POLYMER BASED IMMUNE MODULATION FOR THE GENERATION OF AN

ANTI-TUMOR IMMUNE RESPONSE

by

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ABSTRACT OF THE DISSERTATION

Polymer Based Immune Modulation for the Generation of an Anti-Tumor Immune Response

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Increasing emphasis is being placed on overcoming tumor-associate immune escape mechanisms that facilitate disease progression. Understanding this immune tolerance and developing methods to overcome it provide advanced understanding to further develop vaccine strategies. In the current work we have engineered a polymer based therapy that enhances cellular immunity capable of generating anti-tumor activity. We have determined that a poly(lactic-co-glycolic acid) (PLGA) based delivery system encapsulating tumor associated antigen (ovalbumin, OVA) and the TLR9 agonist CpG motif DNA can initiate an effective type 1 mediated response. Local administration of the polymer therapy on E.G7-OVA lymphoma bearing mice significantly delayed tumor progression by eliciting a strong local and systemic IFN-γ mediated anti-tumor response. It was found that this response worked independently of antigen specific CTLs, which were active in the tumor draining lymph nodes yet incapable of retarding tumor formation. In analyzing the dendritic cell response to this polymer system, it was demonstrated that this delivery system indeed increased the Th1 phenotype of dendritic cells as measured by an increase in cell surface expression of CD80, CD86 and MHCII and secretion of the cytokines IL-12 and IFN-γ. In addition, introducing the immune modulator 1-Methyl Tryptophan (1-MT) in concert with the antigen and adjuvant enhanced the dendritic cell Th1 profile when delivered via the PLGA vesicle. Taken together, these studies demonstrate the immunological mechanisms and benefits of a PLGA based delivery system.

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CHAPTER 1: INTRODUCTION

1.1 IMMUNOLOGY AND ANTIGEN PRESENTING CELLS: DENDRITIC CELLS

The human body has internal defense mechanisms to protect itself against invading pathogens. Via two main responses, the immune system provides this defensive shield. The innate immune response provides the first line of protection against invading pathogens by nonspecific defense mechanisms. This is mainly composed of general and nonspecific protection against foreign pathogens. It includes factors such as the skin, mucous membranes, temperature, chemical mediators (i.e. cytokines) and cellular phagocytosis. Cells of the innate response, such as antigen presenting cells (APCs), play the role of scavengers to phagocytose foreign matter, process the material, and subsequently interact with the adaptive immune response. This adaptive (or acquired) immune response, also known as the specific defense mechanisms, is then tailored to combat foreign pathogens by directing the immune response to particular antigens associated with the pathogen. This response occurs through both cell and antibody mediated pathways and is what aids in conferring long term and enhanced immunity against an antigen and its associated pathogen. T lymphocytes are one family of cells of the adaptive defense mechanism that produce specific responses to modulate the immune response. CD8+ T cells, for example, recognize and destroy cells expressing foreign antigen while CD4+ T cells help activate or enhance immune responses.

As implied, these adaptive and innate immune responses do not function independently of each other. There are dynamic and complex cellular and biochemical interactions between the innate and adaptive systems. APCs of the innate immune response, for example dendritic cells (DCs), phagocytose and subsequently degrade foreign pathogens,

such as in the form of protein. These degraded proteins are processed into peptides and presented on the extracellular regions of the major histocompatibility complex (MHC) I or II. Based on this and other stimuli received from the pathogen, DCs are differentiated to various phenotypes. For example, CpG motif DNA or lipopolysaccharide (LPS) from bacteria will stimulate DCs to upgregulate a number of processes, including increased cell surface expression of CD80 and CD86 molecules and increased secretion of IL-12 and TNF-α to name a few[1,2]. In conjunction with the antigen harboring MHC complexes, these costimulatory molecules, CD80 and CD86, will bind receptors on T cells and initiate a cascade of events. In the classical inflammatory model, the binding of these costimulatory molecules along with MHC complexes result in the active killing and removal of cells expressing the antigens first processed by the DCs. However, binding of CD80/CD86 to different receptors on T cells, namely CD28 or CTLA-4, result in differential stimulation of T cells. Interactions with CD28 result in positive signals that activate and expand the effector T cells[3,4]. In contrast, binding of the CTLA-4 molecules results in a negative response by attenuating T cell activity[5,6]. These divergent responses generated by DCs are essential for the regulation of the overall T cell response and aid in maintaining T cell homeostasis.

It is now increasingly clear that DCs play a powerful role in immune system modulation and can interact in various ways with the many T cell subsets. DCs original known role of activating T cells to direct the killing and removing of pathogenic cells is now only a partial known role of these potent APCs. As previously implied DCs interact with CD4+ and CD8+ T cells in order to initiate or augment antigen specific immune response. Some of the costimulatory molecules, i.e. CD80/CD86, utilized in these interactions though can

also result in inhibition, as previously discussed with the CTLA-4 pathways[7]. More evidence is being presented that demonstrate the ability of DCs to also stimulate a subset T cells now termed T regulatory cells (Tregs), originally exposed as a subset of lymphocytes that provided a degree of tolerance[8]. It is now believed that there are both CD4+ and CD8+ Tregs, each performing distinct yet complementary functions[9]. In general, these Tregs mitigate the T cell milieu, inducing immune tolerance. As depicted in Figure 1.1, these Tregs are known to inhibit effector T cell stimulation and activity, via cell to cell contact and soluble factors. Interestingly however, it is being shown that DCs can interact and activate both branches of Tregs[10,11]. Typically, immature DCs pulsed with high levels of antigen in the absence of inflammatory signals can differentiate, activate, and/or expand CD4+CD25+ Tregs[12,13]. However, detailed studies also indicate that there is a fine control over DC stimulation and that different DC subsets stimulate different T cells. For example, LPS, which typically is shown to mature DCs to activate CD8+ and CD4+ Tcells, can have differential effects on DC function depending on timing and exposure. As a result, LPS induced DCs can paradoxically also expand Treg populations[14]. Supporting this is the response of DCs to CpG DNA. Although normally seen as a stimulating factor, CpG DNA at high doses can induce elevated IL-10 secretion from DCs, a known negative regulator[15]. Despite these findings demonstrating the DC and Treg interface, one aspect that eludes science is how DCs interact with Tregs to arrest their activity and thus reverse anergy. Considering the power and versatility of DCs with regards to their ability to modulate T cell activity, understanding the various DC subsets and their behaviors becomes increasingly significant.

As science progresses, we are learning that there are more subsets of DCs than the simply immature vs. mature populations, creating a misnomer. In conjunction, as more T cell subsets are discovered, we are learning how these different DCs can interact with the various T cells. The implications for understanding and being able to control these pathways span various fields of biomedical sciences. It is thus crucial to understand the DC activation process and what their immune modulatory properties are. Doing so will provide great benefits in viral, cancer, and bacterial therapies. Knowing how the innate and adaptive immune response process antigen and danger signals will provide great insight into developing better therapies.
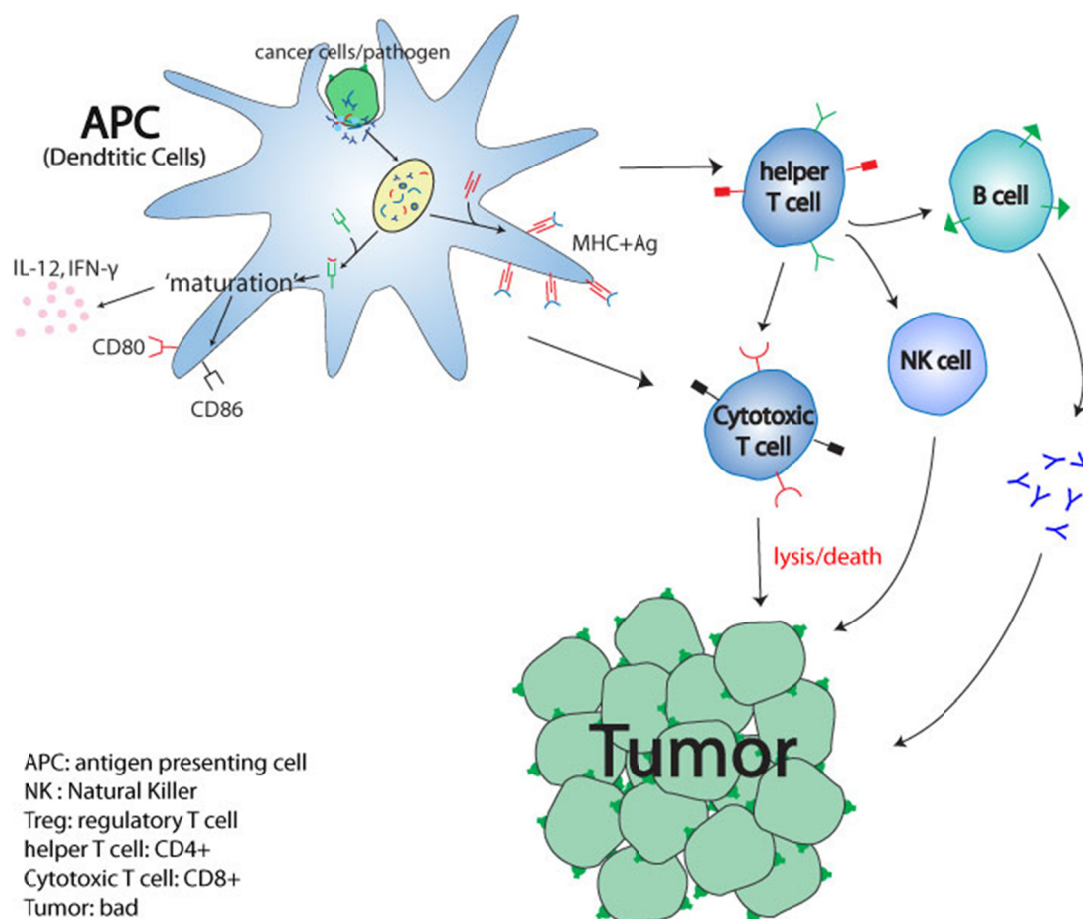
Figure 1.1 Brief summary of cancer immunology. Antigen presenting cells (APCs) are powerful immune cells capable of eliciting active immunity against foreign and host pathogens. After processing antigens, they interact with T cells to initiate an array of responses. In this study, this response is lysis of target cancer cells. Unfortunately there are various mechanisms that inhibit and block this cascade, such as Tregs, minimizing the potential effector activity.

1.2 CANCER IMMUNOLOGY AND IMMUNOTHERAPY

According to the National Cancer Institute (NCI), there is a 40.35% chance that an individual will be diagnosed with cancer at some point in his/her lifetime, independent of age, sex, and race[16]. Even more staggering is the statistic that there is a 21.21% chance that a person will die from cancer. As a result, cancer is the second leading cause of death

in America, second only to heart disease[16]. The number of cancer diagnoses has also increased in the past 30 years, yet many arguably attribute this to improved diagnostic methods. Fortunately the incidence of cancer related deaths has been decreasing over this time period, showing promise in current therapies[16]. To further reduce these statistics, advance and multifaceted approaches are required. As science sheds more light into the complexities of cancer and tumor progression, we are learning that attacking cancer in a monotypic approach, e.g. with a single drug, will not be adequate in most cases. A combinatorial approach will be needed that attacks the cancer as well as the supporting deficiencies that facilitate its progression.

There are many immunotherapeutic methods currently under investigation which address the problem of cancer treatment, including, but certainly not limited to, antibodies, viral vaccines, cell based therapies, and polymer delivery systems[17,18,19]. In general, the goal of these approaches increases the immunogenicity of the cancer and/or creates an antitumor response by increasing the activity of, among others, effector T cells. These approaches have been found to successfully induce and/or augment the immune response, which is necessary in any immunotherapeutic approach. The current study adopts one of these approaches, a polymer based delivery vehicle, to modulate the immune system to combat cancer. The versatility of polymer based delivery systems has allowed them to be used to deliver chemotherapeutic payloads directly to cancer cells[20] or packaged with stimulants such as CpG motif DNA to stimulate dendritic cells of the immune system[21]. These polymer systems have the ability to prime an immune response against, for example, tumor antigens, providing an advantageous platform for use in cancer therapies.

As with most experimental therapeutics, there are problems that limit the full potential of the therapy. It is now apparent that much of the limiting factors are a result of the disrupted immune response caused by cancer. For example, tumor progression, relapse and/or therapeutic evasion may be due to tolerance and immune inhibition initiated directly by the cancer[22,23,24]. In many systems, regulatory T cells (Tregs) are activated and suppress key immune cells such as dendritic and/or CD4+ / CD8+ T cells that are necessary to successfully mount an anti-tumor response. Tregs can be stimulated by dendritic cells, yet have conversely been demonstrated to inhibit the dendritic cell and effector T cell interactions. In addition to these cell dependent mechanisms, there are pathways mediated by soluble factors or cytokines that can also disrupt the natural activity of these same cells. IDO, IL-10, TGF-β, and VEGF are some examples of mediators that are either secreted by cancer cells or immune system cells and contribute to tumor progression and immune suppression[25,26,27,28]. Cancer cells and the tumor microenvironment have established ways to evade and/or mitigate the immune response. To circumvent this, therapies will need to target various branches of the immune response. In attempts to develop these therapies, tumor models are required that display this immune escape mechanisms. It is critical to study the effects of a therapy on these *in vivo* tumor models to gain a deeper understanding of its effects.

Several studies have fortunately begun to investigate the role of therapeutic enhancement by blocking immune suppression. For example, there are antibodies available that can inhibit Treg activity[29]. When applied in combination with tumor specific therapies, one study reportedly induced increased specific tumor immunity compared to therapy alone[30]. However, even in these types of combinatorial studies, complete tumor regression is

rarely seen. For obvious reasons, an antibody based therapy works only for as long as the antibody is available. Once cleared, if the proper immune response was not generated, relapse will occur. These studies however, as many others, fail to understand how a therapy modulates the immune response on a tumor bearing host, where the immune system is greatly altered. Considering this, elucidating the effects any immune modulator has on a cellular and holistic level becomes critical.

1.3 IMMUNE MODULATION: 1-METHYL-TRYPTOPHAN

Among approaches to increase our understanding of Treg control is the identification of specific biochemical pathways that can be blocked and subsequently lead to Treg inhibition and greater effector T cell stimulation. For example, activation of indoleamine 2,3-dioxygenase (IDO) pathways is considered to be an important tolerogenic mechanism in tumor progression, suppressing T cell immunity and increasing Treg based immune suppression[31]. IDO production from stimulated dendritic cells has been shown to directly inhibit T cell proliferation and other studies have demonstrated that IDO is critical in allowing dendritic cells to activate Tregs, ultimately minimizing effector T cell activity[32,33,34]. There is thus undoubtedly an IDO mediated balance between dendritic cell activation of Tregs and effector T cells. Although literature provides evidence of IDO regulated suppression, the *in vivo* role of IDO and its interactions with dendritic cells and T cells remains arguably elusive[35].

Current evidence therefore suggests that an efficient immunotherapy must not only induce maturation of dendritic cells to stimulate effector CD4+ and CD8+ T cells, but also block IDO expression to inhibit Treg activation. It is a hypothesis that by activating

dendritic cells while silencing the effects of IDO one can overcome the immune tolerance induced by Tregs and simultaneously stimulate effector T cells. There have been multiple methods used to inhibit IDO, such as 1-methyl-tryptophan (1-MT) supplementation and IDO-siRNA, both leading to a decrease in Treg suppression and subsequent increase in antitumor T cell activity[36,37]. Using a polymer based delivery system components such as these can be readily encapsulated and delivered to suppress IDO. Although the use of 1-MT, for example, is widely used for IDO inhibition, little is known of the role 1-MT plays on modulating the immune response. As previously described, the immune response can be greatly altered as a result of the tumor microenvironment. Although 1-MT may be effective at ultimately blocking Tregs, literature is uncertain what effects it has on DC stimulation and the immunological responses. One goal of these studies was to expound our understanding of its effects on the immune system independent of IDO.

1.4 POLYMER BASED IMMUNOTHERAPY

Therapies that utilize polymers, more particularly polymers that can be engineered to act as delivery vehicles, have been studies for decades[38]. In addition to being studies in laboratories, polymer carriers are being used widely in clinical medicine[39,40]. More particularly, polymers created into submicron and nanometer scaled vesicles have attracted increased attention over the years. For example, poly(lactic-co-glycolic acid), PLGA, has been widely studied, characterized, has gained FDA approval, and thus been used in clinical trials. Due to PLGAs many benefits over traditional and even newer therapies, literature is filled with different applications for this polymer based systems. It has been used to encapsulate and deliver proteins, viruses, bacteriophages, small

molecules, DNA and RNA[40,41,42,43,44,45]. As the name implies, PLGA is composed of varying portions of lactic acid and glycolic acid. Upon degradation via enzymes or hydrolysis, the acid monomers are released, but due to their presence in many metabolic pathways, the polymer is considered biocompatible/bioresorbable and thus has low toxicity. In relation to their ability to deliver components to cells, *in vivo* and *in vitro*, they have far reaching benefits aside from ensuring a controlled delivery of encapsulated components to cell. These PLGA delivery systems help prevent enzymatic and protetolytic degradation, naturally target macrophages and dendritic cells *in vivo*, can be modified to include targeting moieties, prolong and enhance the delivery of antigens to dendritic cells, and increase antigen presentation and cross-presentation of antigens on dendritic cells [46,47,48,49]. These PLGA molecules provide a power platform for adoption in many different biomedical studies. In the current study we adopted PLGA to engineer nanometer scaled vesicles for the delivery of key components to dendritic cells with the aim of modulating the immune response.

In most applications that target immune cells with the goal of increasing immunity require delivery of multiple components simultaneously; allowing cells to capture the payload and process it. Using a polymer based vehicle has been a popular choice to accomplish this in the past. In many studies, polymers have been shown to deliver components to mature dendritic cells and consequently stimulate T cells with antigen specificity, a critical component to creating antitumor therapies[21]. Numerous studies as these have utilized polymers as a vehicle to stimulate dendritic cells for purposes of inducing immunogenic responses against cancer. Inclusive in these approaches are encapsulating the tumor specific antigen, which has been accomplished by using tumor

lysate or encapsulating a single specific antigen[30,50], as well as a stimulant such as the TLR9 agonist CpG DNA or MLPA[51].

Using this PLGA based delivery system, we engineered a combinatorial therapy and demonstrated its stimulatory effects on dendritic cells and the immunological effects on tumor bearing mice. We used the well characterized antigen ovalbumin (OVA) as the tumor associated antigen, expressed by the lymphoma cell line E.G7-OVA. As an immune stimulant we chose to encapsulate the adjuvant CpG motif DNA, a TLR9 agonist commonly found in bacterial DNA known to initiate an inflammatory response in dendritic cells. We first demonstrated this delivery system's ability to initiate an anti-tumor immune response and overcome immune tolerance of a tumor bearing mouse. By monitoring tumor growth, CTL activity and the Th1 response, it was found that the polymer system could prime for a Th1 response and consequently retard the tumor growth. With this fundamental understanding of the *in vivo* immune response, we next demonstrated how the combination of components affects dendritic cells on an individual cells basis, analyzing cell surface molecules and secretion of key cytokines. It was determined that the polymer system was able to generate a robust Th1 response from dendritic cells. Finally, as a step towards modulating Treg activity, the IDO inhibitor 1-methyl-tryptophan (1-MT) was encapsulated along with the antigen and adjuvant and delivered to dendritic cells. When combined, these components elicit and even higher Th1 response. Taken together, this combination of components, delivered via PLGA, generates a strong, antigen specific, Th1 based, immune response capable of fighting tumor growth and prolonging survival in a tumor bearing host.

CHAPTER 2: LOCAL INJECTIONS OF A POLYMER DELIVER SYSTEM ELICITS A TH1 MEDIATE ANTI-TUMOR IMMUNE RESPONSE

2.1 ABSTRACT

Overcoming tumor-associated immune escape has increasingly become a requirement of immunotherapy treatment strategies. Towards this end, we have developed a polymer based vaccine approach for enhancing immunization of tumor-bearing mice with resulting systemic anti-tumor activity. We demonstrate here that a poly(lactic-co-glycolic acid) (PLGA) based delivery system encapsulating tumor antigen (OVA) and the TLR9 agonist CpG motif DNA can initiate an effective type 1 (IFN-γ producing) mediated anti-tumor response in a tumor bearing mouse model. While E.G7-OVA tumors spontaneously generate antigen specific CTLs in draining lymph nodes, these CTLs do not protect against tumor progression. Intratumoral stimulation with the PLGA based therapy failed to enhance the already present CTLs. However, treatment led to the generation of an elevated antigen specific Th1 response as a result of the PLGA based therapy. Administering intratumoral injections of PLGA with OVA and CpG DNA generated an enhanced systemic and local antigen specific Th1 response as measured by elevated IFN-γ levels. This method significantly delayed tumor growth and prolonged survival. These studies provide insight into the immunological mechanisms and benefits of a PLGA based delivery system on a tumor bearing host.

2.2 INTRODUCTION

Studies from our and other laboratories have focused on identifying immune escape mechanisms and developing targeted strategies to overcome these mechanism[18,52,53]. In particular, our work has focused on approaches to modulate the tumor microenvironment to enhance effective systemic immunity. Studies clearly demonstrate that the tumor microenvironment involves a complicated interface between the immune system and the tumor, leading to poor antigen presentation, elevated levels of IL-10 or increased Treg activity[22,23,24,26,54]. The immune escape mechanisms have significant impact on the development of spontaneous anti-tumor immunity as well as potentially inhibiting immunotherapies. In efforts to overcome escape, our and other studies have identified the need to both include tumor antigen as well as immune regulatory mediators in the treatment regimens for optimal therapeutic success.

In light of this, there is a clear need to understand and target alternative branches of the immune response to fight cancer. CD4+ T cells, for example, have been studied for their helper cell properties as well as antitumor activities. These helper cells are known to augment the *in vivo* effects of CTLs, boosting tumor immunity[55,56]. Increasing evidence is now demonstrating the anti-tumor properties of these CD4+ T cells, mediating the killing of tumor cells independent of CD8+ T cells[57,58]. The hallmark cytokine of this Th1 response, IFN-γ, is a key component in this CD4+ mediated tumor killing. IFN-γ is responsible for recruiting NK cells and macrophage and inhibiting angiogenesis[59,60]. Armed with this knowledge, researchers attempt to understand how their therapies affect both CD8+ and CD4+ activity and in turn design better therapies to elicit stronger

responses. As described, the *in vivo* environment greatly alters the immune response and understanding how a therapy can alter it is paramount in generating effective anti-tumor therapies.

In the current study we evaluated the de-novo response at both the local and systemic compartments of a tumor bearing host as a result of a polymer based therapy. Tumor associated antigen in the form of ovalbumin (OVA) and the immune adjuvant CpG motif DNA were encapsulated in poly(lactic-co-glycolic acid) (PLGA) and administered to E.G7-OVA bearing mice. PLGA, an FDA approved resorbable polymer widely utilized as a delivery vehicle offers numerous advantages over soluble delivery of therapeutic agents; enhancing and prolonging antigen presentation, increasing cross presentation, ensuring co-delivery of antigen and adjuvant to antigen presenting cells, and protection from proteolytic degradation[46,47,61,62]. Despite these benefits and evidence of potent immune stimulation from PLGA based systems, there is often a failure to analyze the immunological effects of a this delivery system on a tumor bearing model[50,51,63]. Here we demonstrate the mechanism used to escape immune tolerance and induce anti-tumor behavior in response to a PLGA based therapy. In our studies we find that despite a significant CTL response in the local lymph nodes of tumor bearing mice, tumor growth progresses. It was found that with the PLGA based therapy a Th1 response is significantly elevated and consequently delays tumor growth.

2.3 MATERIALS AND METHODS

2.3.1   Tumor model

The OVA expressing cell line E.G7-OVA (ATCC), derived from the EL4 lymphoma cell, was maintained in TCM media (RPMI 1640 based media supplemented with 10% FBS, 2mM L-Glutamine, essential AA, non-essential AA, 110mg/L Na-Pyruvate, 50IU/mL penicillin/streptomycin). Cells were maintained in 37°C incubators with 5% CO_2. Cells were split two days prior to implantation to ensure consistent growth.

2.3.2   PLGA based delivery system

Poly(lactic-co-glycolic acid) (PLGA) based vesicles were generated using the water/oil/water double emulsion method[48], with the following modification. In brief, 0.1g of PLGA (Sigma-Aldrich, MW 7-17kDa, 50:50 ratio) was dissolved in 0.4mL of chloroform (Sigma-Aldrich). 50mg/mL of OVA (ovalbumin, Sigma-Aldrich) and/or 5mg/mL of CpG DNA (ODN-1668, phosphorothiated TCCATGACGTTCCTGATGCT, IDT) was added to the PLGA mixture at 0.05mL per 0.4mL of chloroform/PLGA. A microtip sonicator (Branson Ultrasonics) created the primary emulsion at 60% magnitude for 5sec pulses and repeated for 4 cycles. This primary emulsion was combined with a 2mL solution of 7% PVA (Sigma-Aldrich, MW 31-50kDa, 87-89% hydrolyzed) and sonicated for another round. Secondary emulsion was added drop-wise to an 8mL bath of a 7% PVA solution under constant stirring overnight at 4°C. Final product was collected by centrifuging at 16,000xg for 2 hours, washed with dH_2O, resuspended in 2% sucrose and lyophilized.

To determine loading efficiency, FITC labeled BSA (Sigma-Aldrich) was dissolved to 50mg/mL and Cy5 labeled CpG DNA (IDT) was dissolved to 5mg/mL and incorporated into the polymer fabrication as before. Lyophilized polymer was weighed and lysed with 3M NaOH. Supernatant was collected and scanned on a fluorescent plate reader at 495nm and 655nm, corresponding to the labeled BSA and CpG DNA respectively. Standard curves were generated and loading concentrations calculated.

Scanning electron microscopy (SEM) was performed on washed and dried PLGA vesicle samples. Samples were first sputter coated with gold for 2min at 30-40mA. Imaging was performed at the indicated magnifications with a 20kV electron beam.

2.3.3 Murine model and therapy

6-8 week old female C57BL/6 mice (Jackson Laboratories) were immunized via s.c. injections into the lower left ventral abdomen with 5mg of polymer in 100μL of PBS (day -14), followed by another 5mg injection of the same polymer after 2 weeks (day -7). Saline solution was used as a sham control. After an additional one week, mice were challenged with $250x10^3$ EG7-OVA cells s.c. in the lower left ventral abdomen (day 0). For the treatment model, $250x10^3$ E.G7-OVA cells were implanted s.c. into the lower left ventral abdomen. On days 3, 5, and 7, i.t. injections of 5mg of the respective polymer were administered. Mice were monitored and the tumor sizes were measured every 2 days with metric calipers by measuring the largest two diameters. Mice were sacrificed when the longest diameter of the tumor reached 1.5cm. Kaplan Meier survival plots were generated using MedCalc commercial software. Mean tumor sizes were calculated and statistical analysis performed using a two sample Student t-test.

2.3.4 ^{51}Cr release assay

Cytotoxic T Lymphocyte (CTL) assays were performed as previously reported with the following modifications[26]. Stimulators were generated from processed spleens of naïve female C57BL/6 mice. Spleens were homogenized, incubated with ammonium chloride buffer (ACK, 0.15M NH$_4$Cl, 1.0mM KHCO$_3$, 0.1mM EDTA) for 5 min to lyse red blood cells, washed with TCM then filtered through a 70μm mesh (BD Falcon). Cells were resuspended in TCM media, supplemented with 2-mercaptoethanol (2-ME), at $4x10^6$ cells/mL an restimulated with or without 300μg/mL of OVA overnight. Effector cells were harvested and processed from the inguinal lymph nodes and spleens of day 16 treated mice. $3x10^6$ stimulator splenocytes were then added to $7x10^6$ effector cells in 2mL total volume. On day 4, 50μL of supernatant was harvested from each condition and analyzed for INF-γ via a sandwich ELISA. On day 5, effector cells were harvested and added to target cells. $2x10^6$ E.G7-OVA target cells were incubated with 100uCi of ^{51}Cr for 1 hour at 37°C and 5% CO$_2$. ^{51}Cr-labeled target cells ($5x10^3$) and effector cells were combined at known effector:target (E:T) ratios in 200μL of TCM media. After 4hrs in 37°C and 5% CO$_2$, 100μL of supernatant was collected and measured for ^{51}Cr with a gamma counter (Packard Bioscience). Percentage of specific lysis was calculated from the formula (experimental release-spontaneous release) x 100/(maximal release in 1% SDS+0.5%NaOH spontaneous release).

2.3.5 ELISA

Purified rat anti-mouse IFN-γ (PharMingen) were diluted in coating buffer (NaHCO$_3$) to 2μg/mL and incubated overnight at 4°C on 96 well flat bottom plates (Nunc). Plates were

blocked with PBS/10%FBS for 2 hours at room temperature. Standards and sample were added at 100µL per well and incubated overnight at 4°C. After washing, biotin anti-mouse IFN-γ (PharMingen) were dissolved to 1µg/mL and added at room temperature for 45min. Wells were washed and avidin-peroxidase diluted to 2.5µg/mL in PBS/Tween/0.1%FBS for 30 minutes at room temperature. Enzyme activity was determined using O-phenylenediamine dihydrochloride reagent (OPD, Sigma) dissolved to 1mg/mL citrate buffer (pH 4.5) with 3% H_2O_2. Reaction was stopped with 3M HCl and color read at 492 nm. Statistical comparison between experimental averages was done using a two sample equal variance Student's *t*-test.

2.4 RESULTS

2.4.1   Polymer delivery system

With the goal of developing a vaccine platform which would allow incorporation of both varied antigenic material and immune-regulating agents, we have adopted the FDA-approved, resorbable copolymer PLGA. Based on the water/oil/water emulsion technique[50], PLGA was utilized to fabricate submicron diameter vesicles. SEM images (Figure 2.1A) visualize the polymer vesicles when fabricated as described, having an average diameter of 500nm. With a starting concentration of 50mg/mL, final encapsulation of the tumor associated antigen OVA was measured to be 11µg of OVA per mg of polymer vesicle. Final CpG DNA encapsulation with a starting concentration of 5mg/mL yielded a final 150pg of CpG DNA per mg of vesicle. To verify the ability of the polymers to be phagocytosed by antigen presenting cells, mainly dendritic cells (DCs), we delivered PLGA particles containing rhodamine labeled dextran to DCs in vitro. As seen in Figure 2.1B, these DCs readily uptake the polymer vesicles.
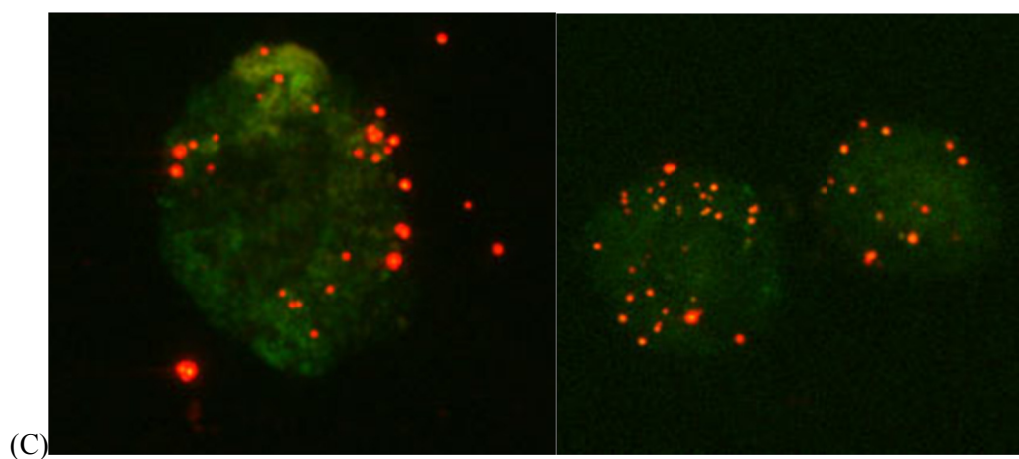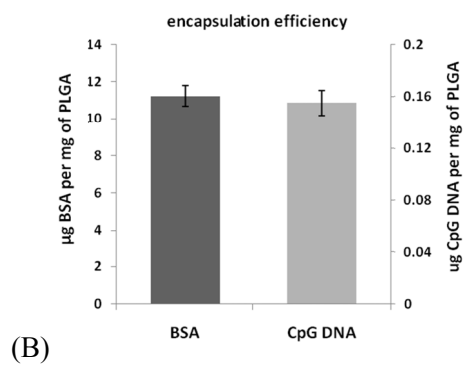
(A)

(B)

(C)
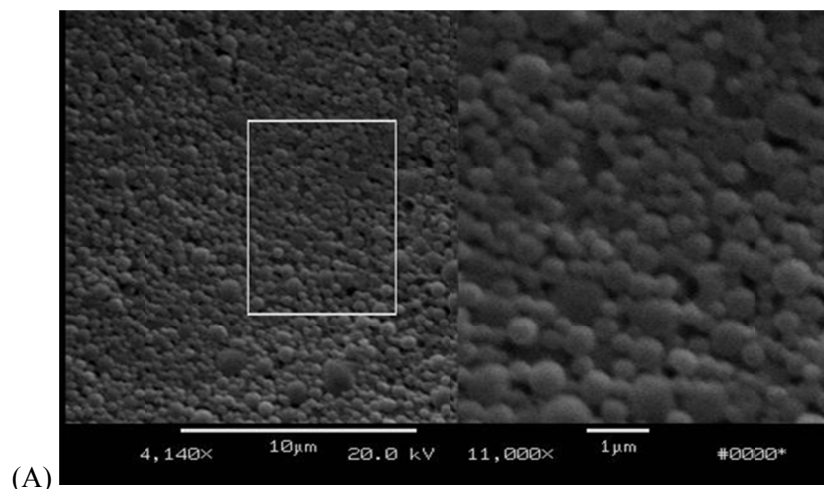
Figure 2.1 Polymer characterization. (A) SEM images at 4,140x and 11,000x magnification provide visuals of the PLGA vesicles. Scale bars indicate 10µm and 1µm for the 4,140x and

11,000x magnification, respectively. (B) To quantify the amount of protein and CpG DNA, fluorescently labeled BSA and CpG DNA were encapsulated within the PLGA vesicles. Standard curves were generated and the concentration of the vesicles were calculated to determine the loading efficiency; µg of BSA or CpG DNA per mg of PLGA vesicle. (C) Bone marrow derived dendritic cells were cultured for 48hrs with PLGA particles containing TMR dextran. Cells were stained with FITC labeled CD11c.

2.4.2 Prophylactic immunization protects against tumor formation

To demonstrate the capacity of the polymer complex to effectively generate tumor-specific immunity, mice were pretreated with the polymer vaccine then challenged with the lymphoma tumor line E.G7-OVA. We chose the combination of tumor antigen OVA and CpG DNA based on the impetus to include tumor antigen with an adjuvant. Unmethylated CpG motif DNA is a popular TLR9 agonist, as it provokes a power defense mechanism from B cells and antigen presenting cells. It is known to create vigorous IFN-γ responses, capable of inducing robust anti-tumor behavior, particularly when used in concert with tumor associated antigen[64,65]. Based on this, mice were pretreated with the polymer vaccine then challenged with tumor. In the ten mice per condition of two independent experiments, we see significant protection against tumor formation after administration of the polymer (Figure 2.2A). In sham mice, tumors were palpable on average by day 7, while tumor onset was delayed until day 9 for CpG DNA treated mice, day 13 for OVA treated mice and day 17 for OVA+CpG DNA treated mice. At the average time of death for non-treated mice, day 17, all mice treated with antigen and adjuvant had either no palpable tumors or below 10mm^2 tumor cross-sections while sham had most tumors above 100mm^2 cross-sections ($p < 0.01$, Figure 2.2B). There were

however no difference between mice treated with OVA alone or OVA with CpG, as the anti-tumor response from either regimen was very effective at delaying tumor onset. In addition, vaccination prolonged survival of mice in each treatment condition compared to sham ($p < .02$, Figure 2.2C). Several tumor free mice as a result of vaccination were seen, demonstrated in the Kaplan-Meier survival curves (Figure 2.2C).
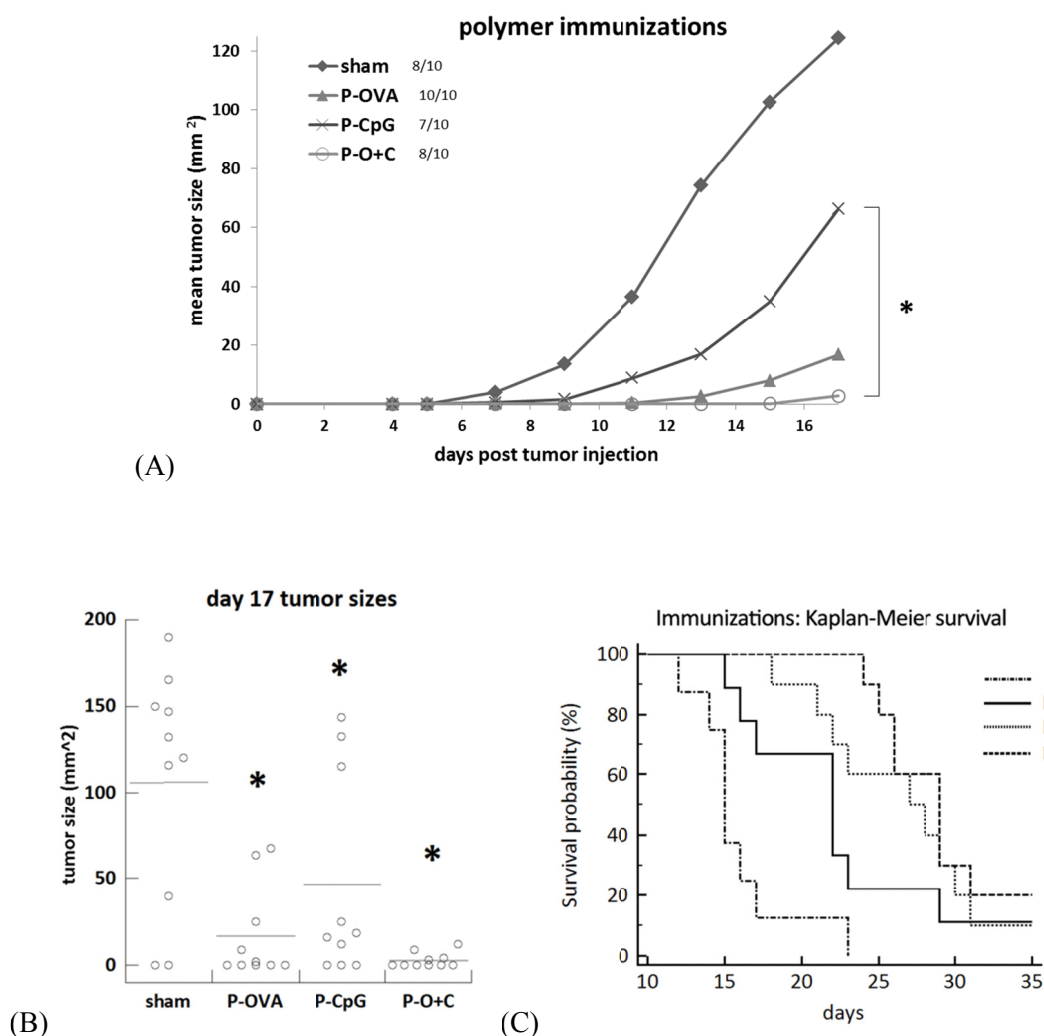

(A)

(B)

(C)

Figure 2.2 Prophylactic treatment with respective polymer vesicles. (A) 5mg of indicated polymer (PLGA with OVA (▲ P-OVA), PLGA with CpG (× P-CpG), PLGA with OVA+CpG (○ P-O+C)) or PBS (♦ Sham) was injected on days -14 and -7, followed by E.G7-OVA tumor

challenge on day 0. Tumor size was monitored and reported as the cross sectional area using largest two diameters. Two independent experiments with 5 mice each (10 total) were conducted and mice with no tumor formation were omitted from growth curves. (B) Day 17 tumor size distribution where each point represents one mouse in each respective condition. (C) Kaplan-Meier survival curves demonstrate the probability of survival in response to polymer treatment. Significance calculated by standard ANOVA followed by Tukey-Kramer post-hoc analysis and reported as $p < 0.05$ (*). For Kaplan-Meier, significance reported as $p < 0.01$ (*) or $p = .02$ (¥) in comparison to sham unless otherwise noted.

2.4.3 Therapeutic treatment significantly delays tumor growth

While prophylactic immunization is able to demonstrate the ability to vaccinate against an antigen specific tumor, the overall goal must be therapeutic studies on established tumors. As a means to drive a therapy towards the clinic, determining the responses of treatment on a tumor bearing host is essential. Towards this end, tumor bearing mice were administered the PLGA based therapy intratumorally and responses investigated. The studies show that a significant delay in tumor growth is observed after administration of polymer treatment (Figure 2.3A). While empty PLGA vesicles had no effect on tumor growth compared to sham (not reported), we did observed that all other polymer treatment conditions delayed tumor growth in comparison to sham (15 mice from 3 independent experiments, $p < 0.01$, Figure 2.3B). Although no significant difference between OVA and CpG DNA treatment was observed, PLGA with both OVA and CpG DNA did significantly delayed tumor growth better than any other polymer treatment ($p < 0.02$, Figure 2.3A). By day 17, all OVA and CpG DNA treated mice had tumors below 50mm^2, while all other polymer conditions had numerous if not most tumors above

50mm^2 ($p < 0.05$, Figure 2.3B). Additionally, polymer with OVA and CpG provided the optimal survival probability, with several mice not forming tumors ($p < 0.01$, Figure 2.3C).

(A)

(B)

(C)
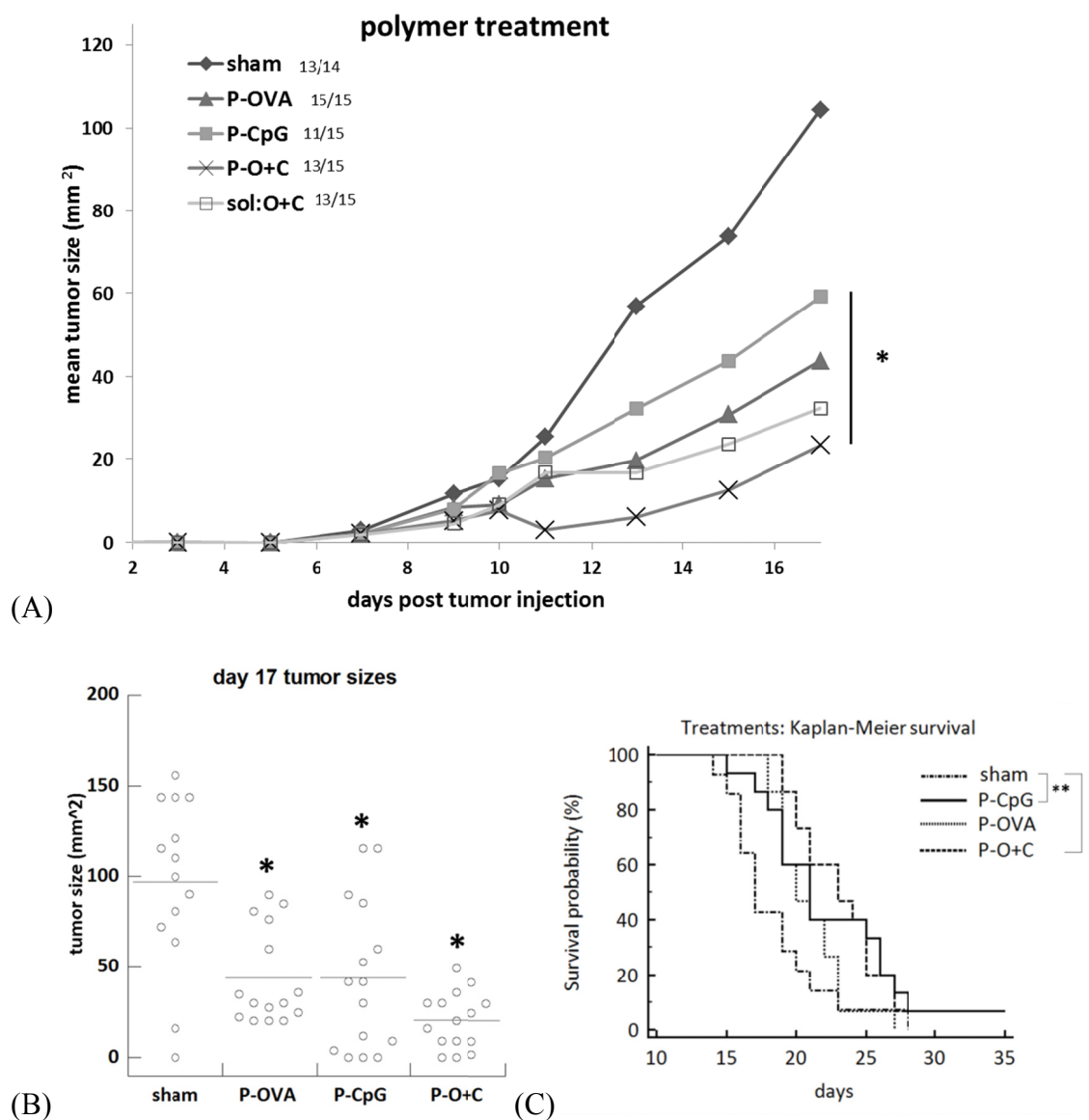
Figure 2.3 Tumor treatment model with polymer vesicles. (A) E.G7-OVA cells were implanted on day 0 and treatments were administered i.t. on days 3, 5 and 7 with the indicated conditions

(PBS (♦ sham), PLGA with CpG (■ P-CpG), PLGA with OVA (▲ P-OVA), PLGA with OVA and CpG DNA (× P-O+C), OVA and CpG in solution/PBS (□ sol:O+C)). Tumor size reported as the cross sectional area using largest two diameters. Three independent experiments with 5 mice each (15 total) were conducted and mice with no tumor formation were omitted from growth curves. (B) Day 17 tumor size distribution where each point represents one mouse in each respective condition. (C) Kaplan-Meier survival curves demonstrate the probability of survival in response to polymer treatment. Significance calculated by standard ANOVA followed by Tukey-Kramer post-hoc analysis and reported as $p < 0.05$ (*). For Kaplan-Meier, significance reported as $p < 0.01$ (*) and $p < 0.05$ (**) calculated by standard t-test.

2.4.4 E.G7-OVA spontaneously elicits local but not systemic CTL response

Given the well-established role of CTLs in anti-tumor responses[66], we analyzed these cells in our current therapeutic system. The studies demonstrate that while CTLs prove effective at killing tumor cells, they fail at inhibiting *in vivo* tumor progression. Figure 2.4 shows a representative result of one of three ^{51}Cr release assays demonstrating the systemic (spleen) and local (tumor draining lymph node) antigen specific CTL response of tumor bearing mice post treatment. We observed that tumors alone lead to a significant local lymph node, but not systemic (spleen), CTL response. Mice can spontaneously generate antigen specific cells to target and kill E.G7-OVA cells. It was also demonstrated that the polymer treatments were unable to increase this response, as there was still no detectable systemic CTL activity and no measurable change in local CTLs after polymer treatment. This divergence should be noted, as polymer treatments were still able to retard tumor growth, providing evidence of the dysfunctional CTL *in vivo* activity.
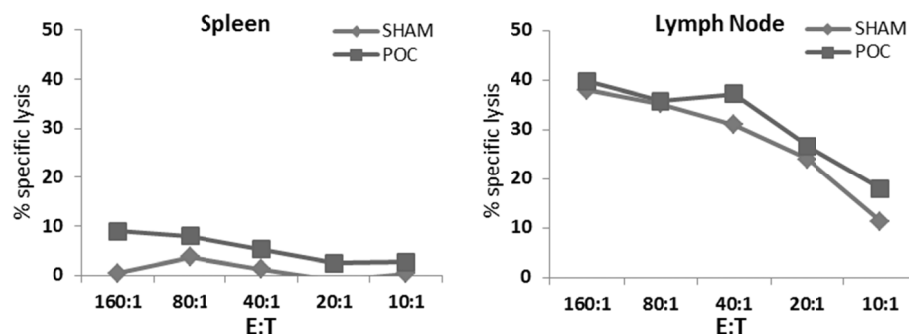
Figure 2.4 Representative systemic and local CTL responses against E.G7-OVA cells. Restimulated spleens and lymph nodes from respective conditions were cultured with ^{51}Cr labeled target cells, E.G7-OVA, at the indicated Effector:Target (E:T) ratios. Systemic (spleen) specific lysis was below detection after OVA restimulation. CTL response was elevated in the tumor draining lymph nodes as a result of restimulation, however no difference is seen between the different treatment conditions. Data representative of three independent experiments run in triplicate.

2.4.5   Polymer delivery system elevates Th1 response

In addition to the known CTL anti-tumor activities, studies have identified and exploited the anti-tumor effects associated with a CD4+ response[57,59]. A CD4+ mediated response is popularly characterized as type 1, IFN-γ producing. Our studies demonstrate that local injections of the polymer vaccine were able to increase the systemic and local type 1 response. We found that sham mice express a low baseline level of IFN-γ in both the spleen and lymph nodes (Figure 2.5A). Mice treated with OVA and CpG DNA via PLGA polymer were able to generate a significant IFN-γ response both systemically and locally as compared to sham ($p < 0.05$, Figure 2.5A). Interestingly, we found that when mice were treated with OVA and CpG DNA in soluble form, there was no induction of a type 1 response, as no significant change in IFN-γ levels was observed (Figure 2.5B).
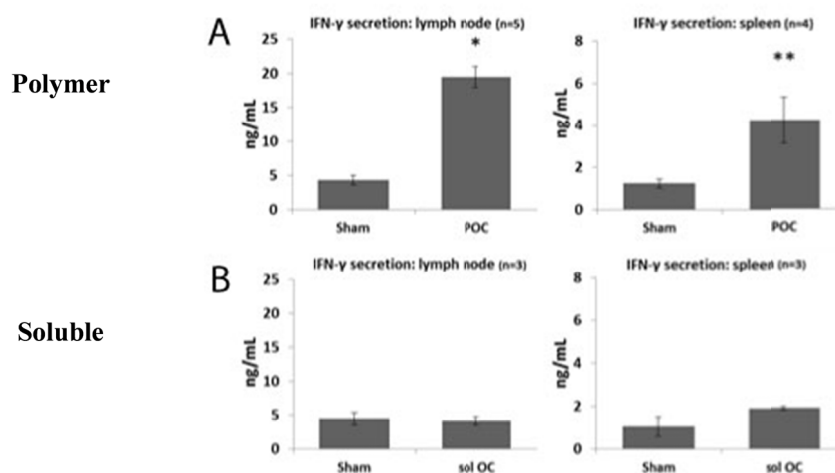
Figure 2.5 INF-γ levels from restimulated spleens and lymph nodes. (A) Systemic and local levels of INF-γ are elevated as a result of the polymer treatment containing both OVA and CpG DNA. (B) Delivery of OVA and CpG DNA dissolved in PBS had no effect on IFN-γ expression levels. Significance reported as $p < 0.01$ (*) or $p < 0.05$ (**) in comparison to sham. Data represents the average of n=3-5 independent experiments run in triplicate.

2.5 DISCUSSION

The studies presented here demonstrate the ability of a polymer delivery system to induce a Th1 based anti-tumor immune response in a tumor bearing mouse model. It was shown that this PLGA based delivery system generated an IFN-γ mediated response to provide protection against tumor growth. We found that although CTLs were present, they were incapable of delaying the growth of the tumors. This was overcome by the PLGA polymers ability to induce a robust Th1 response and significantly delay tumor growth.

This study provides an alternative approach to common immunotherapies for inducing anti-tumor immune responses.

As we and others have shown, there is a niche of mechanisms identified that are responsible for immune escape. It has been well documented that tumors can curb the immune response by producing and/or inducing factors such as IL-10[26,67,68], TGF-beta[69], VEGF[70], and by activating Tregs[71]. Our prior studies in the murine bladder cancer model MB49 showed that IL-10 can render antigen presenting cells deficient and inhibit immune stimulation[26]. It was demonstrated in this MB49 model that local but not systemic CTLs were generated by the tumor. This lack of systemic immunity could be overcome by local administration of a recombinant viral vaccine producing antigen and GMCSF. However, it was unable to induce tumor regression[18]. In the current cancer lymphoma model, E.G7-OVA, tumors induced local CTL activity as well (Figure 2.4) but are also unable to block tumor progression (Figure 2.3A). In an attempt to broaden the therapeutic immunization techniques and also investigate non-viral platforms, we aimed to elicit a similar anti-tumor response by utilizing intratumoral injections of a polymer based delivery system that administers both an antigen and an immune stimulant.

Although methods such as viral vectors provide effective anti-tumor immunity, PLGA was chosen as an alternative vehicle for delivery for its many benefits. PLGA is FDA approved, is easily adaptable to different systems and cost effective. It has high bio-compatibility with minimal toxicity; the original monomers, lactic acid and glycolic acid, are byproducts of various metabolic pathways[72]. In addition, these vesicles are phagocytosed *in vivo* by macrophages and antigen presenting cells (APCs), facilitating

the development of adaptive immunity[48]. As we have shown in prior studies, PLGA based vaccines provide a superior platform to increase the activation of dendritic cells and the overall Th1 response from these profession antigen presenting cells (in press). It was shown that delivery of antigen and adjuvant via PLGA vesicles could increase the Th1 profile of bone marrow derived dendritic cells *in vitro*. Cell surface expression of CD80, CD86, MHCII and secretion of IL-12 were upregulated as a result of treatment. It was hypothesized that this dendritic cell response would provide optimal utility in a tumor study. To demonstrate the ability of this polymer system to induce an antigen specific anti-tumor response, the polymer was adopted for use in the E.G7-OVA tumor bearing mouse model.

Studies were conducted to demonstrate the ability of the polymer system to reverse the anergy observed in tumor bearing mice. It was found in our studies that intratumoral injections of the PLGA vesicles were unable to produce measurable systemic CTLs as previously anticipated and seen in our viral vector based MB49 studies. However, PLGA administration could reverse immune escape by greatly elevating the IFN-γ mediated Th1 response. The PLGA based delivery system was able to induce elevated IFN-γ levels both systemically and locally when OVA and CpG DNA were delivered together. This elevated Th1 response is directly correlated with tumor growth, as mice treated with the polymer vesicles provide the greatest protection against tumor growth in both a prophylactic and treatment study. It is interesting to note that soluble delivery of the same components was unable to generate any increase in IFN-γ secretion both locally and systemically (Figure 2.5B). These results further demonstrate the benefits and motivation of using a polymer based delivery system. Similar findings support this phenomena and

show the advantage of a PLGA based antigen delivery system at enhancing humoral immunity, inducing elevated antibody levels, and generating Th1 responses[73,74,75,76,77]. In particular, elevated IFN-γ is commonly attributed to a strong CD4+ mediate Th1 response[59], and has been shown to act directly on tumor cells to inhibit both proliferation and angiogenesis to facilitate apoptosis[78,79,80]. The current data thus supports this hypothesis that PLGA delivery to a tumor bearing host generates a strong IFN-γ mediated Th1 response that supports combating tumor growth.

In conclusion, this PLGA based delivery system provides an effective means to deliver antigen and adjuvant to initiate anti-tumor responses. Although tumor cells are able to elicit local antigen specific CTL responses in mice without vaccination or stimulation, this alone is insufficient at perturbing tumor growth. The data suggest tumor cells fail to prime for a functional type 1 (IFN-γ) response in normal mice, allowing tumor progression. Treatment with a polymer system such as PLGA provides a means of escaping immune tolerance, in this case by priming and protect via an elevated IFN-γ mediated, antigen specific response. With a broader understanding of the immune responses in tumor bearing hosts and the roles therapies play at modulating those responses, we can better assess and subsequently design therapeutic strategies.

CHAPTER 3. ANALYSIS OF DENDRITIC CELL STIMULATION UTILIZING A MULTI-FACETED NANOPOLYMER DELIVERY SYSTEM AND THE IMMUNE MODULATOR 1-METHYL TRYPTOPHAN

3.1 ABSTRACT

Dendritic cells (DCs) play a pivotal role in immune modulation. Therefore, understanding and regulating the mechanism of DC activation is paramount for functional optimization of any immunotherapy strategy. In particular, the paradoxical ability of DCs to secrete the immune suppressive enzyme indoleamine 2,3-dioxygenase (IDO) and the suppressive cytokine IL10 during the course of, and in response to, stimulation is of great interest. 1-Methyl-Tryptophan (1MT) is a known inhibitor of IDO and has thus been administered in numerous in vitro and in vivo systems to block IDO activity. However, the effect 1MT has on DCs beyond inhibiting IDO, especially in therapeutic models, has rarely been analyzed. In the current study, we have administered 1MT via a nanopolymer based delivery system in conjunction with an antigen (ovalbumin, OVA) and an adjuvant (CpG motif DNA) to determine both the effects of 1MT on DCs and the resulting efficacy of the polymer based treatments. 1MT delivery alone, either via the polymer based delivery vehicle or dissolved in solution, induced no significant change in DC activation as measured by surface expression of CD80, CD86, and MHCII and several secreted products such as IL-12. These same factors were up-regulated however, when 1MT was delivered in conjunction with OVA and CpG. Although soluble delivery of these components increased the levels of expression and secretion of key proteins, a differential effect of DC stimulation was seen as a result of

the polymer delivery system. The T cell suppressive IL10 secretion was lower with the polymer based treatments and IL-12 immune enhancing secretion was elevated when 1MT was supplemented into the polymer system. As a result, including 1MT in the polymers along with OVA and CpG was seen to have additional effects on DC stimulation and was able to shift DCs to a state more indicative of inducing a Th1 type response.

3.2 INTRODUCTION

Dendritic cells (DCs) play a powerful role in immune system modulation and interact in various ways with many T cell subsets. CpG motif DNA or lipopolysaccharide (LPS) from bacteria stimulate DCs to upgregulate a number of critical immunomodulatory molecules, including, for example, increased expression of CD80 and CD86 and increased secretion of IL-12 and TNF-α [1,2]. DCs interact with specific T cell subsets to initiate or augment antigen specific immune responses. However, binding of CD80(B7-1)/CD86(B7-2) to different receptors on T cells, specifically CD28 or CTLA-4, result in differential stimulation of T cells. Interaction with CD28 results in an expansion of T cells[3,4], while binding of CTLA-4 results in an attenuated T cell response[5,6]. These divergent responses are essential for the regulation of T cell activity and in coordination with the cytokines secreted by DCs, aid in maintaining T cell homeostasis. Hence, the classic role of DC activation of T cells for the purpose of killing and removing pathogenic cells is now known to be only a partial immunomodulatory role of these potent APCs.

It is known that DCs can directly facilitate and induce immune suppression, for example via the CTLA-4 pathways[7,81]. DCs can stimulate a subset of T cells now termed T regulatory cells (Tregs), originally identified as a subset of lymphocytes that provided a degree of tolerance[8]. It is believed that both CD4+ and CD8+ Tregs exist, each performing distinct yet complementary functions[9]. DCs can interact and activate both Treg subsets[10,11], typically mitigating the T cell milieu. Studies have begun to investigate methods of blocking this immune suppression, via antibodies against Tregs, which are proving to be effective therapeutic supplements[29,30]. Biochemical enzymes and pathways are also potential avenues for Treg blockade. The indoleamine 2,3-dioxygenase (IDO) enzyme pathway has been described as an important tolerogenic target in tumor progression, suppressing T cell immunity and increasing Treg based immune suppression[31]. Stimulated DCs increase their levels of IDO production and can directly inhibit T cell proliferation and activate Tregs, minimizing effector T cell activity[32,33,34]. With this clear IDO mediated balance within DC activation, there is a need to minimize its activity. Multiple methods have been used to inhibit IDO, the most popular being 1-methyl-tryptophan (1MT) supplementation, which leads to a decrease in Treg suppression and subsequent increase in effector T cell activity[36]. However, the literature is sparse in details concerning the cellular and biochemical effects 1MT has on DCs.

Considering the power and versatility of DCs with regards to their ability to modulate T cell activity, understanding how 1MT affects DC subsets becomes increasingly significant. A major goal in DC therapy is to induce antigen specific immunity while inhibiting IDO activity, providing a strong effector T cell response, while inhibiting Tregs. In the current study, we aimed to develop an efficient and quantitative method to

deliver 1MT in conjunction with antigen and a known adjuvant, CpG motif DNA, to bone marrow derived DCs. The ultimate goal is to provide antigen specific stimulation, while minimizing and/or inhibiting the suppressive capabilities of DCs. In such a process though, 1MT can have collateral effects on DC activation. We sought to elucidate the effects, either negative or positive, of 1MT treatment on DCs by delivering 1MT via a polymer deliver vehicle. DCs were treated with 1MT alone or in conjunction with stimulatory factors and analyzed for secreted products and their cell surface expression of key proteins responsible for costimulation. Our results indicate 1MT may be playing a larger role in DC stimulation than was previously thought.

3.3 MATERIALS AND METHODS

3.3.1 PLGA polymer characterization

Polymer vesicles were generated using a water/oil/water double emulsion method[48], with the following modification. In brief, 0.1g of poly(lactic-co-glycolic acid) (Sigma-Aldrich, MW 7-17kDa, 50:50 ratio) was dissolved in 0.4mL of chloroform (Sigma-Aldrich) using a sonicator water bath to expedite dissolution. Aqueous phase, containing OVA (ovalbumin, Sigma-Aldrich), CpG DNA (ODN-1668 TCCATGACGTTCCTGATGCT, phosphorothiated, IDT) and/or 1MT (1-Methyl-DL-tryptophan, Sigma-Aldrich), or Dextran-TMR (40kDa, Invitrogen) was added to the organic PLGA mixture at 0.05mL per 0.4mL of chloroform/PLGA. To create the primary emulsion, a mirotip sonicator (Branson Ultrasonics) was placed in the first aqueous and organic phase and ran at 60% magnitude for 5sec pulses, followed by 30sec pauses, repeated for 4 cycles. To create the secondary emulsion, the resulting primary emulsion was placed in 2mL of a 9% PVA solution (Sigma-Aldrich, MW 31-50kDa, 87-89% hydrolyzed) and subjected to identical

sonication as the primary emulsion. To finalize the secondary emulsion, all of secondary emulsion was added drop-wise to an 8mL bath of a 9% PVA solution under constant stirring. Solution was kept under constant stirring for at minimum 5 hours to evaporate the organic solvent. Final product was collected by centrifuging at 17,500xg for 2 hours, washed twice with dH_2O, resuspended in 2% sucrose and lyophilized.

Particle size was determined by dynamic light scattering using a Zetasizer (Malvern Instruments Ltd). Antigen loading was determined by first lysing the polymer vesicles in 0.1M NaOH for 24 hrs then analyzing the supernatant with UV spectroscopy at 280nm and 260nm or using a BCA protein assay. Due to interference in the BCA assay, 1MT loading capacity was estimated based on the OVA encapsulation results. To load 1MT into PLGA vesicles at specific amounts, the starting concentrations of OVA used to obtain a desired final loading concentration was used as the starting concentration for 1MT. To determine vesicle weight, 10mg of polymer vesicle was dissolved in 200uL PBS and analyzed via flow cytometry (BD FACSCalibur) at a constant flow rate of 12µL/sec for 30sec. Using BD CellQuest software, polymer count was established and the average weight of each polymer capsule calculated.

3.3.2   DC generation

Dendritic cells were generated from murine bone marrow as previously described with slight modification[26]. Briefly, the femurs and tibias of six to eight-week-old male C57BL/6 mice (Jackson Laboratory) were removed and cleaned of excess tissue then washed with 75% EtOH for 60 seconds. Marrow was flushed out with 1x PBS using a syringe and 27 $^{1/2}$ gauge needle. Bone marrow plugs were dissociated by vigorous inverse

pipetting. Red blood cells were lysed using 5mL of ammonium chloride buffer (0.15M NH_4Cl, 1.0mM $KHCO_3$, 0.1mM EDTA) for 5 min. Cells were washed twice with media (Invitrogen Advanced RPMI 1640, 110mg/L Na Pyruvate, 10% FBS, 50IU/mL penicillin/streptomycin, and 2mM L-Glutamine) and filtered through a 70μm cell strainer. Resulting cell pellet was resuspended to a concentration of $2x10^6$ cells/mL in media with 10ng/mL of GM-CSF and 10ng/mL of IL-4 (R&D) and plated at 5mL per well on 6 well plates (BD). Cells were then incubated at 37°C and 5%CO_2. Every 48hrs floating cells were removed and the media was replaced with fresh media and cytokines. On day 7-9, all cells were harvested using a cell scrapper and characterized as immature DCs for subsequent analysis and experimentation.

3.3.3   Polymer treatments

Bone marrow derived DCs were resuspended at 1x106cells/mL in media at 1mL per well of a 24 well plate (BD Falcon). Prepared polymer vesicles was weighed, sterilized by UV light for 10 minutes, and dissolved in media and added to cell suspensions. LPS (055:B5, Sigma-Aldrich) was used a control for stimulation at a final concentration of 2μg/mL. Cells remained in 37°C and 5%CO2 during the remainder of treatment.

3.3.4   Immunocytochemistry and flow cytometry

DCs were harvested from culture via vigorous inverse pipetting and washed with PBS. Cells were blocked with FC block (BD Pharmingen) for 10min then stained in 100uL with 2ug/mL of antibodies for the following surface proteins; CD80, CD86, CD11c, MHCII, CD8a, CD11b (BD Pharmingen). Expression levels were analyzed via flow

cytometry using a BD FACSCalibur with BD CellQuest or a Coulter Cytomics FC500 and analyzed with the Coulter CXP software. Geometric mean fluorescent intensities are calculated by subtracting the intensities of the respective isotype controls from the Ab treated conditions. Flow cytometry on the Coulter Cytomics FC500 was performed using the CINJ Shared Resource.

3.3.5   ELISA/Bioplex

Supernatants from cell cultures were harvested and run through an ELISA with paired antibodies or run on a Bio-Rad Bioplex Suspension Array System. For the ELISA, a standard sandwich assay was performed. For the Bioplex system, 50uL of sample was used and the protocol for the assay was followed as per manufacturer's recommendations, analyzing samples in duplicate. Statistical comparison between experimental conditions was done using a two sample equal variance Student's t-test. Differences were considered significant at $p < .05$ and thus reported.

3.4 RESULTS

3.4.1   Polymer delivery system

We chose to utilize the FDA-approved, resorbable copolymer PLGA due to its versatility and well characterized properties, that allow one to deliver any specified tumor associated antigen and/or immune stimulant at desired concentrations. Based on the water/oil/water emulsion technique[50], particles were constructed in an array of sizes starting as low as 200nm with highest sonication power settings available. Decreasing sonication power to 10% increases the vesicle average diameter to 500nm. The set time of exposure to sonication strength ensures a homogenous population at the reported

average vesicle size, while shorter exposures results in large polydispersity and heterogeneous sizes. Longer exposures would further decrease the polydispersity, however this causes excessive heat to develop within the system. Using dynamic light scattering, we verified the reproducibility of particle size generation (Figure 3.1A) with an average polydispersity below 0.1, demonstrating near homogeneity. Lyophilized polymer was weighed and lysed, and it was determined that up to 10μg of OVA could be encapsulated per mg of vesicle when the starting concentration during particle generation is at 100mg/mL. Starting concentration was incrementally decreased to 25mg/ml (Figure 3.1B), yielding vesicles containing as low as 2μg per mg of particles. Starting concentrations higher than 100mg/mL induced aggregation and prevented individual particle formation, while concentrations lower than 25mg/mL proved difficult with regard to measuring encapsulation efficiency.

To first verify the ability of the polymer vesicles to be internalized by DCs, tetramethyl rhodamine (TMR) labeled dextran was encapsulated and delivered to DCs over a 30 hour period, shown in the flow cytometry plot of Figure 3.1C. Throughout the incubation period, polymer vesicles are seen entering the cells, as indicated by an increase in fluorescent levels. As seen in the fluorescent microscopy images of Figure 3.2, polymer vesicles are in fact within the cells in high numbers.
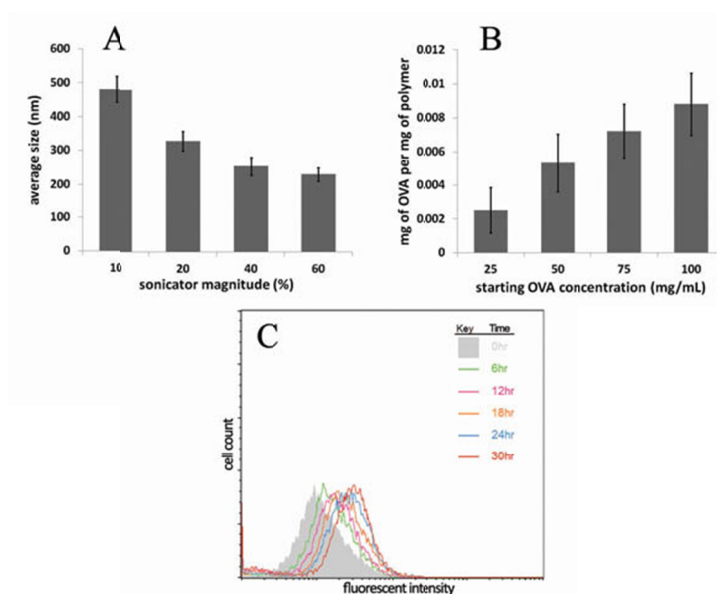
Figure 3.1 PLGA polymer characterization A) PLGA polymer vesicle sizes based on sonication power settings B) By modulating the starting concentration of OVA, the final encapsulated concentration can be controlled an measured C) Polymer encapsulating TMR-rhodamine was delivered to DCs over 30 hours and analyzed via flow cytometry to determine uptake of the polymer vesicles over time.
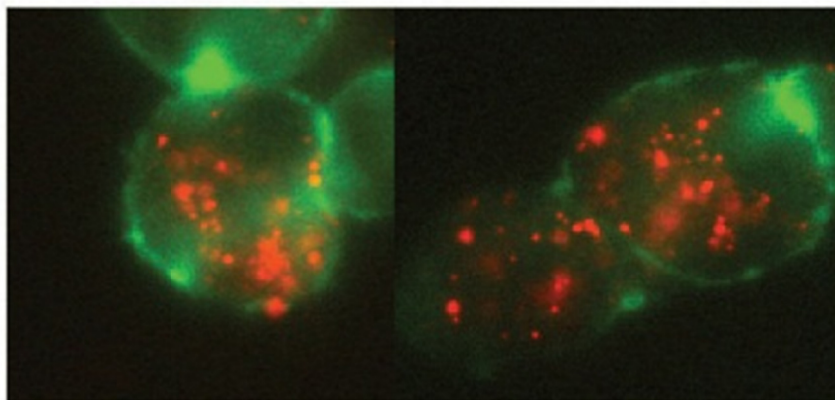
Figure 3.2 Representative images of polymer uptake by DCs. Rhodamine labeled dextran was encapsulated within the PLGA particles then delivered to DCs. After 24hrs DCs were stained with FITC conjugated anti-CD11c and visualized via fluorescent microscopy.

3.4.2 Dendritic cell stimulation

CpG motif DNA (ODN 1668) and whole OVA protein was first delivered in solution to DCs to determine baseline stimulation capabilities. Delivering OVA alone in solution resulted in no change in levels of any cell surface protein (data not shown). As seen in the histograms depicted in Figure 3.3, OVA with CpG induced stimulation of DCs, as indicated by a large increase in CD80, CD86, MHCII and CD11b expression as compared to cells cultured in media alone. CpG delivered alone in solution resulted in the same stimulation as OVA+CpG (data not shown). Although variation was seen in the geometric mean fluorescent intensities in response to the different doses of CpG with OVA delivered, the differences observed were not significant to demonstrate a concentration dependent effect at those ranges. In addition, concentrations below 0.5mg of polymer equivalent did not produce significant changes in most surface markers. To

ensure comparison between the delivery methods though, overlapping values of 0.5mg and 1mg for both soluble form and polymer delivery are reported. Supernatants were also collected and analyzed for secretion profiles, with data normalized to non-stimulated and LPS treated cells. Figure 3.4 shows that OVA+CpG treated cells increased their secretion of IL-12(p40 and p70), IFN-γ, TNF-α, MCP-1 and IL-10.

While 1MT is known to inhibit IDO activity, the effects it has on DC stimulation is still unclear. To gain insight into the effect of 1MT on altering DC maturation, 1MT was administered to cells in solution, either alone or with OVA and CpG. 1MT delivered alone significantly altered CD11b expression, as indicated by higher fluorescent intensities, with the largest effects seen at highest concentrations (Figure 3.3). 1MT delivered in conjunction with OVA and CpG did not alter the expression of any markers as compared to OVA+CpG. 1MT alone was also unable to alter the secretion of any measured cytokine and no significant change in secretion was observed when 1MT was added in conjunction with OVA and CpG.
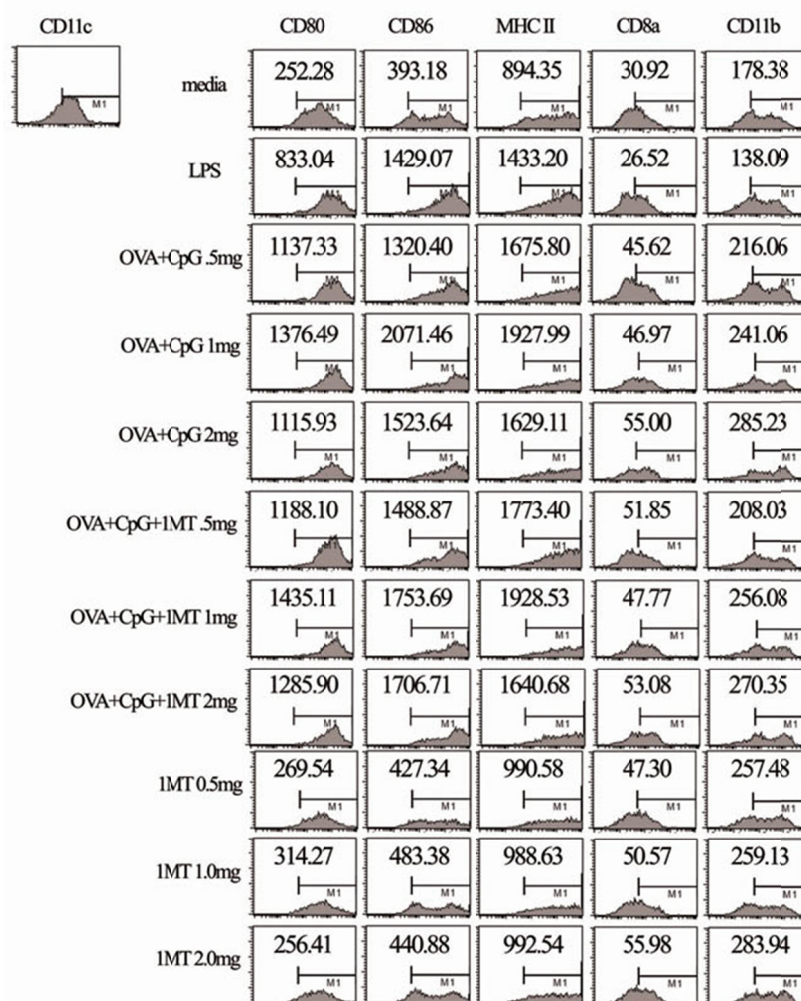
Figure 3.3 Representative flow cytometry histograms of cell surface expression of specified markers and corresponding geometric mean fluorescent intensities. Cells were treated for 48hrs with OVA, CpG and/or 1MT in solution form at concentrations equivalent to 0.5, 1.0, and 2.0mg of PLGA polymer. Cells were dual stained for CD11c and indicated surface molecules with the geometric mean intensities of Abs reported on CD11c+ cells. Cells treated with OVA alone reported identical histograms as the negative control (media) and cells treated with CpG alone showed identical histograms as the OVA+CpG condition (data not shown).
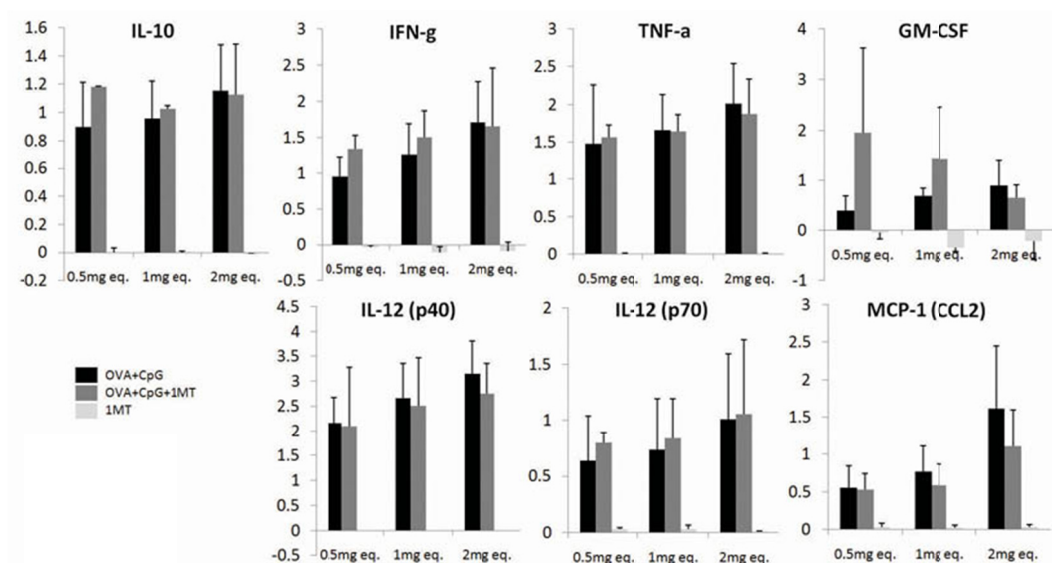
Figure 3.4 Secretion profiles of DCs. Cells were cultured at 1×10^6/mL in 1mL volume and treated for 48hrs with OVA and CpG in solution (■), OVA, CpG and 1MT in solution (■), and 1MT alone in solution (■) at the indicated polymer equivalent concentrations. Supernatants were harvested and run on ELISAs or the Bio-plex assay. Concentration values were normalized to untreated and LPS treated cells. Error bars indicate standard error on n=2-3 independent experiments.

3.4.3   Polymer based stimulation

Although delivering antigen and adjuvants in solution stimulates DCs to a mature phenotype, a polymer based delivery vehicle provides a controlled environment to deliver desired components at precise amounts to cells. In effect, desired DC stimulation can be achieved and pathways can be altered that are not possible via soluble delivery. Thus, a polymer based delivery method has proven efficacious in many systems and provides an advantageous therapeutic approach[82,83]. In verifying this approach for the current system, the first aim was to decouple the effects of delivering OVA, CpG and/or 1MT to DCs.

DCs were treated with different variations and concentrations of the polymer vesicles. Figure 3.5 shows representative histograms of CD86 expression on CD11c+ DCs treated with up to 500μg of PLGA vesicle per 10^6 cells. In analyzing the immunological effects of PLGA itself, PBS was encapsulated (PLGA-PBS) and delivered to DCs. We noticed no significant change to DC phenotype or secretion profiles in response to empty PLGA particles. Only when doses up to 5mg of vesicles were administered per $1x10^6$ DCs did the empty vesicles elicit a small response from DCs (data not shown). From this we can confirm that any changes in DC properties and/or function resulted from encapsulated components and their route of delivery. OVA and CpG was then encapsulated in PLGA (PLGA-OVA, and PLGA-CpG), but also did not change DC CD86 expression profiles throughout the administered doses. However, DCs treated with PLGA containing both the CpG DNA and OVA (PLGA-CpG+OVA) displayed an increase in CD86 expression. The DC population expressing elevated levels of CD86 was expanded, with more cells expressing higher amounts of CD86 as the dose of polymer vesicle was increased.
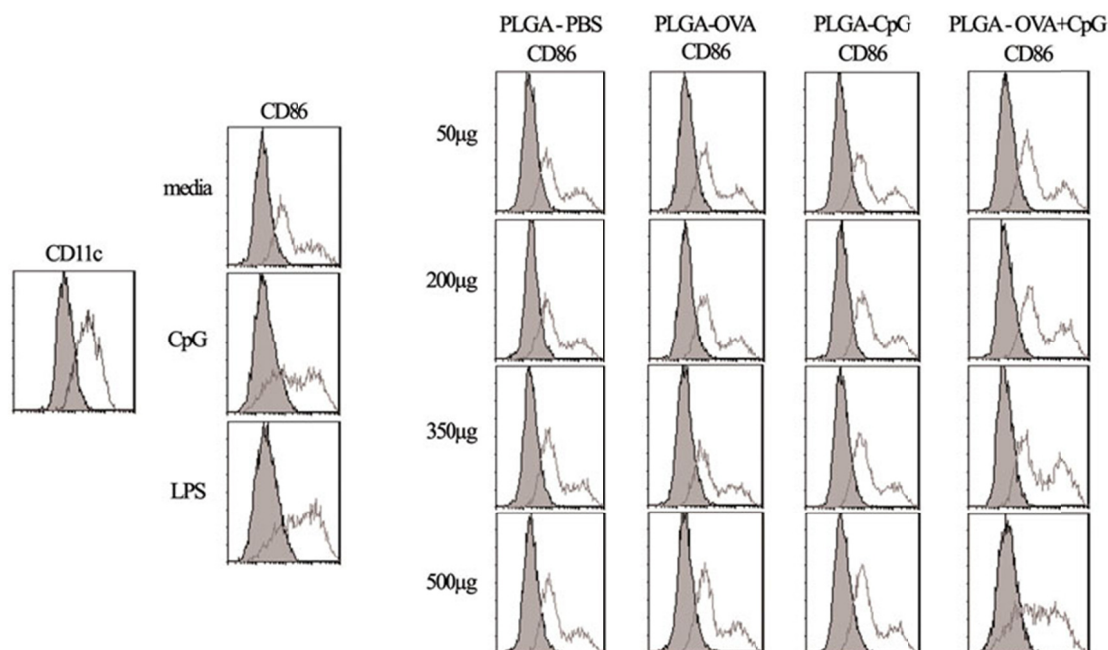
Figure 3.5 Representative flow cytometry histograms of CD86 expression on CD11c+ cells. DCs were treated for 48hrs with PLGA polymer encapsulating PBS (PLGA-PBS), OVA (PLGA-OVA), CpG (PLGA-CpG), or OVA+CpG (PLGA-OVA+CpG). As controls, cells were either not treated (media), or cultured with CpG or LPS in solution at 2μg/mL. Cells were dual stained for CD11c (one representative example of CD11c expression shown) and CD86 and gated on the CD11c+ population.

To further expand on these findings and determine the effects of delivering 1MT via the polymer system, DCs were analyzed for CD80, CD86, MHCII, and CD11b expression in response to the polymer treatment. PLGA containing OVA, CpG and/or 1MT were administered in increasing amounts to DCs, from 0.2mg to 1.0mg per $1x10^6$ cells. Figure 3.6 shows representative histograms of these DCs treated with the respective vesicle conditions. Although the polymer containing OVA and CpG was able to induce

stimulation with increasing dose, including 1MT with the OVA and CpG helped increase the levels of CD80, CD86 and MHCII to even more elevated levels. Secretion profiles of these DCs (shown in Figure 3.7) corroborate this finding, as significant increases in IL-10, IL-12 and MCP-1 were observed as a result of adding 1MT to OVA and CpG in the polymer vesicles. This increase was more evident at the higher concentrations of vesicles. As with delivery in soluble form, 1MT alone delivered via the polymer vesicles had no significant change in any surface protein throughout the doses (Figure 3.6). However, there was arguably some effect in the secretion profiles of DCs treated with 1MT via the polymer vesicles, as shown in the IFN-γ and GM-CSF profiles (Figure 3.7), but variability between experiments was high and thus not statistically significant.
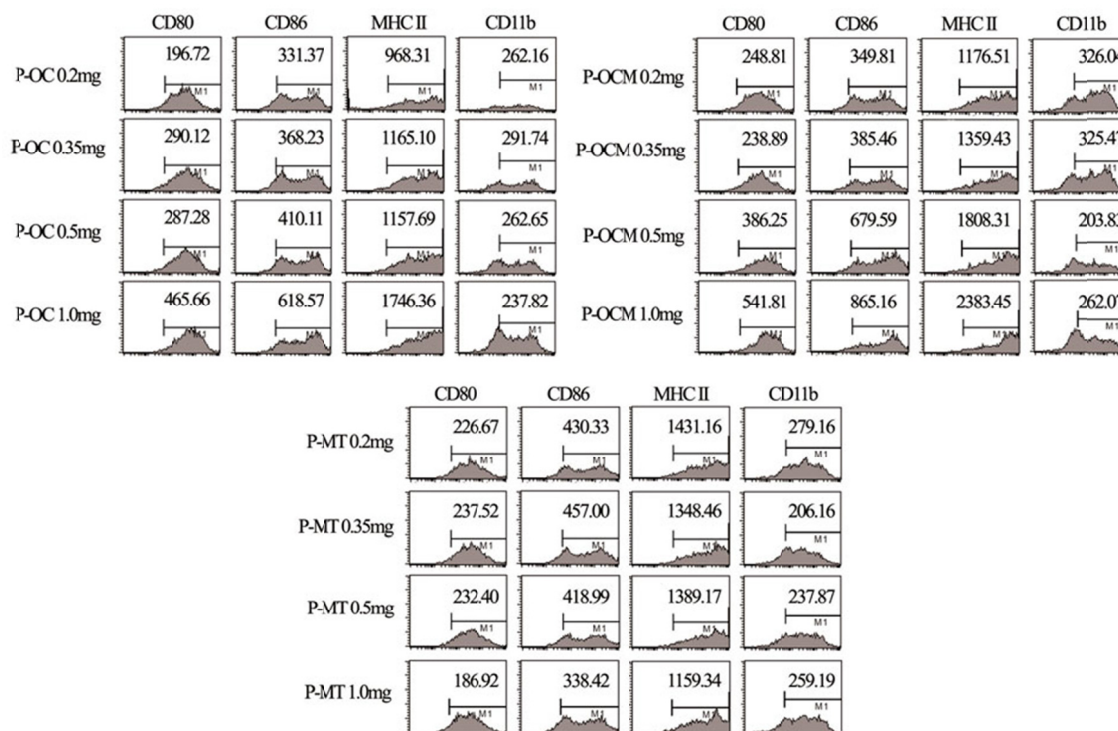
Figure 3.6 Representative flow cytometry histogram of cell surface expression of specified markers and corresponding geometric mean fluorescent intensities. DCs were treated for 48hrs with 0.2 to 1.0mg of polymer vesicles containing OVA+CpG (P-OC), OVA+CpG+1MT (P-OCM), and 1MT (P-MT). Cells were dual stained for CD11c and the following Abs; CD80, CD86, MHCII and CD11b. Cells were gated for the CD11c+ population to obtain the geometric mean fluorescent intensities of the reported Abs.
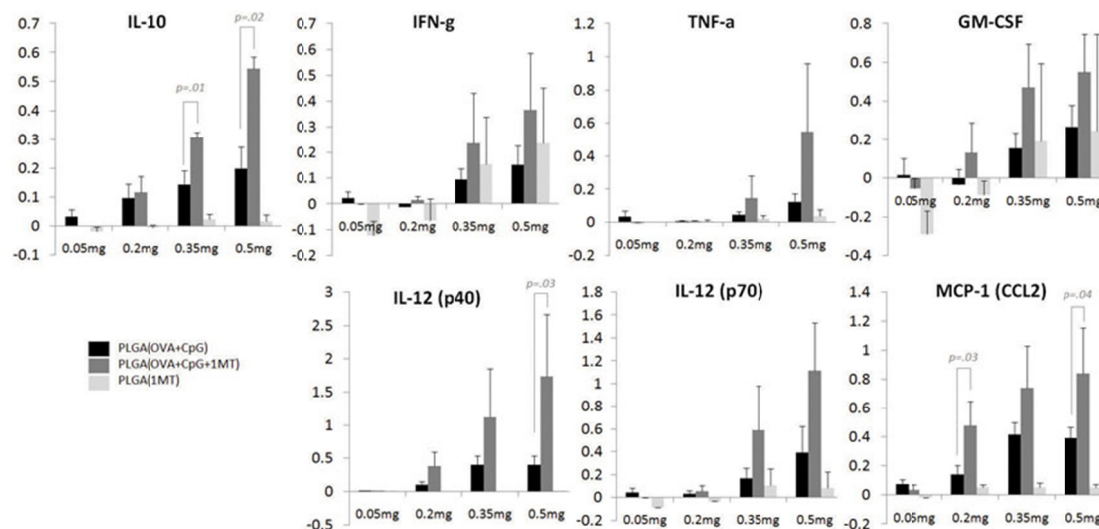
Figure 3.7 Secretion profiles of DCs in response to polymer treatment. Cells were cultured at 1×10^6/mL in 1mL volume and treated for 48hrs with OVA and CpG in PLGA (■), OVA, CpG and 1MT in PLGA (■), and 1MT alone in PLGA (■).Supernatants were harvested and run on ELISAs or Bio-plex assays. Concentration values were normalized to untreated and LPS treated cells. Error bars indicate standard error on n=2-3 independent experiments with *p* values determined by an unpaired one tail equal variance Student's *t*-Test.

Although the levels of CD86 and MHCII increased as a result of DC treatment both via the polymer vesicles and soluble components, it is interesting to note the ratio of expression of theses markers. It is well known and characterized that T cell binding of DCs occurs in at least two stages, with the initial stage being the binding of MHCII to T cell receptors (TCR), followed by a second stage of CD86 binding to CD28[84,85,86]. As shown in Figure 3.8, we see an increase in this MHCII to CD86 ratio as a result of the polymer deliver system. This is seen throughout the doses, both with and without

inclusion of 1MT. Even with this increased ratio though, CD86 remains highly elevated in these groups, leading to a DC phenotype able to potentially increase antigen presentation via an elevated MHCII and provide significant costimulation via CD86.
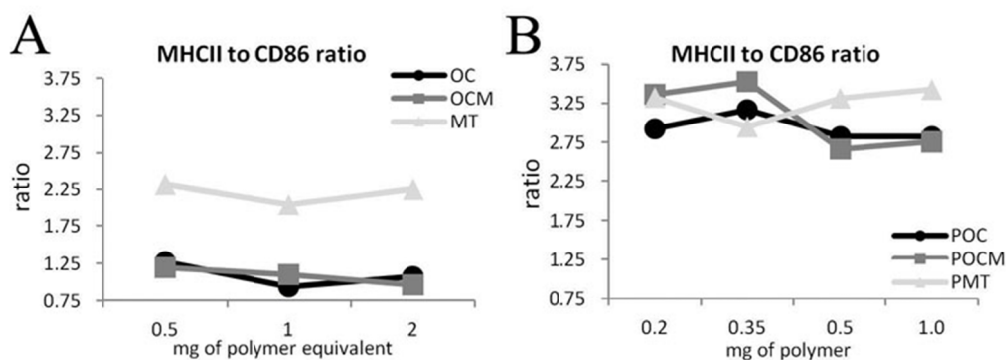
Figure 3.8 Representative expression ratios of MHCII to CD86 on DCs stimulated with OVA, CpG, and/or 1MT either in solution or in polymer form. The ratios of mean fluorescent intensities of binding Abs for MHCII and CD86 (MHCII/CD86) were compared on $1x10^6$ CD11c+ DCs treated (A) in solution form with OVA+CpG (OC,●), OVA+CpG+1MT (OCM,■), and 1MT (MT,▲) at the indicated polymer equivalent amounts, or (B) in polymer form containing OVA+CpG (POC,●), OVA+CpG+1MT (POCM,■), and 1MT (PMT,▲) at the indicated polymer amounts.

3.5 DISCUSSION

PLGA has been widely used as a deliver vehicle for drugs, proteins, DNA and adjuvants for a wide array of applications[87]. Utilizing this FDA approved polymer as a delivery vehicle, detailed and well documented control over polymer properties can be attained. Via the double emulsion method, polymer vesicles were made ranging from 250-500nm in diameter (Figure 3.1). Vesicle sizes greater than 500nm are attainable, but endocytosis

efficiency and the potential deviation from the observed processing of encapsulated components may vary at larger sizes. Smaller particles, i.e. nanoparticles, offer an advantage of greater cellular uptake as compared to larger microparticles. It has been shown that 100nm size nanoparticles have 2.5 times greater uptake compared to 1mm particles and 6 fold higher uptake compared to 10mm particles[88]. Larger particles will also degrade differently, both within cells and in tissue, altering the timing of release and potential stimulation. To minimize this variation, polymer vesicles with average diameter of 250nm were used throughout our studies. Although a limit for protein encapsulation was found, the limit for DNA encapsulation was not analyzed. Ranges up to a starting concentration of 20mg/mL was used and proved successful (data not shown), but a starting concentration of 5mg/mL was sufficient to create polymer vesicles that stimulated DCs. This property is one potential hypothesis for the reason CpG DNA alone in the polymer vesicle was unable to stimulate DCs. Perhaps increasing this DNA starting concentration would allow for vesicles containing CpG alone to stimulate DCs, and will thus be an aim of future studies. From the current results though, we have demonstrated the ability to create a wide range of polymer compositions containing antigen and adjuvant at various ratios. This allows us to fabricate a specifically tailored system to expand various DC populations.

As seen in Figure 3.5, particles with either OVA or CpG DNA alone did not induce any significant change in CD86 expression, nor in the cytokine secretion profiles (data not shown). In solution though, this same CpG DNA was able to stimulate DCs to increase CD80, CD86 and MHCII expression (Figure 3.3), as well as a vast array of cytokines (Figure 3.4). When these concentrations of CpG DNA and OVA were encapsulated

simultaneously within the polymer vesicle, the resulting polymer vesicle was able to stimulate DCs. Figures 3.6 and 3.7 show that these polymer based vesicles were able to stimulate DCs to a highly mature state. There are several hypotheses which could explain why CpG requires OVA within the polymer to stimulate the DCs; OVA helps protect CpG from degradation once internalized within the cell and/or OVA helps increase the loading capacity of CpG DNA within the polymer. Yet another possibility lies in the fact that the internalization and intracellular pathways targeted may differ in DCs as a result of polymer delivery. These results and hypotheses are not unique though, as others have reported similar events. For example, it has been shown that there is enhanced CTL activity against OVA from immunized mice as a result of coencapsulating both OVA and CpG, as compared to decoupled delivery or inoculation in soluble form[21]. It has also been shown in studies that delivery of antigen via a polymer vesicle such as PLGA both enhances and prolongs the cross presentation of exogenous protein on MHCI complexes, a result absent when antigen is delivered in soluble form[47]. These findings demonstrate the importance and hidden benefits of delivering components to DCs in a combined and controlled manner such as through a polymer delivery vehicle.

This benefit of utilizing PLGA was further exemplified when 1MT was introduced into the polymer system. 1MT is commonly utilized to inhibit or minimize the activity of IDO either within DCs or Tregs. It has thus been proposed as an agent for use in antitumor therapies to abrogate the immunosuppressive effects of Tregs. In relevance to this study, reports show that stimulating DCs with CpG can induce immune suppression of T cells via the IDO pathway, but can be reversed with 1MT treatment[89]. Although delivering CpG can stimulate DCs to increase costimulatory molecules and induce an inflammatory

cytokine milieu, CpG DNA can also paradoxically increase IDO secretion, thus potentially creating a suppressive environment. This finding, coupled with the understanding that secreted IDO can encourage Treg activity, leads to a need to block IDO activity during DC activation, antigen presentation and costimulation. However, very little work has been performed to analyze the effects 1MT has on DCs in addition to directly blocking IDO activity. To emphasize the importance of this, studies have claimed that 1MT delivered with LPS or TNF-α decrease the stimulation levels of DCs as measured by, among other things, CD80 and CD86 expression and secretion of key cytokines[90,91]. Hence, 1MT may be inhibiting beneficial factors required for a successful therapy. We therefore sought to analyze the delivery of 1MT in a therapeutically relevant system; PLGA containing antigen (OVA) and adjuvant (CpG DNA).

We observed that IL-10 was increased significantly as a result of the polymer based treatment, even more so when 1MT was introduced into the polymer vesicles. It should be noted though that these levels were lower than those levels observed with the soluble delivery method. Although it is not abnormal for levels of IL-10 to increase after stimulation, IL-10 is an accepted immune suppressant and has been known to skew lymphocytes from a Th1 to a Th2 response. In addition, our group has reported an IL-10-dependent inhibition of DC function in a model of tumor-induced immune suppression[26]. Effects of increased IL-10 would consequently be detrimental in most systems. For example, IL-10 has been demonstrated to not only have autocrine effects, but can down-regulate CD86 and MHCII expression and can decrease the amount of IL-12 secreted from DCs[92,93,94]. These effects would normally further encourage Th2 responses. However, as a result of the polymer based delivery system, a deviation from this pattern

was observed, unlike that seen in soluble delivery. Introducing 1MT into the polymer vehicle with OVA and CpG was also able to greatly increase IL-12 secretion (Figure 3.7) and not only maintain but increase the expression of CD86 and MHCII (Figure 3.6), seemingly reversing the effects of having elevated IL-10 levels. Whether this is a result of 1MT inhibiting IDO or influencing another alternative biochemical pathway will require further studies.

It is also unknown what effects increasing the MHCII to CD86 ratio will have on T cell interactions (Figure 3.8) without further studies. It has been shown that when this ratio is elevated, a decrease in immune competence can be observed[95]. Although an increase in this ratio was observed as a result of the polymer based treatments, CD86 was still significantly high. Perhaps this elevated level of both cell surface proteins, in addition to the increased ratio and elevated IL-12 secretion, will provide an overall stronger TCR binding and subsequent costimulation, allowing superior antigen priming and/or activation of T cells. Summarized in Table 1, these DCs generated as a result of the polymer based treatment provide an advantageous environment to skew lymphocytes to a Th1 phenotype. Cell surface proteins CD80, CD86, and MHCII are elevated along with secretion of IL-12. Although IL-10 is also elevated in each OVA and CpG treatment, the fold increase is significantly less than LPS stimulated cells. The resulting DC population generated by the polymer based delivery system will potentially greatly improve the efficacy of antigen specific therapy. This hypothesis would require further testing, where cytotoxic T cell assays and mixed lymphocyte reactions would help verify the claim.

condition	CD80	CD86	MHCII	CD11b	IL-10	IL-12
LPS	3.8 ±.8	4.7 ±1.1	1.7 ±.4	.99 ±.2	61.6 ±-28	129 ±16
1MT	1.1 ±.2	1.1 ±.1	0.9 ±.1	2.7 ±1.3	1.0 ±.6	.8 ±.1
OVA+CpG	3.9 ±.9	3.9 ±.7	2.0 ±.3	1.7 ±.7	17.6 ±4	159 ±21
OVA+CpG+1MT	3.9 ±1.2	3.7 ±.4	1.9 ±.2	1.6 ±.6	24 ±3	134 ±33
P-1MT	1.1 ±.1	1.4 ±.1	1.3 ±.03	.9 ±.02	1.1 ±.6	.8 ±.1
P-OVA+CpG	1.6 ±.5	1.8 ±.4	1.4 ±.3	.9 ±.01	9.1 ±7	62 ±19
P-OVA+CpG+1MT	2.1 ±.4	2.6 ±.6	1.9 ±.6	.98 ±.06	32 ±13	213 ±94

Table 3.1 Summary of the fold increase of DC surface protein expression (CD80, CD86, MHCII, CD11b) and cytokine secretion (IL-10, IL-12) in response to respective treatment condition; lipopolysaccharide (LPS), 1-methyl tryptophan (1MT), ovalbumin and CpG DNA (OVA+CpG), ovalbumin and CpG DNA and 1-methyl tryptophan (OVA+CpG+1MT), and each within the PLGA vesicle (P-1MT, P-OVA+CpG, P-OVA+CpG+1MT). Values and corresponding standard errors represent the fold increase of protein expression verses non treated cells.

In light of these results, the effects of 1MT delivery via PLGA on DCs extend beyond direct IDO inhibition. When designing a therapeutic system that includes 1MT, attention should now also be focused on the effects 1MT has on DC stimulation properties. Our results indicate that including this compound with OVA and CpG in PLGA enhanced several aspect of the delivery system. PLGA encapsulating OVA, CpG and 1MT was able to generate a DC phenotype indicative of a Th1 inducing environment. The functional effect of these treated DCs on T cell stimulation is currently unknown. Experiments are currently underway to verify their functional capabilities. This study verifies the efficacy of delivering 1MT and the advantages of doing so in a PLGA based polymer delivery vehicle and warrants further exploration of stimulating DCs in a controlled and multi-faceted manner.

CHAPTER 4: IMMUNOLOGICAL INSIGHT INTO THE EFFECTS OF 1-MT

4.1 Introduction

The PLGA based delivery system was able to induce elevated IFN-γ levels systemically and locally when OVA and CpG DNA were delivered together. Soluble delivery of the same components however was unable to induce any increase in IFN-γ secretion. Aside from providing a controlled and protected delivery mechanism, PLGA based delivery systems, as confirmed here, elicit stronger immune responses than soluble delivery. Others have reported similar findings that demonstrate the advantage of PLGA based antigen delivery at enhancing humoral immunity, inducing elevated antibody levels, and generating Th1 responses[73,74,75,76,77]. In particular, elevated IFN-γ is commonly attributed to a strong CD4+ mediate Th1 response[59], and has been shown to act directly on tumor cells to inhibit both proliferation and angiogenesis, facilitating apoptosis[78,79,80]. The current data thus supports the hypothesis that delivery of OVA and CpG via the PLGA vesicles generates a strong IFN-γ mediated Th1 response.

While increasing the immunogenicity of tumor associated antigens, such as through polymer delivery systems, has proven efficacious, the field of cancer immunology has also recognized the important role of regulatory T cells (Tregs). Particularly in cancer models, Tregs are known to attenuate the response of ACPs, B cells, NK cells, CD8+ T cells and other CD4+ T cells[96,97,98,99,100]. While they are upregulated in cancer models and obviously present a challenge, Tregs have proven successful targets for improving antitumor responses[101,102,103,104,105]. In the current lymphoma model, Tregs are upregulated and comprise up to 15% of the T cell population in tumor draining lymph

nodes[106,107,108]. Blocking these Tregs would thus potentially increase the effectiveness of our PLGA therapy. To do so, we encapsulated and delivered 1-methyl-tryptophan (1-MT) on its own or in concert with OVA and CpG DNA. 1-MT is a known IDO inhibitor and has antitumor effects but only recently has it been utilized in a combinatorial treatment regime[109,110]. We observed in the detailed *in vitro* studies that 1-MT delivered via the PLGA based polymer can expand the Th1 phenotype from dendritic cells. It was thus a goal of this work to analyze the effects 1-MT had on modulating the immune environment and enhancing therapeutic efficacy.

4.2 Delivery of 1-MT reverses Treg induced CD4+ inhibition

Bone marrow derived dendritic cells were generated as before and stimulated with the polymer delivery system. After 48hrs, dendritic cells were treated with mitomycin-c for 1hr then co-cultured at a 1:5 ratio with syngeneic CD4+ T cells with or without CD4+CD25+ T cells. T cells were obtained from spleens of previously OVA immunized mice. Spleens were harvested and T cells isolated with the Miltenyi Biotec cell separation systems using kits for CD4+ T cells and for CD4+CD25+ Regulatory T cells. CD4+ T cells were stained with CFSE dye per manufacturer's recommendations prior to co-culture (CellTrace CFSE Cell Proliferation Kit, Invitrogen). T cell proliferation was monitored after 5 days of incubation by measuring the decrease in fluorescence intensity associated with cell division.

Dendritic cells treated with PLGA encapsulating OVA and CpG DNA (POC) or PLGA encapsulating OVA, CpG DNA and 1-MT were able to induce significant stimulation of CD4+ T cells, as demonstrated in Figure 4.1. As controls, dendritic cells were not

stimulated (media, m) or incubated with PLGA encapsulating 1-MT. These conditions did not stimulate CD4+ T cells. When Tregs were introduced into the co-culture (CD4 + CD4CD25), there was a decrease in proliferation of CD4+ T cells from cultures where dendritic cells were treated with only OVA and CpG DNA (POC). However, by including 1-MT with OVA and CpG DNA (POCM), CD4+ T cells were able to escape this inhibition and proliferate. This data demonstrates the multi-functional benefit of the polymer system, where an adjuvant, antigen, and immune modulator can be encapsulated and delivered to dendritic cells to both stimulate CD4+ T cells and provide some protection form CD4+CD25+ Tregs.
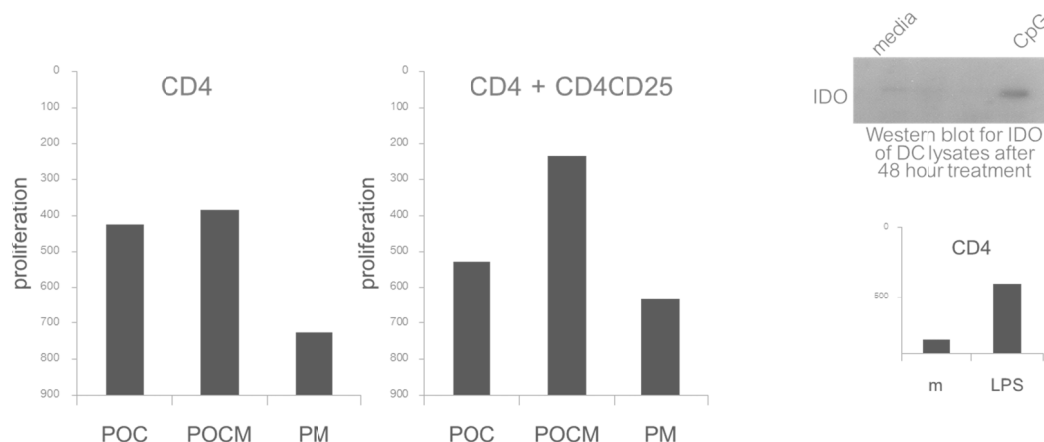
Figure 4.1 T cell proliferation in response to dendritic cells treated with polymer vesicles. Bone marrow derived dendritic cells were incubated with the indicated polymer conditions for 48hrs (PLGA with OVA and CpG DNA (POC), PLGA with OVA, CpG DNA, and 1-MT (POCM), and PLGA with 1-MT (PMT)). After 1hr incubation with mitomycin-c, dendritic cells were co-cultured at a 1:5 ratio with CFSE treated CD4+ T cells or with CFSE treated CD4+ T cells with the inclusion of CD4+CD25+ regulatory T cells. Increased proliferation is measured as the decrease in average fluorescent intensity.

4.3 Delivery of 1-MT to tumor bearing mice alters the immune response

1-MT delivered alone via the PLGA vesicle was able to moderately delay the growth of tumor, yet had significantly less effect than OVA and CpG DNA (Figure 4.2). In addition, there is no difference in local CTL activity from the different treatment modalities (Figure 4.3A). Systemic CTL activity was still not measurable in any treatment condition. Being that 1-MT does not increase CTL activity (Figure 4.3A) and is unable to elicit a systemic or local IFN-γ response (Figure 4.5), 1-MT is curbing a different branch of the immune response; such as inhibiting IDO and subsequently blocking Tregs. When 1-MT is included with OVA and CpG DNA in the PLGA vesicles, there is no statistically significance in tumor sizes. Analyzing tumor sections on days 9 and 17 via H&E staining (Figure 4.4), significant scar tissue is observed, due to the rapid growth and turnover of E.G7-OVA cells, but no major infiltration of immune cells or macrophages.
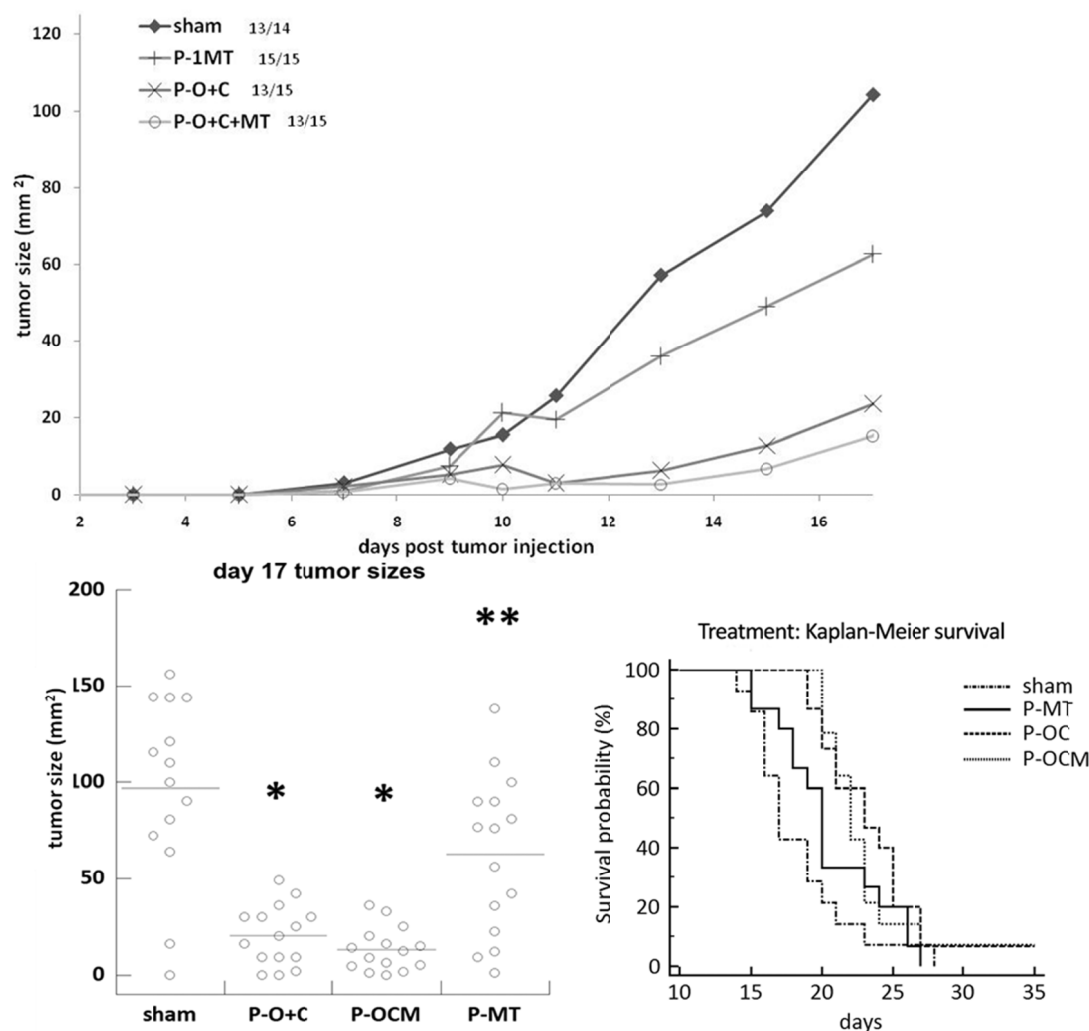
Figure 4.2 Tumor treatment model with polymer vesicles. (A) E.G7-OVA cells were implanted on day 0 and treatments were administered i.t. on days 3, 5 and 7 with the indicated conditions (PBS (♦ sham), PLGA with 1-MT (+ P-1MT), PLGA with OVA (▲ P-OVA), PLGA with OVA and CpG DNA (× P-O+C), PLGA with OVA and CpG DNA and 1-MT (○ P-O+C+MT), OVA and CpG in solution/PBS (□ sol:O+C)) . Tumor size reported as the cross sectional area using largest two diameters. (B) Day 17 tumor size distribution where each point represents one mouse in each respective condition. A significance of $p < 0.01$ (*) or $p < 0.05$ (**) was observed in comparison to sham. (C) Kaplan-Meier survival curves demonstrate the probability of survival in response to polymer treatment.

A)

B)

C)
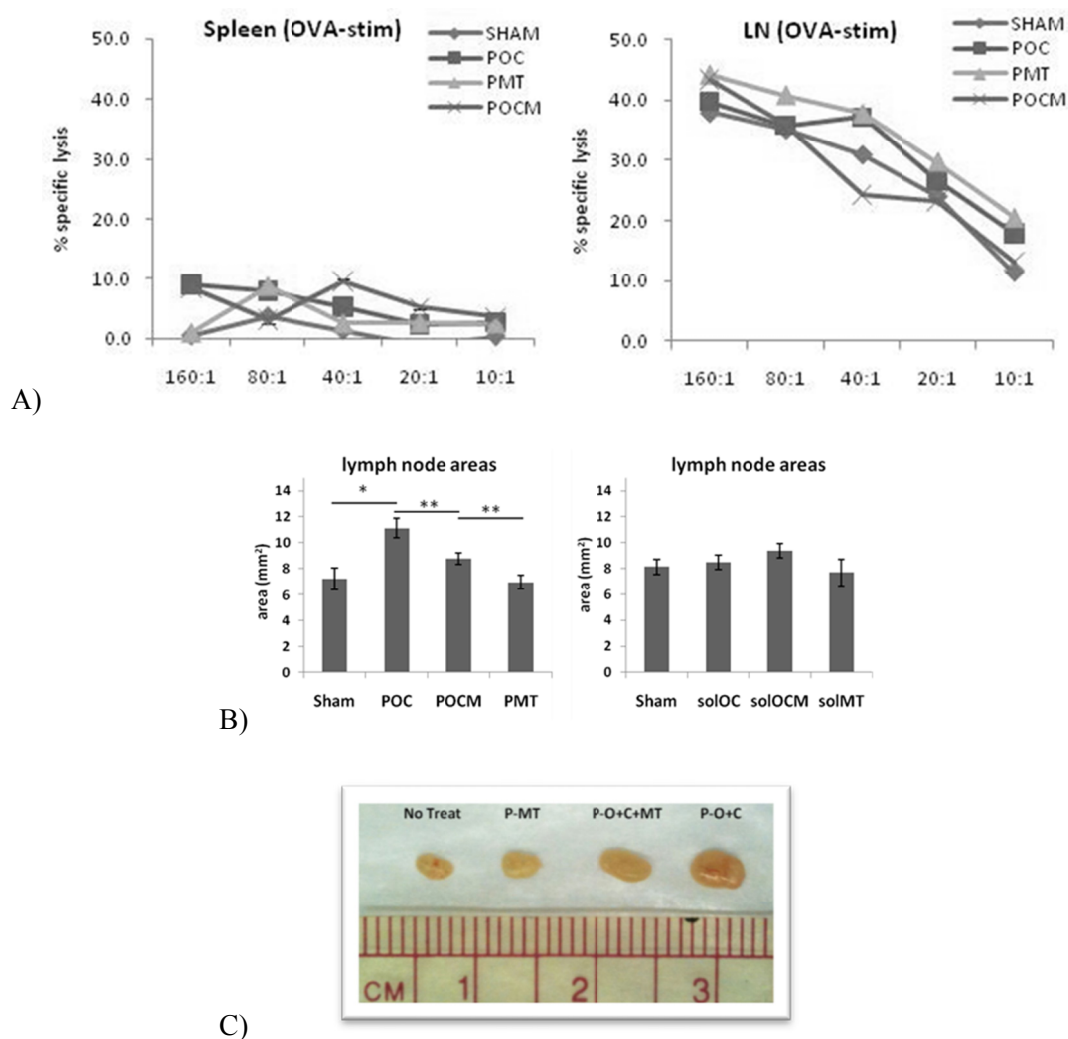
Figure 4.3 Systemic and local CTL responses against E.G7-OVA cells. A) Systemic specific lysis was absent with or without OVA restimulation. CTL response was elevated in the tumor draining lymph nodes as a result of restimulation, but did not vary between treatment and sham conditions. Although the gross immune response is elevated in the various treatment conditions (B and C), as indicated by the increased sizes of tumor draining lymph nodes, no significant difference is seen in the lysis ability of CTLs between the treatment conditions.
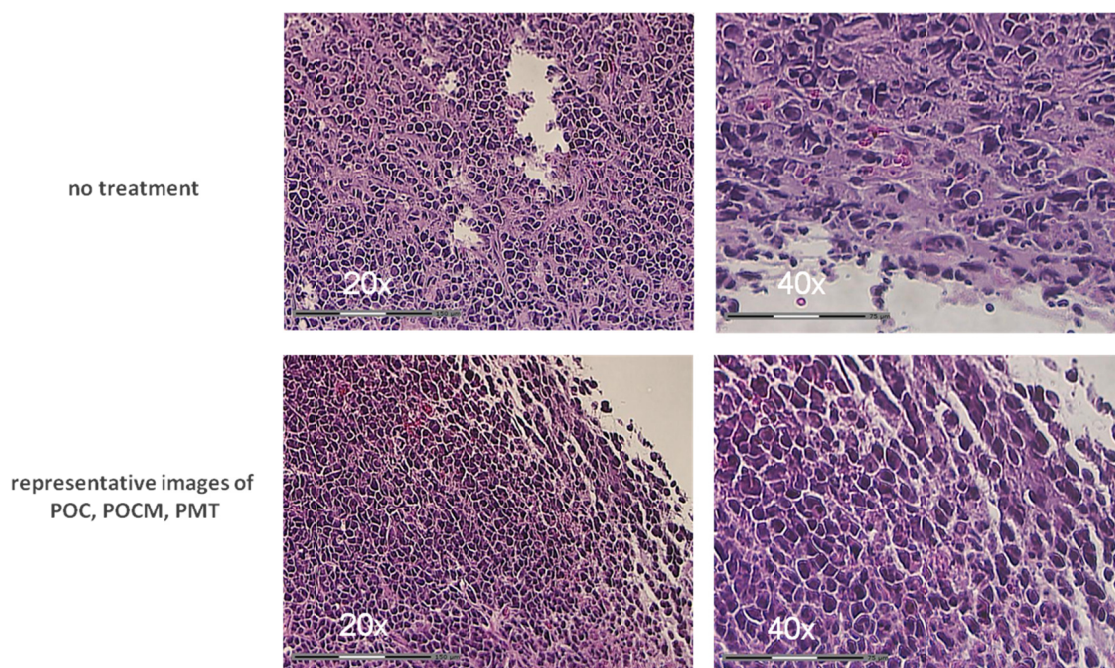
Figure 4.4 Tumor section tissue staining. 20x and 40x magnification of H&E staining on tumor sections were analyzed for gross immune infiltration. No significant presences of immune cells were seen in the sections taken in any of the treatment conditions. Although scar tissue is prevalent, infiltrating cells such as macrophages or T cells are not visible in large numbers in mice treated with the polymer system.

Considering the magnitude of the antitumor response seen from delivering OVA and CpG DNA, it is not surprising that including 1-MT did not result in any significant reduction in tumor growth. Literature has demonstrated the benefit of first depleting Tregs in a pretreatment modality, resulting in greater therapeutic potential[30]. Although pretreatment regimens, in this case with 1-MT, may be beneficial, adverse effects may go unseen if delivered independently. For example, even though there is a delay in tumor growth when 1-MT is added to OVA and CpG DNA, 1-MT essentially blocks the local IFN-γ response and curtails the systemic IFN-γ response (Figure 4.5). From the current

data it is uncertain why adding 1-MT blocks IFN-γ production yet allows tumor delay. It can be hypothesized that it is the combination of treatments that resulted in the observed effects. While OVA and CpG elicit strong antitumor activity, the Th1 responses are partially curtailed by 1-MT. Perhaps this is compensated for by the alternative benefit of 1-MT. Further studies would be required to verify this. Interestingly, literature is void of an explanation as to why 1-MT may impede IFN-γ production. With the growing interest in utilizing 1-MT for its beneficial immune regulatory properties, such as the several ongoing 1-MT based clinical trials, science needs a better understanding of how 1-MT functions and what it does. These studies provide a first step into revealing the many alternative roles 1-MT has on *in vivo* tumor models.
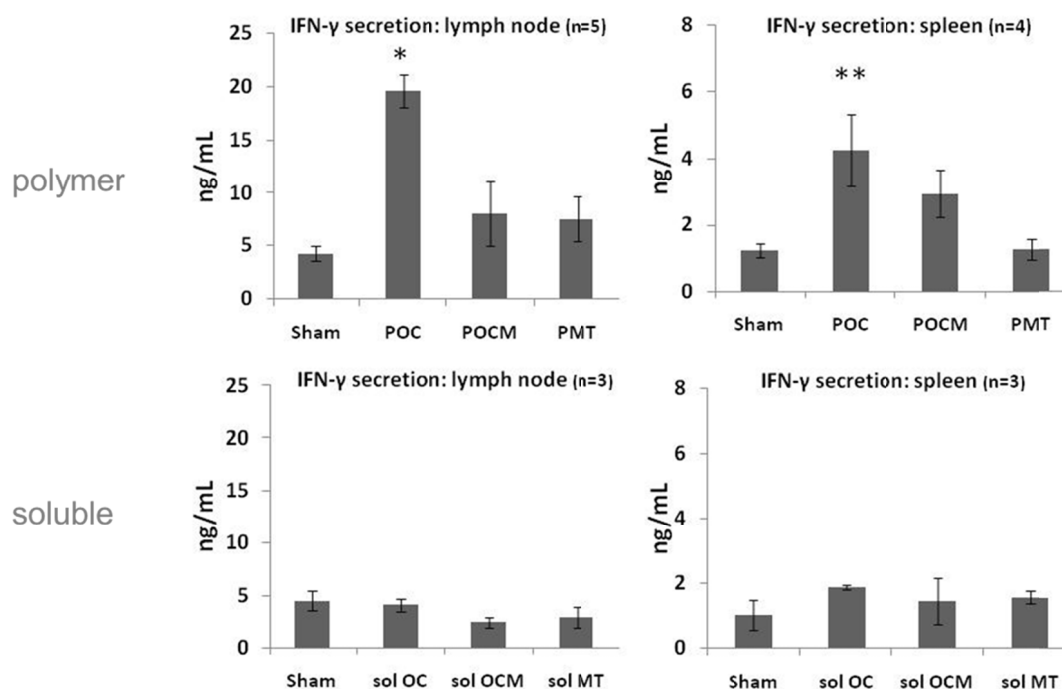

Figure 4.5 Local and systemic INF-γ levels from 1-MT treated mice. INF-γ was elevated in the polymer treatment condition containing both OVA and CpG DNA. However, when 1-MT was included, IFN-γ levels were greatly reduced. Similar trends were observed in spleen cells, yet the

amount of IFN- γ was overall significantly lower and 1-MT did not have as great of an attenuation effect. Delivery of OVA, CpG DNA or 1-MT dissolved in PBS induced little expression of IFN-γ as compared to the polymer delivery system. In the polymer system, a significance of $p < 0.01$ (*) or $p < 0.05$ (**) was observed in comparison to sham.

CAHPTER 5: CONCLUSIONS

5.1 KEY FINDINGS

It was found that the lymphoma tumor line E.G7-OVA is capable of eliciting an antigen specific, CTL response. Although a syngeneic model, the murine hosts are capable of generating a spontaneous anti-tumor response in the local but not systemic environment. These CTLs are functionally ineffective, as tumors progress rapidly after implantation. Additionally, there is no significant local or systemic Th1 response in the tumor bearing hosts, as levels of IFN-γ are low.

With administration of the polymer delivery system, we observe a significant reduction in tumor growth and prolonged survival. Delivering OVA and CpG DNA independently had some effect on tumor progression, but was dwarfed by the ability of OVA and CpG DNA delivered together to delay the growth of a tumor. The polymer system however was unable to change the antigen specific CTL response, as the local activity remained the same and there was no measurable systemic effect.

Delivering OVA and CpG DNA via PLGA was able to induce a Th1 response that provided a means to counteract the immune escape exhibited by the tumor. In both the systemic and local compartments levels of antigen specific IFN-γ were elevated as a

result of the polymer treatment. These results directly correlate with the delay of tumor growth and the demonstrated ability of the polymer system to vaccinate against tumor challenge. The polymer system generates an anti-tumor Th1 response that functions independently of CTLs to reduce tumor growth.

In vitro data supports this Th1 response as DC increase their Th1 phenotype as a result of polymer treatment. In a dose dependent manner, OVA and CpG DNA delivered to DCs via PLGA increase their Th1 phenotype. A marked increase in the levels of CD80, CD86, MHCII, IL-12 and IFN-γ are observed after polymer treatment. Soluble delivery of OVA and CpG increased these cytokines and cell surface molecules as well, but also elevated Th2 markers such as IL-10 to a much greater level than with polymer delivery. This data further demonstrates the benefits of utilizing the PLGA delivery system to administer OVA and CpG to generate a Th1 response.

Lastly, delivery of 1-MT via PLGA has the ability to increase the Th1 response *in vitro*. While 1-MT had no effect on DC stimulation when delivered on its own, it greatly enhanced the Th1 phenotype of DCs as a result of treatment. There was a significant increase in CD80, CD86, MHCII and IL-12 levels as a result of the combinatorial treatment.

5.2 FUTURE OUTLOOK

PLGA based delivery systems have been used for a vast array of applications. Here we have detailed one such application where PLGA was utilized to induce an immune response on systemic level generated from a single dendritic cell population. It was

demonstrated that the delivery system could generate a robust and antigen specific Th1 based immune response within a tumor environment. As a result, this immune response was able to delay the growth of the tumor and prolong survival. Analyzing the discreet cellular response to this therapy further demonstrated that a Th1 response was induced within dendritic cells to initiate the cascade of adaptive immunity.

Although conclusive in its ability to initiate an effective anti-tumor response, this PLGA delivery system opens a broad door for future studies. To begin, the studies presented in this work contribute to the characterization of immunological responses in tumor bearing mice. Broader understanding of the complex interaction within the immune system in a tumor bearing host provides insight into advancing treatment strategies. The studies shown were developed from previous known regimens, as they were well characterized or accepted. However, there are numerous methods to advance the treatments. For example, testing different routes of injection, providing continued administration of the polymer and providing increasing doses are all feasible methods to investigate the therapeutic potential of the polymer system. For example, upon further investigation it was found that by co-encapsulating both CpG DNA and OVA an increase in the amount of CpG DNA was observed within the PLGA vesicles (Figure 5.1). It is hypothesized that the ionic interactions of the largely positive OVA and highly negative CpG DNA induce aggregation and therefore increase the encapsulation efficiency of CpG DNA. This would result in increased stimulation of dendritic cells, as was observed in the *in vitro* studies where CpG DNA alone in the PLGA was unable to stimulate dendritic cells (Chapter 3).
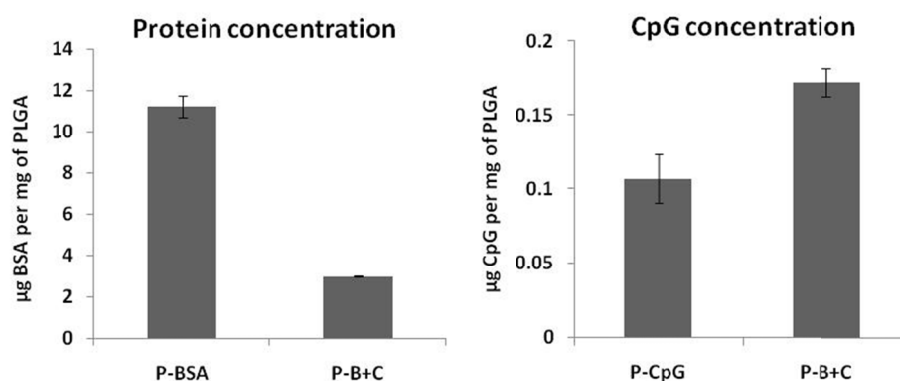
Figure 5.1 Encapsulation efficiencies as a result of co-encapsulation. FITC labeled BSA (50mg/mL) and Cy5 labeled CpG DNA (5mg/mL) were dissolved together and incorporated into the polymer fabrication process. Lyophilized polymer was weighed and lysed with 3M NaOH. Supernatant was collected and scanned on a fluorescent plate reader at 495nm and 655nm, corresponding to the labeled BSA and CpG DNA respectively. Standard curves were generated and loading concentrations calculated as a result of co-encapsulation.

Due to the highly versatile platform, alternative tumor models can be adopted for use. Ovalbumin is not a natural tumor antigen and thus easily distinguished in the sea of murine protein. Using a less immunogenic antigen or a human tumor line will provide an approach closer to clinical relevancy. The current studies aid in demonstrating the capabilities of the PLGA delivery system and how it affects the immune response. Transfer to a clinically relevant model would provide further validation of the efficacy of this platform. These necessary *in vitro* and *in vivo* tests provide initial indications of therapies mechanisms and potential. With further development, PLGA based treatments

can be used to treat a variety of cancers and other debilitating diseases. Albeit a tedious path, the promising results presenting in this body of work provide a first step towards translating therapy from research and development into applications within a clinical setting.

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