïve B cells circulate through the follicles of the lymph nodes and spleen where they encounter foreign proteins brought there by direct lymphatic drainage.[3]

A few naïve B cells will express a BCR that is specific for a single antigen. In the presence of a particular antigen (i.e. infection), naïve B cells with B cell receptors specific for the antigen bind the antigen. This, in turn, stimulates the intracellular portion of the BCR to transduce a signal that causes the B cell to migrate to the edge of the lymph node, that is, the T cell area.[4] This binding also results in the B cell internalizing the antigen by receptor mediated endocytosis. Within the endosome of the B cell, the foreign protein is degraded into peptides, followed by the loading of immunogenic peptide epitopes onto MHCII. This ultimately leads to expression of the peptide:MHCII complex on the surface of the B cell. In this way, B cells are acting as a type of antigen presenting cell. Still localized at the T cell border of the lymph node, the peptide:MHCII expressing B cell(s) will bind to CD4+ (helper) T cells that have T cell receptors (TCRs) specific for the same antigen (peptide:MHCII complex) expressed by the B cell. This interaction causes activation of the antigen-specific CD4 T cell, which will then secrete cytokines that stimulate the antigen-specific B cell to proliferate and differentiate into effector cells. The result is in an exponential increase in the number of B cells specific for the particular antigen. In addition, secondary lymphoid organs begin to form germinal centers within the lymph node.

A differentiated B cell has one of three potential effector cell fates; short-lived plasma cell[5], germinal center (GC) B cell[6], or memory B cell.[7] Upon initial activation of the naïve antigen-specific B cell, the outcome of becoming a short-lived plasma cell or GC B cell is governed by transcriptional regulation. B cells that up-regulate the transcription factor Blimp-1 exit the germinal center and differentiate into short-lived, antibody-secreting plasma cells. During the course of the initial infection, plasma cells or effector B cells produce and secrete large quantities of antibodies, the secreted form the B cell receptor. This process can occur within a few hours of the initial antigen exposure. Short-lived plasma cells are generally disseminated into blood and tissues, where they secrete millions of copies of the clonal antibody. The antibodies secreted from the short-lived plasma cells are typically the IgM (shorter half-life) isotype, which have a relatively lower affinity. However, short-lived plasma cells and associated secreted

antibodies are essential for clearance of the initial infection (i.e. acute response to antigen exposure).

B cells that up-regulate the Bcl-6 transcription factor migrate into the germinal center of the follicle and interact with another type of CD4 T cell, the follicular helper T cell.[8] The interaction of the B cell with the follicular CD4 T cell promotes proliferation of the B cells and stimulates somatic hypermutation[9], such that those that acquire "positive" mutations develop a higher affinity for the antigen.[10] Somatic hypermutation is the occurrence of hundreds of thousands of mutations within the complementarity-determining regions of the immunglobulin genes within the B cell nucleus, which encode for the variable region of antibodies. B cells that have undergone somatic hypermutation must compete for available antigens over the course of the immune response (or infection). Specialized dendritic cells within the germinal center follicles of the secondary lymphoid organs, called follicluar dendritic cells, present antigen to the B cells. The progeny B cells with the highest affinity for the specific antigen are selected to survive. Those, which bind antigen with lower affinity, are deleted due to the competition for the limited antigen. With each round of selection, the secreted antibodies have greater affinity for the antigen. This process is referred to as affinity maturation.[11] As a result of somatic hypermutation and affinity maturation, B cells that develop the highest affinity B cell receptors out-compete those without the receptors and emerge from the germinal center as either memory B cells or long-lived plasma cells. These two effector cell types are the core of immunological B cell memory.

The long-lived plasma cells migrate to the bone marrow, where they take up residence and continuously produce and secrete large quantities of clonal antibodies. Along with affinity maturation, plasma cells undergo isotype switching, generally from an IgM to an IgG isotype. As such, these cells persist as effector cells that secrete IgG (longer half-life) antibodies of high affinity for their antigen. Unlike memory B cells, long-lived plasma cells express little or no

BCRs. Thus, long-lived plasma cells are not stimulated either to divide or increase the rate of antibody production in the presence of antigen (i.e. they persist independent of antigen).

Memory B cells are localized primarily within the secondary lymphoid organs. Like the long-lived plasma cells, they have undergone the processes of affinity maturation and somatic hypermutation, and thus express a high affinity B-cell receptor (BCR). However, they do not secrete antibodies in the absence of antigen. In response to secondary infection or antigen re-exposure, they mediate rapid recall responses by quickly dividing and differentiating into antibody-secreting plasma cells, while also simultaneously replenishing the memory B cell pool. This amplified and exquisitely selective antibody production is the hallmark of the secondary antibody response.

Antibodies

Antibodies are a key component of adaptive immunity and the response to vaccination. Antibodies are the secreted immunoglobulins that are initially present at the surface as a B cell receptor. Biochemically, antibodies are Y-shaped proteins consisting of four polypeptide chains, of which two are referred to as heavy (longer) chains and two as light (shorter) chains. At the end of the two "arms" of the antibody, both the light and heavy chains combine to form the variable, antigen specific, portion of the antibody. Together, the end of one heavy and one light chain create a pocket, uniquely shaped with the appropriate chemical moieties, to bind a specific antigen. The stem of the Y-shaped antibody is identical in all antibodies of the same class, is independent of antigen specificity, and is called the constant region.

The known classes of antibodies include immunoglobulin M (IgM), IgD, IgE, IgA, and IgG. The constant region is the same for each isotope of immunoglobulin. The different constant regions among the classes of antibodies allows the antibody to interact with cells of the innate immune system, including natural killer (NK) cells, complement proteins, neutrophils and mast cells, which in turn, are involved in the destruction or removal of the bound antigen. Along with

affinity maturation, antibody class switching is simultaneously occurring during the antigen-dependent phase of B cell development. Antibody class switching is a mechanism that changes a B cell's production of antibody from one class to another, for example, from the IgM isotype class to the IgG isotype class. During this process, the constant region portion of the antibody heavy chain is changed, but the variable region of the heavy chain remains unchanged. The IgG class of antibodies is the most abundant antibody isotype found in the blood and extracellular fluid, representing approximately 75% of serum immunoglobulins in humans. IgG plays a vital role in the immune response to bacteria, fungi, and viruses and does so by a variety of mechanisms that include opsonization (coating the pathogen for recognition by cytotoxic immune cells), binding and immobilizing the pathogen, and triggering the initial step of antibody-dependent cellular cytotoxicity.

T Cell-mediated immune responses

T-lymphocyte

Equally important to antibodies in the humoral response are T lymphocytes, which are the key cells in the cell-mediated arm of the adaptive immune system. Two general subtypes of T lymphocytes exist, CD4-expressing (CD4) T cells and CD8-expressing (CD8) T cells. CD8 T cells are often referred to as cytotoxic T lymphocytes due to their ability to exert direct cytotoxic effects on antigen-expressing target cells. CD4 T cells are generally known as helper T cells due to their role in boosting the antibody response to specific antigens by interaction with antigen-specific B cells. However, numerous other roles for distinct subtypes of CD4 T cells exist, including some direct cytotoxic function that have been shown to play a role in anti-tumor immunity. Like naïve B cells with their B cell receptor, each naïve T cell has a T cell receptor (TCR) expressed on its surface that is specific for a given antigen. Unlike B cell and BCRs, TCRs can only recognize antigens when bound to the surface of a major histocompatibility complex

(MHC). Thus, the antigen that is recognized by a given TCR is a complex of a short immunogenic peptide bound to an MHC protein molecule.

MHC molecules exist in two forms, MHCI and MHCII. MHCI is expressed on all nucleated cells, including cancer cells as well as antigen presenting cells (APCs) of the immune system. In contrast, MHCII expression is limited to APCs, including B cells, macrophages, and dendritic cells (DCs). MHCI proteins bind CD8 T cell epitopes and are presented as a complex with the epitope on the surface cells. The peptide:MHCI complex on the surface of a cell is then recognized and bound by a naïve CD8 T cell specific for the antigen complex. MHCII proteins bind CD4 T cell epitopes and are presented as a complex with the epitope on the surface of APCs. The peptide:MHCII complex expressed on the surface of the APC is then recognized and bound by a naïve CD4 T cell specific for the antigen complex. Thus, as a rule, peptide-MHCI complexes are recognized by TCRs on CD8 T cells, while peptide-MHCII complexes are recognized by TCRs on CD4 T cells (white blood cells of the immune system, also called T-helper cells,) which send signals to CD8 killer cells to destroy the invading organisms.

The CD8 and CD4 peptide epitope bound by MHCI and MHCII proteins, respectively, is referred to as minimum peptide epitope. This name reflects that it is the shortest amino acid sequence, the final digest, able to fit into the peptide binding groove of the MHC protein. Minimum peptide epitopes that are bound by MHCI are either 8 or 9 amino acids, while minimum peptide epitopes that are bound by MHCII proteins are approximately 14 to 17 amino acids. In most cells, including cancer cells, MHCI molecules only bind to peptides derived from intracellular proteins that are cleaved in the cytosol and trafficked into the endoplasmic reticulum (ER) where the binding occurs. Peptide-MHCI complexes are then transported in vesicles to the plasma membrane and expressed on the cell surface. Therefore, for CD8 epitopes within a protein to gain access to the MHCI pathway, they must enter the cell by pinocytosis or be located within the cell itself, for example an intracellular infection or certain cancer proteins. CD4 epitopes gain access to the MHCII processing pathway by undergoing endocytosis into the endolysosomal

system. Inside the endolysosomes, the proteins are digested, and the endolysosome merges with another vesicle carrying an MHCII molecule.[12] Thus, the pathways by which CD4 and CD8 epitopes are presented on their respective MHC molecules differ.

Dendritic cells

Dendritic cells are exceptional as antigen presenting cells. DCs can internalize extracellular proteins into endolysosomes and translocate them into the cytosol for processing and peptide binding to MHCI molecules in a process called cross-presentation.[13] This is shown in Figure 1. Antigen cross-presentation is critical to the development of effective anti-cancer vaccines, because it can process CD8 antigens that enter into DCs by receptor-mediated endocytosis, a common pathway for entry of exogenous proteins into immune cells.[14] Thus, the ability of DCs to express both MHCI and MHCII molecules as well as process and present antigen by any of three different pathways is what has led to their designation as "professional APCs".

DCs are part of the innate immune system. They are widespread throughout the body and are often localized at entry points of infection, e.g. skin. In this strategic location, DCs can continuously sample their environment for the presence of foreign bodies, which includes the draining lymph nodes. These acting sentinel DCs are in their immature state and are specialized for phagocytosis. Upon uptake of extracellular material, the DC will direct the immune system towards a path of either tolerance, in response to "self" antigens, or activation, in response to "foreign" antigens. In either case, DCs are able to stimulate the appropriate immune response by up-regulating MHC proteins as well as other co-stimulatory molecules. The co-stimulatory molecules are then recognized as immunostimulatory or immunosuppressive by other cells of the immune system, thereby leading to an "activation" or a "tolerant" immune response, respectively.

Following uptake of foreign antigens, DCs will up-regulate activating co-stimulatory molecules, such as CD80 and CD86, along with the appropriate MHC protein. These changes are

the hallmarks of the transition of DCs into their mature state, and their ability to phagocytose material is decreased coincident with increased specialization for T cell activation. The mature DCs present peptide-MHCI or peptide-MHCII complexes to CD8 and CD4 T cells, respectively, as well as the appropriate co-stimulatory molecules. The co-stimulatory molecules are recognized and bound by the CD28 ligand on the surface of T cells. Simultaneous binding of the pepide:MHC complex on the DC surface with the TCR and CD80/86 co-stimulatory molecules on the surface of DCs with CD28 on the T cell surface is required for T cell activation. With activation, T cells undergo extensive proliferation with their progeny migrating from the lymph nodes to the site of the foreign material.

Phases of the Immune Response

The immune response to an acute exposure of foreign peptide:MHC (antigen) complexes involves three distinct phases. The first, expansion phase, occurs over the initial seven days and involves rapid proliferation and differentiation of antigen-specific naïve T cells into effector T cells. Antigen-specific T cells that have proceeded through an entire proliferative cycle, approximately ten to thirteen cell divisions, are then classified as antigen-specific *effector* CD8 T cells based on their ability to traffic to sites of infection and producing anti-inflammatory lymphokines. A single clone of effector cells specific for a single antigen often develops heterogeneity in their cytotoxic patterns. The cytokines secreted by effector T cells include interferon gamma (IFN-γ), tumor necrosis factor beta (TNF-β), and/or interleukin 2 (IL-2). In addition to the differences in cytokine production and secretion, effector T cells secrete a variety of toxic mediators, which may include perforin or granzyme.[15] Effector T cells, generated following antigenic stimulation of naïve cells, extravasate into peripheral tissues to rapidly control infection by elaboration of their effector functions and target cell identification and eradication. Unlike memory T cells, which are able to persist in the absence of antigen, effector T cell functions are maintained only in the presence of antigen.[16]

The expansion phase is followed by a contraction phase that results in the elimination of approximately 90 percent of the antigen-specific effector T cells over a two to three week period. The effector cells undergo apoptosis and are cleared from the circulation by macrophages of the reticuloendothelial system (RES). The surviving effector cells undergo further differentiation into memory cells.[17]

Following the contraction phase, the memory or maintenance phase occurs. Here, the pool of stable, highly polyfunctional memory T cells are found to persist indefinitely by continuous replication and self-renewal. Maintenance of the antigen-specific memory population can occur in both the presence and absence of antigen. In the absence of antigen, memory T cells persist in response to homeostatic signals, such as the cytokines IL-7 and IL-15, which are secreted in the secondary lymphoid organs.

Effector & Memory T cells

In the recent decade, much research has gone into studying the heterogeneic and qualitative nature of antigen-specific memory cells. Although the number of distinct types of memory T cells continues to increase, a generally accepted finding is that two types of memory T cells exist, the effector memory (T_{EM}) and central memory (T_{CM}) T cells.[18] Phenotypically, T_{EM} and T_{CM} cells are characterized by the expression of L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7), which play a key role in determining the trafficking properties T cells. Central memory CD8 T cells, characterized by high levels of expression of CD62L and CCR7, are localized primary within the secondary lymphoid organs and have limited effector capabilities. They are reported to have stem cell-like properties of self-renewal. Thus, upon re-exposure to their cognate antigen, they undergo rapid proliferation and differentiation into effector CD8 T cells. In contrast, effector memory CD8 T cells, characterized by a lack of expression of CD62L and CCR7, proliferate much more rapidly upon re-exposure to antigen and are localized primarily in the peripheral tissues. Their location at the site of disease allows for the rapid attack upon

encountering target cells. In the case of cancer, newly dividing cancer cells could be identified and eradicated much more rapidly due to the presence of the antigen-specific effector memory CD8 T cells.

For immunotherapy of GBM, migration through the blood stream to the location of a tumor is a critical step. Whereas the brain was once believed to be an immune privileged site, an abundance of new evidence has demonstrated the ability of innate immune cells effector T cells, plasma cells[19] and antibodies to traffic into the brain and to the site of the tumor.[20,21]

Cancer Vaccines

Prophylactic vs therapeutic vaccines

Vaccines may be either prophylactic, intended to prevent disease, or therapeutic, intended to treat disease. Prophylactic vaccines, such as measles, small pox, and polio have a long history, whereas the use of therapeutic vaccines is a much more recent development. The aim of therapeutic cancer vaccines is to harness the specificity and expansive capabilities of the immune system by stimulating a small number of tumor-specific lymphocytes to proliferate into a large number of tumor-specific effector lymphocytes.

The potential impact of therapeutic vaccines for treatment of active disease states is beginning to be realized in both the scientific and medical communities. This is evident by the introduction of Sipuleucel-T[22] for treating prostate cancer, which is the first therapeutic vaccine ever to receive FDA approval (2010). Presently, there are more than 100 of such vaccines in ongoing phase III trials (clinicaltrials.gov "search terms: cancer AND vaccine). A major advantage of therapeutic vaccines over other types of immunotherapy is the potential for immune memory. As described above, the T lymphocytes of the immune system undergo activation and proliferation, with the peak response in the number of antigen-specific T cells occurring at approximately day 7 in response to acute exposure to foreign antigens,. This is followed by a contraction phase over the next 3-5 days that results in the elimination (apoptotic death) of

approximately 90% of the antigen-specific T cells. The remaining 10% of the antigen-specific memory cells[23] are capable of responding much more rapidly upon re-encounter with the same antigen. Theoretically, memory B and T cells, along with innate immune cells, would be able to continuously survey the entire body for new or metastasized tumor cells. Memory cells thus provide the potential for achieving a complete cure for cancer. That is, complete elimination of all cancer cells for a life time. No other therapy holds this promise. The key to realize the full potential benefit of therapeutic cancer vaccines lies in the phenomenon of immune memory.

Although therapeutic vaccines are formulated in a similar manner to prophylactic vaccines, the treatment objective is unique. The aim of prophylactic vaccines is to develop an immune response to a disease or infection, so that if the pathogen is encountered, then the immune system is already primed to mount an effective immune response and eliminate the pathogen. Therapeutic vaccines, in contrast, are intended to stimulate an immune response against an already established disease. While the potential for development of prophylactic cancer vaccines exists, much work remains in identifying and delivering antigens that lead to the development of efficacious immune memory.

Adjuvants in vaccine formulations

In formulating vaccines, an adjuvant is often included. Adjuvants are designed to non-specifically activate the cells of the immune system. With the growing interest in both prophylactic and therapeutic vaccines in the past decade, development of novel adjuvants is a key goal to create a more potent and efficacious response to the antigen in the vaccine. Adjuvants are classified as one of three types, particulate formulations, immunostimulatory, or a combination of immunostimulatory molecules formulated within particulate systems.[24] Particulate formulations or delivery vehicles function as adjuvants by more effectively presenting antigens to the immune system.

In contrast, immunostimulatory molecules directly activate innate immune receptors of the immune system. Immunostimulatory adjuvants contain "danger signals", which are pattern-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs).[25] Both PAMPs and DAMPs are pathogen-associated products with conserved molecular motifs or signatures of cell stress/tissue damage and are recognized and bound by pattern-recognition receptor (PRRs).[26] PRRs are expressed by many different cells of the immune system as well as by some non-immune cells. PRRs include the class of toll-like receptors (TLR)[27], which comprise proteins that play a key role in the innate immune system. TLRs are transmembrane receptors expressed by the phagocytic cells of the immune system, including macrophages, dendritic cells, and neutrophils. TLRs may span the cell membrane with the ligand binding site facing externally or span the endosomal membrane with the ligand binding site facing into the endosome. Ten total TLRs (TLR-1 through TLR-10) have been recognized to date and are distinguished by their activation of a particular set of signaling pathways and the resulting biological effects. When activated, they stimulate signaling that leads to the activation of key transcription factors, most commonly nuclear factor-κB (NF-κB).[28] These transcription factors, in turn, stimulate gene expression programs that lead to secretion of cytokines and chemokines, enhanced antigen presentation capacity, and migration to lymphoid tissues where the DCs interact with T cells and B cells to initiate and shape the adaptive immune response.[29]

In this thesis, resiquimod was studied as a potential immunostimulatory molecule. Resiquimod is an analogue of the RNA base guanine and TLR-7 and TLR-8 agonist. TLR-7 and TLR-8 are natural receptors for viral RNA and are expressed on the endosomal membrane. Thus, resiquimod can mimic the immunostimulatory effects of a naturally occurring viral infection.

Antigens in vaccine formulations

The other major component of vaccines is the antigen. This perhaps is considered most important because it is responsible for the specificity of the immune system response.

Prophylactic vaccines most commonly contain a protein or carbohydrate antigen.[30] Protein antigens, minimal peptide epitopes, and cell lysates are the most commonly used antigens in therapeutic vaccines.[31] One advantage of whole protein antigens is that they often contain multiple antigenic epitopes capable of being recognized by MHCI or MHCII proteins or B lymphocyte receptors (BCRs). As such, they are capable of simultaneously activating both CD8 and CD4 T cells as well as the humoral arm (antibodies) of the immune system.

Minimal peptide epitopes activate CD8 when complexed with MHCI and CD4 T cells when complexed with MHCII proteins.[32] Finally, whole cell lysates are commonly used as the antigen source for cancer vaccines. The lysate is derived from cells taken from either the individual's own tumor, called autologous cell lysate vaccines, or from the tumor of another unrelated individual with the same type of cancer, called allogeneic cell lysate vaccines.[33] As with whole protein antigens, which are also present in cell lysates, whole cell lysates are able to activate both the cell-mediated and humoral arms of the immune system. An additional advantage of lysates is that they contain multiple proteins and other types (i.e. carbohydrate) of antigens. Many of which are unique to the tumor as a result of genetic mutations during cancer cell growth. This latter type of antigen is referred to as a neoantigen[34],[35] and has become a promising new target for therapeutic vaccines.

Delivery vehicles in vaccines

A third and critical aspect of a vaccine is the control of the availability of the antigen. The use of polymers as vaccine delivery vehicles has evolved to a prominent area of research in the last couple of decades. Polymer vehicles can not only act as an adjuvant but also function as controlled-release depots and/or as immune cell targeting particles.[36]

The use of biodegradable polymers as delivery vehicles is a very promising approach and has continued to gain momentum in the drive towards the improved design of next-generation vaccines. Poly-(lactic-co-glycolic acid; PLGA) is the most widely used polymer for this

application due to its biocompatibility, large body of research, and FDA approval status. However, despite the encouraging status of therapeutic vaccines in clinical trials, only a single phase I trial involves the use of a polymer-based therapeutic vaccine (clinicaltrials.gov as of 2/21/13). The apparent disconnect may be a result of the relatively poor understanding of the interaction between polymeric biomaterials and the immune system. Although the majority of polymer-based vaccines are well designed based on rationale chemical approaches, a clear immunologically-guided rationale appears lacking.

The rationale for polymer-based delivery of vaccines is driven by two main aspects. The first is that polymer-based vaccines can be formulated to molecularly mimic pathogenic viruses via their ability to undergo phagocytosis and to persist within antigen presenting cells. This should provide a more potent immune response. The second aspect is the depot effect by which the polymer provides a persistent source of antigen and adjuvant to the immune system. The degradation of a polymer *in vivo* is largely determined by its backbone chemistry but also molecular weight, which is further governed by the monomer composition and distribution of monomers within the copolymer backbone. Characterization of these properties is required to validate the synthetic strategy employed. The self-assembly behavior of the copolymer in aqueous solution can dictate its ability to encapsulate various peptides/drugs as well as modulate the release of the loaded compounds from the polymer. Additionally, manipulation of the fractional composition of monomers may produce control over the relative size of assembled structures in solution.

The ease of phagocytosis is primarily dependent on formulation of the polymer vehicle (encapsulating vaccine components) into appropriately sized particles, often referred to as particulate antigens. The optimal size of the particulate antigens for uptake by APCs is generally in the range of 50-1000 nm.[37,38] Following internalization by APCs, particulate antigens have demonstrated the ability to persist within the cytosol, providing for increased and prolonged rates of antigen cross-presentation and sustained production of important cytokines including

interleukin-2 (IL-2)[39] and interferon gamma (IFN-g).[40] Shen H et al.[41] reported that nanoparticles (500 to 1000 nm), generated from a biodegradable polymer, poly-(D,L-lactide-co-glycolide) (PLGA), and encapsulating OVA protein, were able to persist within the cytosol for 4 days. This represents a significant increase in time available for antigen cross-presentation to occur as soluble antigens typically persist for only a couple of hours after uptake. Another report[42] suggests that the particles can become entrapped within the partially acidic endosomal environment and thereby promote antigen cross-presentation upon fusion with vesicles originating from the endoplasmic reticulum and carrying MHCI proteins. In addition, particulate antigens have a demonstrated ability to generate increases in the magnitude, duration, and cytotoxicity of antigen-specific CD8 T-cells *in vivo*.

The depot effect embodies the concept that a persistent source of antigen/adjuvant to the immune system will provide a sustained immune response. The sustainment of the immune response will then, theoretically, translate into improved eradication of the diseased cells. This strategy serves as the basis behind Freund's Complete Adjuvant (FCA)[43]. FCA is composed of light mineral oil, mannide monooleate (a surfactant agent), and heat-killed and dried mycobacterial cells.[44] FCA still remains the most potent antibody-stimulating adjuvant known to date. Despite its unmatched adjuvant effect, the use of FCA is not permitted for human use because it has been associated with a variety of lesions, including localized injection site lesions and diffuse granulomas.[45] This led to the development of Incomplete Freund's Adjuvant (IFA), which was first used by Jonus Salk in the 1950s in an attempt to improve influenza vaccines.[46] IFA differs from FCA in that it lacks the killed mycobacterial cells. [47] The removal of the killed mycobacterial cells significantly improves the safety profile of the emulsion, which has received approval by the Food & Drug Administration for use in humans.

Both FCA and FIA are prepared by emulsifying an aqueous solution and mannide monooleate oil to produce a water-in-oil emulsion. Antigen, buffered in water, is incorporated into the aqueous phase of the emulsion. Upon injection, the mixture forms a depot in the

extracellular environment and provides for controlled-release of antigen for 3-4 weeks. Maintaining the antigen in a single location is reported to prevent exposure to effector cells of the immune system. A reaction to the injection can lead to encapsulation of the vaccine with fibrous tissue, which limits release of the antigen. The formation of a stable water-in-oil emulsion is a critical step in the effectiveness of both adjuvants. The potential of IFA for use as a single-dose vaccinations in underdeveloped countries and to induce persistent, high titer protective antibodies has been very promising.

Interestingly, the adjuvant activity of IFA, without the incorporation of a TLR agonist, was traditionally believed to function by three different mechanisms of action; these include establishing an antigen depot with slow antigen release, providing a vehicle for antigen transport throughout the lymphatic system to immune effector cells, and interacting with antigen-presenting cells including phagocytes, macrophages, and dendritic cells.[48] However, recently, this has been questioned. There is a debate as to whether the adjuvant effect of IFA exists solely due to the slow release of antigen or there is an intrinsic adjuvant effect of the IFA itself.

Many polymer-based vaccines have been prepared with the intent to incorporate both of the approaches described above.[49] Most commonly, biodegradable polymers are used to encapsulate, or incorporate by similar means, the vaccine antigen and adjuvant. Studies report both the increased uptake of the micro- or nanoparticles by the cells of the immune system and also the sustained release of the antigen and adjuvant from the polymer. However, the release studies are performed in vitro, and thus the rates of release are dependent solely on diffusive release of antigen and adjuvant from the polymer. It is likely that the *in vitro* biodegradation and release kinetics are poor predictors of their *in vivo* behavior. Often, *in vitro* time release profiles of weeks to months are reported. In vivo, however, the phagocytic cells of the immune system take up the entire vaccine particle within hours after injection. Moreover, evidence demonstrating the ability of nano- and/or microparticles to persist within the cytosol of the DC for only 4 days,

the reported *in vitro* release rates of antigen and adjuvant are irrelevant to the *in vivo* performance.

To date, few examples wherein both strategies are effectively used exist. Irvine, et al,[50] compared intranodal versus intramuscular injections of both PLGA nanoparticles and large, ~10 – 20 μm, microparticles containing fluorescently labeled polyI:C. The authors observed that 24 hours after injection, the uptake of fluorescently labeled polyI:C by macrophages and DCs in the lymph node was increased in the nanoparticles as compared to the microparticles injected intranodally, with the percent of cells containing fluorescently labeled polyI:C being 20-25% versus 1-2% in the two groups, respectively. The particles injected intramuscularly showed minimal uptake, and no difference was observed between groups. At 96 hours post-injection, the intranodally injected microparticles continued to provide sustained release of polyI:C into the lymph node, translating into prolonged activation of immune cells. The rationale was that the size of the microparticles was beyond the limit for uptake by phagocytic immune cells, and the sustained release afforded by the PLGA polymer was maintained *in vivo*. Conversely, the release kinetics of adjuvant from nanoparticles failed to be maintained *in vivo* because of the rapid uptake of the particles by the cells of the immune system. Although these experimental observations offer a unique look at the potential for combining both advantages of polymer delivery, there remains a significant challenge in translating the vaccine to the clinic.

CURRENT STATE OF CANCER VACCINES AND HYPOTHESIS

Current state of cancer vaccines

Antigen

Despite the promising potential of therapeutic vaccines, their development has yet to achieve the level of success seen with adaptive cell therapies[51] or monoclonal antibodies.[52] One key reason for this discrepancy is the existing gaps in knowledge as to the magnitude, duration, and qualitative nature of immune response required to eliminate cancer cells.

It is thought that an *effective cancer vaccine* must produce large numbers of tumor antigen peptide-MHCI-specific CD8 T cells capable of killing tumor cells. As a result, CD8 T cells have been the focus of many therapeutic vaccines because of their direct cytotoxic activities against target cells. Various modified forms of vaccines (i.e. cell lysates, peptides, proteins) have been utilized in an attempt to find the "ideal" vaccine components. Presently, there is great interest in the use of minimal peptide epitopes for activation of cytotoxic CD8 T cells against cancer cell antigens. However, targeting minimal peptide epitopes has not been shown to stimulate a sufficiently powerful immune response to overcome the aggressive nature of cancer cells nor their intrinsic ability to escape the cells of the immune system.[53,54] In contrast, attacking the cancer from a number of angles, i.e. an orchestrated attack from both arms of the immune system, has been shown to produce improvements in overall survival in preclinical models of GBM[55] as well as other cancers.[56]

The same has proven true in human trials, as the vast majority of CD8 T cell-targeted cancer vaccines have failed to produce an increase in patient survival.[57,58] However, this may occur because of tumor down-regulation of the relevant protein as a means of avoiding CD8 T cell recognition.[59] The vast majority of therapeutic vaccines, including polymer-based approaches, have failed to account for the important role of helper and effector CD4 T cells[60] and antibodies.[61] Only recently has it been suggested that tumor antigen-specific CD4 T cells are also required for effective tumor control.[62,63]

Delivery systems

Much work has gone into studying various methods of antigen (and adjuvant) delivery, such as dendritic cell vaccines and polymer vehicles, in order to alter timing, to mimic naturally occurring infections, and to target specific immune cells. Despite the advances in the chemical design of vaccine delivery vehicle, there remains a clear gap in knowledge as to how the rate and duration of release of antigen and adjuvant from the vehicle or the uptake of the particulate

vaccine affects the magnitude, duration, and/or qualitative nature of the theoretical immune response. Thus, although the majority of polymer-based vaccines are well designed based on rationale chemical approaches, a clear immunologically-guided rationale is often lacking.

Persistent antigens and exhaustion

The effect of persistent antigen on the therapeutic functioning of the immune response is a fine balance between stimulation and exhaustion. In many cases, high levels of persisting antigen, from either vaccination or uncontrolled infections, have been shown to cause the immune system to become "exhausted".[64,65] In the case of many chronic infections, *high and systemic* levels of persisting antigen have been shown to cause peptide-MHC-specific T cells to suffer a decrease in their cytotoxicity and/or an up-regulation of an immunosuppressive state. The interplay of functional efficacy and exhaustion of the immune system has been shown to be a product of the magnitude and duration of persistence antigen as well as the qualitative nature of the immune response.

In chronic viral infections, there is an indication of continual progression of immune system exhaustion with time, although this is seemingly dependent upon the degree of pathogenicity and localization of the disease. In a study of the immune response to HIV[66], the authors established that clearance of the pathogen resulted in reduced immunity and protection from repeat infection, suggesting that in this, and potentially other disease states, sustained protection from reinfection may depend on continuous exposure to low levels of antigen.[67] Similarly, the Jenkins' lab at the U of MN showed that *Salmonella enterica* infection, which persists at low levels within the mesenteric lymph nodes for prolonged periods, sustains the number and function of peptide-MHCII-specific memory CD4 T cells. This effect was lost upon eradication of the infection with antibiotics or if the infection became systemic. This suggests that sources of persistent antigen may be most beneficial when confined to a localized space.[68] These same findings were true in a murine model of *Leishmania majo*r (*L. major*) infection, where

protection from repeat infection (or primary infection following vaccination) was found to correlate strongly with the presence of polyfunctional CD4 T cells, independent of the frequency of these cells.

With regards to the qualitative nature of the immune response, antigen-specific CD4 T cells have the ability to "rescue" exhausted CD8 T cells during chronic viral infections.[69] This suggests that CD4 T cells have a vital role in generating and maintaining the quality of the CD8 T cell response. Assessment of the CD8 T cell response in the absence of a CD4 T cell epitope fails to account for the impact that CD4 T cells have in forming the overall response to vaccination (or infection). Therefore, incorporation of a CD4 T cell epitope with a CD8 T cell epitope may improve the immune response against difficult targets such as tumors. Taking all of these studies together, there is compelling support for the contention that the failure of a vaccine to stimulate a "complete" immune response, involving CD4 and CD8 T cells as well as antibodies, adversely affected the efficacy and potential clinical translation of therapeutic vaccines.

To achieve a "complete" immune response, polymer-based vaccines, such as poly (lactic-co-glycolic acid) or PLGA, have been designed with entire protein antigens incorporated into the matrix. This approach has the potential to activate both the humoral (antibody) and cellular (both CD8 and CD4 T cell) arms of the adaptive immune response. However, despite the probable generation of an antigen-specific CD4 T cell response in these studies, research has been limited to the analysis of antibody and/or CD8 T cell response.[70,71,72] This is likely due to the widespread, commercial availability of tools for studying these responses. In contrast, quality assessments of CD4 T cells are limited to a great extent by the lack of quality experimental tools as well as the comparatively small number of well-defined CD4 T cell epitopes in cancer.

Hypothesis

The progress achieved with immunotherapy in other malignancies has failed to be realized in GBM. Despite the development of numerous vaccines capable of generating an

immune response against tumor antigens, none has yet demonstrated any meaningful advances in overall survival. We believe that the disconnect between the anti-tumor immune response and tumor regression/survival is in part due to the lack of a well-defined kinetic profile for an effective therapeutic immune response to cancer. This disconnect exists in the type, quality, quantity and duration of the anti-tumor immune response. Therefore, we attempted to identify a relationship between the magnitude and duration of the immune response to a controlled-release vaccine and the resulting therapeutic benefit in a mouse model of glioblastoma multiforme.[73]

The approach involved the use of a novel, biodegradable and biocompatible semi-solid polymer vehicle, poly-(ε-caprolactone)-poly-(ortho ester) [PCL-POE] (figure 2). The novelty of this polymer is that the antigen and adjuvant are both readily mixed into the semi-solid at room temperature and, upon injection into the extracellular space, a depot forms. When administered subcutaneously, the polymer itself is able to persist over a period of 3 to 4 weeks, allowing for a slow and continuous release of the antigen and adjuvant into the extracellular space. Thus, the role of persistent antigen (and adjuvant) on the immune response and on overall survival in a model of GBM could be studied. We hypothesized that the slow release of antigen and adjuvant from the novel PCL-POE polymer would extend the presence of effector T cells and antibodies in the lymphatic system and tissues after injection, thereby resulting in an increase in overall survival in our mouse model of glioblastoma multiforme. To confirm this hypothesis, we tested the following specific aims:

1. Establish the ability of the PCL-POE polymer to provide sustained release of both antigen and adjuvant *in vitro*
2. Establish a correlation between persistent release of antigen and adjuvant from the PCL-POE polymer and the extended duration of the innate and adaptive immune responses
3. Establish the ability of the extended duration of the innate and adaptive immune responses to increase overall survival in a mouse model of glioma

MATERIALS AND METHODS

In vitro release studies

The *in vitro* release of OVA protein and RES from the PCL-POE semi-solid polymer was measured as follows. First, 1000 µg of OVA and 200 µg of RES were mixed with 200 µl of the polymer and placed into 10 mL of PBS in a 50 mL conical tube. The sample was then incubated at 37°C while rotating at 200 revolutions per minute (RPMs). At each indicated time point, 2 mL of supernatant was collected and replaced with 8 mL of fresh PBS. Mini dialysis centrifuge tubes with a MW cut-off of 3500 kDa were used to separate the OVA protein from RES. Both compounds are completely soluble in 100% methanol, which used as the dialysate fluid. The dialysis fluid was replaced at 4, 8 and 18-24 hrs. The dialysate was collected and the OVA protein within the dialysis tubing was collected by repeated rinsing with sterile water. All samples were dried to a powder form by a combination of rotary evaporation and speed vacuuming. The amount of OVA contained in each sample was quantified using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific Pierce). The amount of RES in each sample was measured using liquid chromatography-mass spectrometry (LC-MS/MS).

We followed the manufacturer's protocol for the Microplate Procedure. The technique allows for use of a smaller volume (10-25µL) of protein sample with sensitive detection. Ovalbumin was used as the standard. Concentrations from 5 µg/mL to 500 µg/mL were used to create the standard curve. The r-squared value for the standard curve was 0.993. Each sample was suspended in 250 µL of sterile water, and 25 µL of each standard or sample was added to a single well of a 96 well ELISA plate (Thermo Scientific Pierce), and 200 µL of the blue colorimetric reagent. The samples were then incubated at 37° for 30 minutes. The reaction involved the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The second reaction results in an intensely purple-colored reaction product, the absorbance of which is measured at 562 nm using a plate reader. All samples and standards were run in triplicate.

The concentration of resiquimod was determined using a specific and sensitive LC-MS/MS assay. RES standards were diluted in methanol, dried under a gentle stream of nitrogen, and reconstituted in 100 µl mobile phase. RES samples were diluted in 500 µl mobile phase. The sample was then diluted further to obtain a concentration in the linear range of the LC-MS/MS assay (a 1:400 dilution). Following reconstitution in mobile phase, the standards and samples were vortexed and transferred to auto sampler vials. A 5 µl sample was injected onto a Zorbax Eclipse SB-C18 column (4.6 x 75 mm, 3.5 µm particle size; Agilent Technologies, Santa Clara, CA). The aqueous mobile phase was 25 mM ammonium formate with 0.1% formic acid, and the organic mobile phase was acetonitrile (75:25; v/v) and was delivered at a flow rate of 0.5 ml/min total with a total run-time of 8 min. The ionization was conducted in positive mode, and the m/z transition was 196.295 → 197.795. The retention time of RES was 4.47 minutes. The assay was sensitive and linear over a range of 1-500 ng/ml, with the coefficient of variation being less than 20% over the entire range.

Flow cytometry

All antibodies, except where indicated, were obtained from eBioscience. Phycoerythrin (PE)-conjugated K^b-OVA peptide–MHC multimer staining was performed with dextramer (Immudex).

Single cell suspensions (4–5 × 10^5 cells in 100 µl of whole blood) were stained for 30 minutes at room temperature with 100 ng/µL PE-conjugated K^b-OVA dextramer. For identification of surface T cell surface markers, the samples were stained for 45 minutes on ice with the following antibodies; perCP-Cy5.5-conjugated anti-mouse CD8α (clone 53-6.7), Alexa Fluor 700-conjugated anti-mouse CD44 (clone IM7), allophycocyanin (APC)-e780-conjugated anti-mouse CD3ε (clone 17A2), APC-conjugated anti-mouse CD4, (Clone GK1.5), fluorescein isothiocyanate (FITC)-conjugated anti-mouse PD-1 (CD279) (clone J43), Pacific Blue-conjugated anti-mouse LAG-3 (CD223) (clone C9B7W), and KLRG1 PE-Cy7-conjugated anti-

mouse (clone 2F1). In experiments designed to detect nuclear proteins the cells were surface stained with some of the aforementioned antibodies, then treated with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), and stained for 1 hour on ice with an Alexa Fluor 647-conjugated anti-mouse Ki67 (clone SolA15) antibody. After each stain, cell samples were washed twice with PBS prior to analysis. Appropriate isotype controls were used.

For dendritic cell maturation assays, single cell suspension of the lymph node were stained on ice with the following antibodies for identification of surface markers; FITC-conjugated anti-mouse CD11c (clone N418), PE-Cy7-conjugated anti-mouse MHCII (clone M5/114.15.2), APC-conjugated anti-mouse CD86 (clone GL1), and Pacific Blue-conjugated anti-mouse CD80 (clone 16-10A1; BioLegend). Samples were analyzed on a BD FACSCanto II flow cytometer. Data were analyzed with FlowJo software (TreeStar).

Cell lines and culture

All GL261 cell lines were stably transfected with Luciferase maintained by selection with 1mg/mL puromycin. The GL261 cell line grown in 5 % oxygen was maintained as neurosphere cultures in DMEM/F12 (1:1) media supplemented with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/ml), B27 and N2 supplements, 0.1-mg/ml normocin, and EGF-F and FGF-F cytokines. Cytokines were replenished twice per week. Cells were maintained at 5 % oxygen for a minimum of two weeks prior to use. The GL261 cell line grown in 20 % oxygen was maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% with fetal bovine serum and were maintained by selection with 1 mg/mL G418. All culture media contained 100 U/mL penicillin/streptomycin and 0.1mg/mL normocin.

Mouse models

Mouse experiments were performed in accordance with University of Minnesota Animal

Care and Use Committee guidelines. C57BL/6 (B6) mice and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were purchased from the Jackson Laboratory and were maintained in a specific pathogen-free facility. For all experiments, the age of the mice was between six and eight weeks.

For studies of the immune response to persistent antigen, we used C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice for our positive control mice. These transgenic mice have 100% of their T cell receptors (TCRs) specific for the SIINFEKL:2-K^b peptide:MHCI complex. Thus, we are able to utilize non-transgenic mice of the same genetic background to study their response to OVA protein vaccine.

For our survival studies, we used whole cell lysates grown in 5% O_2 as our vaccine antigen, because the GBM tumor model was grown from a mouse glioma cell line, GL261, which does not express the OVA antigen. As such, it better mimics the natural growth of a GBM than an OVA-expressing GL261 cell line. For orthotopic glioma models, single cell suspensions of a glioma cell line, GL261, was suspended in PBS and treated with 1 µL On-column DNase (Sigma) per 100 mL of cells. A maximum of 1 µL of cell suspension was inoculated in the right striatum, 2.5 mm lateral and 0.5 mm anterior of bregma, and 3 mm deep from the cortical surface of the brain. The number of cells used in the inoculation was 15,000 GL261 cells in a 1 µL injection. Tumor development and growth were assessed routinely by bioluminescent imaging. Per protocol, glioma bearing mice were euthanized, when they became symptomatic.

Vaccine Preparation

For the polymer vaccinations, 800 mg of whole ovalbumin protein (Anaspec) and 200 mg of resiquimod (prepared by the laboratory of Dr. Dave Ferguson, Department of Medicinal Chemistry, University of Minnesota) were placed into 100 µL of PCL-POE, and a uniform solution was obtained by stirring for 2 minutes. All vaccination samples were used within one hour of preparation. Control (solubilized) vaccinations were diluted in PBS to a final concentration of 200 µg Ovalbumin and 50 µg resiquimod per 100 µL of PBS. For control

vaccinations, resiquimod was solubilized in 2% DMSO/PBS. Ovalbumin was diluted in PBS.

Lysate vaccines were prepared from GL261 cultures grown in 5% oxygen. Cells were collected, counted, and re-suspended at a concentration of 10 million tumor cells per 1 mL PBS. Each sample was treated with liquid N2 for 30 seconds followed by lyophilization for 48 hours using (machine, company) and then stored at -80°C. On the day of vaccination, cell lysate powders were either re-suspended along with resiquimod at a final concentration of 2.5 million tumor cells and 50 μg resiquimod per 100 μL PBS (for soluble vaccines) or mixed with resiquimod into 100 uL of PCL-POE at final concentrations of 10 million tumor cells and 200 μg resiquimod per 100 μL polymer. Polymer vaccines were mixed for 2 minutes and were used within one hour of preparation. Just prior to each individual vaccination, the preparation was again mixed using a double-syringe luer-lock system.

Vaccination protocol

Mice were vaccinated with either 100 μL of polymer or soluble (control) vaccine. All vaccinations were administered as subcutaneous injections at the base of the left hind leg near the inguinal lymph node. For OVA-containing vaccine, soluble vaccines were administered once daily for four consecutive days. Polymer vaccines were given as a single dose. All vaccines were prepared at the time of vaccination. For cell lysate experiments, soluble vaccinations were given once weekly (beginning 7 days post-inoculation) for 4 consecutive weeks. For both sets of experiments, polymer vaccines were given either once (day 7 post-inoculation) or twice (days 7 and 28 post-inoculation).

Dendritic Cell Maturation Assay

For analysis of DC maturation, mice were sacrificed at one and two weeks post-vaccination. Draining lymph nodes were harvested and single cell suspensions were prepared. Single cell suspensions were resuspended to a concentration of $4–5 \times 10^5$ cells per 100 μl of PBS.

Samples were stained with surface antibodies against CD11c, CD80, CD86, and MHCII in the dark on ice for 45 minutes. Samples were then washed twice with PBS and suspended in 100 µL of PBS and analyzed.

ELISA

Detection of plasma anti-OVA antibodies was performed with a standard enzyme-linked immunosorbent assay (ELISA). Whole Ovalbumin protein (Fisher) was plated at 0.5 µg/ml in PBS (100 µL/well) in 96-well flat bottom plates (company) and incubated overnight at 4ºC in the dark. Each well was washed with 2% fraction V BSA/PBS (Sigma Aldrich) (100 µL/well) for 60 min at room temperature in the dark. Wells were then washed one time with 0.5% fraction V BSA/PBS (200 µL/well). Plasma samples were thawed and added to each well at dilutions of 1:10, 1:100, or 1:1000 in PBS (100 µL/well) and incubated for 2 hrs at room temperature in the dark. Wells were then washed three times with 0.5% fraction V BSA/PBS (200 uL/well) and one time with PBS (200 uL/well). Next, 100 µL of a 1:2500 goat anti-mouse IgG (H + L) polyclonal alkaline phosphatase-conjugated secondary antibody (Thermo Scientific Pierce) was added to each well, and the plate was incubated for 1 hour at room temperature in the dark. Wells were then washed three times with 0.5% fraction V BSA/PBS (200 uL/well) and one time with PBS (200 uL/well). Thereafter, 100 µL of a 1 mg/mL p-nitrophenyl phosphate/diethanolamine buffer solution (Thermo Scientific) was added to each well. The reaction was allowed to proceed at room temperature in the dark for 20 min, and the absorbance was read at 405 nm using a plate reader.

Model protein antigens

For our studies of the immune response to the controlled release of vaccine components by PCL-POE polymer, we utilized a model protein antigen, chicken ovalbumin (OVA). We chose OVA as our model antigen because reagents exist that allowed us to track the CD8 T cell

response specific for the predominant CD8 epitope, SIINFEKL, contained within the OVA protein. The reagent was a dextramer of K^b:SIINFEKL (Immudex). Use of a multimeric complex of a specific peptide:MHC molecule results in increased reliability and improved detection of an antigen specific T cell population.

Measurement of the OVA-specific CD8 T cell response

For our studies, identification of SIINFEKL (OVA)-specific CD8 T cells was performed using whole mouse blood. On the indicated day post-vaccination, either 100 µL of blood was collected via the periorbital vein or the mouse was sacrificed and secondary lymphoid organs removed for cell harvesting. The latter method was used for repeat immune monitoring because a greater number of total immune cells could be collected from the secondary organs relative to that in 100 µL of blood. Thus, improved accuracy in measurement of the cells of interest was achieved. For periorbital blood collection, mice were anesthetized with continuous flow of 0.4% isoflurane and a single drop of local anesthetic, proparacaine hydrochloride 0.5 %, was placed onto the site of collection. The respiration and heart rates of the mice were monitored during anesthesia. Sterile 100 µl capillary tubes were used for collection, and the collected blood was placed into conical tubes containing heparin. Mice were treated with an antibiotic ointment (Triple Antibiotic Ointment®; Boynton Pharmacy) at the collection site and aroused by removal from isoflurane. Mice were monitored for a minimum of 1 hour after wakening and until normal feeding and activity resumed. After collection of immune cells by either method, samples were treated with 10 µL of PE-conjugated K^b:SIINFEKL dextramer (Immudex) per 1,000,000 total cells. Samples were vortexed and incubated at room temperature in the dark for 30 minutes. The samples were stained with fluorescently-labeled antibodies for CD8, CD3 and other markers of interest at a 1:100 v/v concentration, and incubated on ice for 45 minutes in the dark. After staining, samples were washed to remove unbound antibody, centrifuged at 1000 x g for 5

minutes and the supernatant removed by aspiration. Samples were re-suspended with 100 µL of PBS and the percentage of OVA-specific CD8 T cells was analyzed by flow cytometry.

Measurement of non-specific CD8 and CD4 T cell responses

For identification of rapidly dividing CD8 or CD4 T cells, which served as an indicator of response to treatment after treatment with lysate antigen, samples were stained after treatment with lysate antigen with anti-mouse antibodies against CD8α, CD3ε, and CD44 in the dark at 4ºC for 45 minutes, followed by washing with PBS. Ki67 expression marker was used as the intracellular marker for identification of proliferating cells. Ki67 was detected by first permeabilizing and then fixing the cells with a FoxP3 Cell Permeabilization Buffer Kit (eBioscience). Samples were suspended in 400 µL of a 1x concentration of the permeabilization buffer and incubated at 4ºC for 1 hour in the dark. Samples were then washed twice with PBS and stained with a 1:100 concentration of anti-mouse Ki67 in the permeabilization buffer for 1 hour at 4ºC in the dark. Samples were then washed twice with PBS and resuspended in 100 µL of PBS and analyzed.

Statistical analysis

Statistical analysis and curve fitting were performed using Prism software (Graphpad). Significance analysis was performed with Student's unpaired t-test. For the Kaplan-Meier survival curves, the log rank test was used. The significance of the results are expressed with the p values, using the following notation: *:$p<0.05$; **:$p<0.01$; ***: $p<.001$.

RESULTS AND DISCUSSION

The ultimate goal of this research was to identify an effective vaccine for the treatment of glioblastoma multiforme. The approach involved the use of a polymer vehicle that provided a slow, continuous release of antigen and adjuvant. In addressing this goal, a careful and systematic approach was followed, where several aspects of the immunological response were investigated. This included *in vitro* release studies to characterize the release of the model protein antigen, ovalbumin (OVA), and the immunostimulant drug, resiquimod (RES), from the polymer. With vaccine administration, the time course of the biological response was assessed by examination of dendritic cell maturation via their up-regulation of MHC-II, CD80, and CD86 levels, antigen-specific IgG and IgM antibodies, tumor lysate-specific CD8 and CD4 T cells, and ultimately the effect on cancer progression and survival in the mouse animal model.

Time-release profile of antigen and adjuvant from PCL-POE polymer *in vitro*

The in vitro release of 1000 μg OVA and 250 μg resiquimod from the PCL-POE polymer was examined. In these studies, the polymer is insoluble and has a higher density than water, so it remained as a semisolid mass at the bottom of the centrifuge tube in the release study. In Figure 3a, the cumulative percent release of Ovalbumin (OVA) is given as a function of time over a period of 28 days. As can be seen, the cumulative percent release initially increased in a linear manner for the first four days where just less than 20 % (ca 200 μg) of OVA was released. Thereafter, the release rate progressively decreased yielding a curvilinear relationship reaching nearly 50 % release at 28 days. In Figure 3b, the corresponding results for resiquimod are given. As with OVA, the release was linear for the first four days with about 60% (ca 150 μg) release and then slowed over the remainder of the experiment. Nearly 100% was released by the 28th day.

Comparing the release of vaccines components from the PCL-POE polymer showed that while approximately sixty percent of Ovalbumin protein was released over 4 weeks, release of

Resiquimod, a much smaller and hydrophobic molecule, was much more complete in the same time frame. Both showed a more rapid linear release rate over the first 4 days. This burst release was followed by a slower, apparently linear release for the remainder of the study. The similar time course of OVA and RES from the polymer is suggestive of a comparable mechanism in the control of the release. Important for the immunological response, the burst release in the initial phase corresponds to a time profile that closely approximates the time over which pathogens are rapidly replicating during an acute infection or that of a rapidly growing tumor. The slower phase of release closely resembles that of a chronic infection or a slow growing tumor.

While the release profile from polymer vehicles is readily measured *in vitro*, experimental difficulties have prevented the accurate, quantitative measurement of the release *in vivo*. In general with polymer vehicles, the *in vitro* release kinetics is often a poor predictor of *in vivo* behavior. For a nano- or microparticulate polymer vaccine, the *in vitro* release rates may be significantly different from that observed *in vivo* as a result of phagocytosis and intracellular digestion of the polymer by dendritic cells (DCs), macrophages and other non-professional APCs. Given the presence of widely expressed esterases, PCL-POE degradation will occur *in vivo* but not during the *in vitro* release measurement. Thus, with the differences in the degradation rate of the polymer, we would expect that the rate of release from the PCL-POE polymer would be more rapid *in vivo* because the release of vaccine components will be a product of both polymer degradation and molecule diffusion within the polymer.

The advantage of the novel PCL-POE semi-solid polymer used in this research is that upon injection it approaches a solid state. As such, it is able to persist *in vivo* for a much longer time than polymer particles, because cells of the immune system are unable to phagocytose the intact semi-solid. This is analogous to the injection of Freund's oil-in-water emulsion that results in an initial reaction at the injection site, which ultimately led to encapsulation of the injected material within fibrous support tissue. By sheltering the antigen in a single location, the polymer depot is able to prevent exposure to effector cells of the immune system and thereby allow for

prolonged release of antigen and adjuvant with a rate determined solely by the rate of release from the polymer. Support for this assertion was obtained by visual inspection of the polymer mass in mice following injection. Here, we were able to substantiate that the polymer remained visible at 3-4 weeks reflective of the slow degradation *in vivo* (data not shown). Although this confirmed that the polymer itself persisted for a defined period of time *in vivo*, additional release coincident with polymer degradation would alter the kinetics of antigen and adjuvant release relative to that observed *in vitro*. Nevertheless, the similarity in the time course for each component would be expected to remain the same despite the additional release occurring with polymer degradation.

Guided by the results from the in vitro release study, the immunological response was then assessed. In this study, OVA antigen was incorporated into PCL-POE with either of two adjuvants, RES, or the established T cell adjuvant, polyinositic-polycytidylic acid (polyI:C). These two different formulations were then injected as a single subcutaneous injection. As a positive control, the two different antigen/adjuvant combinations were also suspended in PBS and injected as four consecutive daily boluses (figure 4). The immunological response was evaluated by measuring the percentage of OVA-specific CD8 T cells in the blood over time by flow cytometry.

The results are given in Figures 5a, b, and c, which provide the percent of OVA-specific CD8 T cells measured in mice as a function of time. In these Figures, the dashed line at about 0.5% represents the mean percent of naive OVA-specific CD8 T cells in unvaccinated B6 mice.

Figure 5a provides the data for OVA+polyI:C in PBS given subcutaneously as four repetitive once daily bolus injections and OVA+polyI:C in PCL-POE given as a single subcutaneous injection. The majority of mice receiving OVA+polyI:C as repetitive injections achieved a peak in percent OVA-specific CD8 T cells on day 7 as expected, with values ranging from about 1.7 to 7 %. This was followed by the typical contraction and then maintenance phase over the next three months. In mice vaccinated with OVA+polyI:C in PCL-POE polymer, the

peak OVA-specific CD8 T cell response occurred between days 7 and 21 for the individual mice, with the percentage of OVA-specific CD8 T cells ranging from 1 to 3 %. While the peak percent of OVA-specific CD8 T cells in the group of mice vaccinated with polymer was significantly lower than mice receiving repetitive injections, there was no increase in the duration of OVA-specific CD8 T cells in the blood in this group. The appreciable variability in the response precluded the ability to detect any statistical difference in the peak response, during the contraction phase (rate of decline) or in the maintenance phase among these treatment groups.

In Figure 5b, the results are provided for the formulations: OVA+RES in PBS administered subcutaneously as four repetitive once daily doses as well as OVA+RES in PCL-POE administered as a single subcutaneous injection. The total amount of OVA and RES was equivalent in these groups. As can be seen in the figure, the observed time course when RES was incorporated into the polymer was atypical. Specifically, the peak OVA-specific CD8 T cell response was lower on day seven, but the percentage of OVA-specific CD8 T cells in the blood stream remained elevated for a longer period in comparison to the group receiving four repetitive daily boluses of vaccine with the same (RES) adjuvant. In one case, the CD8-T cell response continued to increase and reached about 7 % at the last time point monitored in this experiment. Approximately half of the mice receiving OVA+RES in PCL-POE polymer achieved a peak in percent OVA-specific CD8 T cells ranging from approximately 1 to 5 %. The time of the peak response in these mice ranged from day 7 to 30 post-vaccination. The other half of mice receiving polymer vaccinations did not achieve an OVA-specific CD8 T cell response beyond that of saline-treated mice. In mice vaccinated with OVA+RES in PBS given as repetitive injections, only a single mouse achieved a 1.4 % of OVA-specific CD8 T cells, which was above that of saline treated mice.

In Figure 5c, the OVA-specific CD8 T cell response of PCL-POE polymer plus OVA with RES is compared to polyI:C. Two differences are apparent from the time profiles. First, the group of mice vaccinated with polyI:C developed an OVA-specific CD8 T cell response more

typical of an acute exposure to antigen. This is characterized by a distinct peak early in the response and followed by a rapid contraction phase to near baseline percents of OVA-specific CD8 T cells in the blood. In contrast, the group receiving resiquimod in the PCL-POE+OVA stimulated the activation and maintenance of OVA-specific CD8 T cells for up to two to three months. Moreover, the peak response occurred as late as 1 month post-vaccination and in one case continuing to increase to 7 % at 3 months post-vaccination, the last time point measured in this study.

To interpret this data, both the kinetics of an immune response to an acute infection (bolus dosing) as well as the physicochemical properties of each adjuvant were considered. Resiquimod is a small molecule drug that is an analogue of the nucleotide base guanine. It has a molecular weight of ~350 kDa, is fairly hydrophobic with an octanol/water partition coefficient of nearly 20 (log P=1.3) and is completely insoluble in non-acidic aqueous solutions. In contrast, polyI:C is nucleotide polymer of the nucleotide base, cytidylic acid, and the base precursor, ionositic acid. The molecule is quite large and polydisperse, with the number of bases in the chain ranging from 2000 to 8000 pairs. It is also quite hydrophilic with a high aqueous solubility. Because the immunological response of OVA-specific CD8 T cells is directly related to the time course of the concentration of these agents, it would appear that the greater hydrophobicity of RES relative to polyI:C is an important factor for the initial delay in the peak as well as the prolonged, enhanced immunological response.

Measuring the Immune Response to Vaccination

In considering the observed immune response to the controlled release of vaccine antigen and adjuvant by the novel PCL-POE polymer, it is important to examine the properties of the model protein antigen, chicken ovalbumin (OVA), in more detail. OVA was chosen, because reagents are readily available that allow tracking of the CD8 T cell response specific for the predominant CD8 epitope, SIINFEKL, contained within the OVA protein. This reagent consists

of a multimer of biotin-conjugated MHCI molecules specific for SIINFEKL. The SIINFEKL peptide is loaded into the MHCI protein, and the monomer units are bound together by conjugation to a streptavidin core (Figure 6). The purpose of having a multimer is that T cells rarely are specific for a single peptide:MHC complex. That is, perhaps as little as 0.1% of the cells in the blood of a typical B6 mouse are specific for any given peptide:MHC complex.[74] With a complete expansion of these antigen-specific T cells by vaccination, characterized by approximately 11 doubling events over a 6 to 7 day period, this number could increase to between 20,000 and 200,000+ cells. Despite such a large expansion, the relative percentage of these cells in the blood remains low due to dilution and the localization of T cells to the secondary lymphoid organs. For these reasons, detection of a population of T cells by a monomer peptide:MHC complex has proven difficult and unreliable (REF). Therefore, use of a multimeric complex of a specific peptide:MHC molecule results in increased reliability and improved detection of an antigen specific T cell population.

PCL-POE/OVA/RES vaccine-induced maturation of dendritic cells

Dendritic cells (DCs) are specialized antigen presenting cells (cf Figure 1). They circulate within the skin and extracellular spaces of the body. In their immature state, they specialize in phagocytosis, continuously sampling the surrounding environment for the presence of foreign antigens. Dendritic cells undergo maturation and activation by exposure to microbial agents or inflammatory mediators (i.e. adjuvants), marked by their increased expression of major histocompatibility complex (MHC) molecules and co-stimulatory molecules such as CD80 and CD86. Upon phagocytosis or pinocytosis of a foreign body or antigen, the antigen enters into either the endolysosomal (phagocytosis) or cytosolic (pinocytosis) compartments. In both cases, the foreign protein antigen is digested by protease enzymes, and peptide epitopes are loaded onto either an MHCII or MHCI molecule, respectively. Dendritic cells are often referred to as professional antigen presenting cells, because they have the unique ability to transfer digested

proteins and peptides that have entered via the endosomal pathway into the cytosol for loading onto MHCI molecules. This is an important pathway when the antigen is a whole protein (i.e. Ovalbumin) because whole proteins contain both CD4 and CD8 epitopes, requiring loading onto MHCII and MHCI molecules, respectively. The DC, now in a mature state, presents the peptide:MHC complex on its surface, along with co-stimulatory molecules, and is primed to interact with and activate antigen-specific CD4 or CD8 T cells. As described earlier, the T cells continuously re-circulate throughout the secondary lymphoid organs in search of their cognate antigen. The primed DC simultaneously scans the paracortical (T cell) area of a lymph node in search of T cells with T cell receptors specific for the presented peptide:MHC complex. Upon interaction with the DC, the naïve antigen-specific T cell is activated to proliferate and develop into an antigen-specific effector T cell.

Evidence of the intrinsic ability of various polymer vehicles to activate the innate immune response, especially dendritic cells, has been well documented in the literature.[75, 76] Based on these considerations, the next set of experiments was carried out to assess the innate immune response of OVA/RES in the PCL-POE polymer system. Mice were vaccinated (a) with OVA antigen and resiquimod in the PCL-POE polymer administered as a single subcutaneous injection, (b) with OVA and resiquimod in PBS given subcutaneously and (c) repetitively as four daily bolus injections (positive control), or (d) with a single injection of PCL-POE polymer alone (figure 7). The innate immune response was evaluated by measuring the expression of the markers of dendritic cell maturation, MHCII, CD80, and CD86. At each time point, mice were sacrificed, their lymph nodes harvested, and dendritic cells were identified by expression of CD11c.

The results are given for a single mouse that was representative of the average response of each group, and Figure 8 depicts the percentage of dendritic cells expressing MHCII, CD80, and CD86 on days 7 and 14. On day 7 post-vaccination, MHCII expression was increased in all treatment groups relative to the saline (negative) control group. In the group of mice vaccinated

with saline, MHCII was expressed on approximately 7 % of dendritic cells, while in the three treatment groups; MHCII was expressed on 32 to 44 % of the dendritic cells. All three groups had statistically significant greater expression of MHCII as compared to saline treated mice ($p < 0.05$). The pair-wise difference between treatment groups was not statistically significant.

CD86 expression followed a similar pattern as MHCII on day 7. Mice vaccinated with saline expressed CD86 on 15 % of dendritic cells, while mice in the three treatment groups expressed CD86 on 32 to 41 % of cells. There was no significant difference between any of the groups in the percent expression of CD80 on dendritic cells. There was, however, a small, clear population in the PCL-POE polymer only group that showed an increased expression of CD80, with 28 % of DCs expressing CD80 as compared to 20 % in the saline treated group.

On day 14, the percentage of dendritic cells expressing MHCII was still somewhat higher in the groups receiving four repetitive boluses of OVA and resiquimod in PBS (positive control) and PCL-POE polymer alone. The percent of DCs expressing MHCII in these groups was 21.9 and 22.8 %, respectively, compared to 17 % in the group receiving saline. The group of mice receiving OVA and resiquimod in the PCL-POE polymer, as a single subcutaneous dose, continued to display a higher percent of DCs expressing MHCII, which was at 31.2 %. This difference was a statistically significant ($p < 0.05$) relative to all other treatment groups. The percentage of DCs expressing CD86 remained greater in mice receiving OVA and resiquimod in PCL-POE polymer and PCL-POE polymer alone, both at approximately 30 %, while the group of mice receiving four repetitive boluses of OVA and resiquimod in PBS had only 20 % of DCs expressing CD86, which was equivalent to that seen in the saline control group. By day 14, expression of CD80 by DCs had returned to near baseline levels in all groups, although there remained a small but distinct population of DCs in the group of mice vaccinated with OVA and resiquimod in PCL-POE polymer that still had an elevated expression, but the difference was not significant.

The ability of the PCL-POE/OVA/RES vaccine to stimulate prolonged dendritic cell maturation is significant, because it is the first step required for activation of a prolonged T cell response. The maturation of the DCs essentially acts as the rate-determining step for the initial activation of antigen-specific naïve T cells. Of further interest was the finding that, in mice receiving polymer alone, there was also a prolonged period of DC maturation. This indicates that the polymer itself is being recognized by the innate immune system (DCs, macrophages, neutrophils) as foreign, resulting in an adjuvant-like response to the polymer. The intrinsic adjuvant activity of the PCL-POE polymer is not unique, since other polymers also demonstrated with effect when used as a vaccine vehicle.[77,78]

Generation of OVA-specific Antibodies in Response to Vaccination with OVA and Resiquimod in PCL-POE Polymer

Long-term antibody production is one of the hallmarks of effective vaccination and is an important characteristic of immunological memory.[79] There have been many mechanisms proposed to explain the phenomenon of long-term antibody production and maintenance following infection or vaccination. The phenomenon can arise from low level, persistent exposure to antigen (i.e. a low-grade chronic infection) or from multiple and repeated exposures to antigen.[80] Both have the common requirement of continual stimulation of antigen-specific memory B cells to proliferate and differentiate into antibody-secreting plasma cells. Based on the *in vitro* release profile with the caveat in extrapolating the data, it seemed reasonable to expect that low levels of OVA and RES would persist in vivo following the initial burst release. If true, then the exposure to these antigens would resemble that seen with many chronic infections. Maintaining persistent antigen levels is important for the B cell and antibody responses as evident in the work of Murphy and Ohlfest, et al[81], in which the presence of plasma cells along with IgG were observed in the brain of vaccinated mice with established gliomas. Thus, the next set of

experiments addressed the impact of vaccination with the PCL-POE/OVA/RES vaccine on the persistence of antibody production and maintenance in the blood stream.

The anti-tumor antibody response was evaluated by measuring the anti-OVA IgG and IgM levels following vaccination. In this study (Fig. 9), mice were vaccinated with (a) OVA antigen and resiquimod in the PCL-POE polymer administered as a single subcutaneous injection, (b) OVA antigen alone in the PCL-POE polymer administered as a single subcutaneous injection, (c) OVA and resiquimod in PBS given subcutaneously and (d) repetitively as four consecutive daily bolus injections (positive control), or (e) saline. The anti-OVA IgG and IgM antibody levels measured using an enzyme-linked immunosorbent assay (ELISA). At each time point, plasma was obtained from whole blood collection in heparinized conical tubes. The samples were run in triplicate and an average result was recorded for each individual mice. The results of this study are shown in Figure 10, where the mean and standard error of the mean (SEM) of each treatment group are shown.

Mice treated with OVA and resiquimod in PCL-POE polymer by single subcutaneous injection developed an anti-OVA IgG and IgM antibody response that was markedly increased over that of any other treatment group. The antibody response peaked at approximately five weeks (day 34), although as early as day 20. It remained elevated at relatively high levels for a period of two to three months before undergoing the contraction phase. The increase in anti-OVA antibodies in this group was statistically significant at each time point measured from day 20 and throughout the remaining time points. The antibody response for 250 days post-vaccination was measured, and mice treated with OVA and resiquimod in PCL-POE polymer had a statistically significantly higher maintenance level of anti-OVA antibodies compared to all other treatment groups.

These results are very encouraging in demonstrating that the PCL-POE system results in the presence of persistent antigen levels as reflected in the B cell and antibody response. Additional experiments are needed to determine the effect of the rate and duration of exposure to

persistent antigen on the rate of conversion of naïve antigen-specific B cells into memory B cells. Such studies will provide clarification of the effect of persistent vaccine antigen on the kinetics of this physiological process. In addition, studies of the rate of change of antibody affinity with time would allow for further understanding how persistent vaccine antigen alters the relative portions of antigen-specific naïve B cells that differentiate among the three different B cell effector pathways.

OVA-specific CD8 T-cell response to Vaccination with OVA and Resiquimod in PCL-POE Polymer

To study the effects of sustained release of OVA on the T-cell mediated arm of the immune system, the percent of OVA-specific CD8 T cells was measured at specified time points over a four month period in a *non-tumor* vaccination model (Fig 9). Mice received (a) a single dose of OVA and resiquimod in PCL-POE polymer, (b) a single dose of OVA alone in PCL-POE polymer, or (c) four consecutive daily doses of OVA and resiquimod in PBS. All vaccinations were given subcutaneously (SQ). At each time point, whole blood was collected from individual mice and stained with antibodies against CD3, CD8, CD44, and OVA dextramer. Samples were examined by flow cytometry, and the percent of OVA-specific CD8 T cells was obtained using the gating scheme shown in figure 11. The results are shown in figure 12 and are expressed as the mean (+/-) SEM percent of OVA-specific CD8 T cells as a function of time.

Mice vaccinated with OVA and resiquimod in PCL-POE polymer had a slightly slower onset to peak response at day 13, with the average percent of OVA-specific CD8 T cells just above 1% of all CD8 T cells in the blood. This average percent was maintained within the range of 0.7 to 1.2 percent over the remaining time. While no statistically significant differences were seen between groups, mice receiving polymer-OVA-RES maintained, on average, peak levels of OVA-specific CD8 T cells for a period of 2 to 3 months before the onset of the contraction phase. The group of mice receiving OVA alone in PCL-POE polymer showed a mean peak percent of

OVA-specific CD8 T cells of 1.2 % on day 7 post-vaccination. This was followed by contraction and maintenance phases typical of an acute infection. Surpisingly, mice vaccinated with four repetitive once daily boluses of OVA and resiquimod had a response that was similar to saline treated mice.

In this study, mice receiving OVA and resiquimod in PCL-POE polymer appeared to be divided into two groups based on their response to the vaccine. Thus, the mice were divided into "responders" or "non-responders", based on the development of a peak percent of OVA-specific CD8 T cells above 1 %. The data for mice characterized as responders is plotted in figure 13 where the percent of OVA-specific CD8 T cells is given as a function of time for each individual mouse. Mice, considered to be responders, achieved a peak OVA-specific CD8 T cell response of at least 1 %. At each time point measured, whole blood was collected from individual mice and stained with antibodies against CD3, CD8, CD44, and OVA dextramer. Samples were subjected to flow cytometry, and the percent of OVA-specific CD8 T cells was obtained using the gating scheme shown in figure 11, and the percentage of OVA-specific CD8 T cells in the blood is plotted versus time. The initial peak percent of OVA-specific CD8 T cells in the blood ranged 1 to 5 % and occurred between 2 to 6 weeks post-vaccination. Three of four mice continued to maintain the percent of OVA-specific CD8s in their blood for the remainder of the measured time points. In mouse 2, the percentage of OVA-specific CD8 T cells in the blood continued to rise, reaching a secondary peak of approximately 7 % at 3 months post-vaccination, the last time point measured.

There are a couple of potential explanations for the observed differences between responding and non-responding mice, discounting human error upon vaccine preparation or injection, and inconsistency in the rate of polymer degradation or OVA and resiquimod release. One possibility would be the occurrence in natural heterogeneity in the immune response to a vaccine. The natural tendency towards heterogeneity of an immune response to vaccine is implicated at multiple points in the development of the immune response. The number of naïve

antigen-specific CD8 T cells present in an antigen naïve mouse can vary by fifty to one hundred percent between mice. The average number of OVA-specific naïve CD8 T cells in Bl-6 mice can vary from approximately 50 to 200 cells.[82] In addition, antigen-specific naïve CD8 T cells will proliferate between 10 and 13 times in response to antigen. This can lead to a 10-fold difference in the peak number of antigen-specific CD8 T in the blood stream on day 7 post-vaccination. These possibilities are supported by the clear difference in the magnitude and duration of the OVA-specific CD8 T cell response observed between "responders" and "non-responders". Mouse 2, a "responder", is a perfect example of the difficulty we faced in predicting the release of OVA and resiquimod from the PCL-POE polymer *in vivo* and the associated CD8 T cell response observed. In this mouse alone, it is possible that the "secondary" peak we observed at around three months post-vaccination is a combination of a re-activation of OVA-specific memory CD8 T cells due to a "secondary" burst release from the PCL-POE polymer.

Another potential explanation for the heterogeneity of the responses is the unknowns created by working with a novel slow-release vaccine. Given the uncertainties associated with the rate and extent of antigen and adjuvant release from the polymer, it is possible that different mice may have experienced peak responses at unmonitored time points or that the trafficking of the cells to the tissues may have occurred at different times relative to vaccination in the individual mice. However, this possibility is less likely given that the majority of the mice showed either a solid response or lack thereof at each time point measured. That is, in only one mouse did we observe a response at one time point and not at the others or vice versa.

Due to the lack of a quality reagent for detecting OVA-specific CD4 T cells, we limited our analysis of the OVA-specific immune response to that of OVA-specific B cells (and anti-OVA antibodies) and OVA-specific CD8 T cells. However, the magnitude and persistence of the anti-OVA antibody response we observed is a good, albeit indirect, marker for the existence of an OVA-specific CD4 T cell response. The delay in the development and peak of antigen-specific antibodies occurs because B cells specific for a protein antigen cannot be activated to proliferate,

form germinal centers, or differentiate into plasma cells until they encounter a CD4 (helper) T cell that is specific for one of the peptides derived from that antigen. As discussed below, the prolonged time frame for activation and proliferation of CD4 T cells in mice treated with polymer vaccine suggests that the delay, seen in the peak antibody response as well as the prolonged duration of response, is likely a combination of secondary exposure of antigen-specific memory B cells to their cognate antigen as well as the initial activation of naïve antigen-specific B cells and their simultaneous interaction with activated helper CD4 T cells specific for the same antigen. The potential role of antigen-specific CD4 T cells on the stimulation of the humoral immune response in response to persistent release of antigen and adjuvant warrants further exploration as the necessary reagents become available.

Overall Survival in Response to Vaccination with PCL-POE/lysate/RES Vaccine in a Mouse Model of Glioma

To address the impact of the PCL-POE polymer-based vaccine on overall survival in a mouse model of glioma, mice were inoculated with a GL261 murine glioma cell line. Because this cell line contains thousands of potential antigens, developing a vaccine which would target these potential antigens required that we rely on another type of antigen, whole cell lysates, for incorporation into the vaccine. Although lysate-based vaccines were most clinically relevant at the time of these experiments, analysis of any antigen-specific T cell response to any peptide:MHC complex was not possible because the specific antigens within the lysates had yet to be identified.

For this study (Fig. 14), we used an orthotopic glioma model in which we inoculated mice with a 1 µL suspension containing 15,000 GL261 cells into the right hemisphere of the brain, according to the coordinates provided. Tumor development and growth were assessed routinely by bioluminescent imaging (BLI). Glioma-bearing mice were euthanized when they developed symptoms such as hunching, listlessness or cranial deformation. Tumor presence and

rate of growth was verified by BLI imaging on days 3 and 7 post-inoculation, prior to treatment group randomization or vaccination. Mice were excluded from the study if there was an absence of tumor on either day or if the tumor intensity by BLI imaging was less than 10^5 photons/cm^2/sec.

Whole cell lysate vaccinations were derived from GL261 cells grown in 5% O_2.[83] Mice were vaccinated seven days after inoculation of GL261 glioma tumor cells (Fig. 14). Mice were divided into groups on the day of vaccination based on bioluminescent imaging (BLI) data. Mice were categorized as having either a low, medium, or high intensity tumor. Mice from a single intensity group were then divided randomly into treatment groups so that each treatment group contained mice with an average and range of tumor sizes (intensities). Treatment groups include (a) saline, (b) lysate and RES in PBS (lysate/RES; positive control) given as four repetitive once weekly doses, (c) polymer and lysate only (PCL-POE/lysate) given once, and (d) lysate and resiquimod in PCL-POE polymer (PCL-POE/lysate/RES) given once. Mice in the PCL-POE/lysate/RES group received either one or two doses of the vaccine. Both groups received a single dose of polymer containing 10 million killed cells and 200 μg of RES on day 7 post-inoculation. The group receiving 2 doses was vaccinated a second time two weeks following the first vaccination, again receiving 10 million cells and 200 μg of RES. Mice receiving lysate and resiquimod in PBS were given the same total dose of 10 million killed cells and 200 μg of RES. This dose was divided into four separate vaccinations and mice were vaccinated on days 7, 14, 21, and 28 post-inoculation. All vaccinations were administered subcutaneously.

In the glioma survival model (Figure 15a), complete remission (cure) was observed in one of six mice (16 %) receiving lysate only in PCL-POE polymer, in one of six mice (16 %) receiving two doses of lysate and resiquimod in PCL-POE polymer, and in three of fourteen mice (21 %) receiving one dose of lysate and resiquimod in PCL-POE polymer. The mice achieving complete remission were followed for an additional fifteen months. No signs or symptoms of relapse was observed in any of the mice at the time of sacrifice. No mice in any other group

survived beyond 81 days post-inoculation. Interestingly, there was no difference seen among any of the treatment groups in the length of median survival. Thus, mice appeared to either achieve complete remission (cure) or experienced no benefit from treatment. Bioluminescent imaging (BLI) data of mice receiving a single vaccination of lysate and resiquimod in PCL-POE polymer is shown in figure 15b. The results shown are from one of three independent experimental replicates. Mice achieving complete remission were sacrificed, and none showed any signs or symptoms of disease at the time of sacrifice.

Perhaps surprisingly, observation of the survival data revealed that the value of a second vaccination with lysate and resiquimod in PCL-POE polymer was minimal, as percent overall survival in the group receiving one vaccination of lysate and resiquimod in PCL-POE polymer was 21 % as compared to 16 % of mice receiving a second dose. However, this data must not be over-interpreted given that the number of mice in the group receiving a second dose was only six and was only included in the last of the three replicates of the experiment. The second vaccination did appear to induce a temporary regression in a small percentage of mice treated (Fig 15c). However, despite the apparent regression induced by the second vaccination, the tumor quickly advanced within a period of eight to twelve weeks following the second vaccination resulting in death in all but the sole mice achieving complete remission. The lack of any significant improvement in survival with a second vaccination may indicate that past a certain point, providing further persistent antigen and adjuvant does not lead to further improvement in survival. However, additional replicates of this experiment may be necessary to better assess the value of adding a second dose of the polymer vaccine.

Finally, we observed that the group of mice receiving lysate and resiquimod in PBS as four repetitive bolus vaccinations consistently experienced a faster rate of tumor growth (Figs 15d & 15e) and death than mice receiving saline control. These results were replicated in the three independent experiments. We believe that one potential explanation for this unexpected finding was the result of the different pharmacokinetics of resiquimod when given as a bolus vaccine as

compared to its incorporation into the PCL-POE semi-solid polymer. We hypothesized that the increase in local concentration and higher dose of RES resulting from a bolus dose of vaccine resulted in more widespread activation of TLR7/8 expressing cells of the immune system. In addition to neutrophils and dendritic cells of the innate immune system, which coordinate the T cell response to antigen, additional cells of the immune system have been found to express TLR7/8. One type of immunosuppressive cell, myeloid derived suppressor cells (MDSCs), has been shown to express the TLR7/8 receptor and to be activated in response to RES exposure.[84,85] An increase in the activation of MDSCs was shown to result in poorer survival rates in numerous pre-clinical[86] and human cancers.[87,88] It is possible that delivery of RES by its incorporation into the polymer vehicle resulted in lower local concentrations of RES and therefore a decrease in the number of MDSCs and/or an increase in the ratio of DCs to MDSCs that are activated. Future studies to determine the effect of RES doses and vaccination regimens on the preferential activation of different immune cells will be necessary to optimize the overall potential of this adjuvant and the timing of its release from polymer vehicles.

Non-specific CD4 and CD8 T-cell response to PCL-POE/lysate/RES Vaccine

As discussed above, the GL261 glioma cell line contains thousands of potential antigens. Therefore, we utilized a whole cell lysate vaccine developed from the same tumor cell line in order to stimulate an anti-tumor T cell response specific for GL261 antigens. Although lysate-based vaccines are a clinically relevant form of anti-cancer vaccine, analysis of the antigen-specific T cell response to any specific peptide:MHC complex is not possible because the specific antigens within the lysates have yet to be identified. Therefore, to understand the link between the vaccine-induced immune response kinetics and overall survival, a method for detecting the T cell response to the lysate vaccine was needed. For this, we relied upon a non-specific method for detecting activated (proliferating) T cells, which allowed measurement of the CD4 T cell response in addition to the CD8 T cell response.

The method relies upon use of the nuclear protein Ki67. When cells are actively proliferating, the nucleus up-regulates the protein Ki-67, which is strictly a marker of proliferating cells. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis) but is absent from resting cells (G_0).[89] Thus, Ki67 is an excellent marker for determining the so-called growth fraction of a given cell population.[90] In an average, healthy cell population, such as CD4 or CD8 T cells, the average expression of Ki67 ranges from three to ten percent of the cells.[91] In cells that are activated to proliferate, as would be the optimal response of immune cells to a vaccine, the percentage of cells of a single type, ideally CD4 or CD8 T cells that are expressing Ki67 would be expected to increase sharply beyond that of non-proliferating cells. Therefore, we used Ki-67 as a marker for the ability of the PCL-POE/lysate/RES vaccine to activate CD4 and CD8 T cells.

Although the mice are housed and treated under sterile conditions, the immune system is continually responding to minor antigens, and thus there remains the possibility that non-tumor specific T cells are proliferating in response to non-tumor antigens. Therefore, use of a saline treated group of mice as a negative control allowed detection of the relative percentage of CD4 and CD8 T cells that were activated in response to PCL-POE/lysate/RES vaccination.

In this study, we vaccinated tumor-bearing mice seven days after they had been inoculated with glioma cells (Fig. 14). Groups of mice that underwent immune analysis were vaccinated with (a) saline, (b) lysate and RES in PBS (lysate/RES; positive control) given as four repetitive once weekly doses, (c) polymer and lysate only (PCL-POE/lysate) given once, and (d) lysate and resiquimod in PCL-POE polymer (PCL-POE/lysate/RES). The results of this study are shown in figures 16a and 16b. Figure 16a shows the percentage of proliferating, Ki67+ CD8 T cells, at two week intervals starting at day 7 post-vaccination. The group of mice vaccinated with lysate and resiquimod given as four repetitive once weekly doses showed a weak peak at day 7

post-vaccination that was very similar to the increase in KI67+ proliferating CD8 T cells that was seen in the saline treated group. However, this group developed a “secondary” peak on day 21 post-vaccination that was statistically greater compared to the saline treated group ($p < 0.05$). The marginal increase in the percent of proliferating CD8 T cells observed in saline treated mice is likely the result of local inflammation at the injection site.

Mice treated with lysate and resiquimod in PCL-POE polymer developed a peak percent of Ki67+ cells on day 7 post-vaccination that was greater than that of mice receiving soluble vaccinations. It was our expectation that mice receiving the four consecutive bolus lysate/RES vaccines would have a higher percentage of proliferating CD8 T cells on day 7 post-vaccination because, as previously stated, this is the time of the peak response in the blood after an acute infection, which is most closely resembled by the bolus vaccinations. However, the percentage of KI67+ cells in this group underwent a contraction phase after the day 7 peak, reaching baseline levels at 5 weeks post-vaccination. This represents a stark contrast to the extended duration of time over which OVA-specific CD8 T cells were maintained in the blood following initial vaccination with OVA and resiquimod in PCL-POE polymer. This difference may be a consequence that vaccinations were injected into tumor-bearing mice. The glioma-specific CD8 T cells that were activated and had undergone rapid proliferation and differentiation to effector CD8 T cells would acquire the ability to traffic into the tissues; in this case, the brain and to the site of the target glioma cells. Thus, the percentage of cells present in the blood in the presence of disease is not the best indicator of the immune response to the vaccination. However, given the trafficking of effector cells via the blood stream on their way to the target site, the data obtained from blood analysis still provides some value, because the correlation between the percentage of proliferating CD8 T cells in the blood and overall survival in glioma can be assessed.

The CD4 T cell response to vaccination is shown in figure 16b. Mice vaccinated with GL261 lysate and resiquimod in PCL-POE polymer developed a slow response to vaccine, exhibited by the percent of Ki67+ cells in the blood on day 7 that was less than that of saline

treated mice. However, mice in the polymer group developed a delayed peak response, demonstrated by a statistically significant increase in the percentage of proliferating CD4 T-cells in the blood on day 21 as compared to mice vaccinated with weekly bolus injections of GL261 lysates and resiquimod. Mice treated with weekly bolus injections of GL261 lysates and resiquimod showed no difference in the percent of Ki67+ CD4 T cells in the blood at any time point compared to saline treated mice.

The CD8 and CD4 T cell responses were consistent with previous reports showing that antigen-specific CD8 T cells are best activated by a single, acute dose of vaccine (or exposure to pathogen), while CD4 T cell responses are slower to develop a response to the same level of antigen exposure.[92] CD8 T cells are intrinsically programmed to have a faster rate of cell division than CD4 T cells due to the expression kinetics of cell-cycle derived growth factors.[93] In addition, the duration of antigen exposure required to stimulate rapid proliferation of naïve antigen-specific CD8 T cells has been shown to be much less than that required for naive antigen-specific CD4 T cells.[94, 95] The results observed in the above experiment are consistent with these findings. It is possible that vaccinating with repetitive bolus doses of lysate and resiquimod vaccine once weekly resulted in an initial CD8 T cell response similar to that of an acute infection. Over the first week post-vaccination, memory CD8 T cells would start to develop, such that each of the next three weekly vaccinations would stimulate a secondary immune response to the lysate antigens, exemplified by the later peak in percent of Ki67+ CD8 T cells observed in this group on day 21 post-vaccination. The group achieving the greatest percent of Ki67+ CD4 T cells also experienced the peak response on day 21 post-vaccination. Again, this observation coincides with the evidence discussed above in that CD4 T cells have a slower intrinsic response to antigen exposure and also require a longer duration of antigen exposure in order to undergo a rapid proliferation response.

The difference between the soluble and polymer vaccines and the timing of their injection allow further inferences regarding the rate and mechanisms of antigen and adjuvant release from

the PCL-POE polymer *in vivo*. It seems likely that, following an initial burst release of lysate antigen and RES, the remainder of each is released at a slower and continuous rate. It remains possible that the slow and persistent release of the antigen and adjuvant is providing a secondary exposure of antigen to antigen-specific memory T cells that have formed. It is also reasonable that the number of memory cells was slowly accumulating with time as additional antigen (lysate)-specific naïve T cells are activated upon their initial exposure to antigen. This creates a scenario in which the PCL-POE/lysate/RES vaccine is continuously activating and re-activating antigen-specific cells with the persistent release of antigen and adjuvant from the polymer. Future studies of the effect of a slow release polymer on proliferating CD4 and CD8 T cells are required in order to best understand the kinetics of the immune response.

Correlation Between Vaccine-induced T Cell Response and Overall Survival

Although the primary aim of this work was to develop an efficacious vaccine for treatment of glioma, we additionally assessed the connection between the magnitude of the CD4 and CD8 T cell responses and overall survival in our mouse model of glioma. Using data from our survival experiment and immune response monitoring, we plotted the average percentage of Ki67 expressing CD8 (Fig. 17a) and CD4 (Fig. 17b) T cells over time in mice characterized as survivors, those in which a relapse was not observed following complete remission, and non-survivors. Data shown are compiled from the survival and immune response data from the experiment shown in figure 14. As the immune response to mice receiving two doses of PCL-POE/lysate/RES vaccine was not monitored, the mouse achieving complete remission in that group was not included in this analysis. Although there was no statistically significant correlation between the percent of Ki67+ CD8 T cells and survival, there was an observable difference between survivors and non-survivors on day 14 post tumor inoculation, 7 days after the initial vaccination. The percent of Ki67+ CD8 T cells in the blood of survivors declined over the next

few weeks, showing no difference in response compared to the non-survivors at any other time point measured.

Mice undergoing complete tumor remission (survivors) had an observable increase in the percent of Ki67+ CD4 T cells in the blood at each time point measured as compared to non-survivors. The biggest difference was seen on day 21 post-vaccination (day 28 post-tumor cell inoculation), as the percent of Ki67+ CD4 T cells in the blood of survivors was approximately 17 % as compared to approximately 12 % in non-survivors. This difference reached statistical significance ($p < 0.05$).

The peak in percent of proliferating CD4 T cells observed in the group vaccinated with lysate and resiquimod in PCL-POE polymer appeared to have an effect on overall survival. In contrast, the peak in percent of proliferating CD8 T cells observed in mice receiving repetitive once weekly bolus vaccinations of lysate and resiquimod did not appear to have a positive effect on survival. This finding is of particular interest because evidence from Nelson, et al[96] demonstrated that in a chronic infection of Salmonella typhimurium, continuous and local "release" of antigen at low levels resulted in a more stable antigen specific CD4 T cell response in which the development and persistence of memory CD4 cells was prolonged compared to that of an acute exposure to antigen. In addition, studies of cancer and chronic viral infections have shown that the presence of a strong CD4 response is an important factor in alleviating the onset of CD8 T cell exhaustion.[97,98]

CONCLUSION

Glioblastoma multiforme is a primary and devastating malignancy of the central nervous system. GBM, along with many other solid tumors, continue to have extremely poor prognoses despite the extensive research dedicated to finding a cure. Immunotherapy is a growing area of research that presents a novel approach for achieving a cure. The central premise for the use of anti-tumor vaccines is the potential of complete eradication cancer cells at the tumor site. The potential for tumor-specific immune cells to identify tumor cells beyond the visible mass provides support to the idea that a complete cure is possible. The reasons behind the current lack of success of anti-cancer vaccines are innumerable, ranging from poor choice of vaccine components to the presence of the immunosuppressive tumor environment. Here we have attempted to address the role of antigen persistence and altering the quantitative and qualitative nature of the immune response through the use of a novel PCL-POE polymer vaccine.

We have shown that the *in vitro* release of antigen and adjuvant from PCL-POE polymer was dependent upon the physiochemical characteristics of the antigen and adjuvant incorporated. We observed a burst release of both antigen and adjuvant that was likely the result of the high concentration of antigen and adjuvant that were incorporated into the polymer. In the both cases, the initial burst release was followed by a much slower phase of release over the next three weeks.

Incorporating antigen and adjuvant into the PCL-POE polymer as a vaccine allowed measurement of the effects of antigen and adjuvant release rates on the innate and adaptive immune responses. With the innate immune system, we observed that the slow release provided by the PCL-POE polymer stimulated the maturation of dendritic cells for a period of at least two weeks following a single vaccination. This was exemplified by the increased expression of MHCII and co-stimulatory molecule CD86 on the surface of the DCs. We also observed an intrinsic adjuvant effect of the polymer itself, as injection of the polymer alone caused an increased expression of MHCII and co-stimulatory molecule CD86 on the surface of the DCs.

Persistent antigen and adjuvant release from the polymer led to the development of a potent and long-lasting anti-OVA antibody response. This was perhaps most striking with the PCL-POE polymer vaccine. Given the vital role of antibodies in containing and eliminating disease, we believe that this finding supports further investigation of the cause and effect relationship between the rate and extent of antigen and adjuvant release and the stimulated antibody response. Future studies to determine the rate of conversion of naïve antigen-specific B cells into the effector cells, including memory B cells, short-lived plasma cells and long-lived plasma cells will be necessary in order to understand the effect of persistent vaccine antigen on the kinetics of this physiological process. In addition, studies of the rate of change of antibody affinity with time would allow for further understanding how persistent vaccine antigen alters the relative portions of antigen-specific naïve B cells that differentiate down the three different B cell effector pathways.

Finally, we have shown that mice vaccinated with antigen and adjuvant delivered by the PCL-POE polymer experienced a significant, potentially translatable increase in overall survival in a mouse model of glioma. Although the median survival was not improved with this treatment, twenty percent of mice treated with the polymer vaccine achieved long-term remission and a probable cure. This finding is consistent reports emanating from current clinical trials of immunotherapies in humans.[99] Of further interest was the finding of a correlation between the percent of proliferating CD4 T cells in response to vaccination and overall survival. This finding warrants further investigation given the evidence showing the positive effect of persistent antigen on the CD4 T cell response, the value of a strong CD4 response is an important factor in alleviating the onset of CD8 T cell exhaustion, and the direct role that CD4 T cells have shown in elimination of cancer and infected cells.[100,101]

Future work is necessary to better understand the effect of persistent antigen on the quantitative and qualitative nature of the anti-tumor immune response. In addition, considerable effort will be needed to unravel the effect of altered immune response kinetics, stimulated by

persistent antigen and adjuvant, on the mechanisms and effectiveness of tumor cell killing. Nevertheless, these findings have helped to clarify the link between persistent antigen provided by a polymer-based vaccine and survival in glioma and hopefully will stimulate further work to reach the ultimate goal of curing glioblastoma.

Figures

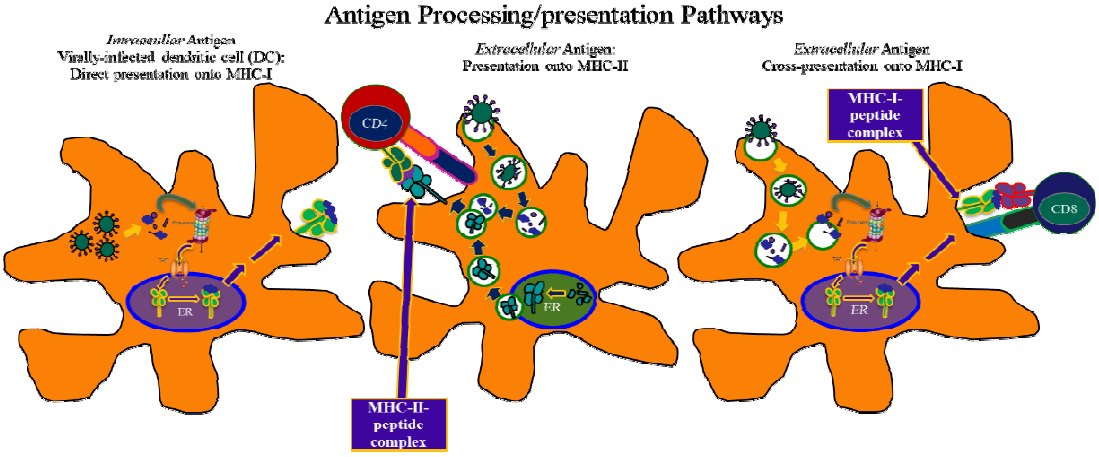

Figure 1) Schematic diagram of the antigen processing and cross presentation pathway in dendritic cells (DCs). Left) Viruses are internalized and processed in DCs; antigens are then shuttled to the endoplasmic reticulum (ER) and cross-presented by DCs through the MHC-I and MHC-II complexes. (Middle) CD4 cells (T-helper cells) recognize MHC-II presentation causing proliferation of CD4 cells and signaling CD8 cells. (Right) CD8 cells (killer cells) recognize MHC-I presentation and kill the organism.

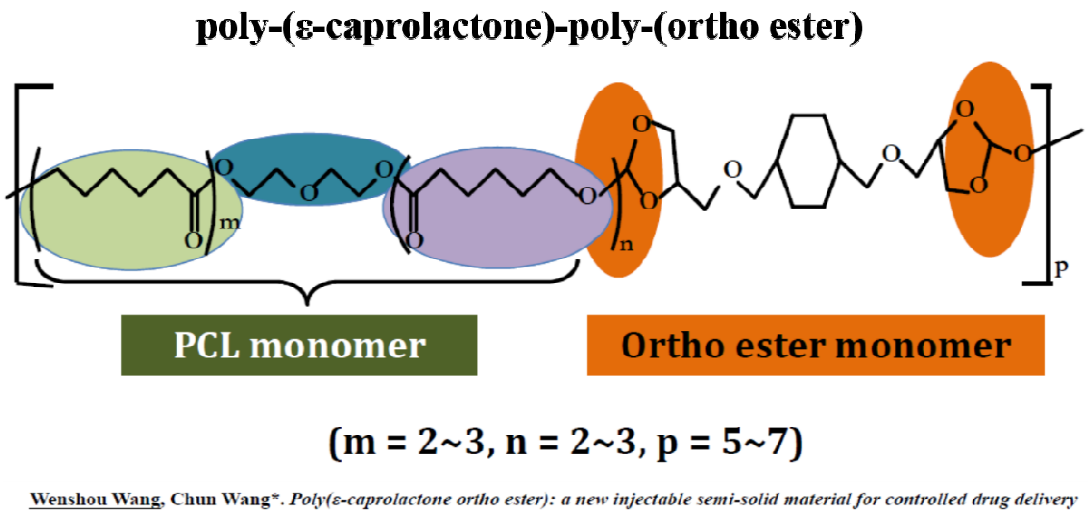

Figure 2) Schematic diagram of poly-(ε-caprolactone)-poly-(ortho ester) [PCL-POE] polymer

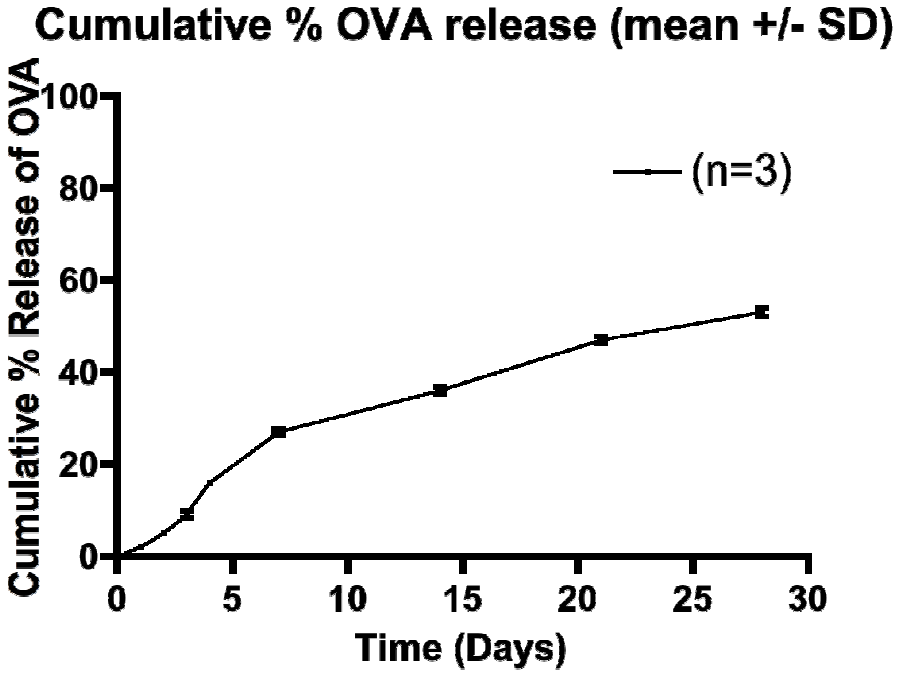

Figure 3a) Cumulative mean (+/- SD) percent release of Ovalbumin protein (OVA) from the PCL-POE polymer as a function of time. Experimentally, 1000 µg of OVA and 200 µg of RES were combined with 200 µl of the polymer and then placed into 10 mL of PBS in a 50 mL conical tube. The sample was then incubated at 37 °C while rotating at 200 RPMs. At each indicated time point, 2 mL of supernatant was collected and replaced with 2 mL of fresh PBS. The amount of OVA contained in each sample was estimated by the Bradford BCA (Pierce) protein quantification kit. The experiment was performed in triplicate.

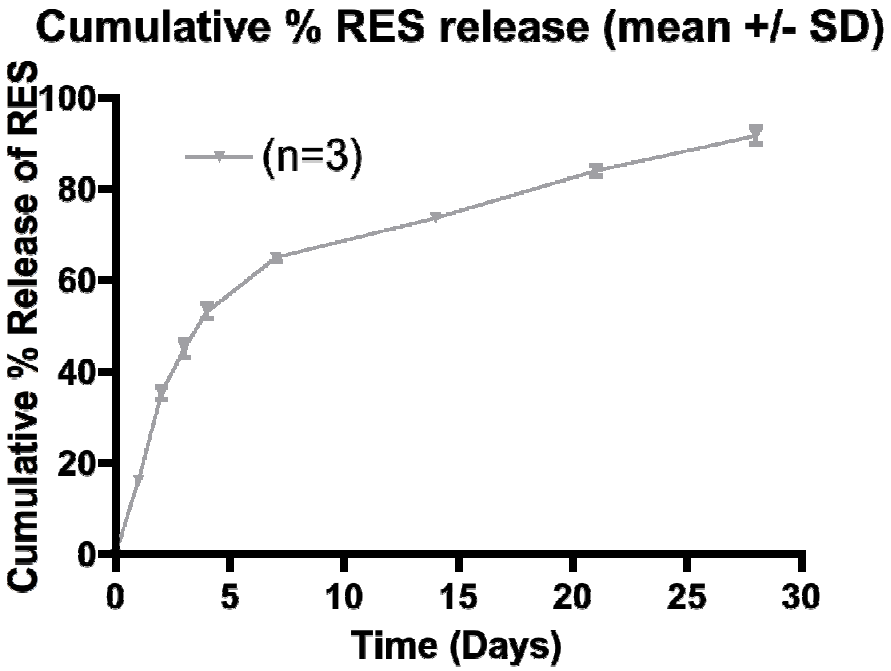

Figure 3b) Cumulative mean (+/- SD) percent release of resiquimod from the PCL-POE polymer as a function of time. Experimentally, 1000 µg of OVA and 200 µg of RES were combined with 200 µl of the polymer and placed in to 10 mL of PBS in a 50 mL conical tube. The sample was then incubated at 37° C while rotating at 200 RPMs. At each indicated time point, 2 mL of supernatant was collected and replaced with 8 mL of fresh PBS. This process was repeated for each time point. The amount of RES in each sample was measured using liquid chromatography-mass spectrometry (LC-MS). The experiment was performed in triplicate.

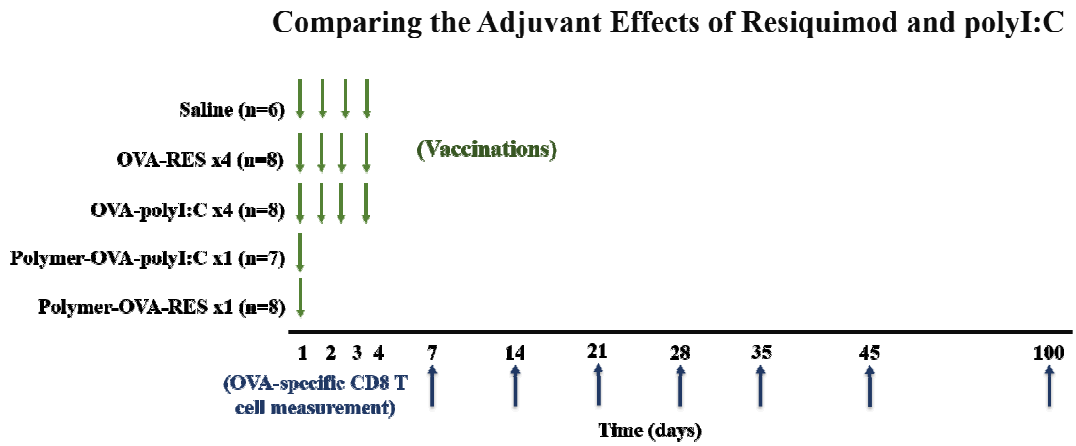

Figure 4) Schematic diagram of the experimental protocol used in comparing the OVA-specific CD8 T cell response to two adjuvants, resiquimod and polyI:C given as a bolus or polymer vaccine. Resiquimod treatment groups included OVA 200 µg and RES 50 µg in PBS (OVA-RES x4; positive control) given as four repetitive once daily doses, PCL-POE polymer and OVA 800 µg plus resiquimod 200 µg (polymer-OVA-RES) given once. PolyI:C treatment groups included OVA 200 µg and polyI:C 50 µg in PBS (OVA-polyI:C x4; positive control) given as four repetitive once daily doses, PCL-POE polymer and OVA 800 µg plus polyI:C 200 µg (polymer-OVA-polyI:C) given once. All vaccinations were given subcutaneously. OVA-specific CD8 T cell percentages were measured by flow cytometry on days 7, 14, 21, 28, 35, 45, and 100 post-vaccination.

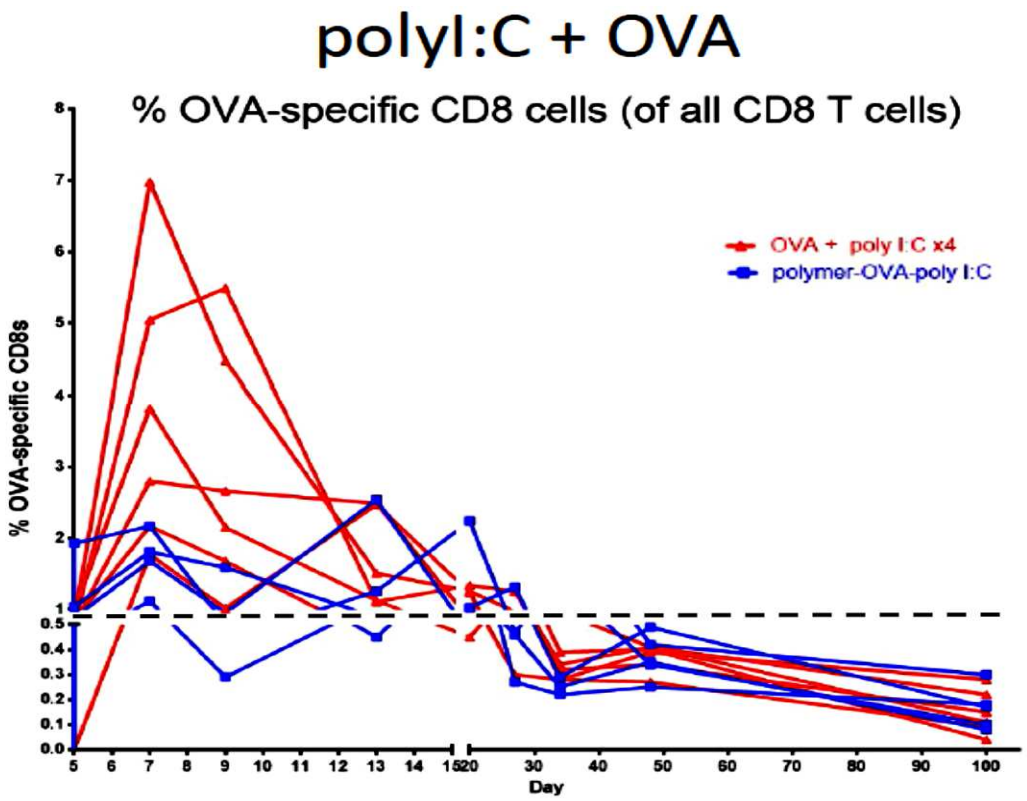

Figure 5a) Percent OVA-specific CD8 cells as a function of time for mice vaccinated with either (Red) OVA+polyI:C in PBS given subcutaneously as four repetitive once daily bolus injections or (Blue) OVA+polyI:C in PCL-POE given as a single subcutaneous injection. Total doses for OVA and polyI:C were equivalent in both groups.

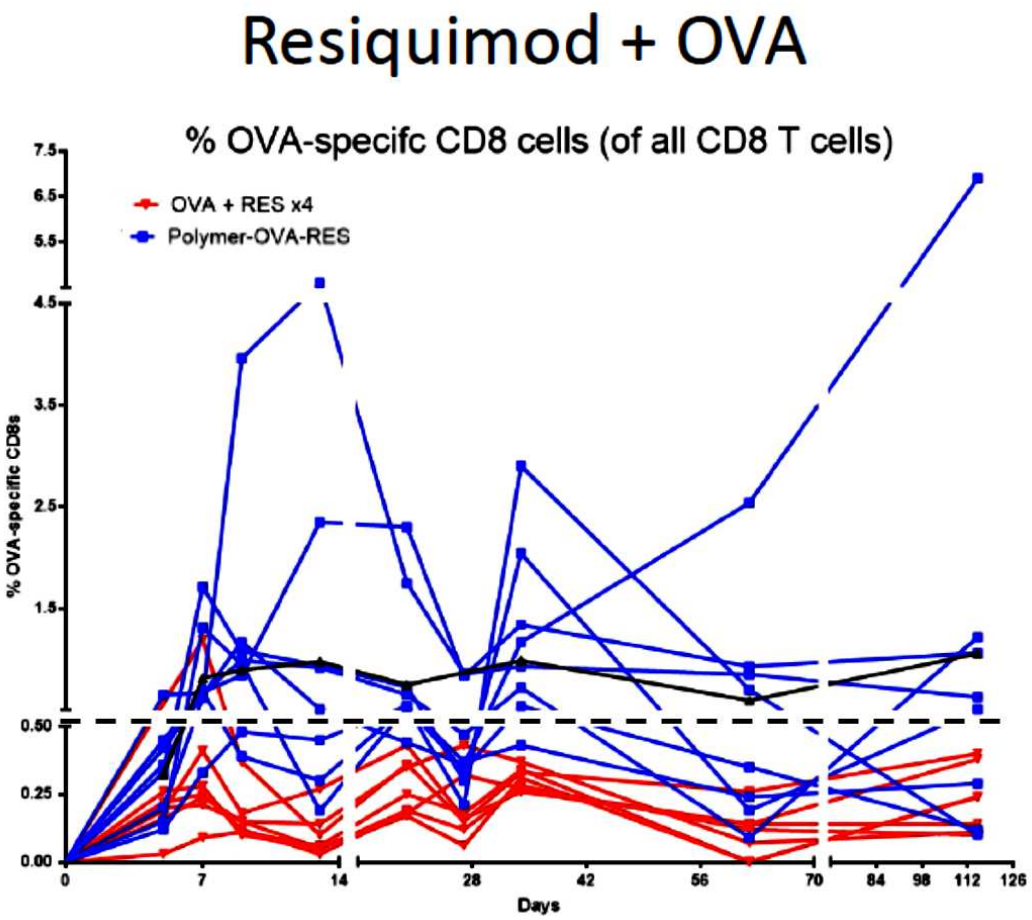

Figure 5b) Percent OVA-specific CD8 cells as a function of time for mice vaccinated with either (Red) OVA+RES in PBS given subcutaneously as four repetitive once daily bolus injections or (Blue) OVA+RES in PCL-POE given as a single subcutaneous injection. Total doses for OVA and RES were equivalent in both groups.

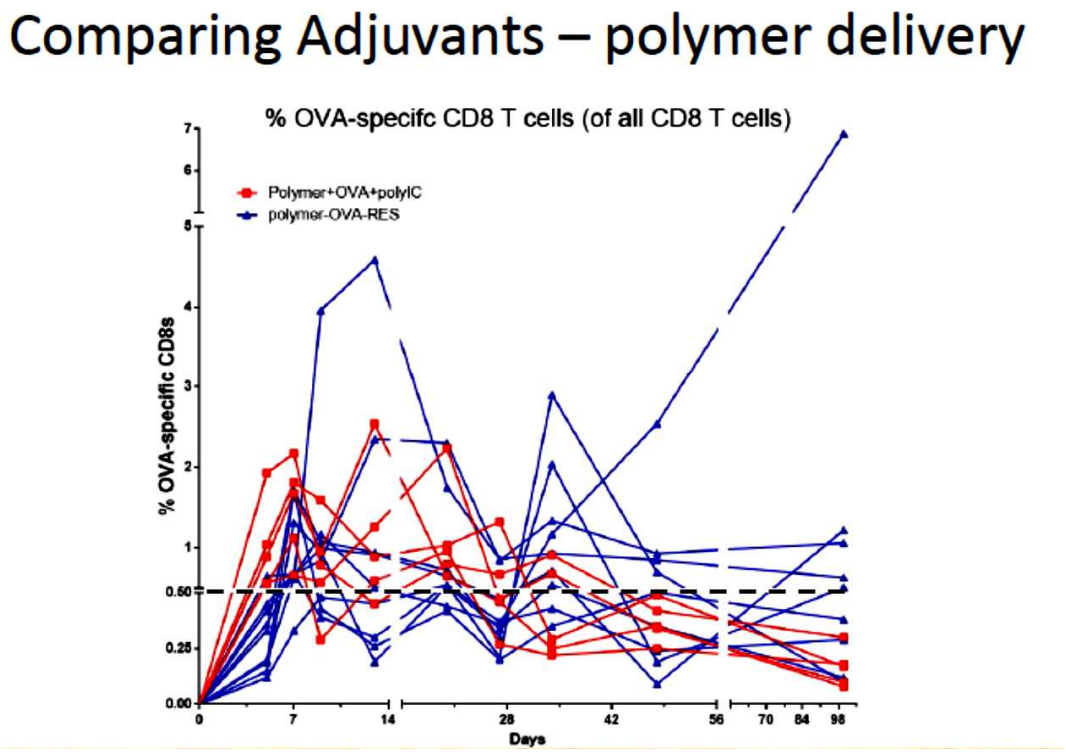

Figure 5c) Percent OVA-specific CD8 cells as a function of time for mice vaccinated with PCL-POE polymer containing OVA protein and either (Blue) RES or (Red) polyI:C adjuvant. Both vaccinations were given as a single subcutaneous dose, and the total dose of OVA and the dose of either RES or polyI:C were equivalent in both groups. The dashed line represents the mean percent of naïve OVA-specific CD8 T cells in a saline treated B6 mouse.

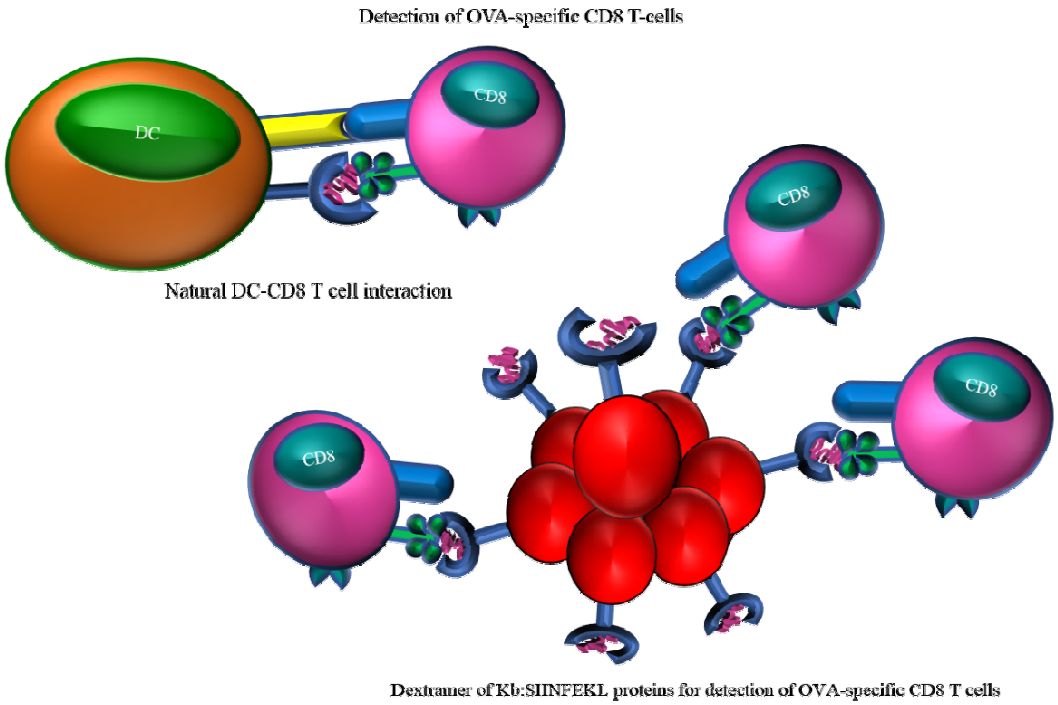

Figure 6) Schematic diagram of K^b:OVA dextramer method of identification of OVA-specific CD8 T cells. The K^b:OVA dextramer reagent used for identification of OVA-specific CD8 T cells consists of a multimer of biotin-conjugated MHCI molecules specific for SIINFEKL. The SIINFEKL peptide is loaded into the MHCI protein, and the monomer units are bound together by conjugation to a streptavidin core. The dextramer reagent mimics the OVA:MHCI-specific CD8 T cell receptor binding to the OVA:MHCI presented on the surface of dendritic cells.

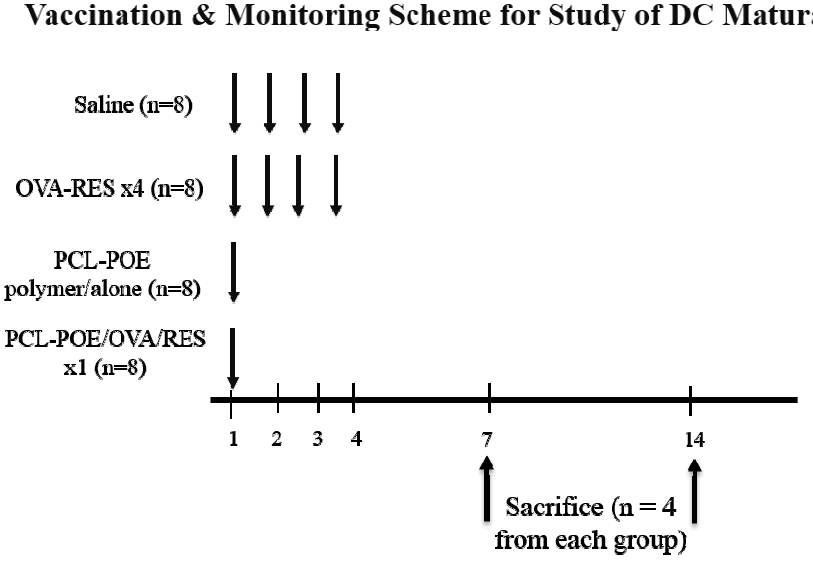

Figure 7) Schematic diagram of the experimental protocol used for vaccination and measurement of dendritic cell maturation *in vivo*. Treatment groups include saline, OVA 200 µg and RES 50 µg in PBS (OVA-RES x4; positive control) given as four repetitive once daily doses, PCL-POE polymer and OVA 800 µg only (polymer-OVA) given once, and PCL-POE polymer and OVA 800 µg plus resiquimod 200 µg (polymer-OVA-RES) given once. On days 7 and 14 post-vaccination, mice were sacrificed and the draining lymph nodes were harvested. The maturation of DCs, characterized by the percent expressing MHCII, CD80, and CD86 was measured by flow cytometry. DCs were initially separated by expression of CD11c.

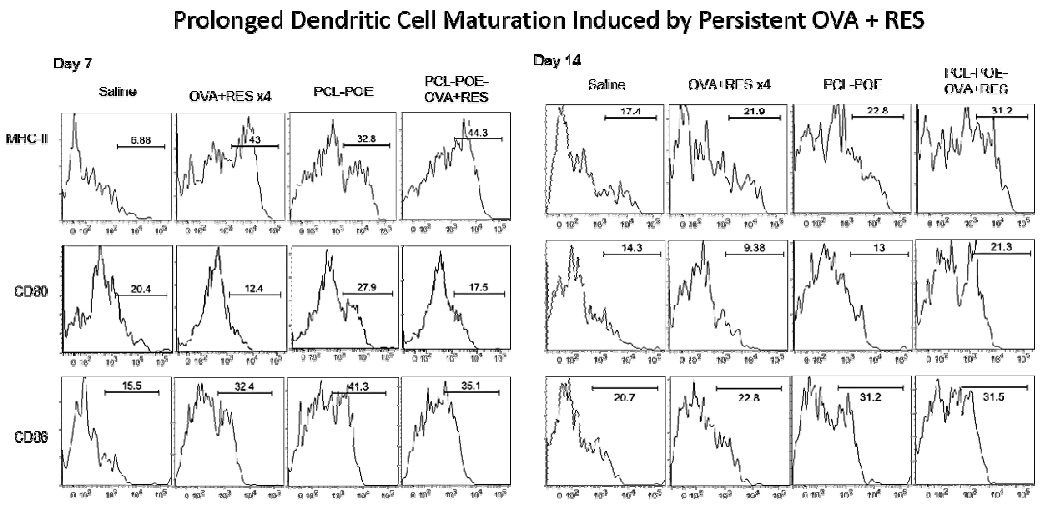

Figure 8 (a) Vaccination and Monitoring Scheme for Study of Dendritic Cell Maturation. (b) Induction and maintenance of DC maturation by vaccination with PCL-POE/OVA/RES, where the percentage of dendritic cells expressing MHCII, CD80, and CD86 is given on days 7 and 14 post-vaccination.

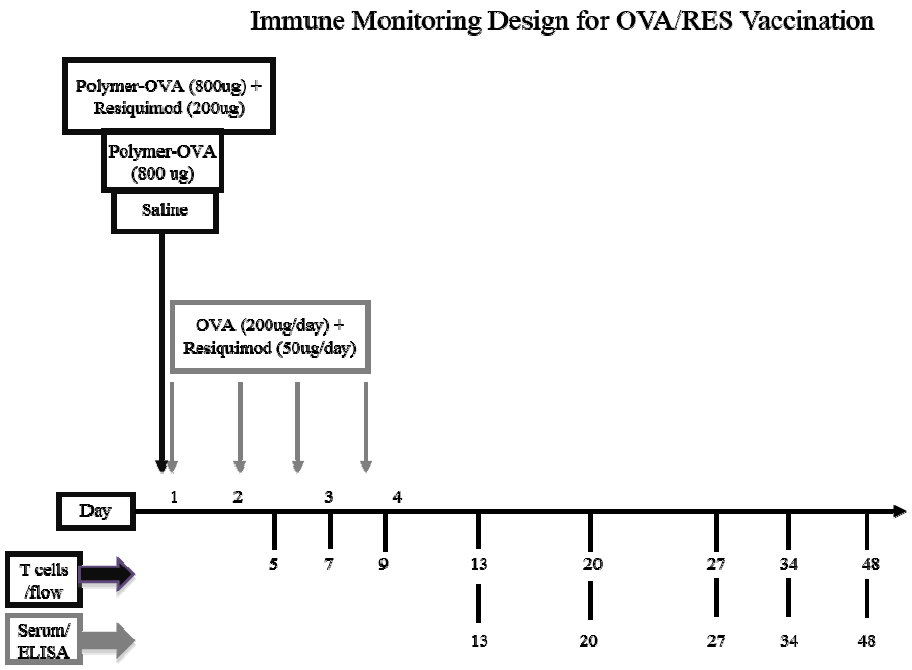

Figure 9) Schematic diagram of the experimental protocol used for vaccination and anti-OVA antibody and OVA-specific CD8 T cell monitoring of mice vaccinated with OVA and RES as a bolus or incorporated into the PCL-POE polymer. Treatment groups include saline, OVA 200 µg and RES 50 µg in PBS (OVA-RES x4; positive control) given as four repetitive once daily doses, PCL-POE polymer and OVA 800 µg only (polymer-OVA) given once, and PCL-POE polymer and OVA 800 µg plus resiquimod 200 µg (polymer-OVA-RES) given once. All vaccinations were given subcutaneously. Anti-OVA antibodies were measured by ELISA on days 13, 20, 27, 34, and 48 post-vaccination. OVA-specific CD8 T cell percentages were measured by flow cytometry on days 5, 7, 9, 13, 20, 27, 34, and 48 post-vaccination.

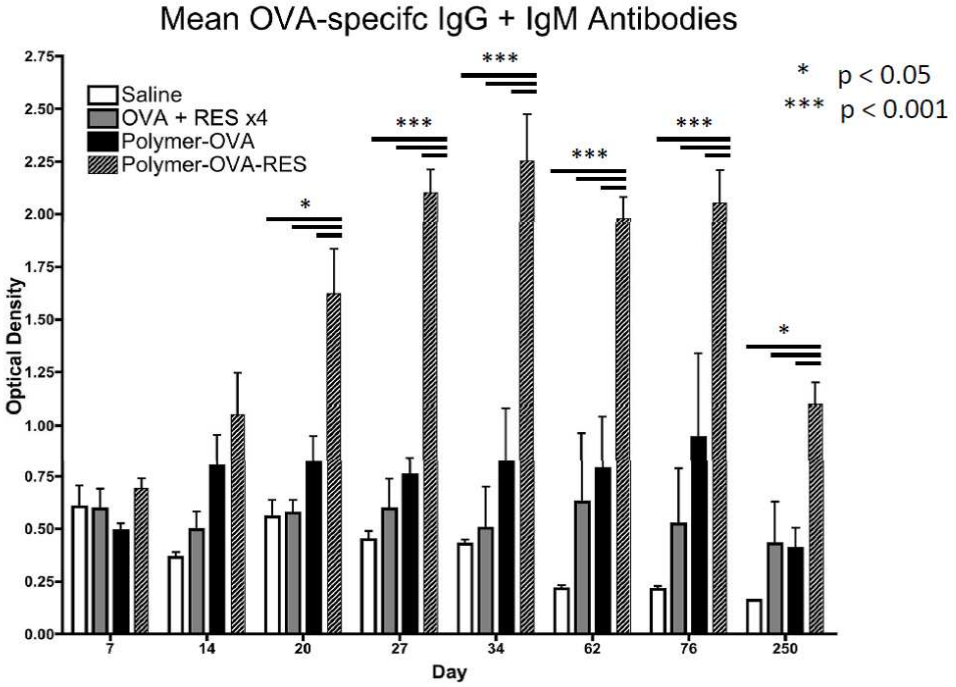

Figure 10) Optical density measurements reflecting OVA-specific IgG and IgM antibodies. C57BL/6J (B6) non-tumor bearing mice were vaccinated with OVA antigen and resiquimod in the PCL-POE polymer administered as a single subcutaneous injection, OVA antigen alone in the PCL-POE polymer administered as a single subcutaneous injection, OVA and resiquimod in PBS given subcutaneously and repetitively as four consecutive daily bolus injections (positive control), or saline. The total dose of OVA and/or resiquimod given was the same in all groups. At the time points indicated, plasma was collected from individual mice and analyzed for the presence of OVA-specific IgG and IgM antibodies by ELISA. All samples were run in triplicate and an average result recorded for each individual mice. The results shown are the mean and standard error of the mean (SEM) of each treatment group.

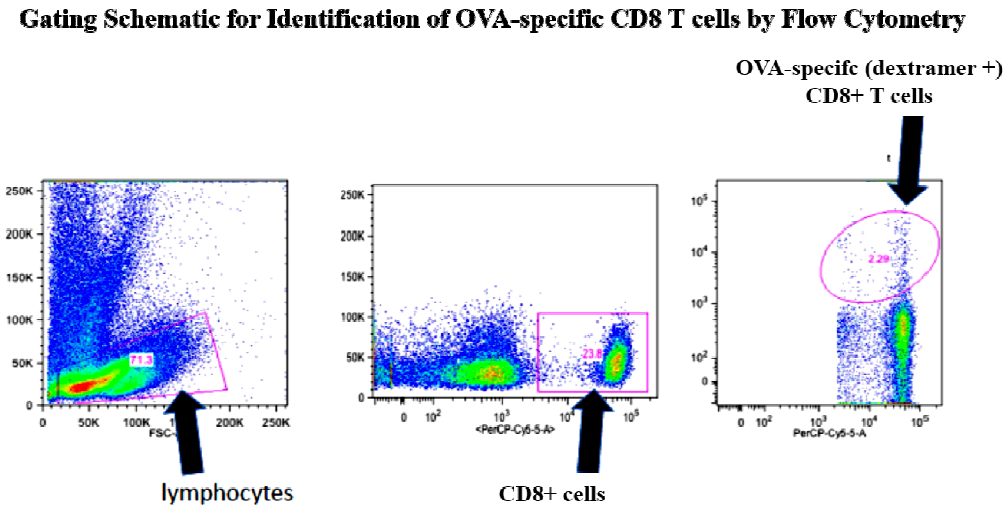

Figure 11) Representative figures indicating the gating zones used in the analysis of the flow cytometry data for identifying (left) lymphocytes, (center) CD8+ cells, and (right) OVA-specific CD8 T cells.

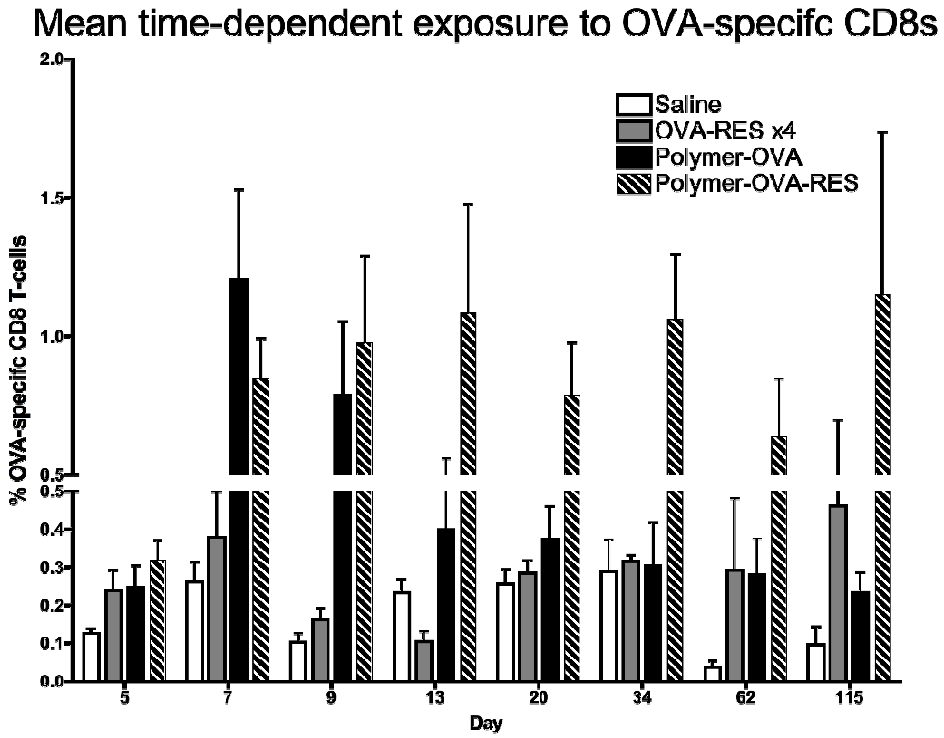

Figure 12) OVA-specific CD8 T cell response in blood of vaccinated mice with values expressed as the mean + SEM percent of OVA-specific CD8 T cells as a function of time.

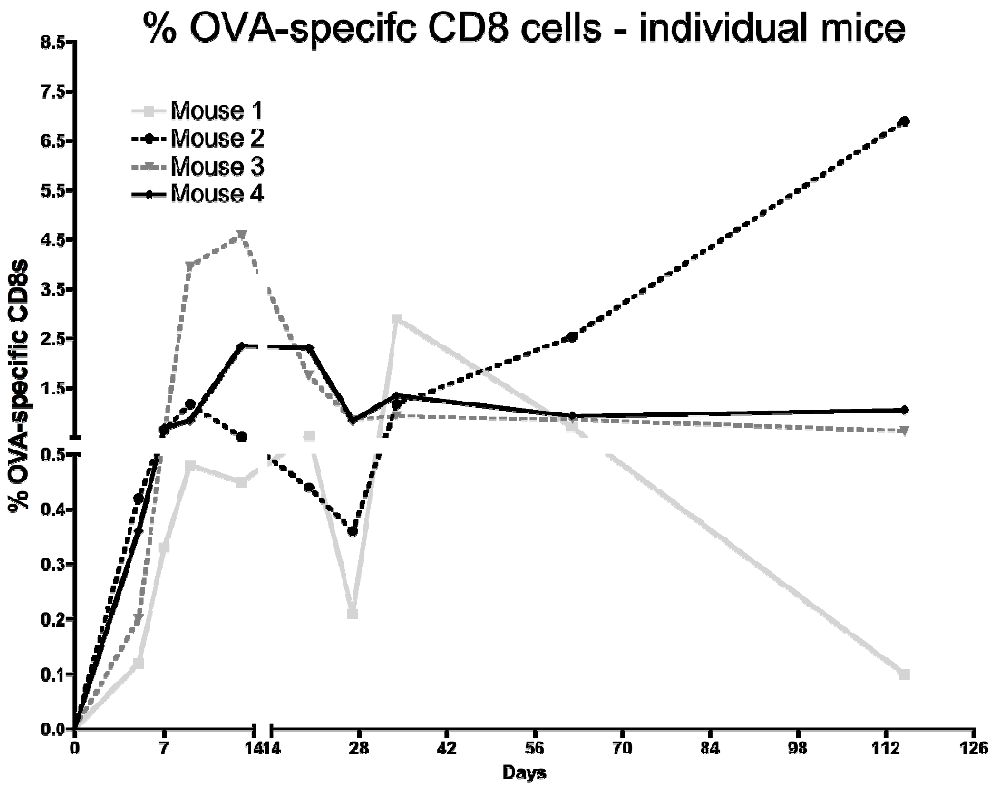

Figure 13) Time-dependent percentages of OVA-specific CD8 T cells in the blood of "responders". C57BL/6J (B6). Non-tumor mice were vaccinated and those considered as responders achieved a peak OVA-specific CD8 T cell response of at least 1 %. At the time points indicated, whole blood was collected from individual mice and analyzed by flow cytometry according to the indicated protocol.

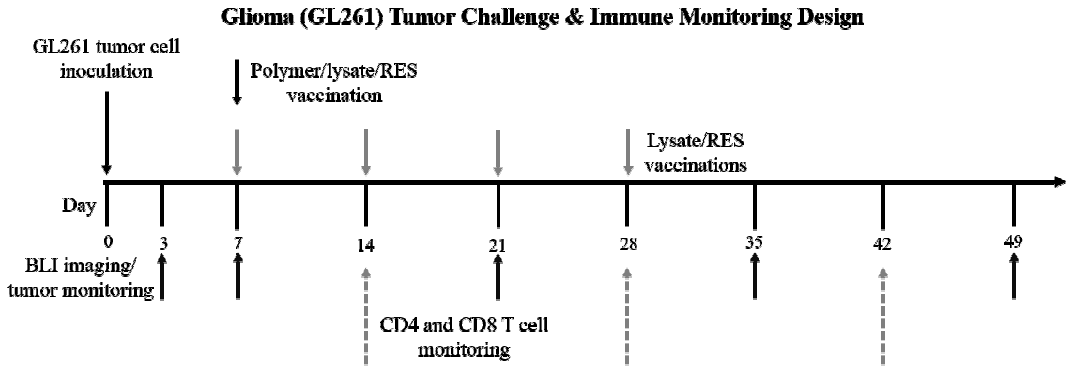

Figure 14) Schematic diagram of the experimental protocol for Glioma Tumor Challenge and Immune Monitoring in Tumor-bearing mice. Mice were inoculated with 15,000 GL261 cultured tumor cells on day 0. Tumors were confirmed with bioluminescent imaging on days 3 and 7 post-inoculation. Whole cell lysate vaccinations were derived from GL261 cells grown in 5% O_2. Treatment groups include saline, lysate and RES in PBS (lysate/RES; positive control) given as four repetitive once weekly doses, polymer and lysate only (PCL-POE/lysate) given once, and lysate and resiquimod in PCL-POE polymer (PCL-POE/lysate/RES).

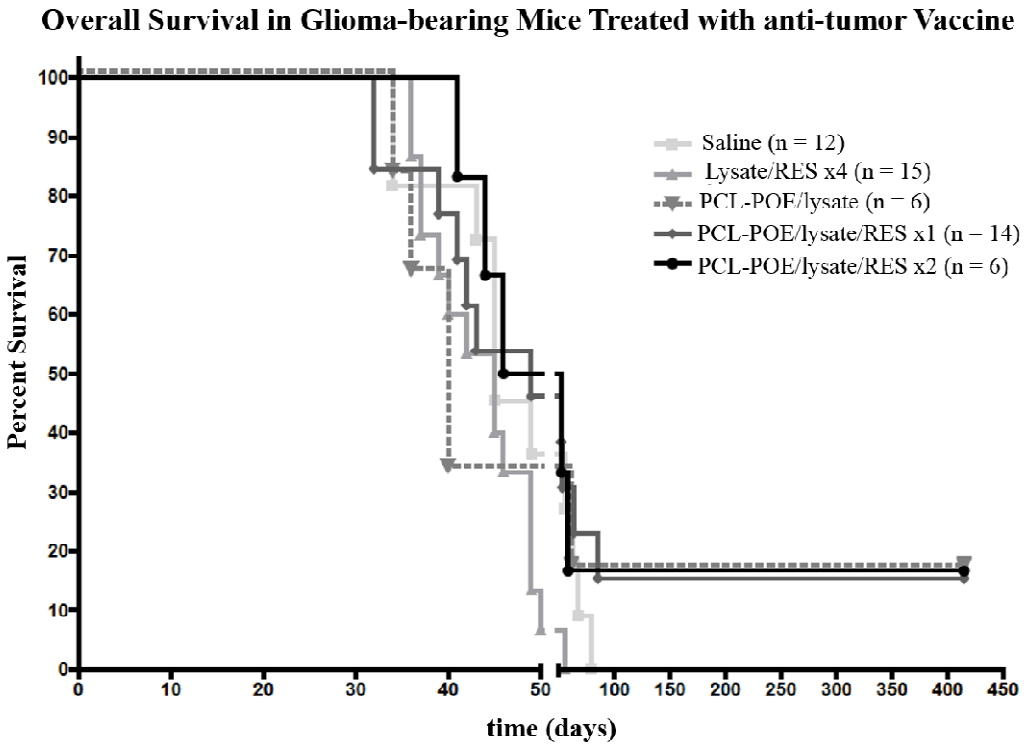

Fig 15a) Overall Survival in Glioma-bearing Mice Treated with Experimental Vaccine. Results shown are pooled data from three independent experiments. Mice were inoculated with glioma tumor cells and treated as described.

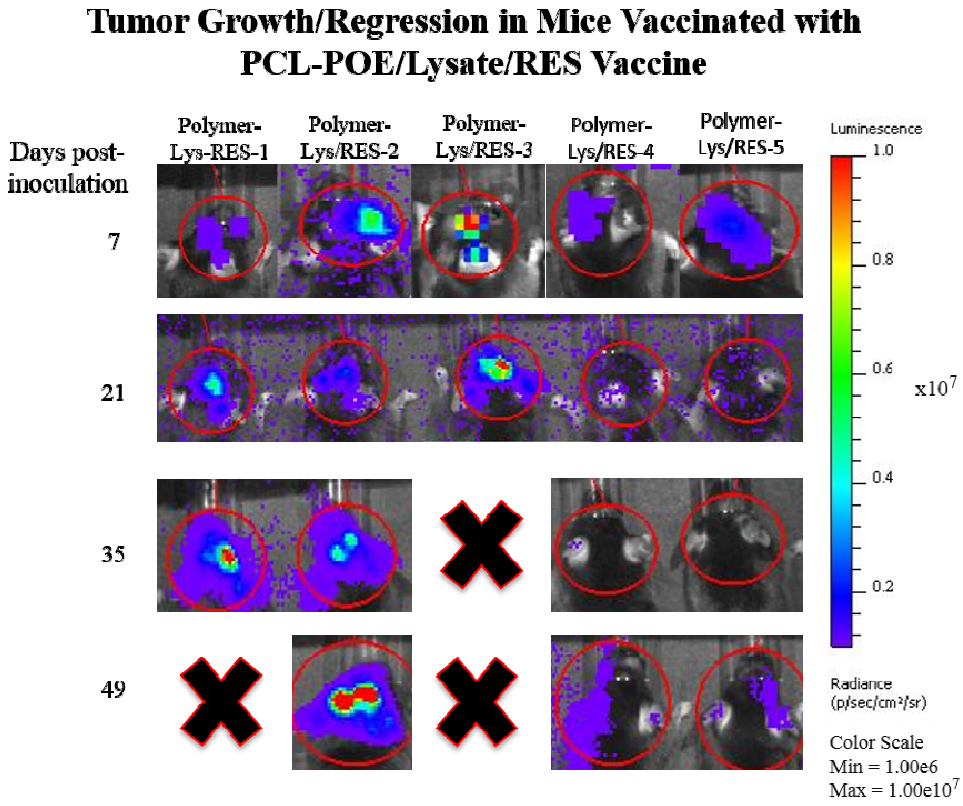

Figure 15b) Bioluminescent Imaging Data of Mice Given a Single Vaccination of Lysate and resiquimod in PCL-POE polymer. Images shown are the results from one of three independent experimental replicates. The two mice on the far right were long-term survivors.

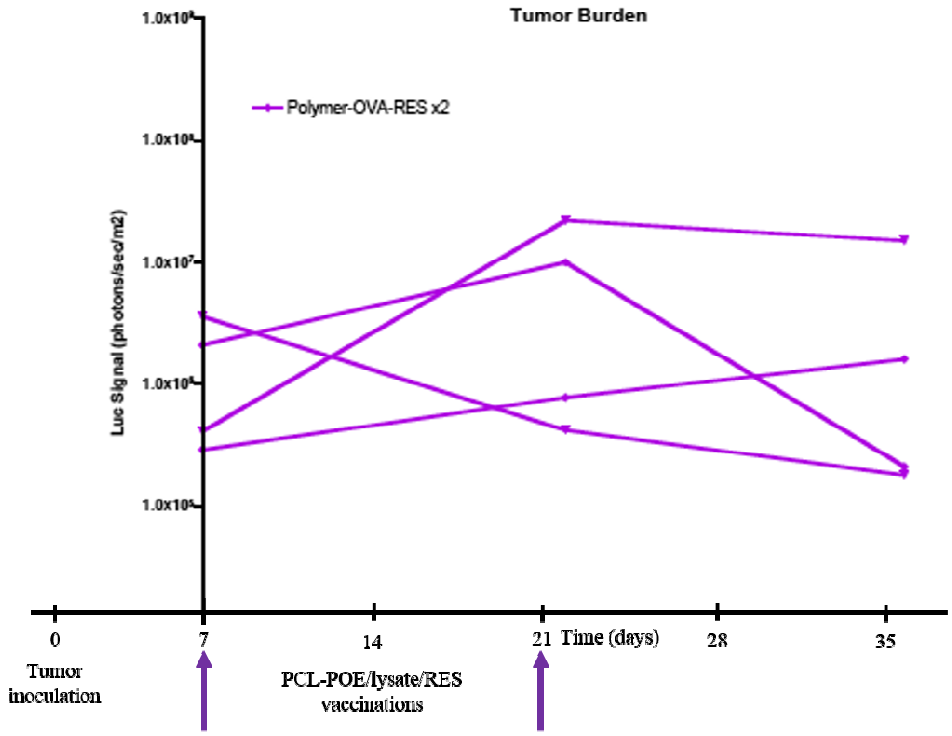

Figure 15c) Time-dependent bioluminescent imaging intensity data of mice treated with two doses of lysate and resiquimod in the PCL-POE polymer.

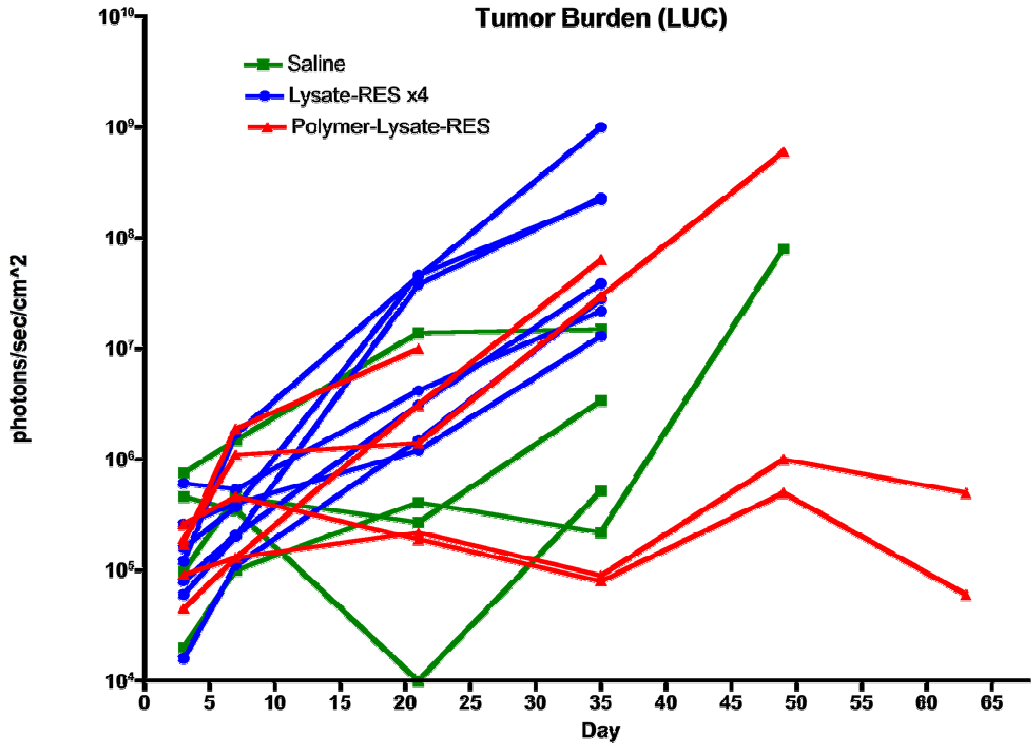

Figure 15d) Time-dependent bioluminescent imaging intensity data of mice treated with lysate and resiquimod in the PCL-POE polymer, lysate and resiquimod given as four repetitive once weekly boluses, and saline.

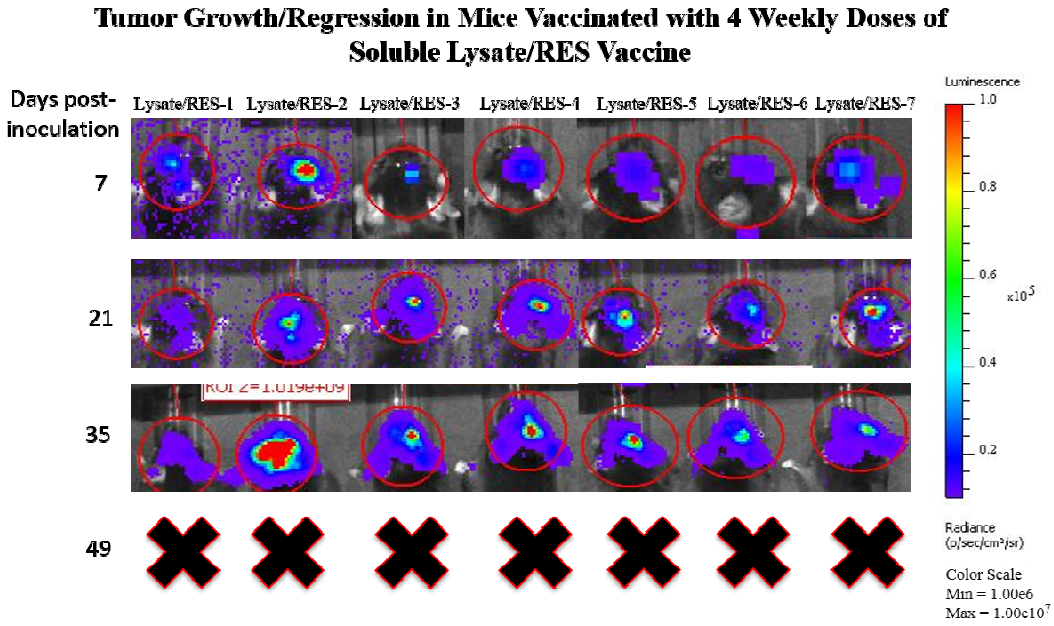

Figure 15e) Time-dependent bioluminescent images of mice treated with lysate and resiquimod given as four repetitive once weekly boluses.

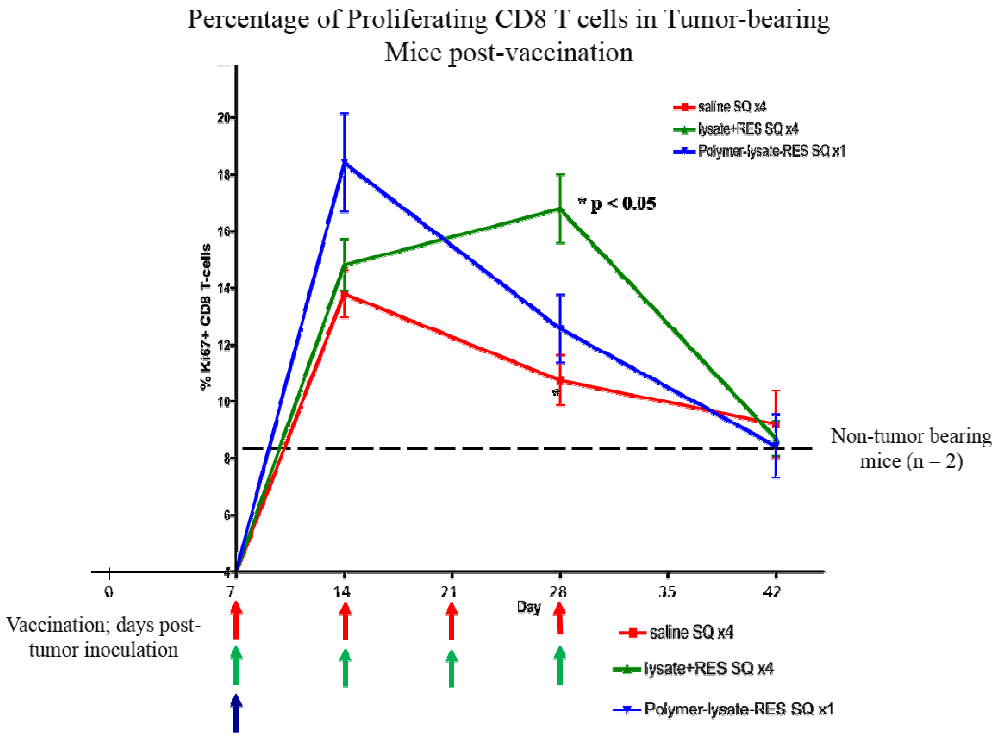

Figure 16a) CD8 T cell Activation and Proliferation in Response to Treatment with Whole Cell Lysate Vaccines. Tumor-bearing mice were vaccinated seven days after they had been inoculated with glioma cells as shown in figure 12. Mice were vaccinated with saline, lysate and RES in PBS (lysate/RES; positive control) given as four repetitive once weekly doses, polymer and lysate only (PCL-POE/lysate) given once, or lysate and resiquimod in PCL-POE polymer (PCL-POE/lysate/RES). The results shown are the percentage of proliferating, Ki67+ CD8 T cells, at two week intervals starting at day 7 post-vaccination. The dashed black line represents the average number of Ki67+ cells in untreated B6 mice.

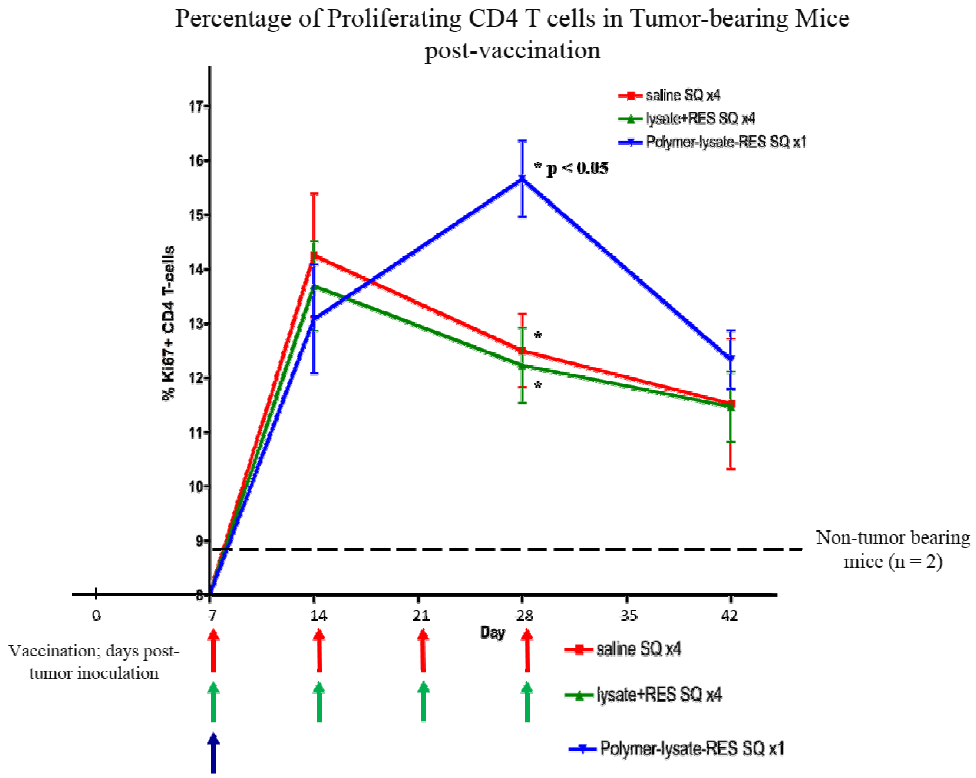

Figure 16b) CD4 T cell Activation and Proliferation in Response to Treatment with Whole Cell Lysate Vaccines. Tumor-bearing mice were vaccinated seven days after they had been inoculated with glioma cells as shown in figure 12. Mice were vaccinated with saline, lysate and RES in PBS (lysate/RES; positive control) given as four repetitive once weekly doses, polymer and lysate only (PCL-POE/lysate) given once, or lysate and resiquimod in PCL-POE polymer (PCL-POE/lysate/RES). The results shown are the percentage of proliferating, Ki67+ CD8 T cells, at two week intervals starting at day 7 post-vaccination. The dashed black line represents the average number of Ki67+ cells in untreated B6 mice.

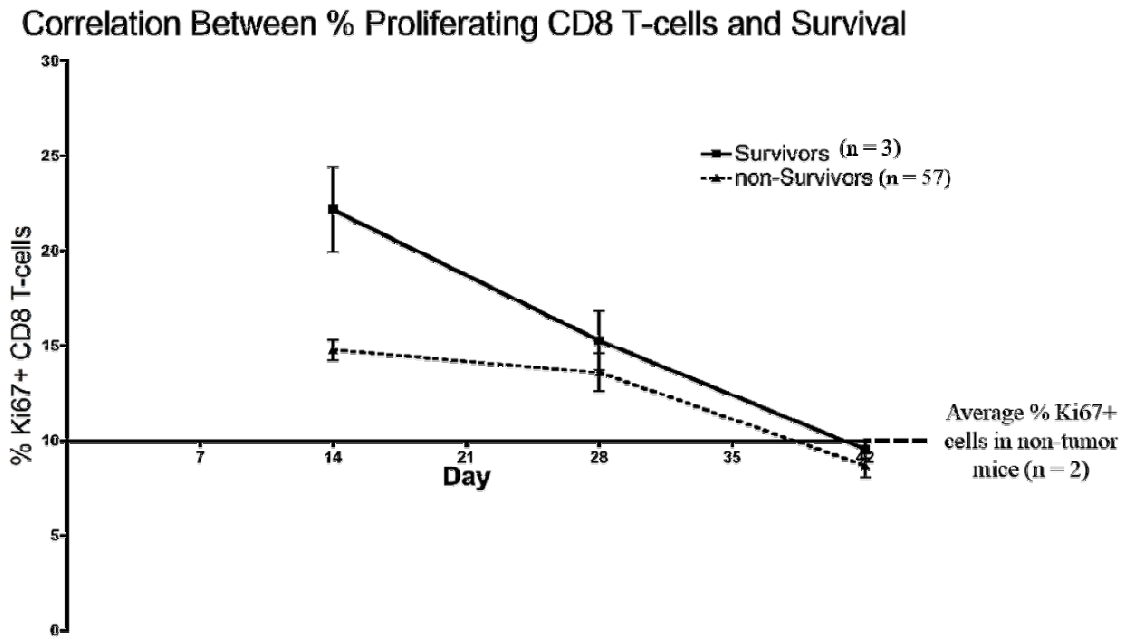

Fig 17a) Correlation Between the Percentage of Ki67+, proliferating, CD8 T cells and Survival in a mouse glioma model. Data shown are compiled from the survival and immune response data from the experiment shown in figure 12. Data are plotted as the percent of Ki67+ CD8 T cells in the blood over time, separated by survivor status. The dashed line is the average percentage of Ki67+ CD8 T cells in the blood of untreated mice.

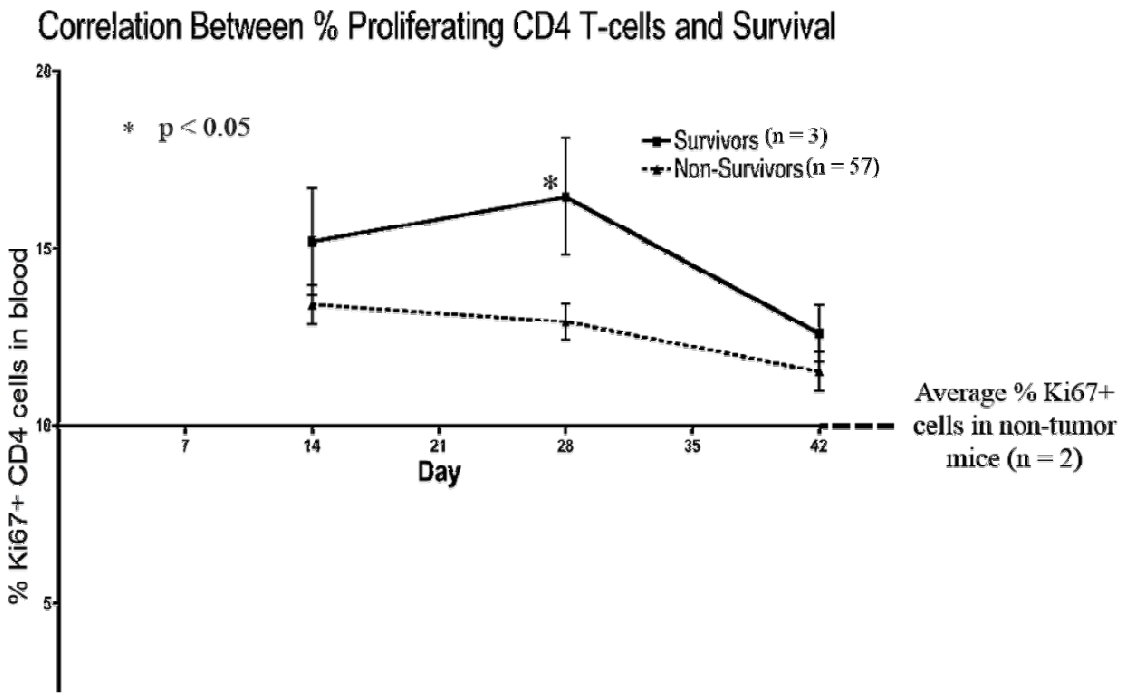

Fig 17b) Correlation Between the Percentage of Ki67+, proliferating, CD4 T cells and Survival in a mouse glioma model. Data shown are compiled from the survival and immune response data from the experiment shown in figure 12. Data are plotted as the percent of Ki67+ CD4 T cells in the blood over time, separated by survivor status. The dashed line is the average percentage of Ki67+ CD8 T cells in the blood of untreated mice.

Bibliography

[1] Wrensch M, Minn Y, Chew T, Bondy M, Berger MS. Epidemiology of primary brain tumors: Current concepts and review of the literature. Neuro Oncol 2002;4:278-99.

[2] Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Cur-schmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO. European Organisation for Research and Treatment of Cancer Brain Tumor and Radio-therapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005;352:987-96.

[3] Pape KA, Catron DM, Itano AA, Jenkins MK. The humoral immune response is initiated in lymph nodes by B cells that acquire soluble antigen directly in the follicles. Immunity. 2007;26:491–502.

[4] Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. Science. 1998;281:96–99.

[5] Smith KG, Hewitson TD, Nossal GJ, Tarlinton DM. The phenotype and fate of the antibody forming cells of the splenic foci. Eur J Immunol. 1996;26:444–48.

[6] Ho F, Lortan JE, MacLennan IC, Khan M. Distinct short-lived and long-lived antibody-producing cell populations. Eur J Immunol. 1986;16:1297–1301.

[7] Taylor JJ, Jenkins MK, Pape KA. Heterogeneity in the differentiation and function of memory B cells. Trends Immunol. 2012;33(12): 590–597.

[8] Dent AL, Shaffer AL, Yu X, Allman D, Staudt LM. Control of inflammation, cytokine expression, and germinal center formation by BCL-6. Science. 1997;276:589–92.

[9] Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell. 1991;67:1121–29.

[10] Tarlinton DM, Smith KG. Dissecting affinity maturation: a model explaining selection of antibody forming cells and memory B cells in the germinal centre. Immunol Today. 2000;21:436–441.

[11] Teng G, Papavasiliou, FN. Immunoglobulin Somatic Hypermutation. Annu. Rev. Genet. 2007;41:107–20.

[12] Neefjes J, Jongsma MLM, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat. Rev. Immunol. 2011;11:823-36.

[13] Scholz C, Tampe R. The peptide-loading complex – antigen translocation and MHC class I loading. Biol. Chem. 2009;390:783-94.

[14] Kovacsovics-Bankowski M, Rock KL. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. Science. 1995;267:243–246

[15] Harty JT, Badovinac VP. Influence of effector molecules on the CD8+ T cell response to infection. Curr. Opin. Immunol. 2002; 14, 360–65.

[16] Slifka MK, Rodriguez F, Whitton JL. Rapid on/off cycling of cytokine production by virus-specific CD8+ T cells. Nature 1999;401:76-79.

[17] Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat. Immunol. 2003;4:835–42.

[18] Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2002;2:251-62.

[19] Murphy KA, Erickson JR, Johnson CS, Seiler CE, Bedi J, Hu P, Pluhar GE, Epstein AL, Ohlfest JR. CD8+ T cell-independent tumor regression induced by Fc-OX40L and therapeutic vaccination in a mouse model of glioma. J Immunol. 2014;192(1):224-33

[20] Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. J Clin Invest. 2010;120(5):1368-79.

[21] Sallusto F, Impellizzieri D, Basso C, Laroni A, Uccelli A, Lanzavecchia A, Engelhardt B. T-cell trafficking in the central nervous system. Immunol Rev. 2012;248(1):216-27.

[22] Kantoff PW, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N. Engl. J. Med. 2010;363:411-22.

[23] Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat. Immunol. 2003;4:835–42.

[24] Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. Nat. Med. 2013;19:1597-1608.

[25] Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int. Immunol. 2009;21:317–37.

[26] Nolte MA, Leibundgut-Landmann S, Joffre O, Sousa CR. Dendritic cell quiescence during systemic inflammation driven by LPS stimulation of radioresistant cells in vivo. J. Exp. Med.2007;204:1487–1501.

[27] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783–801.

[28] Dalpke A, Heeg K. Signal integration following toll-like receptor triggering. Crit Rev Immunol 2002;22:217-50.

[29] Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J. Ex. Med. 2009;206(7):1589-1602.

[30] D'Argenio DA, Wilson CB. A decade of vaccines: Integrating immunology and vaccinology for rational vaccine design. Immunity. 2010;33:437–440

[31] Lazoura E, Apostolopoulos V. Insights into Peptide-Based Vaccine Design for Cancer Immunotherapy. Curr. Med. Chem. 2005;12(13):1481-94.
[32] Lazoura E, Apostolopoulos V. Rational Peptide-based vaccine design for cancer immunotherapeutic applications. Curr. Med. Chem. 2005; 12(6):629-39.
[33] Stathopoulos A, Samuelson C, Milbouw G, Hermanne JP, Schijns VE, Chen TC. Therapeutic vaccination against malignant gliomas based on allorecognition and syngeneic tumor antigens: proof of principle in two strains of rat. Vaccine. 2008;26(14):1764-72.
[34] Rathe SK, Johnson JE, Silverstein KA, Erdmann JJ, Watson AL, Popescu FE, Ohlfest JR, Largaespada DA. MMuFLR: missense mutation and frameshift location reporter. Bioinformatics. 2013;29(18):2353-4.
[35] Qu HQ, Jacob K, Fatet S, Ge B, Barnett D, Delattre O, Faury D, Montpetit A, Solomon L, Hauser P, Garami M, Bognar L, Hansely Z, Mio R, Farmer JP, Albrecht S, Polychronakos C, Hawkins C, Jabado N. Genome-wide profiling using single-nucleotide polymorphism arrays identifies novel chromosomal imbalances in pediatric glioblastomas. Neuro Oncol. 2010;12(2):153-63
[36] Irvine DJ, Swartz MA, Szeto GL. Engineering synthetic vaccines using cues from natural immunity. Nat Mater. 2013; 12(11): 978–90.
[37] Champion JA. Role of Particle Size in Phagocytosis of Polymeric Microspheres. Pharm. Res. 2008;25:1815-21.
[38] Tabata Y and Ikada Y. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage. Biomaterials. 1988;9:356-62.
[39] Shen H, Ackerman AL, et al. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology. 2006;117:78-88.
[40] Waeckerle-Men Y. Encapsulation of proteins and peptides into biodegradable poly (d,l-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine. 2006;24:1847-57.
[41] Shen H, Ackerman AL, Cody V, Giodini A, Hinson ER, Cresswell P, Edelson RL, Staltzman WM, Hanlon DJ. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology. 2006;117(1):78-88.
[42] Prasad S, Cody V, Saucier-Sawyer JK, Fadel TR, Edelson RL, Birchall MA, Hanlon DJ. Optimization of stability, encapsulation, release, and cross-priming of tumor antigen-containing PLGA nanoparticles. Pharm Res. 2012;29(9):2565-77.
[43] Freund J, McDermott D. Sensitization to horse serum by means of adjuvants. Proc Soc Exp Biol Med 1942;49:548-553.
[44] Freund J, Casals J, Hosmer EP. Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. Proc Soc Exp Biol Med 1937;37:509-513.
[45] Broderson JR. A retrospective review of lesions associated with the use of Freund's adjuvant. Lab Anim Sci 1989;39:400-405.
[46] Salk JE, et al. The Use of Adjuvants in Studies on Influenza Immunization. II. Increased Antibody Formation in Human Subjects Inoculated with Influenza Virus Vaccine in a Water-In-Oil Emulsion. Am. J. Hyg. 1952;55:439-56.
[47] Herbert WJ. 1967. Some investigations into the mode of action of the water-in-mineral-oil emulsion antigen adjuvants. In: Symposium Series of Immunobiology Standardization. Vol. 6. Basel NY: Karger. p 213-220.
[48] Stills HF. Adjuvants and Antibody Production: Dispelling the Myths Associated with Freund's Complete and Other Adjuvants. ILAR Journal. 2005;46(3):280-93.
[49] Moon JJ, Suh H, Bershteyn A, Stephan MT, Liu H, Huang B, Sohail M, Luo S, Um SH, Khant H, Goodwin JT, Ramos J, Chiu W, Irvine DJ. Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nat Mater. 2011;10(3):243-51.
[50] Jewell CM, López SC, Irvine DJ. In situ engineering of the lymph node microenvironment via intranodal injection of adjuvant-releasing polymer particles. Proc Natl Acad Sci USA. 2011;108(38):15745-50.
[51] Weber JS. At the Bedside: Adoptive cell therapy for melanoma clinical development. J. Leukoc Biol. 2014 [epub ahead of print]
[52] Prieto PA, Yang JC, Sherry RM, Hughes MS, Kammula US, White DE, Levy CL, Rosenberg SA, Phan GQ. CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. Clin Cancer Res. 2012;18(7):2039-47.
[53] Eguchi J, Hatano M, Nishimura F, Zhu X, Dusak JE, Sato H, Pollack IF, Storkus WJ, Okada H. Identification of interleukin-13 receptor alpha2 peptide analogues capable of inducing improved antiglioma CTL responses. Cancer Res. 2006;66(11):5883-91.
[54] Bioley G, Guillaume P, Luescher I, Bhardwaj N, Mears G, Old L, Valmori D, Ayyoub M. Vaccination with a recombinant protein encoding the tumor-specific antigen NY-ESO-1 elicits an A2/157-165-specific CTL repertoire structurally distinct and of reduced tumor reactivity than that elicited by spontaneous immune responses to NY-ESO-1-expressing Tumors. J Immunother. 2009;32(2):161-8.
[55] Murphy KA, Erickson JR, Johnson CS, Seiler CE, Bedi J, Hu P, Pluhar GE, Epstein AL, Ohlfest JR. CD8+ T cell-independent tumor regression induced by Fc-OX40L and therapeutic vaccination in a mouse model of glioma. J Immunol. 2014;192(1):224-33

[56] Friedman KM, Prieto PA, Devillier LE, Gross CA, Yang JC, Wunderlich JR, Rosenberg SA, Dudley ME. Tumor-specific CD4+ melanoma tumor-infiltrating lymphocytes. J Immunother. 2012;35(5):400-8.

[57] Hailemichael Y, Dai Z, Jaffarzad N, Ye Y, Medina MA, Huang XF, Dorta-Estremera SM, Greeley NR, Nitti G, Peng W, Liu C, Lou Y, Wang Z, Ma W, Rabinovich B, Schluns KS, Davis RE, Hwu P, Overwijk WW. Persistent antigen at vaccination site induces tumor-specific CD8+ T cell sequestration, dysfunction and deletion. Nat. Med. 2013;online:1-10.

[58] Ardon H, Van Gool SW, Verschuere T, Maes W, Fieuws S, Sciot R, Wilms G, Demaerel P, Goffin J, Van Calenbergh E, Menten J, Clement P, Debiec-Rychter M, De Vleeschouwer S. Integration of autologous dendritic cell-based immunotherapy in the standard of care treatment for patients with newly diagnosed glioblastoma: results of the HGG-2006 phase I/II trial. Cancer Immunol. Immunother. 2012;61(11):2033-44.

[59] Sampson JH, Archer GE, Mitchell DA, Heimberger AB, Herndon JE, Lally-Goss D, McGehee-Norman S, Paolino A, Reardono DA, Friedman AH, Friedman HS, Bigner DD. An epidermal growth factor receptor variant III-targeted vaccine is safe and immunogenic in patients with glioblastoma multiforme. Mol. Cancer Ther. 2009;8(10):2773-79.

[60] Zeng G. MHC class II-restricted tumor antigens recognized by CD4+ T cells: new strategies for cancer vaccine design. J Immunother. 2001;24(3):195-204

[61] Eisen HN. Affinity enhancement of antibodies: how low-affinity antibodies produced early in immune responses are followed by high-affinity antibodies later and in memory B-cell responses. Cancer Immunol Res. 2014 May;2(5):381-92.

[62] Tran E, Turcotte S, Gros A, Robbins PF, Lu YC, Dudley ME, Wunderlich JR, Somerville RP, Hogan K, Hinrichs CS, Parkhurst MR, Yang JC, Rosenberg SA. Cancer immunotherapy based on mutation-specific CD4+ T cells in a patient with epithelial cancer. Science. 2014;344(6184):641-5.

[63] Effective Anti-Neu-Initiated Antitumor Responses Require the Complex Role of CD4+ T Cells. Mortenson ED, Park S, Jiang Z, Wang S, Fu YX. Clin Cancer Res. 2013 Mar 15;19(6):1476-86.

[64] Wherry EJ. T Cell Exhaustion. Nat Immunol. 2011;12(6):492-99.

[65] Wherry EJ. T Cell Exhaustion. Nat Immunol. 2011;12(6):492-99.

[66] McNeil AC, et al. High-level HIV-1 viremia suppresses viral antigen-specific CD4+ T cell proliferation. Proc. Natl. Acad. Sci. 2001;98:13878-83.

[67] Uzonna JE, et al. Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation of disease. J. Immunol. 2001;167:6967-74.

[68] Nelson RW, et al. CD4+ T Cell Persistence and Function after Infection Are Maintained by Low-Level Peptide:MHC Class II Presentation. J. Immunol. 2013;190:000-000.

[69]. Aubert RD, et al. Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. PNAS. 2011;108(52):21182-87.

[70]. Maloy KJ, Donachie AM, O'Hanag DT, Mowat AM. Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly-(lactide-co-glycolide) microparticles. Immunology. 1994;81:661-7.

[71]. Puras G, Salvador A, Igartua M, Hernandex RM, Pedraz JL. Encapsulation of Ab1–15 in PLGA microparticles enhances serum antibody response in mice immunized by subcutaneous and intranasal routes. Eur. J. Pharm. Sci. 2011;44:200-6.

[72]. Waeckerle-Men Y, Uetz-von Allmen E, Gander B, Scandella E, Schlosser E, Schmidtke G, Merkle HP, Groettrup M. Encapsulation of proteins and peptides into biodegradable poly-(d,l-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine. 2006;24:1847-57.

[73] Wiesner SM, Decker SA, Larson JD, Ericson K, Forster C, Gallardo JL, Long C, Demorest ZL, Zamora EA, Low WC, SantaCruz K, Largaespada DA, Ohlfest JR. De novo induction of genetically engineered brain tumors in mice using plasmid DNA. Cancer Res. 2009;69(2):431-9

[74] Jenkins MK, Moon JJ. The role of naive T cell precursor frequency and recruitment in dictating immune response magnitude. J Immunol. 2012;188(9):4135-40.

[75] Shen H, Ackerman AL, Cody V, Giodini A, Hinson ER, Cresswell P, Edelson RL, Staltzman WM, Hanlon DJ. Enhanced and prolonged cross-presentation following endosomal escape of exogenous antigens encapsulated in biodegradable nanoparticles. Immunology. 2006;117(1):78-88.

[76] Waeckerle-Men Y. Encapsulation of proteins and peptides into biodegradable poly (d,l-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine. 2006;24:1847-57.

[77] Ali OA, Tayalia P, Shvartsman D, Lewin S, Mooney DJ. Inflammatory cytokines presented from polymer matrices differentially generate and activate DCs in situ. Adv Funct Mater. 2013;23(36):4621-28.

[78] Anish C, Khan N, Upadhyay AK, Sehgal D, Panda AK. Delivery of Polysaccharides Using Polymer Particles: Implications on Size-Dependent Immunogenicity, Opsonophagocytosis, and Protective Immunity. Mol Pharm. 2014 Jan 29. [Epub ahead of print]

[79] Ahmed R, Gray D: Immunological memory and protective immunity: understanding their relation. Science 1996;272:54-60.

[80] Slifka MK, Ahmed R: Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity**.** Trends Microbiol. 1996;4:394-400.

[81] Murphy KA, Erickson JR, Johnson CS, Seiler CE, Bedi J, Hu P, Pluhar GE, Epstein AL, Ohlfest JR. CD8+ T cell-independent tumor regression induced by Fc-OX40L and therapeutic vaccination in a mouse model of glioma. J Immunol. 2014;192(1):224-33.

[82] Moon JJ, et al. Naive CD4+ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity. 2007;27:203–13

[83] Olin MR, Andersen BM, Litterman AJ, Grogan PT, Sarver AL, Robertson PT, Liang X, Chen W, Parney IF, Hunt MA, Blazar BR, Ohlfest JR. Oxygen is a master regulator of the immunogenicity of primary human glioma cells. Cancer Res. 2011;71(21):6583-9.

[84] Lee M, Park CS, Lee YR, Im SA, Song S, Lee CK. Resiquimod, a TLR7/8 agonist, promotes differentiation of myeloid-derived suppressor cells into macrophages and dendritic cells. Arch Pharm Res. 2014;

[85] Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nature Immunology 2002;3:499

[86] Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Seminars in Cancer Biology. 2006;16:53–65.

[87] Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. Journal of Immunology 2001;166:678–89.

[88] Khaled YS, Ammori BJ, Elkord E. Increased levels of granulocytic myeloid-derived suppressor cells in peripheral blood and tumour tissue of pancreatic cancer patients. J Immunol Res. 2014;2014:879897.

[89] Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J. Cell. Physiol. 2000;182 (3):311–22.

[90] Iatropoulos MJ, Williams GM. Proliferation Markers. Exp Toxicol Pathol. 1996;48(2-3):175-81.

[91] Inwald EC, Klinkhammer-Schalke M, Hofstadter F, Xeman F, Koller M, Gerstenhauer M, Ortmann O. Ki-67 is a prognostic parameter in breast cancer patients: results of a large population-based cohort of a cancer registry. Breast Cancer Research and Treatment. 2013;139(2):539-52.

[92] Foulds KE, et al. CD4 and CD8 T cells are intrinsically different in their proliferative responses. J. Immunol. 2002;168:1528–32.

[93] Gudmundsdottir H, Wells AD, Turka LA. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. J. Immunol. 1999;162:5212–23.

[94] Wong P, Pamer EG. Antigen-independent CD8 T cell proliferation. J. Immunol. 2001;166:5864–68.

[95] Kaech SM, Ahmed R. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nat. Immunol. 2001; 2:415–22.

[96] Nelson RW, McLachlan JB, Kurtz JR, Jenkins MK. CD4+ T cell persistence and function after infection are maintained by low-level peptide:MHC class II presentation. J Immunol. 2013;190(6):2828-34.

[97] Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, Wherry EJ. Molecular and transcriptional basis of $CD4^+$ T cell dysfunction during chronic infection. Immunity. 2014;40(2):289-302.

[98] Church SE, Jensen SM, Antony PA, Restifo NP, Fox BA. Tumor-specific CD4+ T cells maintain effector and memory tumor-specific CD8+ T cells. Eur J Immunol. 2014;44(1):69-79.

[99] Prieto PA, Yang JC, Sherry RM, Hughes MS, Kammula US, White DE, Levy CL, Rosenberg SA, Phan GQ. CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. Clin Cancer Res. 2012;18(7):2039-47.

[100] Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, Wherry EJ. Molecular and transcriptional basis of $CD4^+$ T cell dysfunction during chronic infection. Immunity. 2014;40(2):289-302.

[101] Church SE, Jensen SM, Antony PA, Restifo NP, Fox BA. Tumor-specific CD4+ T cells maintain effector and memory tumor-specific CD8+ T cells. Eur J Immunol. 2014;44(1):69-79.