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Evaluation of Immunogene Therapy Using a Plasmid Encoding IL-15 Delivered by Electroporation in a 3D Tumor Model and a Mouse Melanoma Model

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Evaluation of Immunogene Therapy Using a Plasmid Encoding IL-15 Delivered by Electroporation in a 3D Tumor Model and a Mouse Melanoma Model

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: HARV Bioreactor, Gene Delivery, B16.F10, HaCaT, 3D Spheroid,

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DEDICATION

I would like to dedicate this dissertation to my Mom Blanca Marrero, Dad Ramon A. Marrero, Sister Enid M. Marrero, Brother Ramon A. Marrero, Uncle Alberto Ortiz, Nephew Ian Marcos Hinchey and boyfriend Jesse H. Arbuckle for all of their support and love that helped me achieve my goals.
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ABSTRACT

Melanoma is an aggressive disease with few effective treatment options. Non-toxic, anti-tumor therapies and prophylactic approaches are currently being investigated to identify treatment options that will control and remove late-stage melanoma.

The overall goal of this project was to establish an effective delivery method for a plasmid encoding human interleukin (phIL-15) into mouse melanoma cells (B16.F10) using the gene transfer technique electroporation (EP). The EP delivery phIL-15 was optimized using an in vitro 3D tumor model. The purpose was to translate these IL-15 delivery conditions into an in vivo mouse melanoma model to study IL-15 signal transduction and stimulate immune cells to destroy tumor antigens as well as promote an anti-tumor immune memory response.

The in vitro 3D tumor model and the mouse model demonstrated similar expression patterns when delivering phIL-15 with different EP conditions. Intra-tumoral delivery using 500V/cm 20ms enhanced gene delivery and increased IL-15 protein expression compared to 1300V/cm 100µs. There was also a visible increase in transfection efficacy between tumor cells compared to skin cells when delivering pmIL-12 and phIL-15 plasmid constructs in vivo. The plasmid+EP groups 1300V/cm and 500V/cm stimulated increased expression of cytokines IL-1β, IL-6, INFγ, MIP-1β and TNFα. These EP groups also promoted tumor regression by up-regulating CD8+ T cells and CD4+ T cells which targeted melanoma. Regression and survival studies demonstrated that 73.3% of mice cleared B16.F10 cells when treated with phIL-
15+1300V/cm and pVax+500V/cm. In addition, 53% of the mice responded to the phIL-15+500V/cm treatment group. Furthermore, 75% of the mice from group phIL-15+500V/cm survived secondary inoculation and tumor challenge. In conclusion, plasmid with encoding gene insert phIL-15 delivered by EP has the potential to act as an anti-tumor therapy because it promotes melanoma regression and enhances mouse survival through innate and adaptive cell-mediated immune responses.
INTRODUCTION

Melanoma Disease Target

Melanoma is a poorly immunogenic and aggressive cancer localized in the epidermis, it is very dangerous when left untreated, but the survival rate increases if detected early and removed completely\(^1\). Currently, melanoma’s 5 year survival rate is ≤10\% for stage IV metastatic cells that have spread to other organs, ≤30\% for stage IIIC metastatic cells that have spread to nearby tissue and lymph nodes, ≤45\% for stage IIC ulcerated tumors, at least 1mm thick and cells have not migrated to the lymph nodes and ≤95\% for stage IA tumors that are not ulcerated, nor undergoing mitosis and are less than or equal to 1mm thick\(^2-3\). Melanoma is sometimes difficult to identify and lesions are located in places that can easily be ignored. Melanoma cells are melanocytes that have undergone genetic mutations and exhibit enhanced oncogenic properties. Melanoma transformation can develop from UV irradiation activating p53 gene mutations causing melanocytes to lose their cell cycle control. Many pathways are affected by excessive UV exposure (Figure 1) such as continuous autocrine stimulation by growth factors\(^4-5\) which causes prolonged MEK/ERK activation resulting in cell proliferation and differentiation\(^6\) and constitutive oncogenic g-protein N-RAS activity\(^7\) that binds to receptor tyrosine kinase (RTK). Additional oncogenic mutations are found in kinase activating domain V599E of B-RAF\(^7-9\) which enhances DNA proliferation and cell survival. Mutations cause loss of receptor function and enhanced melanogenesis, for example mutations in melanincortin-1-receptor constitutively bind α-Melanocyte
Stimulating Hormone (αMSH) causing increase in tumor survival\(^4,10-14\). Additionally, melanoma mutations cause down regulation of tumor suppressor genes PTEN/AKT and inhibitory pathways P53/APAF-1 in non-metastatic melanoma\(^7\) which increases cell survival. Also, melanoma evades the immune response by using immune suppressor factors to decrease cytokine production stimulated by the JAK/STAT pathway\(^{14}\).

Figure 1: Melanoma Development and Oncogenic Mutations
(Figure Referenced in Literature \(^4-18\)).

Melanoma formation also occurs when there is a continuous stimulation by mitogenic growth factors promoting excessive melanocyte formation into a population of pigmented cells called nevi or moles\(^{15-16}\). Nevi formation is dependent on distinct growth factors like bFGF different from the growth factors stimulating other melanocytes found ubiquitously in the epidermis. When CDKN2A/P16\(^{INK4}\) cell cycle inhibitor is up-regulated
it controls nevi proliferation\textsuperscript{4,14-17}. Nevi that do not undergo senescence and replicate uncontrollably form many nevi or moles that may give rise to melanoma due to a defect in CDKN2A/P16\textsuperscript{14,18} (Figure 1).

Melanocytes are highly migratory cells that move towards keratinocytes when the epidermis is exposed to UV radiation. The melanocytes function by transferring their melanin to keratinocytes to shield against UV exposure\textsuperscript{19}. Melanocytes also elevate anti-apoptotic properties like BCL-2 and αMSH which contribute to cell immortalization\textsuperscript{10,20}. These properties associated with up-regulating melanocyte protection from UV exposure are the same properties that protect melanoma cells from cell death and immune attack. Once melanoma invasively migrates to deeper layers of the skin and invades the bloodstream it causes secondary disease and spreads to the liver, lung, and brain\textsuperscript{2,14}. Treatment options for late stage melanoma are limited and the aggressive disease rapidly floods the body causing irreversible damage. The current treatment options are surgery, chemotherapy, cytotoxic drugs and radiotherapy\textsuperscript{2,7}. Melanoma is highly resistant to chemotherapy and alkylating agents that cause cytotoxic DNA cross-linking. Melanoma is resistant because cells can undergo increased DNA repair and detoxify chemotherapeutic drugs by up-regulating alkyl transferases like glutathione S-transferase to chemically reduce cytotoxic cross-linking\textsuperscript{2}. Melanoma can also impair drug transport and drive out the chemical via P-glycoprotein efflux pumps. Melanoma is known to be resilient to cytotoxic drugs at doses which cause toxicity in patients. Scientists are investigating other alternative therapies to target melanoma such as cytokine immune therapy\textsuperscript{2,7}. 
**Current Melanoma Gene Therapy**

Some of the cytokines that have been studied for their potential use in immune therapies are INFα, IFNγ, IL-2, IL4, IL6, IL-12, IL-18, IL-21, GMCSF, IL-12, and TNFα. In 2007, a study using FDA approved high dose recombinant protein INFα and IL-2 was administered to patients with advanced stage melanoma (II, III and IV). The results showed limited tumor response 10-15% and resulted in high platelet toxicity. Currently, investigators are developing adjuvant therapies that will promote anti-tumor efficacy dependent on the stage of tumor growth. One idea is to target early stage (I/II) melanoma in patients not at risk of relapse and increased survivability with resection, is to use low dose recombinant proteins to target early stage existing disease. The approach is to induce a protective immune response against new primary melanoma.

Another strategy is to mount a protective immune response against tumor cells by employing an adjuvant delivery system that will transflect genes encoding cytokines into specific cells and signal surrounding immune effector cells to enhance their own cytotoxic effect against intermediate and late stage melanoma.

**Gene Delivery Techniques**

There are many gene delivery techniques that are used to promote the introduction of nuclei acid into cells, these include viral vectors, gene gun, charged polymers, liposomes, calcium phosphate precipitation, ultrasound, intracellular microinjections, pressure perfusion, and electroporation (EP). The purpose is to use a safe and minimally toxic system that can effectively target specific cells and deliver specific genes which would direct downstream events leading to an enhanced immune response. The injection of naked DNA into tissues or cells is not an efficient
transfection approach. This efficiency can be significantly improved using physical approaches such as gene gun or EP. Both devices facilitate transport of DNA through the cell membrane and cytosol allowing them to reach the nucleus at high proficiency. The pressure perfusion method (hydrodynamic) forces large volumes of plasmid DNA into a vein. A typical target using this technique is the liver and the high injected volume inflates the organ and leads to efficient gene delivery and high expression. While successful, this approach is not easily translated to the clinic. Although recombinant viral vectors can deliver DNA efficiently and achieve high levels of protein expression, they are accompanied by some safety concerns. These include immune response to the virus, possible insertional mutagenesis and over expression of a gene resulting in high toxicity. An alternative is to use a non-viral vector that will deliver therapeutic genes into the cell’s nucleus without damage as well as limiting systemic toxicity. Delivery of plasmids using mechanical devices such as EP, is safe, promotes efficient, transient delivery and allows short-term gene production for downstream stimulus. The goal is to deliver a gene encoding a cytokine to stimulate pathways of the immune response and promote antigen recognition by effector cells that can up-regulate multiple arms of the immune response as well as a protective response by memory cells.

Many laboratories such as the Heller Lab utilize gene delivery systems to employ anti-tumor therapies into existing disease. Based on previous studies with interleukin 12 (IL-12), intra-tumoral delivery of the gene using EP enhanced DNA delivery into mouse melanoma (B16.F10) improved protein production by tumor cells compared to injection of IL-12 plasmid only. These studies showed that local delivery of the plasmid to the tumor reduced tumor burden, prevented tumor metastasis and increased mouse survival. The study also showed up-regulation of innate and adaptive immune
responders such as IFNγ by ELISA and CD8+/CD4+ T cells by immunohistochemistry. The delivery of IL-12 was able to reduce tumor size using EP intra-tumoral delivery and prevent melanoma lung colonization when delivering IL-12 into the gastrocnemius muscle. The mice responded well to treatment, had no deleterious side effects and did not succumb to melanoma metastasis\textsuperscript{28-30}. The purpose of the subsequent study was to expand upon the knowledge gathered from the IL-12 studies and characterize EP delivery conditions for human interleukin-15 (IL-15). The goal was to test the delivery of IL-15 primarily \textit{in vitro} and then translate the information \textit{in vivo}. Activation of immune responses against B16.F10 antigens and evaluation of memory response could be studied following delivery of plasmid encoding IL-15 (pIL-15). Evaluation of those results would contribute to understanding what effector cells caused reduction in tumor burden and increased mouse survival\textsuperscript{31}.

\textbf{Electroporation}

Electroporation is a technique that provides temporary permeabilization of cells allowing the entry of molecules and desired genes\textsuperscript{32-36}. “Poration” occurs when electric fields are applied across charged electrode plates and creates potential energy at the poles to help feed molecules or DNA into cells\textsuperscript{32,37}. It is suggested that DNA is introduced into the cells by different mechanisms, such as electrophoretic forces\textsuperscript{26,32,35-37}, endosomes envagination\textsuperscript{38}, and binding of DNA to the membrane with lateral diffusion\textsuperscript{34}. To promote DNA entry into the cells, it has been suggested that the electric fields cause deformation of the DNA to compensate for the bulky supercoiled size. The pulses orient the DNA parallel to the electric field and facilitate movement of the DNA into the cytosol within milliseconds\textsuperscript{33}. The “pore” radius depends on the size of the DNA
providing passage of excess DNA found in the extracellular matrix. The plasma membrane regulates resealing of the “pores” a few minutes after the pulses to reduce cellular damage by reverting to normal transmembrane potential. Once inside the cell, it is suggested that the DNA uses the cells’ transcription and translation factors to produce protein. The process of DNA introduction into the cell is still being investigated; studies are attempting to unravel the process of uptake and translocation of the DNA into the nucleus for protein expression.

Electroporation has been utilized to enhance DNA delivery of many types of genes into different cells and promote specific up-regulation of protein signaling. This technique uses the protein stimuli in cells to up-regulate different down-stream pathways that are beneficial to target disease or restore signaling in cells. To promote specific immune responses the delivery system must ensure efficient transfection of DNA into target cells. Delivery of the plasmid and resulting expression can be controlled by regulating EP parameters. EP is controlled by adjusting pulse parameters such as pulse duration, length and frequency as well as plasmid concentration. The applied electric field must exceed a minimal threshold to destabilize the membrane and ensure DNA transfer.

The delivery of luciferase using low electric fields with long pulse durations (600V/cm 5ms 1Hz 8 pulses) demonstrated increased expression of luciferase compared to the combination of high electric fields with short pulse durations and very low electric field with longer pulse durations (1200V/cm 100µs 2Hz 1 pulse and 140V/cm 50ms 2Hz 8 pulses, respectively). Researchers found that long, low voltage electric pulses enhance DNA movement into cells compared to combination pulses. Additionally, the route of plasmid administration, orientation of the electric fields and
direction of pulses influence the magnitude of transfection when delivering to a particular tissue type. It has been suggested that delivery to the liver, spleen, and kidney show more luciferase and β galactosidase expression than muscle and skin\textsuperscript{41}. While this one study demonstrated higher expression with the specific EP parameters tested in liver, spleen and kidney, it is quite possible that utilizing different pulse parameters could lead to higher levels in muscle and skin. Many factors influence EP delivery such as cell size, cellular characteristics, relative conductivity since EP is limited to the area the field is applied, the amount of plasmid available and direction of pulses applied. The application of electric fields in multiple directions enhances delivery of the gene to many cells at one time reducing cell damage\textsuperscript{39,41-42}. The electrode applicators utilized to deliver pulses to the cells are also tissue specific. Penetrating needle electrodes are used for muscle and tumor, while multi-electrode arrays and plate electrodes are used for skin and muscle \textsuperscript{39}. It is important to determine EP parameters for specific cells and tissues to select appropriate delivery conditions that would result in the appropriate protein expression and down-stream signaling.

3D Modeling and Tissue Engineering

There is a concerted effort to develop an \textit{in vitro} model that mimics cellular properties similar to cells \textit{in vivo}. It is important to develop models that will help elucidate the cellular physiology, pathology, drug interactions, cellular behavior, and protein signaling\textsuperscript{43}. Two dimensional (2D), monolayer cell cultures have provided extensive information on cellular behavior, cell signaling and function. However, cells seeded in a 2D monolayer culture differ from cells cultured \textit{in vivo}, because they are morphologically different than structures observed \textit{in vivo} which may lead to physiological differences \textsuperscript{44-45}. 

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It is suggested that cells cultured in a monolayer may differ from cells *in vivo* because of the way they are cultured, specifically altering cell interaction, communications, differentiation, growth, migratory properties, metabolism, adhesive properties, protein expression, protein secretion, cytoskeleton organization, intracellular and extracellular signaling characteristics, receptor stimuli, agonist and antagonistic properties\(^45-49\). It has been demonstrated that tumor cells grown in a 2D culture are less likely to be drug resistant than cells grown in a 3D model\(^45,50\). One method used to test the differences in cellular properties is to develop a 3D model sitting in a synthetic micro-environment that will allow cells to mimic their natural state\(^51\). Generation of a 3D tissue-like construct has become a useful tool in cell culture providing the ability to study cell-cell interactions from homogeneous or heterogenous cell populations. This model gives researchers the opportunity to study cellular functions independently from other factors found *in vivo* that enhance or inhibit cellular processes\(^43-44,52-53\).

The key is to generate a 3D model that can sustain cells in suspension, provide passive diffusion of oxygen, nutrients and allows cells to behave similar to their cell cycle *in vivo*. A microgravity bioreactor was engineered to promote cellular aggregation in a free-floating synthetic environment constantly rotating at less than 1 gravitational force (1g)\(^51,52\). The bioreactor allows cells to be submerged in media and is equipped with a silicon membrane to ensure oxygen and carbon dioxide exchange for the cells. This device known as the high aspect ratio vessels (HARVs) was engineered by NASA in1986 and Synthecon in 1990, to promote 3D growth of cells in a microgravity environment. The cells residing in the vessel experience minimal interference and shearing, while growing in a state of free fall or solid body rotation\(^52,54-58\). The rotation of the vessels concentrate cells in one location allowing aggregation and cell-cell contact
amongst epithelial adherent cells, immune cells, stem cells, melanoma, sertoli cells, and pancreatic islets.

Cells grown in suspension forming 3D cellular aggregates are a good model to study tumor development and cell interactions. Tumor cells residing in a 3D construct, mimic cells in vivo they can organize themselves, polarize and support tumor cell behavior. Tumor cells can organize into small masses mimicking the properties of tumor lesions in nature, proliferating in an oxygenated or hypoxic environment containing viable cells residing on the surface while dying, necrotic cells reside in the center. Tumor cells grown in a 3D model instead of in a 2D culture will provide advantages to understanding the pathology of tumors and answer questions about drug targeting, gene therapy, angiogenesis, migration, tumorigenesis, cells cycle pathways, evasion of apoptosis and invasive properties. Although this is not a completely organized natural system it is a good template for understanding the biology of tumor cells growing in a synthetic 3D microenvironment that can be translated and further tested in vivo.

The construction of a 3D tumor model must contain cell types that will properly interact and appropriately signal. These signals include growth factors, protein regulators, and adherent proteins that enhance synthesis of extracellular matrix and scaffolding. These proteins will also help promote growth, differentiation, and survival of cells involved in the 3D model. For instance, melanoma is a common skin cancer that is studied in vivo to identify early and late stage treatment protocols. This is an interesting model to study because it is poorly immunogenic and resistant to chemotherapy. The tumor cells cultured in the 3D model will possibly mimic cellular behavior and help isolate cellular mechanisms that attribute to the disease pathology. The information gathered from the model may reveal signaling pathways that can be
targeted using chemotherapeutics or optimize expression levels of genes using EP delivery methods to regulate down-stream signaling\textsuperscript{45}. A good tumor model is one that contains complementing cells that normally interact such as keratinocytes from the epithelium with melanoma that reside in the skin. Melanoma are melanocytes that act to protect the skin, but that have undergone genetic alterations, mutations and up-regulation of oncogenic properties\textsuperscript{45,51}.

\textit{In vivo}, the basal layer is the germinal layer of the epidermis and contains proliferative, non keratinized keratinocytes that house melanocytes\textsuperscript{14}. The keratinocytes communicate with melanocytes and melanoma through their dendrite-like processes. Keratinocytes provide survival signals to melanocytes in the epidermis and prevent apoptosis, promote melanocyte differentiation, and migration\textsuperscript{14}. As mentioned previously, keratinocytes are constantly controlling and communicating with melanocytes as well as melanoma. Melanocytes need keratinocytes because if they migrate to the dermis they can undergo apoptosis in the absence of keratinocyte survival signals\textsuperscript{14,65}. The interesting feature of melanoma is that they have the ability to escape keratinocyte control. The keratinocytes control melanoma by preventing escape from deregulation and invasion. At early stages, keratinocytes express adhesive proteins E-cadherin to limit melanoma migration\textsuperscript{66}. One evasion tactic used by melanoma is the down-regulation of E-cadherin and up-regulation of N-cadherin through PI3K pathway\textsuperscript{67} as well as reducing the integrins in keratinocyte for melanoma migration. E-cadherin is expressed by epidermal cells Langerhans, keratinocytes and melanocytes while N cadherin is expressed by vascular endothelial cells and fibroblasts\textsuperscript{66,68}. Some studies show an up-regulation of N-cadherin by melanoma cells themselves to escape keratinocyte control\textsuperscript{66}. Melanoma migrates into deeper layers of the epidermis reaching
the blood stream and metastasizing to distant sites from the primary tumor site\textsuperscript{17,69}. Up-regulation of integrins is a result of UV irradiation in order to promote migration of melanocytes to the area of UV exposure\textsuperscript{70-72}.

Investigators interested in 3D models want to analyze specific processes to clearly understand mechanisms such as receptor up-regulation, protein synthesis, protein phosphorylation, mutation of genes, uncontrolled cancer cell proliferation, and tumor evasion\textsuperscript{56,61}. A 3D tumor model provides an alternative method for analyzing treatment regimens using gene delivery methods and will assist in unraveling additional therapeutic options for melanoma\textsuperscript{45}. Evaluating the effectiveness of cytokine therapy against cancer cells \textit{in vivo} could be first optimized for EP delivery conditions \textit{in vitro} using a 3D model and then translated to \textit{in vivo} testing thus, reducing the use of animals for EP optimization.

\textbf{Interleukin-15}

One of the cytokines that is being studied for delivery to tumors using EP is interleukin-15 (IL-15). IL-15 is a 14-15kDa pleiotropic cytokine\textsuperscript{73-74} that was discovered in 1994 by JD Burton and KH Grabstein. IL-15 protein forms a 4-α helix structure that specifically binds with high affinity to IL-15Rα, but can also bind to IL-2Rβ and IL-2Rγ\textsuperscript{73-77}. The IL-15 gene consists of 9 exons and 8 introns. When translated, the IL-15 protein is 114 amino acids (aa) long encoded from 4 exons and 3 introns containing structural similarities to IL-2\textsuperscript{77}. IL-15 binds IL-15Rα with high affinity; the IL-15Rα contains a positively charged sushi domain that binds negatively charge IL-15\textsuperscript{77-78}. Mouse IL-15 shares 73% aa homology with human IL-15 and human IL-15Rα shares 54% aa sequence homology with mouse. While the human IL-15Rα sushi domain which actively
binder IL-15 shares an 85% aa sequence homology with mouse IL-15 sushi domain\(^{77,79-80}\).

IL-15 and IL-2 share many activities, but differ in functional properties of the immune system\(^{74,76-77}\). IL-2 is mainly a modulator of T cells\(^{77,81}\), while IL-15 protein is a potent producer of NK cells\(^{77,82}\), IFNγ, CTL effector cells and most importantly maintaining the survival of memory CD8+ T cells. IL-15 messenger RNA is expressed by dendritic cells (DC)\(^{77,83}\), monocytes\(^{77,84}\), macrophages\(^{77,85}\), fibroblasts\(^{77,86}\), epidermal skin cells, keratinocytes\(^{77,87}\) and epithelial cells such as lung, heart, kidney\(^{77,88}\), nerve cells\(^{77,89}\), skeletal muscle, and placenta\(^{74,77}\) IL-15 protein is expressed by epithelial cells\(^{77,87}\), fibroblasts\(^{77,86}\), monocytes\(^{84}\), and DC\(^{77,83}\). Although, IL-15 is ubiquitously expressed it is difficult to detect because IL-15 is tightly regulated at the level of translocation, transcription, translation, protein expression, intracellular trafficking, and secretion from the golgi apparatus\(^{77,90-97}\). There are two alternatively spliced isoforms of human IL-15 expression that exist, there is long signal peptide (LSP) that measures 48-aa and short signal peptide (SSP) measuring 21-aa\(^{77,90,94-100}\). Human IL-15 isoforms differ from mouse IL-15 isoform measuring 26-aa in length even though they share a 73% aa sequence homology\(^{77,79,98}\). The IL-15 SSP is sequestered and stored intracellularly in the cytoplasm while IL-15 LSP is found in the golgi, leading to secretion. The role of IL-15 SSP has been elucidated as a type of autocrine regulatory signal that binds to IL-15Rα in the nucleus and regulates IL-15 gene transcription. Messenger RNA for IL-15Rα is also expressed by many cell types including B cells, NK cells, T cells, macrophage, bone marrow and thymic cells, kidney, lung, heart, skeletal muscle, brain, intestine, and liver\(^{77,79,101-105}\). When IL-15 binds to IL-15Rα, cells show low levels of signal transduction by the receptor\(^{77,106-108}\), in turn when IL-15 binds to IL-2Rβγ there is
an increase in signaling transduction. IL-2Rβγ recruits JAK kinases and activates STAT transcription. The phosphorylated STAT proteins translocate to the nucleus and activates gene expression of inflammatory responses, which signals differentiation and expansion of T cells\textsuperscript{77,109-111} (Figure 2).

IL-15Rα exists in two forms, membrane bound and soluble. The cleaved soluble form was detected in the mouse serum and cell supernatant from cultured human glioblastoma and mouse fibrosarcoma\textsuperscript{77,80,112-113}. IL-15 has a short half life due to down-regulation by monocyte or dendritic cell’s negative feedback inhibition pathway\textsuperscript{77}. Soluble IL-15Rα acts as an agonistic binding IL-15 to prolong its half-life and regulates IL-15 concentration within a tissue over a period of time\textsuperscript{77,114-115}. On the other hand, constitutively expressed membrane-bound IL-15Rα can act as an antagonist by binding soluble IL-15 through an autocrine pathway regulating excessive stimulation of IL-15 signaling and limits activation\textsuperscript{77,112-113,115} (Figure 2).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{IL-15 Signaling and Stimulation of the Immune Response (Figure Referenced in Literature\textsuperscript{74-86,109-131}).}
\end{figure}
IL-15 shares some of the same signaling pathways with melanoma, aiming to sustain tumor growth and reduce apoptosis. These pathways including Src family kinases PI3K and AKT, anti-apoptotic BCL-2, AP-1, and Ras/Raf/Mek/MAPK\(^{106}\). More importantly, IL-15 stimulates pro-inflammatory pathways NF-κB, to target tumor cells such as melanoma\(^{77,106,110}\). This information could help elucidate why IL-15 is so tightly regulated when stimulated by immune effector cells. Membrane associated IL-15/IL-15Rα complex expressed on monocytes allows for trans endosomal recycling to enhance memory CD8+ T cell stimuli\(^{77,116-117}\) or trans-presentation of IL-15 to stimulate adjacent CD8+ T cells and NK cells\(^{77,105,118-119}\). It is suggested that this is the main signaling function of IL-15Rα during trans-presentation and up-regulation of the adaptive immune response and memory response\(^{77,105,119}\) (Figure 2).

**IL-15 Stimulates the Immune Response**

IL-15 induces a strong innate immune response recruiting DC, macrophage, NK cells, augments INFγ production, and in turn up-regulates the adaptive response by enhancing costimulatory molecules CD40, CD86, MHC class I, CD8 T cells CTL, MHC class II CD4+T cells, Th1 cytokines, and ultimately promoting the production of long lasting CD8+ T cell memory immune response against the antigens of interest\(^{77,83,120-122}\). IL-15 expands, stimulates functional activities and maintains balance of NK and survival of memory CD8+ T cells\(^{77,81-82,93,123-124}\). IL-15 also regulates B-cell survival and proliferation. B cell follicular DCs produce IL-15 which promotes the production and secretion of IgA, IgG and IgM by CD40L activated B- cells\(^{77,125-126}\). IL-15 produced by monocytes up-regulate proinflammatory cytokines IL-6, IL-8, TNFα\(^{77,112,127}\) which increases phagocytosis, inhibit neutrophil and eosinophil apoptosis\(^{77,128}\). IL-15 also
enhances mast cell development and proliferation\textsuperscript{77,129} and up-regulates adjacent monocyte production of cytokines IL-12\textsuperscript{77,130}, IL-8, and chemokines MCP-1\textsuperscript{77,131} to promote neutrophil phagocytosis of pathogen infection for the body's protection\textsuperscript{77,108} (Figure 2).

IL-15 is also expressed by non-immune cells like fibroblasts, epithelial, endothelial, muscle cells, adipocytes, osteoclasts, neuronal and glial cells\textsuperscript{77}. IL-15 functions to protect these cell types from apoptosis, stimulates proliferation pathways, supports microglial growth, T cell migration in endothelium, reduce proteolysis in skeletal muscle, weakens nitric oxide production from microglial cells, stimulate angiogenesis and inhibits lipid deposition\textsuperscript{77,132-137}.

IL-15 has many useful qualities for clinical therapies to promote or inhibit signaling in many diseases and disorders. It is important to regulate IL-15 expression because over production of IL-15 can lead to hypersensitivity, tissue damage and autoimmunity\textsuperscript{77}. Currently, IL-15 is being used as a target to ameliorate difficulties patients face with rheumatoid arthritis, Lupus, inflammatory bowel disease, diabetes type 1, hypertension, muscle degeneration, and B-cell lymphoma\textsuperscript{77,135,138-145}. IL-15 protein is ubiquitously expressed and it can bind more than one receptor IL-15R and IL-2R. IL-15 signaling can be presented in many forms such as a free soluble protein, soluble receptor bound, or membrane receptor bound. This suggests that regulatory mechanisms of IL-15 can serve as a good mediator to control therapeutic mechanisms against aggressive form of melanoma\textsuperscript{77}.

\textbf{Significance}

Melanoma and other cancers have the ability to increase immunosuppressive genes expression, decrease their adhesive properties, lose MHC expression and lose
expression of T cell co-stimulatory molecules, enhance tumor progression, and build T-cell tolerance against an immune response that could up-regulate effector cells to target tumorogenic epitopes\textsuperscript{22}. It is predicted that a gene therapy approach can promote sufficient levels of a particular cytokine from the transfected cells to stimulate and restore the body’s own natural defenses to destroy cancer cells. By controlling levels of the cytokine it is possible to induce an immune response that recognizes tumor burden, promotes apoptosis, CTL response, and memory response while reducing the potential toxicity often associated with cytokine therapies performed with recombinant protein. It has been previously demonstrated in both preclinical and clinical IL-12 studies that an effective cytokine therapy against melanoma could be established utilizing EP to induce an appropriate immune response with minimal toxicity\textsuperscript{28,146,147}. 
OBJECTIVES

The long-term goal of this study is to establish an immunotherapy using a non-viral gene delivery approach to specifically target advanced-stage cancer cells like melanoma\textsuperscript{28}. Although gene therapy is a relatively young field, it is a promising system to treat existing disease\textsuperscript{24-25}. The purpose of the project is to establish a gene therapy technique entailing the delivery of a gene encoding cytokine human IL-15 using EP into an established mouse melanoma B16.F10 tumors. It is anticipated that the delivery of pIL-15 will stimulate an innate and adaptive immune response removing existing disease and adapting a protective response against continuous tumor antigens\textsuperscript{31}. The short-term goal is to characterize EP delivery methods using mouse melanoma B16.F10 and human keratinocytes HaCaT cells \textit{in vitro} prior to translating the delivery parameters \textit{in vivo}. Melanocytes, melanoma and keratinocytes contain functional properties shared by both human and mouse providing a useful alternative for testing delivery conditions \textit{in vivo}. There are no known macroscopic free-floating tumor models that can adapt EP electrodes for delivery experiments\textsuperscript{59}. The \textit{in vitro} 3D model will be used to examine delivery conditions and compare results to an \textit{in vivo} C57BL/6 mouse model for the delineation of further treatment options \textit{in vivo}. The objective of using a mouse model is to determine the type of immune response attained during EP delivery of IL-15 directly into tumor cells.
Hypothesis

The hypothesis of the following project is the 3D tumor model does mimic natural cellular properties that will provide a platform to establish EP delivery conditions in vitro and translate delivery conditions in vivo. Delivery of IL-15 by EP in vivo will recruit effector cells to the tumor site causing tumor regression and promote a long-term immune response during secondary tumor challenge.

Specific Aims

Specific Aim 1:

To develop a three-dimensional tumor model that could be used to evaluate gene transfer procedures and to examine cell to cell interactions.

The work in Aim 1, included generating an in vitro 3D tumor model to study delivery of different genes using EP to identify the best transfection efficiencies attained when delivering into tumor cells and skin cells. The EP conditions were tested amongst different cell types HaCaT, B16.F10 and human melanoma SKMEL-5 to examine the differences in gene expression. To ensure positive delivery of genes into the cells of the in vitro 3D model and to measure transfection efficiency, green fluorescent protein (GFP) and IL-15 were separately delivered at different EP conditions by varying electric field (EF) strengths, pulse duration and pulse number. The highest protein production obtained in vitro was then compared to patterns of transfection efficiency and protein production in mouse subjects. The 3D tumor model is a useful template to identify practical EP conditions and applicators that provide optimal transfection efficiency of genes into different cell types residing in a synthetic microenvironment. The 3D model helps predict how cells behave when subjected to gene delivery techniques and
translates observations of delivery \textit{in vivo} to target melanoma. The model was used to not only translate delivery parameters \textit{in vivo}, but more importantly reduce the animal subjects for gene delivery trials. Essentially, the purpose of designing a delivery system is to develop an immune adjuvant system that stimulates inflammatory responses against disease targets and prime surveillance cells for continuous recognition of specific epitopes.

\textit{Specific Aim 2:}

\textit{To evaluate the intra-tumor delivery phIL-15 and the resulting protein expression to determine if expression of IL-15 would promote tumor regression and enhance mouse survival in an aggressive mouse melanoma model.}

The work in Aim 2, used the information gathered from preclinical studies\textsuperscript{28} and the 3D tumor model to further test delivery conditions \textit{in vivo}. The purpose was to deliver a plasmid encoding human IL-15 (phIL-15) using EP into established melanoma tumors and determine by ELISA, the expression levels obtained at different time points. Also, test differences in transfection efficacy between tumor cells and skin cells when delivering pmIL-12 and phIL-15 both \textit{in vivo}. It is inferred that EP conditions producing the highest expression level would promote the strongest immune response that would cause tumor regression and increase mouse survival. Three different treatment (Tx) protocols (1Tx, 2Tx and 3Tx) were used to deliver on different days to understand the type of expression levels produced from the tumor cells and implement the protocol that would potentially enhance effector activity that would lead to tumor regression. Established tumors were treated with the 3Tx protocol and tumor volumes were monitored for 50 days. The surviving mice were challenged and monitored for an
additional 50 days to understand whether phIL-15+EP+ could potentially enhance a long-term memory response against secondary tumor inoculation.

**Specific Aim 3:**

*To identify key immune effector cells and cytokines that were directly or indirectly stimulated following intra-tumoral delivery of pIL-15 using EP.*

The work in Aim 3, characterized the signals involved in up-regulating the innate and adaptive immune response against treated tumor cells. In this section, melanoma was established in mice and treated with either the empty backbone pVax or phIL-15 in the presence or absence of EP. The primary response to the treatment protocol was analyzed at different time points to discern the type of immune cells and cytokines present during stimulation. The following assays were performed ELISpot, immunohistochemistry (IHC) and Luminex bead array to determine the types of immune modulators present at the time of tumor regression and contribute to mouse survival. Late stage cancer therapies are scarce and new types of treatment regimens are needed to work quickly against aggressive cancers and prolong patient survival. EP delivery system is a promising technique to control immune response stimuli, mount one’s own immune response against existing disease and improve survival outcome of patients.\(^ {26, 31} \).
MATERIALS AND METHODS

Cell Lines

B16.F10 mouse melanoma cell line (ATCC, Manassas, VA) was used in the in vitro 3D tumor model and inoculated into the mouse model to form solid tumors in vivo. The human melanoma cell line SKMEL-5 (ATCC, Manassas, VA) was also used in the 3D tumor model. Both cell lines were primarily grown in a T275 flask as a 2D monolayer of cells diluted 1:10, B16.F10 were supplemented with McCoy’s media and SKMEL were supplemented with DMEM containing 10% FBS (Atlanta Biologicals Lawrenceville, GA) and 0.2% Gentamycin (Mediatech, Cellgro, Manassas, VA, Mid Sci St. Louis, MO). HaCaT human keratinocytes used in the 3D model to form the scaffolding, were a generous gift from Dr. Mark Jaroszeski (University of South Florida, College of Engineering). The HaCaT cells were supplemented with DMEM media containing 10% FBS and 0.2% Gentamycin. HaCaT cells originated from an immortalized, transformed cell line that had the ability to adhere tightly to the flask and to grow without feeder cells.

All cells were grown in an incubator at 37°C and 5% CO₂ in humidified air. Cells were removed from flasks using trypsin 1x without EDTA (Atlanta Biologicals Lawrenceville, GA). Following removal from the flasks, trypsinized cells were neutralized with media, washed in DPBS (Mediatech, Cellgro, Manassas, VA) and kept on ice until utilized for the 3D model or inoculation in to the mouse model.
Plasmids

The IL-15 plasmid, a generous gift from Dr. David B. Weiner University of Pennsylvania College of Medicine, was cloned using human IL-15 construct (Sequence from Gene Bank, Accessory number NM00585) into pVAX1 cloning vector (Invitrogen, Carlsbad, CA) and was carried out by PCR amplification. The plasmid was engineered to enhance IL-15 protein expression\textsuperscript{149}. The long signal peptide was replaced with an optimized leader sequence 18aa human IgE region to increase secretion levels of IL-15. IL-15 mRNA was also optimized by removing inhibitory up-stream AUGs start codons which normally contains 8-10 AUGs contributing to the tight translational regulation. Also, the replacement of a weak kozak sequence with a stronger kozak sequence designed to enhance protein IL-15 production was introduced into the plasmid\textsuperscript{149-151}. The optimized human IgE IL-15 insert was ligated into a pVax plasmid containing kanamycin resistant gene, a constitutively active human CMV promoter, high copy number pUC Origin of replication and bovine growth hormone polyadenylation site to enhance bacterial growth\textsuperscript{151}. IL-15 plasmid was used in the 3D model studies and in mouse studies. Green Fluorescent Protein pEGFP-N1 (GFP) was purchased from Clonetech (Palo Alto, CA) to study transfection efficiencies in the 3D model. The IL-15 plasmid, the empty backbone p-Vax1 plasmids (control vector) and GFP were commercially prepared by Aldevron (Fargo, ND) to be endotoxin free. Commercially produced plasmids provide lot consistency between experiments. Prior to use, the plasmids were diluted to the appropriate concentration using sterile saline for each experiment.
3D Model System

The High Aspect Ratio Vessels (HARVs) promote 3D cellular aggregation while suspended in a microgravity environment (Synthecon, Houston TX)\(^ {54-55}\). These vessels rotate on their base at 1 gravitational force (1g) providing an optimal environment for cells to grow without restraint, experiencing minimal shear and interference by the vessel walls or disruption from the media. The bubble less system allows cellular expansion to occur in a state of free fall or solid body rotation while floating in media \(^ {52,55-58}\). The vessels are equipped with two luer locks ports and an easy access sampling port. The vessels contain a silicon membrane for passive diffusion of oxygen and carbon dioxide enhancing growth of cells\(^ {44, 57,153-154}\).

HARV Bioreactor Utilization

For long-term use the bioreactor motor systems RCCS-1 or RCCS-4SCQ were disinfected with 70% ethanol and sporicide to prevent rust and contamination by microorganism into cultured cells. In the incubator, the vessels were fastened to the base and rotate in a clockwise motion. To promote aggregate formation, the HARV apparatus was set to 15-17 rotations per minutes (rpm) depending on the density of the cells placed into the HARV. The cells remained suspended in media during the rotation and experienced minimal interference. The pH and oxygen levels between the cells and media remained balanced because the silicon membrane allowed passive diffusion of oxygen and carbon dioxide. This even exchange of gases ensured viability of cells providing the necessary nutrients for cellular replication.
3D Tissue Culture

3-5x10^6 cells of HaCaT were seeded into four T275 flasks (Falcon BD Biosciences Franklin Lakes NJ) grown in DMEM (MediaTech Manassas, VA) containing 0.2% 50mg/ml Gentamycin (MidSci St. Louis, MO), and 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals Lawrenceville, GA). After 2-3 days, confluent cells were Trypsinized 1x with EDTA (Atlanta Biologicals Lawrenceville, GA), collected and viability was determined using trypan blue. On Day 0, 35-50x10^6 live cells were seeded into a HARV in a final volume, dependent on vessel of choice 10ml or 50ml. After 1-2 days HaCaT cells aggregated into a spheroid measuring approximately 1 cm diameter. The HARVs were initially set for 15 revolutions per minute (rpm) for 12h and then increased to 16.5-18.0 rpm depending on size and shape of spheroid and stability of rotation. After 20-24h fresh media was replaced into the HARVs containing the aggregated cells. In parallel, 1-2x10^6 B16.F10 or SKMEL-5 cells were seeded separately into two T275 flasks and harvested for injection on Day 2.

Loading Cells into the HARV

To load the cells into the HARV or to change media, both knobs of the luer locks were turned to the open position and a 14 gauge cannula was attached to a syringe containing HaCaT cells suspended in media. The syringe was fastened to one open port and then cells were slowly injected into the HARV. It was necessary to remove all bubbles from the vessel to prevent disruption to spheroid formation. This was achieved by injecting the media containing cells into one port of the HARV and then slowly tilting the vessel gently forcing the bubbles with a piston-like motion into an empty syringe.
attached to the opposite port. An important factor when handling the vessels is to avoid increased pressure on the membrane\(^57\).

**Injection of B16.F10 into Spheroids**

A monolayer of B16.F10 or SKMEL cells were harvested from T275 flask, washed, counted using a hemocytometer and resuspended in a small volume to concentrate cells in about 2-10x10\(^6\) B16.F10 cells per 25-50\(\mu\)l. A 28 gauge ½ inch needle was used to inject B16.F10 into HaCaT spheroids. Once HaCaT spheroid aggregates formed a 1cm diameter construct, the vessel was removed from the base and the spheroid was manipulated towards the sample port for injection of tumor cells. The adherent properties of the HaCaT cells allowed for the spheroid to accommodate the volume of the introduced melanoma cells. This displacement of volume was easily observed by a slight ballooning of the spheroid during the injection. The spheroid was durable enough to withstand one to three different injection sites, although one injection site was sufficient. The sample port was sealed again and additional media was added to the HARV removing excess bubbles. A mixture of two different media were used to promote the growth of melanoma and Keratinocytes, this included 70% McCoy’s and 30% DMEM for B16.F10 or 70% MEM and 30% DMEM for SKMEL-5. 50% of the media was changed daily in the HARV. To avoid two media changes in one day the CO\(_2\) levels were decreased to 1.5-1.9%. The reusable HARVs were cleaned and sterilized after every use, per manufactures’ instructions (Synthecon, Houston TX). Briefly, the HARVs were soaked in autoclaved deionized water or nanopure water overnight, then the HARVs were soaked in 70% ethanol for 8-16 hours (hr), air dried, wrapped in foil and autoclaved at 105-110°C for 30 minutes (min).
Plasmid Delivery to 3D Spheroid

As mentioned previously, the HaCaT scaffolding forms after two days, the stable spheroids were removed from the HARV and placed into a chamber slide (Fisher Pittsburgh, PA). 500µl of fresh DMEM was used to coat the spheroid, concentrating the media around the delivery area. The EP electrodes were submerged in the media around the spheroid during delivery. A 25-gauge-5/8 inch needle 1ml-syringe (BD Biosciences Franklin Lakes, NJ) was used to inject spheroids with 50µl of a mixture containing 3-5X10⁶ B16.F10 or SKMEL-5 and 1.0mg/ml of GFP or IL-15 plasmid. The EP electrodes were placed around the spheroid and pulsed at different EP parameters, 6-plate electrode (6-PE) 1300V/cm 100µs 6 pulses (P), 500V/cm 20ms 6P, 4-needle 100V/cm 20ms 12P, 4-plate electrode (4-PE) 100V/cm 150ms 8P or 200V/cm 20ms 8P. After EP, 1-2ml of media was added to the chamber slide, covered and placed into a 1.5% CO₂ 37°C humidified incubator. The spheroids were monitored and harvested 24h-48h after transfection.

Stability of 3D Model Construct

HaCaT cells aggregate to form spheroids containing 50X10⁶ cells forming about 1 cm in diameter “tissue-like” construct after 24 hours (h). The peak stability for HaCaT spheroids occurs by 48h. The stability time points were determined by collecting observations for HaCaT spheroid formation up to 15 days (d). The peak stability for HaCaT spheroids injected with B16.F10 or SKMEL-5 cells was 4d after inoculation, totaling 6d for the heterogeneous spheroid. By Day 6, melanoma had grown within and amongst the HaCaT cells. Additionally, we observed that HaCaT spheroid aggregates residing alone in the HARV reached terminal differentiation after 5-7d; viability was
determined using trypan blue. HaCaT spheroids injected with melanoma remained stable in the HARV for up to 15d.

**Double Staining Immunohistochemistry**

(IHC) Paraffin-embedded samples were stained to identify different cell types within the spheroid B16.F10, HaCaT and SKMEL-2. Spheroids containing proliferating B16.F10 or SKMEL-5 cells were fixed in 4% paraformaldehyde or 10% formalin (Fisher Pittsburg, PA) for at least 6-24h, processed and embedded in paraffin wax for preservation. Before staining, the samples were deparaffinized using Xylene (Sigma-Aldrich St. Louis, MO). The recipe for deparaffinization included dipping sections in coplin jars separately containing Xylene, 100%, 95%, and 80% ethanol washes then placed in a humidified chamber. The samples were enzymatically pre-treated for antigen retrieval using 0.4% Pepsin in 0.01M HCL (Sigma-Aldrich, St Louis, MO). After the incubation period, samples were rinsed with 1x phosphate buffered saline (PBS, Sigma St. Louis MO). It was necessary to include an endogenous peroxide quenching step containing 10% hydrogen peroxide in 30% methanol (Fisher, Pittsburg, PA) to avoid non-specific binding of secondary conjugated Strepavidin-HRP antibodies that react with diaminobenzidine (DAB). The sections were blocked with 2.5% normal goat serum diluted (Thermo Scientific, Waltham MA) in 1x tris buffered saline (TBS, Boston Bioproducts, Worcester MA) containing 0.1% BSA (Fisher Pittsburg, PA). For samples labeled with primary antibody of mouse anti-human AE1/AE3 (MAB3412 Milipore, Billerica MA) goat anti-mouse IgG bound to alkaline phosphatase AP (80200 QED Bioscience Inc. San Diego CA) was used as the secondary antibody. For rabbit anti-mouse S100 (AB941 Millipore, Billerica MA) 2.5% bovine serum was used to block for the secondary antibody bovine anti-rabbit IgG HRP (sc-2370 Santa Cruz Biotech, Santa
Cruz CA). In this experiment, AE1/AE3 (1-10mg/ml) was diluted 1:100 in TBS + 0.1%BSA and then the secondary antibody goat anti-mouse IgG AP was diluted 1:20 in TBS + 0.1%BSA. The samples were developed using Fast Red substrate chromogen developer solution, per manufacturer’s instructions (Thermo Scientific, Waltham MA). Briefly, 3ml of Napthol Phosphate Substrate was mixed with 1 drop 40ul of Liquid Fast Red Chromogen. All staining procedures were repeated incubating S100 (1:800) in 2.5% normal bovine serum. Then the samples were incubated with secondary antibody bovine anti-rabbit IgG HRP (1:20) in 2.5% normal bovine serum. A DAB substrate was used per manufacturer’s instructions one tablet 10mg was mixed in distilled water (Acros EASYtablets, Geel Belgium) to develop the stain. The sample slides were counterstained using Hemotoxylin (Sigma, St Louis MO), rinsed, mounted and slides were observed under a light microscope (Leica Wetzlar Germany). Negative controls were included to show specificity of the primary antibodies and the lack of nonspecific binding of the secondary antibodies. For example, the primary antibody was added to the slide but a secondary antibody that did not match was also added and showed no reactivity.

**TdT-FragEL DNA Fragmentation Detection and Immunohistochemistry (IHC) Assay**

The TdT-FragEL DNA Fragmentation Detection assay (Calbiochem San Diego, CA) was performed on paraffin embedded samples to identify cells undergoing cell death followed by a double IHC assay to specifically identify the cells that were undergoing apoptosis. The assay was performed per manufacturer’s instructions. Briefly, a positive and negative control was performed simultaneously with the assay. The positive control, DNAse I (Promega, Madison WI) intentionally nicked the DNA ends and
the negative control was absent of the TdT reaction mix. The samples on the slide were
deparaffinized and rehydrated by immersing slides in Xylene and then in different
concentrations of ethanol (Sigma-Aldrich St. Louis, MO). The sections were
permeabilized using 2mg/ml of Proteinase K (Thermo Sci Waltham, MA) 1:100 in 10mM
Tris pH 8, then blocked for endogenous peroxidases 30% hydrogen peroxide (Thermo
Sci Waltham, MA ) in methanol (Sigma-Aldrich). The sections were equilibrated, labeled
with TdT labeling reaction mix and conjugated with Strep-HRP and developed with DAB
solution. Double IHC staining, the primary mouse anti-human AE1/AE3 antibody was
diluted 1:200 in 2.5% normal goat serum (All chicken, goat and rabbit serum Molecular
Probes, Carlsbad CA) and the secondary goat anti-mouse IgG AP was diluted 1:50 in
2.5% goat serum. All slides were developed using Fast Red. Then, sections were
incubated with primary antibody S100 goat anti-mouse (sc-7849 Santa Cruz Biotech,
Santa Cruz CA) diluted 1:100 in rabbit serum. The sections were incubated with the
secondary rabbit anti-goat IgG HRP (HAF017 R&D Systems, Minneapolis MN) diluted
1:20 in rabbit serum and developed using DAB. Finally, the sections were
counterstained with methyl green, mounted using permount (Fisher, Pittsburgh PA) and
then observed under a light microscope. A methyl green counter-stain distinguished
normal cells from apoptotic cells by producing a tan or blue-green stain for viable cells
and a dark brown stain for dead cells. Viable cells were counted using an ocular lens
micrometer at 200x Magnification (Mag).

**Double Immunofluorescence (IF)**

This assay was performed based on protocol described on the Cell Signal
website (www.cellsignal.com/support/protocols/IF.html). Briefly, the sections were
deparaffinized and rehydrated using xylene and ethanols. The antigen retrieval step
involved using 10mM of sodium citrate pH 6 in deionized water. The slides were placed in a coplin jar and heated in a water bath up to 87°C for 20min. The sections were blocked using cocktail blocking buffer mixed separately from different host serum. Total volume 25ml: mixed 2.5ml 10x PBS, 1.25ml normal chicken or rabbit serum, 21.25ml of deionized water were mixed, and 75μl of TritonX-100 (100%, Sigma St. Louis MO). A primary antibody cocktail was prepared separately and then combined on the slide; a 10ml antibody dilution buffer was prepared using 400μl of 10x PBS, 3.6ml of deionized water mixed, 0.040g of Bovine Serum Albumin (BSA Fisher, Pittsburgh PA) and 12μl TritonX-100. Mouse anti-human AE1/AE3 was diluted 1:100 and goat anti-mouse S100 (sc7849 Santa Cruz Biotech, Santa Cruz CA) was diluted 1:50 in dilution buffer. The cocktail of antibodies were incubated at 4°C overnight. The following day the slides were washed in 1x PBS, an additional wash was added 0.4M high salt 1x PBS solution to reduce background staining. The sections were blocked again to avoid non-specific binding by the secondary antibodies. The rabbit anti-goat IgG FITC was diluted 1:100 and chicken anti-mouse IgG Rhodamine was diluted 1:100 (sc-2777 and sc-2861 Santa Cruz Biotech, Santa Cruz CA). The sections were washed with PBS and high salt PBS. The sections were also re-incubated with rabbit anti-goat IgG FITC diluted to 1:50 to ensure binding of this secondary antibody. The samples were mounted using vectashield medium (Vectashield, Burlingame CA) and excited at two different wave lengths Fluorescein Iso-thiocyanate 490/520nm and Tetramethyl Rhodamine Isothiocyanate 557/576nm for detection. A negative control was performed simultaneously with this assay. The primary antibodies were added but mis-matched secondary antibodies were used to check for cross reactivity.
Proliferation Assay and Extracellular Matrix Staining

A double IHC was performed to stain for proliferating cells and cells synthesizing their own extracellular matrix. An Abcam (http://www.abcam.com) protocol was used to perform staining procedures. Briefly, slides were deparaffinized, rehydrated, antigens were unmasked using 20μg/ml of Proteinase K 30min 37°C and samples were blocked using straight goat serum plus 0.025% triton X. The slides were labeled with primary antibody Ki-67 1:200 (ab15580 Abcam Cambridge MA) or Fibronectin 1:200 (ab2413 Abcam Cambridge MA) and then incubated with secondary antibody goat anti-rabbit IgG-HRP 1:20 (ab6721 Abcam Cambridge MA). The labeled samples were developed using DAB and then visualized under a light microscope. The slides were counterstained using AE1/AE3 1:200 and secondary antibody goat anti-mouse IgG AP 1:200 as mentioned above. A negative control was tested along with the sample slides where the secondary antibody was tested for non-specific binding in the absence of the primary antibody.

Odyssey Western Blots

The western blot was performed using 4%-15% separating ready-gels and transfer blot apparatus (Biorad Hercules, CA) to detect the following protein markers E-cad (MAB3199Z Chemicon Millipore Billerica, MA), Cyto-8 (sc-8020 Santa Cruz Biotech Inc Santa Cruz, CA) and Beta Actin (#4967 Cell Signaling Danvers, MA). Total protein concentrations was determined by BCA protein assay kit (Pierce, Rockford, IL). The 32μg of total protein was loaded into each well of the SDS gel and the odyssey’s two color protein molecular weight marker ranging 10-250kDa. Proteins were separated using 120V for 5mins then 110V for 55mins. The bands were transferred onto a Millipore
Immobilon PVDF Membrane (Licor Lincoln, NE) for 1hr at 110V. The membrane was blocked using Odysseys’ Blocking Buffer (Licor) for 1hr, and then incubated with primary antibody 1:2,000 E-cad or 1:500 Cyto-8 at room temperature (RT) on a shaker overnight. After 16-18h the membrane was incubated with 1:15,000 secondary antibody 0.5mg goat anti-mouse IRDye 800CW NHS Ester for 1hr (Licor). An Odyssey Infrared scanner/reader was used to detect fluorescently labeled bands. A New-Blot PVDF stripping reagent (All reagents and products purchased from Li-cor Biosci Lincoln, NE) was used to analyze the two different primary antibodies. Protein bands were normalized against 1:5,000 Beta Actin (Cell Signaling Danvers, MA).

**GFP Delivery**

To ensure delivery of GFP in the spheroid, spheroids were formed by day 2, injected with a mixture of (1.0mg/ml) GFP+ (2-3x10^6) B16.F10 and immediately electroporated. After 24h, spheroids were collected, snap frozen and sectioned. Sections were labeled with nuclear (1:1,000) DAPI to visualize all cells and then sections were analyzed for GFP expression using an Olympus microscope at 200x Mag. The images show that spheroids that received EP at 500V/cm and 1300V/cm.

**Immunofluorescence Frozen Sections**

Spheroids were collected at 24-48h, snap frozen and cryo-sectioned. Samples were labeled with 1:1,000 DAPI (Sigma-Aldrich St. Louis, MO) for 10mins, blocked with 2.5% chicken serum (Thermo Sci Waltham, MA) for 1hr, incubated with primary antibody 1:100 AE1/AE3 (MAB3412 Chemicon Millipore Billerica, MA) for 2hrs and then incubated with 1:100 secondary antibody chicken anti-mouse IgG Rhodamine (sc-2861 Santa Cruz Biotech. Inc. Santa Cruz, CA) for 1h on shaker at RT. An Olympus microscope with
camera was used to analyze GFP transfection. Leica Stereoscope with digital camera was used to analyze GFP transfected into spheroids macroscopically.

**Flow Cytometry Analysis of GFP**

Spheroid aggregates were pressed through a 40µm Nylon cell strainer (BD Biosciences Franklin Lakes, NJ) and viability was determined by trypan blue (Fisher Sci. Pittsburg, PA). For live/dead gating, 1-3X10^6 cells were incubated with 10µl of Propidium Iodide 250ug/ml (Immunochemistry Bloomington, MN) for 30mins and then sampled using the FACS Aria I to quantitate GFP expression between controls and transfected groups.

**Tumor Production**

Cultured B16.F10 cells were harvested, counted for >90% viability by trypan blue exclusion dye method and resuspended in sterile 1XDPBS and diluted to 20 X10^6 cells/ml prior to mouse inoculation. Female 6-8week-old C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were injected subcutaneously into the shaved left flank with 0.05 ml containing 1x10^6 murine melanoma B16.F10. Tumors cells were incubated in mouse for 6-10 days or until the tumor measured approximately 30-60mm³ in size. Mouse studies were performed to study IL-15 transfection efficiency, establishing plasmid concentrations, tumor treatment schedules, immune response stimulatory assays, regression studies and challenge studies.

**Tumor Treatment**

Mice were anesthetized during all treatments. Anesthesia was administered by placing animals into an induction chamber infused with a mixture of 3% isoflurane and
97% oxygen for several minutes. After animals were anesthetized, the mice were fitted with a standard rodent mask and continuously anesthetized with 2-3% isoflurane in oxygen. Each mouse was closely followed after EP treatment until fully recovered from anesthesia.

Intra-tumoral treatments were performed as described; anesthetized mice were injected with 50µl of 1.0mg/ml-2.0mg/ml of phIL-15 or mouse pmIL-12 plasmid DNA in sterile saline. An applicator containing 6 penetrating electrodes was placed around the tumor slightly penetrating the skin for full contact. Tumor was immediately electroporated with the following conditions: 1300 V/cm of 100 µs 6 pulses (P) or 500V/cm 20ms 6P using a six needle array. The six needle array was selected based on previous studies demonstrating that this electrode design was able to pulse in six different directions encompassing the entire area of the tumor (39).

C57BL/6 mice were intra-dermally injected with phIL-15 or pmIL-12 and immediately electroporated using a 4-PE with 100V/cm 150ms 8p and 200V/cm 20ms 8P conditions. All samples were collected after 12h, 18h, 24h and 48h; snap frozen and stored at -80˚C until processed for ELISA analysis. The mouse IL-12 plasmid was used to confirm EP parameters and compare transfection efficiency and protein production levels between cytokine plasmids. Tumor and skin samples were tested, to understand cytokine delivery outcomes between different tissue types using multiple EP conditions.

**Tumor Measurements**

To determine initial tumor volume and monitor tumor regression, tumors were measured prior to treatments and at periodic intervals following treatment using a digital caliper. Volume was calculated using the following equation \( V=ab^2 \frac{\pi}{6} \): where (a) is the longest diameter and (b) is the shortest diameter perpendicular to (a). The tumor volume
standard deviation (S.D.) was calculated for each group at each selected time point. The mice were followed up to 50 days or until the tumor volume reached 1000 mm$^3$. The mice were humanely euthanized with CO$_2$ under vivarium staff supervision.

**ELISA**

Levels of IL-15 were obtained using a monoclonal human IL-15 Quantikine kit (D1500 R&D Systems Minneapolis, MN) and ELISA performed following the manufacturer’s instructions. Spheroids and media were collected from the 3D model and snap frozen. Retro-orbital bleeding was performed on mice under anesthesia for serum collection and the mice were humanely euthanized for the collection of tumor samples. Tumors were snap frozen and stored at -80°C until analyzed. The sample were thawed on ice, weighed, diluted in 500µl-800µl of phosphate buffered saline (Omnipur EMD4Biosci Darmstadt, Germany) plus Protease Inhibitor Cocktail (Roche Brandford, CT) dependent on sample size and homogenized using a PowerGen 700 (Fisher Scientific Pittsburg, PA). The homogenate was centrifuged at 1000rpm for 5 minutes at 4°C and the supernatant was assayed the same day by colorimetric analysis to measure differences in protein levels. Serum levels were calculated as pg of cytokine per ml of serum (Average pg/mL). Cytokine levels were calculated as pg of cytokine per weight of tumor (Average Total pg/0.1g).

**Western Blot**

Tumor and serum samples were collected to test production of human IL-15. Hong Yang M.D. performed western blot analysis for phIL-15 expression following intra-tumoral delivery using EP. BCA assay was used to test total protein levels of homogenized supernatant. To a 15% separating gel, 40µg of each sample was added to
the wells. The samples were initially separated at 80V for 30 mins then 160V until molecular weight marker separated on the gel. The gel was wet transferred to nitrocellulose membrane (Bio-rad) by electrophoresis at 130V for 1 h. The membrane was blocked for 1hr at RT shaking in 3%BSA then incubated 1:5,000 rabbit anti-human IL-15 polyclonal antibody (PA0672 Cell Sciences Canton, MA) overnight in 3% BSA. The following day the membrane was washed and incubated with 1:1,000 secondary bovine anti-rabbit IgG HRP (sc2370 Santa Cruz Biotech Santa Cruz, CA) in 5% milk for 1h. Protein levels were normalized against internal control rabbit anti mouse β-actin (4967 Cell Signaling Canton, MA) 1:1,000 in 5% milk 1hr and 1:5000 goat anti-rabbit IgG HRP (ab6721-1 Abcam Cambridge, MA)for 1hr. Developed the membrane using Super Signal West Pico chemilluminescent substrate solution (Thermo Sci Pittsburg, PA) for 5 mins, blot then expose to film 30s-1min.

Frozen Immunohistochemistry

Tumor samples were collected from mice and fixed in zinc fixative (BD Pharmingen BD Biosci Franklin Lakes, NJ) at room temperature for 2hrs up to 24hrs. Then samples were embedded in OCT (Tissue Tek Dublin, OH), snap frozen on dry ice and stored at -80°C until cryo-sectioning. The blocks were kept at -16 to-23°C during sectioning, the blocks were sectioned 7-10µm, fixed in cold acetone, then stored at -20°C until staining. The samples were removed from -20°C, air dried for15-30mins and then marked with a pap pen (Biogenex optiplus Hyderabad, India).Sections were rinsed fixed tissue with TBS 2x for 10 mins in humidity chamber with gentle agitation at room temperature on a shaker at 50 rpm. Sections were blocked with 5% normal rabbit serum/TBS for CD4/8+T cells, or goat serum/TBS for NK at room temperature for 1hr. Samples were also blocked with Avidin/Biotin kit for 15mins each (SP 2001 Vector Labs
Burlingame, CA). Primary antibody was added 1:20 purified rat-anti-mouse CD8a IgG2ak (550281 Clone 53-6.7 BD Pharsinghen), 1:20 rat anti-mouse CD4 IgG2ak (220280 Clone RM4-5 BD Pharsinghen) or 1:10 anti-mouse CD49IgM κ pan-NK-ells NK1.1 (108902 Clone DX5 Biolegend San Diego, CA) for 1hr. Washed then incubated with 1:250 secondary antibody IgG rabbit biotinylated antibody (BA-4001 Vector Labs) for CD4+ and CD8+ or 1:1,000 mouse absorbed affinity purified secondary antibody biotinylated Goat Anti-rat IgG, IgM, IgA (612106130 Rockland Gilbertsville PA) for 1hr. Washed then incubated in enzyme 1:300 Alkaline phosphatase streptavidin (SA-5100 Vector Labs) or 1:100 Enzyme Horseradish peroxidase streptavidin 1:100 for 15mins (SA-5004 Vector Labs). Washed and then incubated in Alkaline phosphatase substrate kit red or blue for 7-8mins (SK5100 or SK5300 Vector Lab Burlingame, CA) or DAB kit Easy Tablets. Rinsed with DiH₂O and counterstained using methyl green for 1-2 mins (H-3402 Vector Labs). Washed with tap water and dehydrated with ethanols, dried and mounted using a permanent mounting media (H5000.Vector labs).

**Luminex Bead Assay**

Milliplex (Millipore #MPXMCYTO-70k Billerica, MA) bead assay was used to test multiple cytokine stimulation from tumor and serum samples. These beads were purchased as a 10-plex kit assay measuring expression levels of specific antibody beads including INF-γ, TNF-α, GM-CSF, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, and MIP1β. The color coded polystyrene beads bind to an unknown amounts of cytokines and quantified by Luminex xMap software analysis. Briefly, as per manufacturer's instructions, 96-well plate is prepared by pre-wetting the filter plate using 200μl with wash buffer then vacuum filtered. Normalized samples were diluted for the assay and kept on ice. 25μl of standards, controls were added to 25μl of assay buffer. Matrix buffer PBS+PI was added
to the sample wells, then 25μl of samples were added to the wells, and finally 25μl of premixed beads were added to all wells. The plate was incubated on a shaker at 500-800 rpm overnight. The next day, plates were washed and vacuumed, 25μl of detection antibodies were prepared and added for 1hr with shaking and then 25μl Streptavidin-PE was added directly to detection antibody for 30mins with shaking. The plate was then vacuum filtered. The beads were resuspended in 150μl of sheath fluid and then the plate was read on the Luminex.

ELISPOT Assays:

Spleen and PBMCs were collected at different time points to determine specific anti-tumor responses following treatment with various protocols. Response was based on IFNγ production ex vivo following stimulation with tumor antigens. ELISpot assay was performed as per manufacturer’s instructions (R&D Systems Minneapolis, MN). Briefly, the day after the last therapeutic injection of IL-15 plasmid, samples were collected, prepped for cell culture, red blood cells were lysed with ACK (Quality Biologicals Gaithersburg, MD) and washed. The cells were counted using typan blue, a total of 2X10^6 PBMCs isolated from blood and 30X10^6 splenocytes isolated from spleens. The cells were placed in 24-well plates and incubated overnight to stabilize cells. The next day 300,000 PBMCs and splenocytes were transferred to 96-well V-bottom plates with media and stimulated with target 30,000 B16.F10 cells for 4-6 days. The 96-well plates were coated with antibody IFN-γ on day 3 or 5 (SEL485 R&D Systems) then blocked at RT for 2h. After appropriate incubation and washing, the effector and target cells were added to membrane plates containing IFNγ and incubated for overnight in the incubator at 37˚C 5% CO₂. The plates were washed the following day and then incubated with appropriate detection antibody overnight at 4˚C. The plate was washed on the following
day and then developed using a strepavidin-alkaline phosphatase/substrate system (SEL002 R&D Systems). Positive control concanavalin A (Sigma Aldrich) and recombinant protein IFNγ (Peprotech Rockhill, NJ) were tested and media served as a negative control.

**Statistical Analysis**

The results were analyzed using SPSS (IBM Chicago, IL) software to examine significance between treatment groups and controls samples using a two different tests. In the *in vitro* 3D tumor model experiments a one-way ANOVA post-hoc LSD test was utilized for statistical analysis. In the *in vivo* mouse model measuring phIL-15 and mIL-12 expression a two-tailed Student’s t-test was utilized for statistical analysis. The data was graphed based on the groups mean and standard deviation. 4-6 independent experiments were performed depending on the assay. Results were considered significance at \( p \leq 0.05 \). The experimental variation was analyzed using standard deviation and fold change was compared between the mean of each group tested.
RESULTS

Specific Aim 1

To develop a three-dimensional tumor model that could be used to evaluate gene transfer procedures and to examine cell to cell interactions.

The purpose for developing and utilizing an in vitro 3D tumor model was to evaluate EP conditions that efficiently deliver plasmids into specific cell types and in turn, promote gene expression by the cells’ own transcription/translation factors. The work performed in Aim 1 was focused on creating the 3D tumor model, testing the model’s stability, cellular functionality and over all applicability. The goal was to use the 3D tumor model to develop gene delivery techniques in vitro and translate those methods in vivo to develop a treatment regimen that would induce an immune response against melanoma. The objective was to grow a large synthetic tumor model (spheroid) to test plasmid delivery in vitro and compare results to preliminary studies in vivo and evaluate transfection efficiency and efficacy of delivery parameters. The advantage is that this model would reduce the number of animal subjects otherwise needed to test several preliminary plasmid delivery conditions.

Many different methods have been used to generate 3D tumor models in particular to study melanogenesis. Several different methods have been attempted to generate a large-scale 3D tumor model. A few techniques that were assessed include the liquid overlay method, matrigel/algi matrix (Gibco), cytodex/plastic beads scaffold (Sigma/Solohill) and extracel sponges (Glycosan).
The liquid overlay method consists of an agar/collagen platform that surrounds cells to help stimulate 3D growth and enhance proliferation of cells in a non-adherent matrix (Figure 3). Solidified agar coats the bottom of a plate keeping adherent cells such as B16.F10 cells from attaching. The cells are then coated with a collagen matrix that

**Figure 3: Liquid Overlay Method**
B16.F10 sampled after 26 days in culture. Size: 500µm.

**Figure 4: Algi Matrix**
containing B16.F10 sampled after 17 days in culture. Size: 375µm.

**Figure 5: Cytodex Beads Scaffold**
B16.F10 sampled from HARV after adhering to beads and expanding for 10 days. Size 619µm.

**Figure 6: Plastic Beads Scaffold**
polymerizes around the cells to act as an extracellular matrix that supports cellular aggregation and migration. The system promotes collective “tissue-like” growth of cells in an artificial environment without the need of biological stimulus. It was observed that B16.F10 cells tend to adhere to one another stimulating continuous proliferation and expansion until they form a melanoma mass. In nature, aggressive growth of melanoma needs the presence of a germinal layer for mass formation and cellular expansion. The liquid over lay method thus provides this matrix and substitutes as a scaffolding, extracellular matrix, or basement membrane to promote proliferation and differentiation. The matrix facilitates adherent cells communication for cellular development. In this 3D method, cells aggregate and form several small tumor mass populations on top of the agar, but were limited to \( \mu\text{m} \) in size even after a month in culture. The separate populations formed small mass about 250-500\( \mu\text{m} \) in diameter over a 3-4 week period. The cells behaved similar to melanoma in nature because the aggregated cells formed masses that contained viable cells towards the outside and necrotic cells towards the center. Although, the isolated populations did not aggregate towards each other to form one large mass, they formed several small populations. The cells did form a 3D tumor system similar to \textit{in vivo} tumors, but expansion rates were slow. This approach was
deemed unsuitable as the goal was to generate a tumor system that was about 1 cm in
diameter to enable testing of delivery conditions using electrode applicators containing a
1 cm gap. The objective was to administer electric pulses to enhance delivery to target
cells or tissue in a manner that would simulate the \textit{in vivo} procedure.

Other methods were used to help promote cellular expansion and large-scale
tumor mass formation \textit{in vitro}. To enhance mass formation more cells were required to
be present in the same location. Two methods were tested: the agar/collagen matrix and
algi matrix. In both synthetic materials, the cells were pierced into the matrix. This
allowed for more cells to grow together and expand more quickly in size. The idea was
to increase the number of B16.F10 cells in a location to allow cells the opportunity to
communicate, adhere to one another and expand into a tumor mass at a faster rate
(Figure 4). The cells did aggregate and formed a 3D tumor model as suggested prior
studies\textsuperscript{156}, but the size of the model remained too small to accomplish the goal of this
study and for macroscopic manipulation. A few more matrixes were used to help
promote larger cellular expansion such as an extracel sponge made of a polymer
material that ensures expansion of cells, but was limited to µm in size. Experiments
using the matrices described above were successful in promoting cellular aggregation of
melanoma to a size of 50µm to 500µm. The techniques supplied useful information of
3D modeling on a microscale. The matrices provided structure and scaffolding for
cellular accumulation, but the aggregates were not solid and did not maintain mass
formation, they were easily disturbed when manipulated or transferred. The other
limitation was that cells needed to be in the same location and constantly bathed in fresh
nutrients. Additional experiments were conducted to attempt to obtain tumor sizes closer
to 1cm in diameter.
The necessity to keep cells close to each other was an important feature for cellular expansion and mass formation. The factors that possibly limited cellular expansion using the matrices was reduction in nutrient/oxygen availability to cells and possibly the act of embedding the cells into the matrix limited expansion. The key concepts for mass formation included continuous oxygen exchange, surplus supply of nutrients and cellular contact. A group of NASA scientists developed a bioreactor system (high aspect ratio vessel, HARV) that promoted large-scale cellular aggregation bathing cells in a large volume of fresh nutrients, while growing suspended in an oxygen-rich synthetic environment. The HARV’s horizontal rotation exerts forces onto these dense particles and drives cells towards co-localization. These researchers tested many cell types and found that cells aggregated into 3D constructs using homogeneous or heterogeneous cells populations. The bioreactor provides a microenvironment suitable for cellular growth and development to facilitate natural cellular behavior. The next experiments utilized the bioreactor system to seed B16.F10 cells alone and use their own adherent properties to bind each other and promote mass aggregation. The bioreactors were also seeded with B16.F10 injected into sponge matrix, or grown onto plastic beads or cytodex beads to serve as a scaffold for cellular proliferation and mass formation.

B16.F10 cells were harvested and seeded onto cytodex dextrose coated beads (Figure 5), plastic beads (Figure 6) or extracel sponge (Figure 7) over night for adherence and then injected into the HARV. The use of cytodex beads, plastic beads or extracell sponge was to stabilize melanoma spheroid development (Figure 5-7). The cells were monitored for 10 days and up to 49 days. The beads facilitated mass formation and slightly increased aggregate stability during handling. The cytodex beads strongly adhered cells to the dextrose coating, but limited expansion by concentrating
cells to the beads themselves. The plastic beads and sponge matrix allowed slightly more cells to adhere to one another concentrating them into dense cellular populations, but again limited expansion because the construct was weak and cells would slough off into small sheets. The cells behaved as suggested by NASA/Synthecon bioreactor researchers forming a mass ranging from 600-700µm, but the magnitude and stability of the construct remained unresolved.

B16.F10 cells were injected into the HARV without a scaffold or matrix and instantly these cells aggregated forming a tissue-like construct (Figure 8). The drawback of this method was that the cells were weakly attached to one another and were easily disrupted by stopping the HARV’s rotation or while changing media, leading to detachment of large cellular sheets from the mass. These observations led to the idea that cells must need a scaffold that would keep the cells in a stable mass formation. Based on the mechanics instilled on cells by the HARV, description in the literature on

![Figure 8: B16.F10 Cells Placed into HARV with No Scaffold. Loose cells were observed after 24h.](image1)

![Figure 9: HaCaT Aggregate. HaCaT cells placed into HARV forms a spheroid after 24h. Size 1cm.](image2)
the effects of the HARV has on adherent cells and the observations suggested that a biological scaffold would possibly provide the structure needed to maintain a stable aggregates containing melanoma cells that would enhance cell interaction and communication.

The concept that melanocytes grow in the epidermis and that they are stabilized by keratinocytes gave the idea that adherent keratinocytes would act as the necessary scaffolding needed to promote adherent growth of melanoma cells. The human keratinocytes (HaCaT) were seeded in the HARV at many different concentrations 30-60X10^6 cells/10ml. The HaCaT immediately aggregated into small and large sacs of cells or spheroids after 24hours (h) (Figure 9). The sacs of aggregated cells were tightly adherent to one another and the construct consisted of a smooth external layer, while the cells in the center were mostly loose and dense. Observing the cells under the microscope, the outer cells were sheets of tightly adherent cells and the cells in the center were smaller aggregates of HaCaT populations. These spheroids were so stable after 48h to 72h that interrupting the HARV’s rotation, changing the media or handling did not cause the spheroid to fall apart. These stable aggregates contained adherent

Figure 10: Injecting B16.F10 and HaCaT into the HARV Simultaneously, formed two separate cellular aggregates after Day 2.

Figure 11: HaCaT Layering Effect. Injecting B16.F10 and HaCaT into the HARV simultaneously and adding additional HaCaT cells every day for 6 days.
properties that formed the 3D construct measuring about 1cm in diameter (Figure 9). The spheroid was easily handled and had the potential to be used for plasmid delivery experiments. The large constructs remained intact when transferred from the HARV to a petri dish.

To introduce the B16.F10 cells into the system and promote tumor mass formation, the HaCaT and B16.F10 were seeded into the HARV simultaneously (Figure 10). The B16.F10 cells formed loose secondary aggregates that were unincorporated into the spheroids and were slightly adherent to the outside of the spheroid. Another way to incorporate B16.F10 cells into the HaCaT was to layer the B16.F10 cells with HaCaT by initially seeding a small amount of HaCaT, allowing spheroid formation and then B16.F10 incorporation. This process was repeated after 24h and thereafter, every 48h-72h for 6 days (Figure 11). Unfortunately, the layering-effect also did not incorporate B16.F10 as well as expected. Both attempts at tumor mass formation were unsuccessful because B16.F10 did not incorporate into the system and enhance stability of the construct. The HaCaT spheroid did not form properly when B16.F10 were introduced. It seemed that when two populations of cells with different densities attempted to aggregate in the same space spheroid formation was less likely to occur.

Figure 12: B16.F10 Injection into HaCaT. 3-5X10^6 B16.F10 injected into HaCaT spheroid by Day 2.
Since HaCaT alone spheroids were so stable, the thought was that the HaCaT may be able to withstand an injection of B16.F10 cells into the center of the spheroid (Figure 12). A total of 3-5x10^6 B16.F10 cells were injected into the HaCaT spheroid construct without losing its shape’s integrity. The spheroid stability after 24-72h was impressive; the spheroid was malleable, accommodated volume displacement during injections and was resilient to multiple needle punctures. The spheroid provided the right conditions for B16.F10 cells to expand within the HaCaT spheroid after 5 to 7 days (Figure 13-14). The tan colored cells were the HaCaT and the dark brown pigmented cells were the B16.F10. Figure 13 shows tumor spheroid in suspension and Figure 14 shows a stable tumor spheroid transferred to a petri dish measuring about 0.9 cm. The construction of a 3D tumor model containing a heterogeneous population of HaCaT+B16.F10 cells was successfully developed. This is the first report of a large, free-floating 3D tumor model measuring about 1 cm in diameter. The utilization of bioreactors provided the necessary qualities to promote heterogeneous cellular integration.

Figure 13: B16.F10 and HaCaT Spheroid Residing in HARV remained buoyant and stable after a total of 6 days.

Figure 14: Stability of HaCaT and B16.F10 Spheroid Measuring About 1 cm, remained stable during transfer after a total of 6 days in the HARV. Size 0.9 cm.
With the construction of an appropriate 3D tumor model, it was critical to test the 3D model’s cell viability, proliferative properties, differentiation state, stability, communication, cellular interactions, functionality, and applicability. Cells viability was confirmed by paraffin embedded IHC. In Figure 15, an immunohistochemistry (IHC) analysis was performed on multiple paraffin embedded spheroid HaCaT+B16.F10 samples. It was confirmed through histological evaluation by Jane Messina M.D. (H. Lee Moffitt Cancer Center Tampa, FL) that 80% of B16.F10 stained brown and HaCaT stained red, cells remained viable in this construct. Samples were injected with B16.F10 on Day 2 after the spheroid was formed and 4-6 days later the HaCaT+B16.F10 were collected for staining. Dr. Messina confirmed that the cells’ nuclei were intact, cytoplasm was free of granules and model contained a 3:1 ratio of HaCaT cells to B16.F10. This indicated that the amount of B16.F10 cells grew rapidly over time making up one third of the construct, even though, initially, the spheroid contained 10 times more HaCaT cells than B16.F10 cells (Figure 15).
To provide further evidence of the viability of cells residing in the construct, TdT-FragEL DNA Fragmentation Detection assay (TdT) and double IHC assays were preformed. TdT assay was performed to identify live from dead cells and IHC was

Figure 16: TdT and IHC analysis. B16.F10 and HaCaT sectioned for viability assay and stained with double immunohistochemistry. a. positive control DNasel B16.F10 stained brown, apoptotic nuclei stained dark brown b. B16.F10 brown, HaCaT red viable cells stained tan, or blue-green, samples were collected 4-5 days after B16.F10 inoculation. Bar=100µm Magnification 400x.

Figure 17: Double Immunoflourescence Orientation. B16.F10 and HaCaT cells were labeled with specific fluorescently labeled fluorophores. a. B16.F10 stained green in FITC channel, b. HaCaT staining red in TRITC channel. c. Merged image of B16.F10 and HaCaT, with enlarged magnification of a population of cells. Bar=200µm. 200x + 16x Magnification.
performed to show the specific cell types present such as HaCaT and B16.F10 (Figure 16). Cells that were reactive to the TdT assay had stained dark brown nuclei and those that were non-reactive had tan or blue-green stained nuclei. To confirm the staining protocol a positive control slide was subjected to DNAsel enzyme, which nicked the DNA and allowed TdT labeling to bind free 3’OH groups and produce a dark brown stain. The nuclei that stained dark brown indicated that these cells were dead undergoing a pseudo apoptotic process (Figure 16a). The negative control showed that the nuclei of cells did not stain. In Figure 16b, the viable cells were stained tan or blue, were counted at 100x magnification, from 10 different quadrants, a total of 3 times from 3-4 different samples. It was observed that 80-90% of the B16.F10 cells and HaCaT cells remained viable after residing within the spheroid for 6-7 days (Figure 16b). These stained sections demonstrated that B16.F10 cells were proliferating and grouping together amongst the HaCaT cells forming isolated aggregates as though these cells populations were grouping to form tumor masses.

Observations of the spheroid suggested that cells were interacting and communicating with one another. A double immunofluorescence (IF) assay was performed to evaluate the spatial orientation of HaCaT cells relative to B16.F10 tumorigenic cells (Figure 17). This particular spheroid construct was harvested 5 days after B16.F10 inoculation. Figure 17a, shows the fluorescent excitation of B16.F10 cells attached to S100 and conjugated Fluorescein Iso-thiocyanate (FITC) antibody bound fluorophore and Figure 17b shows excitation of HaCaT cells AE1/AE3 and conjugated Tetramethyl Rhodamine Isothiocyanate (TRITC) antibody bound fluorophore, each cell type fluoresced independently of one another. The final panel in Figure 17c shows the two images merged to analyze the amount of overlap that occurred between the two cell types growing in the HARV. The green fluorescently labeled B16.F10 cells were
surrounded by HaCaT and also integrated with HaCaT cells stained in red (single arrows). The B16.F10 cells appeared as though they were grouped in isolated populations as well as growing on top of the HaCaT cells (single arrows). The HaCaT cells appeared as though they were organized; flattening and stacking making up the walls of the spheroid, while B16.F10 grew on top of the HaCaT (triple arrows). A growth pattern and cellular arrangement amongst these cells were clearly identified in the pictures. B16.F10 cells appeared as though they were growing processes and beginning to display elongated morphological changes (Figure 18a). The development of cellular processes was also observed in a monolayer of cultured cell which indicated cellular growth and differentiation into mature cell types. Figure 18b represents a negative control of FITC in the TRITC channel to show specific fluorescent staining B16.F10 and Figure 18c shows both images merged.

Figures 16-18, provides initial evidence of active cellular proliferation, grouping of B16.F10 cells and possible maturation. To further confirm this type of cellular behavior, an IHC assay was performed on spheroid samples 3 to 7 days after B16.F10 inoculation. The cells were stained using Ki-67 to identify whether B16.F10 cells were undergoing proliferation and to determine if cells are progressing through different phases of the cell cycle (Figure 19).

The cells were stained and then analyzed at 200x for differential nuclei staining. Analysis of nuclei staining demonstrated different shades of brown (Figure 19b), indicating that B16.F10 cells were present at different phases of the cell cycle. The difference in staining ranged from dark to lighter nuclear staining (Figure 19b magnified). Although, the presence of differential nuclear staining was noted, additional nuclear
staining assays are necessary to identify the specific cell cycle stage, this analysis was not performed\textsuperscript{157}. A negative control was included to show that non-specific binding of Ki-67 was not observed (Figure 19a).

The spheroids were also stained to identify whether B16.F10 cells proliferating in the spheroids had the ability to produce their own extracellular matrix. Another set of sections were stained with fibronectin antibody, a component of the extracellular matrix. Fibronectin stains at the surface of cells as depicted in Figure 20. The AE1/AE3 specific staining displayed identifiable round cellular shape of HaCaT and the fibronectin antibody bound to the B16.F10 and HaCaT surfaces displayed an uncharacteristically matrix-like, fibrous shape surrounding the cells. A negative control slide showed that fibronectin antibody was specific to cell surface antigens.

The 3D spheroid was used to promote growth, differentiation, and communication, between B16.F10 and the HaCaT scaffold. The next set of experiments performed were used to determine the model’s stability, potential to stimulate proliferation of a different cell line such as human melanoma SKMEL-5, understand the functional properties between melanoma and the HaCaT scaffold and to determine overall applicability of the model. It has been established that HaCaT cells aggregate to form spheroids after 24h\textsuperscript{59}. These aggregates contain 50X10\textsuperscript{6} cells forming a 1cm in diameter “tissue-like” construct and observations suggest that peak stability for replicating HaCaT spheroids occurs by 48h. The stability time points were determined by observing HaCaT spheroid formation over 15 days (d). The peak stability for HaCaT spheroids injected with B16.F10 cells is 4d after inoculation, totaling 6d for the heterogeneous spheroid. By Day 6, melanoma had grown within and amongst the HaCaT (Figures 13-15, Figure 17). To identify the 3D model’s ability to promote proliferation and aggregation of different cell lines, a human melanoma cell line, SKMEL-
5, was injected into the HaCaT scaffold and monitored for 4-6d. The HaCaT and SKMEL-5 demonstrated the same stability and aggregation patterns as HaCaT and B16.F10. The viability of SKMEL-5 injected into spheroids was determined using the TdT-FragEL DNA Fragmentation Detection assay on paraffin sections (Figure 21). The SKMEL-5 cells were stained brown with melanoma specific antibody S100 and HRP conjugated secondary antibody. The HaCaT cells were stained red with keratinocyte specific antibody AE1/AE3 and AP conjugated secondary antibody to identify between the cells types. Nuclei that stained tan signified that cells were viable and the nuclei that stained dark brown indicated that cells had undergone apoptosis (Figure 21). The level of viability for the SKMEL-5 was >80%, similar to the TdT assay performed on B16.F10 (Figure 16) proliferating in spheroids. During stability experiments, it was observed that HaCaT spheroid aggregates residing alone in the HARV reached terminal differentiation after 5-7d, viability was determined using trypan blue. However, the HaCaT spheroids injected with melanoma extended HaCaT cell proliferation and stability up to 15d in the HARV. These observations suggest that terminal differentiation was delayed due to melanoma injection, evidence of these observations is provided in Figure 22.

Figure 20: Extracellular Matrix Analysis. B16.F10 and HaCaT sectioned to identify extracellular matrix production. Bar=100µm 200x Magnification.

Figure 21: Viability Assay. SKMEL-5 and HaCaT sectioned to analyze viability of human melanoma collected on Day 6. Bar=100µm 400x Magnification.
The 3D model demonstrated that two heterogeneous populations of cells could co-exist and form a viable and stable tumor model, but the functional properties were unknown. These IF sections illustrated also demonstrated dendrite-like processes radiating from melanoma cells when interacting with keratinocytes in the 3D model. This suggests a type of communication occurring between B16.F10 and HaCaT. To test the functional communicative properties of the B16.F10 or SKMEL-5 separately injected into the HaCaT spheroid construct, a western blot analysis was performed to identify up-regulation of adherent proteins E-Cadherin (E-cad) and differentiating protein Cytokeratin 8 (Cyto 8) (Figure 22). The adherent proteins E-cad are known to be up-regulated when keratinocytes and melanocytes or melanoma cells interaction. Keratinocytes promote melanocyte survival and controls melanoma proliferation and

![Figure 22: 3D Model Functionality Test](image)

*Figure 22: 3D Model Functionality Test.* Testing the communication between HaCaT and B16.F10 by the up-regulation of adherent protein E-cadherin and differentiation marker Cytokeratin 8. Samples were collected on Day 2 and 4. HaCaT alone was compared to HaCaT+B16.F10 or HaCaT+SKMEL-5.
The B16.F10 or SKMEL-5 were separately injected into HaCaT and then monitored 2-4d after inoculation, totaling 4-6d. Samples were collected on day 2 or Day 4 after injection into the spheroid, homogenized and the supernatant was tested by the western blot to identify the up-regulation of cyto-8 and E-cad (Figure 22). Positive control MCF-7 breast cancer cells and negative control B16.F10 cells were collected from a monolayer of cultured cells to test primary antibody specificity and to detect protein expression. Each band was normalized against beta actin and fold-change (fold) was determined by comparing each melanoma inoculated spheroid group to control group HaCaT at Day 2 or Day 4. Cyto-8 differentiation marker presents consistently low band densities, <1-fold in groups HaCaT+B16.F10 and HaCaT+SKMEL-5 compared to HaCaT alone on Day 2 and 4. As predicted, there was a 2.3-fold up-regulation of adhesive protein E-cad in HaCaT+B16.F10 group at Day 2 and 1.9-fold increase at Day 4. However there was slightly lower levels of E-cad expression <1-fold in HaCaT+SKMEL-5 group, as suggested in the literature there is a notable reduction in E-cad expression in this cell line\textsuperscript{158}. SK-MEL-5 does not up-regulate expression of E-cad even in the presence of keratinocytes, it is not clear in the literature, if this is due to an inhibitory mechanism. It was observed that the spheroid stability was reinforced while in the presence of HaCaT+melanoma compared to HaCaT alone. These observations were linked to the increased expression of adherent protein E-cad in HaCaT+B16.F10 detected at Day 2 and reduction by Day 4. The increase in E-cad was also linked to the decrease in differentiation marker Cyto 8 at Day 2 and Day 4 in HaCaT+B16.F10. It appears that melanoma had slowed HaCaT terminal differentiation to keep HaCaT cells receptive to melanoma signaling proteins and promote melanoma proliferation\textsuperscript{59, 159}. Overall, comparing Cyto-8 to E-Cad there was a decrease in differentiation, but an increase in adherent protein when injecting B16.F10 into HaCaT. There were brighter
bands of E-cad and Cyto 8 proteins on Day 2 compared to Day 4, suggesting that protein production was controlled by keratinocytes at early stages.

To evaluate the usefulness of the in vitro 3D model the delivery of plasmid DNA with electroporation (EP) into HaCaT and B16.F10 cells was tested. The transfection efficiency of plasmids into the spheroids using different EP parameters was also tested. To determine if there was difference in delivery and protein expression between the cell types, plasmid encoding green fluorescent protein (GFP) was delivered using EP to the 3D spheroid containing only HaCaT cells. GFP expression was analyzed at 24h using a fluorescent stereomicroscope. The results indicated low GFP transfection levels in the HaCaT spheroids when delivered with a 4-needle penetrating electrode (4N) (Figure 23a,b) and with a non-invasive 4-plate electrode (4-PE) (Figure 23c). The EP electrodes and conditions established for the skin were utilized because the HaCaT mimics epidermal cells sitting on the basement membrane in vivo. Testing the skin EP conditions 4N 100V/cm 150ms 12 pulses (P) or 4PE 200V/cm 20ms 8P, on the spheroid did not result in the distribution or intensity level of GFP expression that was predicted. The GFP expression was produced near the surface of the spheroid and specifically those cells in close contact with electrodes (Figure 23b,c).

In the next set of experiments, a mixture of (1.0mg/ml) GFP and 3-5X10⁶ melanoma in a 50µl volume was injected into a HaCaT spheroid to monitor differences in transfection efficacy with different EP parameters. Here, GFP was delivered using a non-invasive 6-PE with EP conditions established for tumor. Increased levels of GFP expression by melanoma cells were observed (Figure 23d-g) compared to the HaCaT alone spheroid in Figure 23b and Figure 23c. A good representation of GFP distribution and transfection efficacy was observed between the HaCaT and B16.F10 cells in vitro. Additionally, there was a difference in transfection levels when delivering
GFP+melanoma into the spheroid using different electrode applicators and EP conditions. For Example, 6-PE EP conditions including field strength of 1300V/cm, pulse width of 100µs and 6P demonstrated lower transfection efficacy than 500V/cm, 20ms and 6P.

At GFP+1300V/cm (Figure 23d), expression was concentrated in two small locations and at GFP+500V/cm many more cells were found to express GFP mainly near the point of injection and some cells opposite the site of injection/EP (Figure 23e-g).

The stereoscopic pictures indicate a difference in delivery and expression between HaCaT and B16.F10. Delivery of GFP+melanoma was further analyzed for GFP expression and distribution by cryosectioning frozen spheroid samples and staining with DAPI (Figure 24).
Sections were analyzed with an inverted fluorescent microscope and the sections demonstrated that there was an increase in GFP expression. There was an increase in GFP expression near the surface and towards the center of the spheroid and an increase in GFP distribution with the GFP+500V/cm group (Figure 24 a,b,c) than with the GFP+1300V/cm group (Figure 24 d,e,f).

![Figure 24: GFP Distribution Analysis. HaCaT+B16.F10 sections analyzed for distribution: Injection of reporter gene GFP and EP a. Spheroid 500V/cm 20ms b. Section 500V/cm 20ms 6P Bar=200µm c. Section 500V/cm 20ms 6P Bar=200µm d. Spheroid 1300V/cm 100us 6P e. Section 1300V/cm 100us 6P Bar=200µm f. Section 1300V/cm 100us 6P Bar=200µm 200x Magnification.]

Although there was visual evidence that there was an increase of GFP expression and distribution in the highest expressing groups GFP+500V/cm, it was not clear which of the cell types HaCaT, B16.F10 or both were actually expressing GFP. To differentiate GFP expression between HaCaT and B16.F10, HaCaT+B16.F10 spheroids were electroporated at GFP+500V/cm and collected after 24h, snap-frozen and cryo-sectioned for immunofluorescent staining (Figure 25a). Sections were labeled with DAPI for all nuclear staining and HaCaT were stained with AE1/AE3 and conjugated red fluorescent antibody TRITC for contrast. The negative slides showed no auto-fluorescence of the cells in the FITC (Figure 25b) or TRITC (Figure 25c) channels. The
positive slides showed specific GFP expression in the FITC channel (Figure 25d) and HaCaT cells showed specific Rhodamine staining in the TRITC channel (Figure 25e) The DAPI and FITC channels were merged to show location of GFP transfection amongst all cells (Figure 25f).

Figure 25: Specific GFP Expression by HaCaT and B16.F10. HaCaT+B16.F10 sections analyzed for cell type transfection: Injection of reporter gene GFP and EP with a-j 500V/cm 20ms. b-c. negative FITC and TRITC controls Bar=200 µm and 100µm. d. positive FITC control Bar=100 µm. e. positive TRITC control Bar=100 µm. f. merging DAPI and FITC channels Bar=200 µm. g-h. merging FITC and TRITC channel Bar=100 µm. i-j. merging of DAPI, FITC, TRITC channels Bar=100 µm 200x Magnification.
Merging of FITC and TRITC channels showed the fluorescent labeled HaCaT specifically stained red colocalized with green fluorescent GFP producing an orange hue meaning that HaCaT were transfected. The cells expressing green GFP protein indicated that solely melanoma cells were transfected with GFP (Figure 25 g-h). Merging the FITC, TRITC and DAPI channels showed localization of all cells transfected with GFP (Figure 25 i-j). Based on these images we visually examined the relative ratios of cells expressing GFP. There were many more solid green melanoma cells that were transfected with GFP compared to combined orange HaCaT cells.

Flow analysis was utilized to quantitate GFP expression levels amongst four groups HaCaT, B16.F10, GFP+1300V/cm and GFP+500V/cm. Spheroids were injected with the GFP+B16.F10 mixture, immediately electroporated, and after 24h, spheroids were collected and processed for same day analysis. The transfected cells were analyzed using FACS ARIA I; P1 gates drawn around the HaCaT+B16.F10 cells, the ARIA I measured 100,000 events (Figure 26a,c,e,g), an H-gate was drawn around live cells and from those live cells a quadratic-gate was used to delineate between GFP positive and negative populations (Figure26b,d,f,h). The total percentages of GFP detected from the total population were as follows; HaCaT 0.29%, B16.F10 0.063%, HaCaT+B16.F10 1300V/cm 0.32% and HaCaT+B16.F10 500V/cm 1.88%. Mean Fluorescent Intensities (MFI) was statistically analyzed using a histogram curve derived from FlowJo software. HaCaT+B16.F10 GFP MFI levels in GFP+1300V/cm and GFP+500V/cm were 4.2-fold and 6.8-fold higher than HaCaT, respectively (Figure 27a-b). Figure 27c demonstrated GFP background levels in both no treatment HaCaT and B16.F10 groups. The GFP+500V/cm group provided 1.6-fold higher level of GFP expression than GFP+1300V/cm group (Figure 27d). The MFI data obtained from the four histograms were plotted onto a graph.
Figure 26: Flow Analysis of GFP Expression. HaCaT + B16.F10 Flow analysis to quantitate GFP expression percentage from total population of cells from FITC channels Area/Height. a-b. HaCaT cells only control GFP 0.29%. c-d. B16.F10 cells only control GFP 0.063%. e-f. HaCaT+B16.F10+GFP expression 1300V/cm 100µs GFP 0.32%. g-h. HaCaT+B16.F10+GFP expression 500V/cm 20ms GFP 1.88%.
The MFIs indicated that both EP conditions expressed higher levels of GFP production than MFI background levels from HaCaT and B16.F10 (Figure 27). HaCaT+B16.F10 transfected with GFP+1300V/cm expressed 3.2-fold higher GFP than HaCaT. The highest expressing group was HaCaT+B16.F10 GFP+500V/cm expressed a significant 7.3-fold more GFP than HaCaT (*p=0.001) (Figure 27).
The next set of experiments tested the transfection efficacy of plasmid encoding human interleukin IL-15 (phIL-15) delivered to the 3D tumor model. The purpose was to demonstrate that the 3D model could be used to predict IL-15 expression patterns compared to delivery results in vivo. The spheroids were injected with mixture of (1.0mg/ml) phIL-15 and 3-5X10^5 B16.F10, electroporated, and collected after 24-48h. Both the spheroid (Average Total pg) and the media (Average pg/ml) were tested for IL-15 protein production using ELISA analysis. The expression of IL-15 was compared between HaCaT only spheroids and HaCaT+B16.F10 spheroids amongst the following groups: no treatment, injection alone, phIL-15+1300V/cm and phIL-15+500V/cm. Low levels of IL-15 expression were detected in HaCaT only spheroid lysate (Figure 28a

![Figure 27b: Flow Analysis MFI GFP Quantitation. MFI graphed n=4 (\*p<0.001) compared to no treatment HaCaT cells alone. Error bars were calculated as standard deviation. Statistical analysis was performed using One Way ANOVA LSD Test.](image-url)
dotted line), from groups phIL-15 injection alone, phIL-15+1300V/cm and phIL-15+500V/cm groups compared to the HaCaT+B16.F10 groups (solid line Figure 28a). In the spheroid lysated there was a <1.2-<2.2-fold increase for phIL-15 injection, phIL15+1300V/cm and phIL-15+500V/cm at 24h and 48h in HaCaT alone. Transfection of phIL-15 into HaCaT+B16.F10, spheroid lysate mixture showed significant 3.1-fold 24h and 2.6-fold 48h increase in expression with the phIL-15+500V/cm group. Fold-change was compared to the no treatment group HaCaT+B16.F10 (solid line Figure 28a). In the media, there was a <1.6 fold increase for phIL-15 injection, phIL15+1300V/cm and phIL-15+500V/cm at 24h and 48h in HaCaT alone (Figure 28b dotted line). Delivery to HaCaT+B16.F10 showed increased levels of IL-15 production in the media at 3.1-fold and 2.3-fold in phIL-15+1300V/cm group and a significant 13.7-fold and 19.5-fold increase with phIL-15+500V/cm at 24h and 48h, respectively, compared to no treatment group HaCaT+B16.F10 (solid line Figure 28b). Slight decrease in expression levels after 48h in both the HaCaT and HaCaT+B16.F10, was possibly due to decrease in live cells after EP, or that HaCaT reached terminal differentiation decreasing overall transfection efficiency (Figure 28b solid and dotted line).
Figure 28: Expression of phIL-15 in the 3D Model. HaCaT + B16.F10 Delivery of human plasmid IL15 to quantitate expression of IL15 protein from the 3D tumor model. a. spheroid lysate HaCaT alone dotted line and HaCaT+B16.F10 solid line n=4-10 (*p<0.001). b. media supernatant from HaCaT alone dotted line and HaCaT+B16.F10 solid line n=4-6 (*p<0.05) all groups compared to no treatment HaCaT or HaCaT+B16.F10. Error bars were calculated as standard deviation. Statistical analysis was performed using One Way ANOVA LSD Test.
**In vitro delivery** of plasmids to a monolayer of cells is not always representative of what happens *in vivo*. It is expected that the 3D model will serve as a good indicator of expression levels when delivering phIL-15 to skin and tumor by utilizing HaCaT alone or HaCaT+B16.F10. To test whether the IL-15 results from the 3D model were indicative of expression patterns *in vivo*, the EP delivery conditions were performed in a C57BL/6 mouse model to compare transfection efficacies between HaCaT+B16.F10 *in vitro* to skin and tumor *in vivo*. Four mice per group were injected intra-dermally with phIL-15 (dotted line Figure 29). At 12h the phIL-15+100V/cm group showed a 1.9-fold increase in IL-15 expression and the phIL-15+200V/cm group demonstrated a 2.6-fold increase in expression compared to phIL-15 injection alone. The skin showed a slight increase of IL-15 expression in groups phIL-15+100V/cm and phIL-15+200V/cm electroporated with skin EP conditions, but were insignificant at 12h. Many individual skin samples in these groups at 12h resulted in zero expression levels compared to ELISA standards. The skin samples at the 18h time point, expressed significantly higher levels of IL-15 than 12h, demonstrating a 17.9-fold increase in phIL-15+100V/cm group and a 38.2-fold increase in phIL-15+200V/cm group of IL-15 expression compared to phIL-15 injection alone. The 24h and 48h time points were much lower and many individual samples expressed zero level of IL-15 plasmid (dotted line Figure 29). Another set of experiments containing four mice per group were inoculated with B16.F10 cells subcutaneously and after 6-10 days, tumors formed. The tumors were injected with phIL-15 and then electroporated using penetrating 6-needle electrode (similar to 6-PE *in vitro*)\(^{161}\) with EP conditions phIL-15+1300V/cm or phIL-15+500V/cm. In the tumor, the phIL-15+500V/cm group had significant 8.9-fold increase in IL-15 expression at 12h compared to injection of pIL-15. The 1300V/cm group was not significant, the highest expression occurred at 12h with 4.7-fold increase compared to injection of phIL-15 (solid line Figure 29).
There was a difference in transfection efficacy between skin and tumor mostly at 12h and a slight difference at 18h and 24h. Expression in both tumor and skin dropped at 48h.

As predicted by the 3D tumor model there was a significant difference in IL-15 expression between the cells types HaCaT only, HaCaT+B16.F10, skin and tumor. There were also differences in expression levels of IL-15 between EP parameters at 100V/cm, 200V/cm, 1300V/cm and 500V/cm as well as the time points tested 12h, 18h, 24h, and 48h. Transfection of phIL-15+EP+ (100V/cm and 200V/cm) into HaCaT and in the skin, revealed that there were similar IL-15 expression patterns at 24h and 48h from the HaCaT only group in the supernatant and in the media in vitro compared to the skin.

**Figure 29: Comparison of phIL-15 Expression to Skin and Tumor In Vivo.** Comparing IL15 expression to in vivo skin dotted line n=12 100V/cm 18h *p=<0.02, 200V/cm 18h *p=<0.001, 24h, *p=<0.03, and 48h *p=<0.02 and tumor samples solid line n=4; 12h 500V/cm *p=<0.02, 18h *p=<0.01, 24h *p=<0.02 compared to injection phIL-15 alone. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.
homogenate \textit{in vivo}. The \textit{in vitro} 3D model demonstrated higher levels of IL-15 expression at 24h in the HaCaT+B16.F10 samples, using EP conditions phIL-15+500V/cm, transfected into mostly B16.F10 cells. This was compared to the \textit{in vivo} model, the IL-15 expression levels were detected primarily in the established melanoma cells at 12h using the EP condition phIL-15+500V/cm. Although, IL-15 expression levels were detected at different time points between the \textit{in vitro} model and the \textit{in vivo} model, the expression patterns were very similar between the cell types and EP conditions used to deliver phIL-15. The highest expression of IL-15 was detected in B16.F10 electroprated at 500V/cm groups \textit{in vitro} and \textit{in vivo}. The results demonstrate that the 3D tumor model serves as a tool to predict EP conditions and plasmid expression levels prior to moving delivery techniques \textit{in vivo}.

Many studies show that IL-15 protein is highly regulated in nature and not easily detected \textit{in vivo}\textsuperscript{162}. The results showed that the 3D tumor model produced higher levels of IL-15 expression from cells secreted into the supernatant at 24h compared to the IL-15 production measured from the tumor lysate at 12h \textit{in vivo}. Compared to Kutzler \textit{et. al}, the optimized plasmid human IL-15 showed greater IL-15 expression from human rhabdomyosarcoma cells\textsuperscript{149}, compared to mouse B16.F10 cells. This is indicative that IL-15 protein was highly regulated \textit{in vitro} and \textit{in vivo} possibly due to cellular and extracellular regulatory mechanisms. The \textit{in vitro} studies also showed that the plasmid does have the ability to express the gene of interest, but some IL-15 protein remains sequestered in the cytoplasm of the B16.F10 cells. The mechanism behind plasmid delivery using EP and IL-15 regulation at the cellular level remains to be further analyzed \textit{in vivo}. 

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Summary

The HARV vessel was used to promote tumor formation of B16.F10 cells in a free-floating environment that did not require a matrix. The 3D tumor model was a stable and functional system that could be used to evaluate plasmid delivery utilizing EP and subsequent protein expression. The delivery of GFP and phIL-15 using EP into the 3D model provided useful information about cellular reaction to different electric fields, pulse lengths and pulse number. The 3D model was used to test plasmid delivery between different cell types and the expression levels attained at different time points. The same expression trend was observed when plasmids were delivered using the in vitro 3D model containing HaCaT+B16.F10 and tumors in vivo. Delivery of phIL-15 with 1300V/cm EP conditions promoted less IL-15 production at 1.0mg/ml than phIL-15+500V/cm at 12h in vivo tumor samples and in vitro HaCaT+B16.F10 at 24h. There were also differences in transfection patterns between skin and tumor in vivo that were also detected in the in vitro 3D tumor model. Transfection of phIL-15+EP+ into the skin showed less IL-15 expression levels compared to tumor in vivo, and the highest expression levels was observed using EP 200V/cm 20ms at 12-18h compared to EP condition 100V/cm. In the 3D tumor model the HaCaT only group showed low levels of expression compared to HaCaT+B16.F10, comparable to results in vivo.

Specific Aim 2

To evaluate the intra-tumor delivery of phIL-15 and the resulting protein expression to determine if expression of IL-15 would promote tumor regression and enhance mouse survival in an aggressive mouse melanoma model.

The work in Aim 1 evaluated the delivery of phIL-15 using a few EP parameters in the in vitro 3D tumor model and determined whether those in vitro results correlated
with *in vivo* experiments. This gave us a better understanding of the delivery conditions needed to obtain appropriate protein expression of the plasmid. In Aim 2, the work was designed to further explore EP conditions used to deliver phIL-15 into tumor and skin, and based on protein expression levels of IL-15 select the best route of plasmid administration and EP parameters to help target established melanoma.

Several EP conditions at different time points were tested. The phIL-15 results were compared to delivery of plasmid encoding mouse IL-12 to show differences between transfection efficacy and expression levels using different plasmids delivered to the tumor. The goal was to evaluate the delivery of phIL-15 intra-tumoral to elicit expression of the protein, promote tumor regression and enhance mouse survival against the aggressively fast growing melanoma.

![Figure 30: Gel Electrophoresis](image)

1% agarose gel testing plasmid aliquots for backbone with and without gene insert.
The first few experiments tested plasmid purification using gel electrophoresis and plasmid functionality using western blot analysis. The purpose was to identify that the plasmids used in all experiments were identical and did not have contaminants that would interfere with results. All plasmid cDNA tested in vitro and in vivo were primarily separated by size exclusion on an agarose gel to verify aliquot content containing empty backbone pVax or plasmid including the gene insert phIL-15. Figure 30 shows backbone pVax and IL-15 plasmid cDNA separated on a standard agarose gel after each plasmid was double digested. Three backbone plasmids and three plasmids with inserts were purified by aldevron between 2005 to 2009, each plasmid was double digested with ECOR1 and XHO1 enzymes. The 2005 plasmids were stock aliquots, the 2007 plasmids

![Figure 31: Testing Functionality of phIL-15 for IL-15 gene expression from 2007 at 24h and 48h. Western Blot performed by Hong Yang M.D.University of South Florida, Tampa, FL.](image)
were used in the majority of the experiments from Aim 1-3 and the 2009 plasmids were also used for experiments from Aim 3. Each plasmid was a product of the stock 2005 plasmid purified by Aldevron and guaranteed endotoxin free. The first three bands on the gel are ladders lambda Hind III, 100 base pair (bp) and 400 bp. The next three bands are cut pVax plasmid ranging about 3kb in size and the last three bands are human IL-15 plasmid cut and separated into backbone and gene insert. The top band is the backbone pVax ranged 2.6kb in size and the bottom band is IL-15 gene insert ranged around 400 bp. The information gathered from the gel indicates that the IL-15 plasmid utilized is the optimized plasmid containing IL-15 insert 396 bp. All plasmids used in experiments were of the correct size and free of contaminants, as guaranteed by Aldeveron.

The functionality of the plasmid was tested by western blot analysis for expression of IL-15 delivered into tumor cells. The plasmid that was constructed to express the IL-15 gene was delivered using EP into mouse melanoma and the presence of IL-15 expression from tumor cells was analyzed (Figure 31). B16.F10 cells were injected subcutaneously into the left flank of C57Bl/6 mice, 6-10 days post B16.F10 inoculations, tumors were monitored and volumes were measured. Mice containing tumor volumes ranging in size from 30-60mm³ were separated into 4 groups including no treatment (Tx), pVax injection (inj) plus EP, phIL-15 inj only or phIL-15 inj plus EP. Each group contained four mice. Tumor bearing mice were administered 1.0mg/ml of phIL-15 or pVax plasmid and then immediately electroporated at 1300V/cm 100µs 6P for those mice in the EP groups. The tumors were collected at 24h and 48h after one treatment (1TX), and then the samples were prepped for western blot analysis. Total protein of each group was determined using the BCA assay and then 100µg of total protein was loaded into the polyacrylamide gel. IL-15 protein expression tested from the four groups
is shown in Figure 31. The total protein loaded into the wells was verified by internal control β-actin. The no treatment group, pVax+EP+ and phIL-15 injection groups showed low background levels of IL-15 production, while the phIL-15+EP+ group showed up-regulation of plasmid IL-15 expression at 24h. At 48h the tumors were tested, the no treatment, pVax+EP+, and phIL-15 injection groups showed low background levels of IL-15 expression and surprisingly the phIL-15+EP+ group also demonstrated low levels of IL-15 production as well. The time period between 24h to 48h demonstrated that IL-15 expression levels were decreased in the tumors. The observations attained from the WB reaffirm the results obtained in Aim 1. Aim 1 tested the EP conditions in the 3D tumor model and in vivo at 12, 18, 24, and 48h, and the ELISA results demonstrated reduced IL-15 protein expression over time. The reduction of IL-15 in the tumor was indicative that IL-15 production is short lived.

The next step was to test a delivery approach using different EP parameters in tissues such as the tumor and skin and at the different time points 12h, 18h, 24h, 48h to understand expression kinetics of phIL-15. The goal is to establish a potential treatment regimen against mouse melanoma (B16.F10), to determine the best route of administration and expression levels of IL-15 when delivered into tumor or skin. Figure 32 demonstrates expression levels of phIL-15 at different time points following delivery into existing B16.F10 tumors using a 6-needle electrode with different EP conditions and different concentrations of the phIL-15. Figure 33 demonstrates the expression levels of IL-15 detected in the serum after EP delivery to the tumor. The EP conditions were chosen based on previous studies28 and the results from the 3D tumor model. A high electric field 1300V/cm with short pulses 100µs and two low electric fields 500V/cm and 200V/cm with long pulses 20ms were utilized in mouse studies. The plasmid concentrations selected for the studies were 1.0mg/ml and increased to 2.0mg/ml based
on low protein concentration observed in the western blot. Previous studies demonstrated low expression levels and suggested that small detectable IL-15 production occurs because it is known to be highly regulated and short lived (163). The groups tested in the mouse studies were phIL-15 injection only, phIL-15+EP+1300V/cm,100µs, 6P, phIL-15+EP+ 500V/cm and phIL-15+EP+ 200V/cm 20ms 6P at 12h, 18h, 24h and 48h n=8-17 for each group (Table 1). Expression levels of hIL-15 were determined from tumor lysate using a sensitive hIL-15 ELISA assay.

Table 1: Number of Animals Used to Evaluate of IL-15 Protein Production.

<table>
<thead>
<tr>
<th>N Values</th>
<th>IL-15 Inj 1.0mg/ml</th>
<th>IL-15 Inj 2.0mg/ml</th>
<th>+1300V/cm 1.0mg/ml</th>
<th>+1300V/cm 2.0mg/ml</th>
<th>+500V/cm 1.0mg/ml</th>
<th>+500V/cm 2.0mg/ml</th>
<th>+200V/cm 1.0mg/ml</th>
<th>+200V/cm 2.0mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>12h</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>12</td>
<td>16</td>
<td>11</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>18h</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>24h</td>
<td>16</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>48h</td>
<td>16</td>
<td>8</td>
<td>17</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>NA</td>
</tr>
</tbody>
</table>

Fold change (fold) of all expression levels were compared to IL-15 injection at either 1.0 mg/ml or 2.0mg/ml. In Figure 32, the highest expression of IL-15 obtained from tumor lysate was collected at 12h in the hIL-15+EP+ 500V/cm 2.0mg/ml group after 1Tx and was found to be 23.6-fold higher than hIL-15 injection at 2.0mg/ml, but was not significant. The phIL-15+EP+ 500V/cm group was not significant because there was one sample that had expression of 602pg compared to the average 92pg. It is not real clear whether this one outlier was increased due to the damaging effects of this specific EP condition or error in the ELISA assay. Although it remains unclear, there was only one mouse from an n of 11 that showed this result.
The following groups were found to have the next highest IL-15 expression levels in tumor lysate, 1.0mg/ml phIL-15+EP+ 500V/cm producing significant 17.6-fold (p=0.001) increase at 12h, 11.1-fold (p=<0.001) increase at 18h 1.0mg/ml and a significant 27.9-fold (p=0.0001) at 18h 2.0mg/ml in IL-15 expression compared to phIL-15 injection. With respect to protein levels for phIL-15+EP+1300V/cm there was a significant increase in expression from 12-24h, containing expression levels with a 10.9-fold (p=<0.0001) at 1.0mg/ml and 19.9-fold (p=0.001) at 2.0mg/ml.

Figure 32: IL-15 Expression in the Tumor In Vivo. Comparing IL-15 expressions from in vivo tumor lysate. Delivery of phIL-15 1300V/cm, 500V/cm and 200V/cm at 1.0-2.0mg/ml compared to injection of phIL-15.*p=<0.04. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.
The phIL-15+500V/cm significantly dropped by 24h, and EP conditions phIL-15+500V/cm and phIL-15+1300V/cm significantly dropped further by 48h. These IL-15 expression levels were similar to expression levels obtained in the WB analysis when delivered with 1300V/cm. In the western blot, protein expression was visible at 24h and no longer detectable at 48h (Figure 31). Expression of IL-15 in the phIL-15+EP+200V/cm group was very low and not significant. For this EP condition, the phIL-15 1.0mg/ml injected and electroporated at 12h, 18h, 24h, and 48h produced very low levels of expression. There was a slight peak at 18h for the 2.0mg/ml 200V/cm group.
since expression levels were so low, delivery of phIL-15 using these EP conditions were no longer tested at 24h or 48h. Figure 33, shows IL-15 expression levels in the serum, there was small difference in expression between the time points. There was a slight significant peak for the phIL-15+EP+500V/cm groups 1.0mg/ml and 2.0 mg/ml in the serum, but overall expression levels were as low as tumor lysate phIL-15+200V/cm group from Figure 33.

In Aim 1, using the 3D model experiments demonstrated a difference in transfection efficacy between the HaCaT and B16.F10 as well as delivery between the skin and tumor (Figure 29). Work in Aim 2, demonstrated that by increasing plasmid concentration for phIL-15 there was an increase in protein expression levels when delivering to tumors in vivo (Figure 32).

![Figure 34: IL-15 Expression in the Skin In Vivo.](image)

Comparing IL-15 expression from in vivo mouse skin. Delivery of phIL-15 at 100V/cm and 200V/cm compared to injection of phIL-15 at 2.0mg/ml.\(^*\)p<0.01. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student's t-test.
The next experiments tested delivery of phIL-15 to the skin and then compared expression levels to the tumor with increased plasmid concentrations 2.0mg/ml, as suggested by the 3D model. Three mice per group were injected intra-dermal with 2.0mg/ml phIL-15 at 4 points on their dorsal side, each site received 100µg of phIL-15 for a total of n=12 per group for evaluating expression levels in skin. Delivery to the skin used a 4 PE applicator injecting phIL-15 with 2.0mg/ml and immediately electroporating, phIL-15+EP+200V/cm group showed a significant (p=<0.001) increase in expression at 12h compared to 1.0mg/ml plasmid dosage (Figure 29), but expression quickly decreased over time (Figure 34). The phIL-15+EP+ 100V/cm maintained significant expression levels 12-24h and then significantly dropped at 48h. The expression levels in

Figure 35: IL-15 Expression in the Serum Post Skin Treatment. Delivering phIL-15 2.0mg/ml and comparing IL-15 expression from in vivo mouse skin serum to injection phIL-15 alone. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.
the skin were lower than expression in tumor lysate at 12-48h. Serum levels also showed very low and insignificant expression levels with an n=3 (Figure 35). Surprisingly, many samples did not demonstrate higher IL-15 expression in the serum especially since the three mice per group received 4 doses of the plasmid injected intra-dermal and electroporated. Based on these observations the skin delivery was no longer pursued as a treatment route for tumor regression studies. The IL-15 expression experiments showed that delivery of phIL-15 to the tumor with EP had a direct correlation of increased expression with a higher concentration of plasmid as observed in the 3D tumor model. Also, there was a difference in expression levels between skin and the tumor. The differences in expression of IL-15 seem to be dependent on plasmid dose, different EP conditions and the different EP electrode applicators used.

Delivery of a plasmid encoding a different cytokine was able to demonstrate a difference in expression levels using varied EP conditions when delivered into tumor and skin. The use of a different cytokine that may not be as highly regulated as phIL-15, was used to demonstrate that the low expression may not have been due to the EP delivery parameters but the gene being delivered. There was a significant increase in expression when delivering mouse plasmid interleukin-12 (pmIL-12) into the tumor using a variety of EP parameters after 1Tx (Figure 36). The 1.0mg/ml of pmIL-12 delivery was able to promote increased IL-12 expression levels in the tumor lysate and serum. There was a significant increase in mIL-12 protein expression at 24h compared to mIL-12 injection (Figure 36) and to the hIL-15 expression levels at 24h (Figure 32). EP was able to ensure delivery of both plasmids and while hIL-15 expression decreased over time, all EP conditions that delivered pmIL-12 maintained expression levels 24h-96h.
The best EP conditions that demonstrated increased mIL-12 expression at 24h utilized a caliper electrode to deliver pmIL-12 with EP condition 667V/cm 100ms 1 pulse (24h \( p=0.001 \), 48h \( p=0.03 \), and 96h \( p=0.02 \)) into the tumor, \( n=12 \). The level of expression was significantly higher amongst all EP conditions and expression levels did not diminish quickly between 24-96h. EP conditions 1300V/cm and 500V/cm using the 6-needle electrode, showed significant expression of mIL-12 \( (p=<0.01, p=<0.04) \) over time compared to injection only. After 24h there was a significant decrease of expression in the 1300V/cm group \( (p=0.01 \text{ 24h}, p=<0.01 \text{ 48h} \text{ and } p=0.01 \text{ 96h}) \), although 500V/cm maintained expression past 48h \( (p=0.01 \text{ 24h}, p=0.004 \text{ 48h} \text{ and } p=0.04 \text{ 96h}) \).

Figure 36: IL-12 Expression in the Tumor \textit{In Vivo}. Delivery of pmIL-12 1300V/cm, 500V/cm, 200V/cm, 804V/cm, 667V/cm compared to injection pmIL-12 1.0mg/ml. **\( p=<0.05 \). Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.
Overall, both groups delivering pmIL-12 with 1300V/cm and 500V/cm showed a dramatic increase of mIL-12 expression compared to hIL-15 expression using the same EP conditions. As demonstrated in the 3D tumor model with GFP and phIL-15 delivery, there were differences in expression between tumor and skin also observed with the delivery of pmIL-12.

As described in Aim1, the lower electric field and longer pulses provided the best genetransfer and protein expression levels 500V/cm and 667V/cm, but caused the most damage. Comparing EP conditions, the least damaging EP parameters used higher electric fields and shorter pulses, 1300V/cm seem to produce high expression early at 24h in tumor lysate and leveled out with the other EP groups at 48h. The 804V/cm conditions expressed slightly lower levels of mIL-12 at 24h n=8 than 1300V/cm n=12,
while 1300V/cm dropped in expression, 804V/cm maintained expression levels possibly because they have slightly longer pulses than 1300V/cm. In addition to evaluating expression in tumor lysate (Figure 36), serum from these mice was also evaluated for IL-12 expression levels (Figure 37). There was significant expression (p=<0.007 24h, p=<0.03 48h and p=<0.02 96h) of mIL-12 in the serum n=7-12. The serum levels were slightly lower than tumor lysate in mIL-12 experiments and decreased over time, but expression patterns correlated with mIL-12 tumor expression. Tumor and serum increased at 24h, and slowly decreased 48h-96h. The information gathered from mIL-12 tumor delivery supports the observations from the in vitro 3D model and in vivo mouse models in Aim 1, suggesting that IL-15 must be highly regulated at the cellular level causing a decrease in expression. Also there was a correlation between lower transfection in the skin and higher transfection in the tumor compared to 3D tumor model cell types. The highest expression levels were observed in the tumor using EP conditions 500V/cm and 667V/cm, low electric field strengths with longer pulses. EP conditions enhanced higher transfection and promoted more expression of mIL-12 in the tumor cells than hIL-15 delivery. Both phIL-15 and pmIL-12 peaked at early time points after 1Tx, slightly decreased and maintained expression levels 48h and 96h, respectively.
Delivery of pmIL-12 into the skin resulted in at least 30x less IL-12 expression compared to tumor levels (Figure 38). The mice were injected at one site on the dorsal side with pmIL-12 1.0mg/ml in a 50µl bubble. The 4PE was used to EP the plasmid bubble injected intra-dermal and then the skin was marked with permanent marker to identify location during tissue collection after 24h-48h. There was a significant increase in expression using the 4PE to deliver pmIL-12+EP+ 200V/cm after 24h (p=<0.01), the skin samples maintained expression up to 48h (p=<0.005) n=8-16.

![Graph](image)

**Figure 38: IL-12 Expression in the Skin In Vivo.** Delivery of pmIL-12 at 100V/cm and 200V/cm compared to injection pmIL-12 1.0mg/ml and measuring IL-12 expression from in vivo mouse skin compared to pmIL-12 injection alone *p=<0.05, **p=<0.01, ***p=0.005. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.

The pmIL-12+EP+ 100V/cm group showed significant expression of IL-12 after 48h (p=<0.05), maintaining the same expression for 2 days after 1TX. The levels of IL-12 in the serum at 24-48h following delivery of pIL-12 to the skin resulted in even less expression of IL-12 at 24h compared to serum levels following delivery to tumor (Figure 39). mIL-12 increased at 24h pmIL-12+EP+200V/cm n=2-3 then dropped quickly by 48h.
The values were analyzed using the student t-test, but results did not present statistically significant expression levels in the skin serum.

![Graph showing IL-12 expression in serum](image)

**Figure 39: IL-12 Expression in the Serum Post Skin Treatment.** Delivery of pmIL-12 1.0mg/ml to skin and measuring expression of IL-12 measured in mouse serum compared to pmIL-12 injection alone. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.

Overall, delivery to the skin resulted in much more defined mIL-12 expression than hIL-15 expression. The mIL-12 data is comparable to the levels of mIL-12 plasmid delivery in the Heller et al publications. One observation that was noted in the in vitro 3D tumor model, discussed in 3D model publications, and also observed in vivo, was that loosely less adherent tumor cells were transfected more readily and expressed more protein compared to adherent, keratinized skin cells. Comparing mIL-12 to hIL-15 protein production showed that high expression levels are lost over time after 1Tx and requires additional boosts to maintained expression. Short lived plasmid expression is beneficial because it reduces the chance of toxicity due to over expression of the protein.
The amount of protein expressed by the tumor cells following DNA delivery is dependent upon the plasmid vehicle, concentration of the plasmid, cells type, EP conditions, EP applicators and number of treatments. hIL-15 expressed lasted longer in the tumor than in the serum, to enhance protein expression and extend systemic stimulus, multiple treatment protocols of hIL-15 should be employed. Based on the expression levels and kinetics, the next sets of experiments were designed to evaluate expression levels of phIL-15 following multiple tumor treatments. Since hIL-15 expression was short lived and peaked at 12h, the treatment protocols were performed on Day 0, 1, 2, and samples collected at 12h, 36h, or 60h to identify expression patterns.

Figure 40: One, Two, Three Treatment Protocol to the Tumor. Delivery of phIL-15 1.0mg/ml into tumor 2Tx and 3Tx on Days 0, 1 and 2 and expression of IL15 measured in tumor compared to phIL-15 injection alone. Error bars were calculated as standard deviation.
with multiple treatment administration. Experiments were performed on 4 mice per group using a 1Tx protocol, a two-treatment (2Tx) protocol occurring on Day 0 and 1 and three treatment (3Tx) protocol on Day 0, 1, 2, delivering 1.0mg/ml phIL-15 with EP conditions 1300V/cm or 500V/cm. The expression levels were similar to the levels of the one-treatment (1Tx) protocol after 12h, 36, and 60h, but in the 2Tx protocol collected at 36, 60h protein levels slightly increased, the phIL-15+EP+ 500V/cm did not increase beyond 7.0 Total pg (Figure 40).

![Graph](image)

**Figure 41: One, Two, Three Treatment Protocol Tested in Serum.**

Delivery of phIL-15 1.0mg/ml into tumor and expression of IL15 measured in mouse serum from multiple treatment protocols on Days 0, 1 and 2, compared to phIL-15 injection alone. Error bars were calculated as standard deviation.

In the 3Tx protocol, at Day 0, 1, 2, samples were collected at 60h and showed a slight increase, but IL-15 production did not increase beyond 7.7 Total pg. Multiple treatments did not increase serum levels of IL-15 (Figure 41), there was no additional
up-regulation of expression detected at these early time points 0, 1, 2 with multiple treatment administrations. In the next experiments the time was spaced further out between EP deliveries to test if expression levels were enhanced.

The following 2Tx and 3Tx treatment regimens were similar to treatment protocols published in Lucas et al., 28. The 2.0mg/ml phIL-15 was delivered using EP on Days 0, and 3, for a 2Tx regimen and Days 0, 3, 6, for a 3Tx regimen with n=12 for each group. There was no real difference in expression in the tumor compared to Days 0, 1, 2 treatments (Figure 42) and serum levels were very low (Figure 43). The 2Tx and 3Tx protocols at Day 0, 3, 6, demonstrated a significant increase of IL-15 expression in EP groups 1300V/cm with a 3.1-fold (p=0.02) and 500V/cm containing a 3.5-fold (p=0.001) increase of IL15 at Day 3, using the 2Tx protocol and significance increase in the 500V/cm group containing a 5-fold (p= 0.04) increase of IL-15 at Day 6, with a 3Tx protocol compared to injection alone. Overall, the multiple treatment protocols did not show enhanced expression levels, but delivery methods were effective in maintaining protein levels in the tumor with each additional treatment. The initial experiments showed increase after 1Tx peaking at 12-24h and then decreased by 48h. It was decided that the 3Tx protocol with treatments at Day 0, 3, 6, would be the best protocol since significant increase in IL-15 expression was detected and based on the decreased tumor volume observations recorded during expression experiments. Some tumors were beginning to regress after 2Tx and 3Tx around Day 4 and Day 7. The injection of IL-15 alone group continued to increase in tumor volume, while the mice treated with hIL-15+EP+ began to decrease.
Figure 42: Two, Three Treatment Protocol Tested in the Tumor. Delivery of phIL15 2.0mg/ml into tumor and detecting expression of IL-15 after multiple treatment protocols on Days 0, 3 and 6. 1300V/cm Day 3 *p=<0.02, 500V/cm Day 3 *p=<0.001, 500V/cm Day 6 *p=<0.04 compared to phIL-15 injection alone. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.

Figure 43: Two, Three Treatment Protocol Tested in Serum. Delivery of phIL-15 2.0mg/ml into tumor and detecting expression of IL15 in the serum after multiple treatment protocols on Days 0, 3 and 6. compared to phIL-15 injection alone. Error bars were calculated as standard deviation. Statistical analysis was performed using the Student’s t-test.
Multiple and adjacent administrations of phIL-15 and EP delivery were necessary to control tumor growth. Previous studies by Heller and Lucas et al.,\textsuperscript{28-29} described that tumors did not regress after 1Tx and 47% of mice experienced tumor regression and mice survived after 2Tx. The best results were attained after administering 3Tx with mouse pIL-12 delivery using EP, there was 80% of mice that experienced tumor regression and survived. Based on these studies, it is important to maintain IL-15 expression to promote continuous tumor regression by administering multiple treatments of phIL-15 and EP to the tumor and more importantly enhance mouse survival. As noted in the 2Tx and 3Tx protocols, the groups obtained multiple treatments with 2.0mg/ml maintained IL-15 expression showing initial signs of tumor volume reduction.

Table 2: Four Separate Regression Studies Measuring Tumor Volume.

<table>
<thead>
<tr>
<th>3 Tx Regression Studies Day 0, 3, 6</th>
<th>ST Exp 1 Day 14 n=4</th>
<th>ST Exp 2 Day 30 n=4</th>
<th>ST Exp 3 Day 60 n=4</th>
<th>LT Exp 4 Day 50 n=15</th>
<th>Primary Response Exp 3, 4 Day 50-60</th>
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<td>No Treatment</td>
<td>25%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>pVax Injection</td>
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<td>0</td>
<td>0</td>
<td>-</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>pVax 1300 V/cm</td>
<td>100%</td>
<td>75%</td>
<td>75%</td>
<td>6/15 40%</td>
<td>9/19 47%</td>
</tr>
<tr>
<td>pVax 500V/cm</td>
<td>100%</td>
<td>75%</td>
<td>75%</td>
<td>11/15 73%</td>
<td>14/19 74%</td>
</tr>
<tr>
<td>pIL-15 1300 V/cm</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>11/15 73%</td>
<td>15/19 79%</td>
</tr>
<tr>
<td>pIL-15 500V/cm</td>
<td>100%</td>
<td>75%</td>
<td>100%</td>
<td>8/15 53%*</td>
<td>12/19 63%</td>
</tr>
</tbody>
</table>

Table 2: Regression Studies Short Term (ST) Experiments (Exp) and Long Term (LT) Experiments.

Table 2, shows a set of 4 different regression studies that were performed to monitored tumor volumes over a period of days such as, Days 14, 30, 50 and 60. Mice
were inoculated with B16.F10, monitored for 6-10 days and tumors ranging 30-60mm³ were treated with the 3Tx protocol on Days 0, 3, and 6. In the three separate short-term (ST) tumor regression experiments, mice tumors were monitored every 3 days to analyze tumor sizes and then tumors were collected for ELISpot and Luminex bead array analysis on Days 14, 30 and 60, as described in Aim 3. The following treatment groups were included in experiments: no treatment, pVax inj, IL-15 inj, pVax+1300V/cm, pVax+500V/cm, phIL-15+1300V/cm and phIL-15+500V/cm. Tumors were monitored in Experiment 1 Day 14, 8 days after the last treatment, in Experiment 2: Day 30, 24 days after and in Experiment 3: Day 60, 54 days after the last treatment. Observations were recorded for comparison regression studies (Table 2). In Experiment 4, a long term (LT) experiment was performed to observe tumor regression and mouse survival up to 50 days. The surviving mice were challenged with injection of 5X10⁵ B16.F10 cells subcutaneously on the opposite flank and monitored for an additional 50 Days. The purpose behind the challenge was to evaluate protection. It was predicted that mice would mount a primary immune response against tumor antigens and would build a stronger, faster secondary response against challenge against B16.F10 inoculation as well as prevent growth of tumors.

In each separate experiment, tumor volume had decreased as early as day 4 and day 7 in pVax+EP+ and phIL-15+EP+ treatment groups, 1300V/cm and 500V/cm. The mice in the no treatment, pVax injection, and IL-15 injection groups had mice tumors that continuously expanded. Some mice became lethargic and grew weak by Day 14 in Experiment 1. In Experiment 2 by Day 21, the tumors had metastasized to the lymph nodes and/or spleen and the metastatic properties of B16.F10 caused labored breathing in the mice. The mice that had tumor volumes greater than 1000mm³ or grew sick because of metastasis were euthanized between Day 16-21 in Experiment 2 and in
Experiment 3. The mice treated with pVax+EP+ or phIL-15+EP+ 1300V/cm and 500V/cm had tumors regress completely as early as Day 14-21, in Experiment 2 and Day 16-23 in Experiment 3. In Experiment 4, many tumors grew deep and some tumors were abnormally shaped due to initial tumor inoculation. In the IL-15+EP+500V/cm group, there were 5 mice that grew deep tumors and had succumb to metastasis, while 2 mice were found dead because of EP condition 500V/cm powerful pulses. The needle placement around the site of injection was important in these EP groups since delivery of IL-15 was necessary to promote expression in enough cells to signal the innate and effector response against fast growing melanoma. Since IL-15 contains a short-half life it is important to deliver to as many cells as possible to overcome the down-regulation of IL-15 protein signaling, and promotes tumor regression in the non responding mice for increased survival. The mice in Experiment 3, Day 60 did not have abnormally shaped tumors or EP complications and all mice in phIL-15+EP 500V/cm survived out to 60 days. Observations indicate that phIL-15 delivered using EP must encompass many tumor cells to properly administer phIL-15+EP+ treatments and avoid tumor escape using the short-lived IL-15 plasmid. The mice from Experiment 3, Day 60 and mice from Experiment 4 Day 50, were combined to determine the total amount of mice that had tumors regress and therefore enhanced mouse survival that were tumor-free (Table 2). Tumor regression was observed in the following groups: the pVax+EP+1300V/cm had 9/19 (47%) of mice respond, pVax+EP+500V/cm had 14/19 (74%), phIL-15+EP+1300V/cm had 15/19 (79%), and ph IL-15+EP+500V/cm had 12/19 (63%) mice that had regressed tumors remained alive (Table 2). The best primary response against tumors was obtained from mice in the phIL-15+EP1300V/cm and pVax+EP+500 V/cm groups.
The mice from Experiment 4 were monitored for 50 days after the 3Tx protocol on Day 0, 3, and 6. Tumor volumes were measured every 3-4 days. The volumes were plotted in Figure 42 a and b. The mice that reacted to treatments demonstrated decreased tumor volume, these mice were considered responders (Figure 44a). Mice that did not respond to the treatments, known as non-responders contained large tumors that continued to grow until the tumor volumes reached 1000mm$^3$ and then these mice were humanely euthanized. The mice that responded to the treatments and survived primary inoculation were plotted on a Kaplan-Meier survival curve (Figure 44c, Table 2). The phIL-15+1300V/cm and pVax+500V/cm showed that 73% of the mice remained alive, while phIL-15 500V/cm showed 53% and pVax+1300V/cm had 40% mouse survival.

**Figure 44a: Non Responders Tumor Volumes Monitored** for primary response up to 50 days after 3Tx on Days 0, 3, 6 a. Nonresponders, mice that have not responded to treatment conditions. n=15. The mice that did not survive were recorded as a percentage.
b. Responders Tumor Volumes Monitored for primary response up to 50 days after 3Tx on Days 0, 3, 6. a. Responders, mice that have responded to treatment conditions. n=15. The mice that did survive were recorded as a percentage.

c. Kaplan-Meier Survival Curve. Demonstrate survival data of mice receiving 3Txs on Days 0, 3, 6. n=15.
The surviving mice from Experiment 4 were challenged at Day 50 on the opposite flank with $5 \times 10^5$ B16.F10 to determine the extent of secondary response against tumor cells (Table 3). The IL-15+EP+1300V/cm had 5/11 (45%) mice were resistant to the second injection of tumor cells compared to the pVax+EP+1300V/cm which had 3/6 (50%) mice that maintained tumor intolerance. The phIL-15+EP+500V/cm had 6/8 (75%) mice protected against secondary challenge compared to pVax+EP+500V/cm 4/11(36%). The best groups that promoted a secondary and long term immune response against melanoma were phIL-15+EP+ 500V/cm followed by pIL-15+EP+1300V/cm.

Table 3: Challenge Study Evaluating Tumor Relapse and Intolerance.

<table>
<thead>
<tr>
<th>Challenge Exp 3Tx Experiment 4 Day 0, 3, 6</th>
<th>Primary Inoculation Day 50 n=15</th>
<th>Secondary Inoculation Exp 4Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15 Injection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pVax Injection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pVax 1300V/cm</td>
<td>6/15</td>
<td>3/6</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>50%</td>
</tr>
<tr>
<td>pIL-15 1300 V/cm</td>
<td>11/15</td>
<td>5/11</td>
</tr>
<tr>
<td></td>
<td>73%</td>
<td>45%</td>
</tr>
<tr>
<td>pVax 500V/cm</td>
<td>11/15</td>
<td>4/11</td>
</tr>
<tr>
<td></td>
<td>73%</td>
<td>36%</td>
</tr>
<tr>
<td>pIL-15 500V/cm</td>
<td>8/15</td>
<td>6/8</td>
</tr>
<tr>
<td></td>
<td>53%*</td>
<td>75%</td>
</tr>
</tbody>
</table>

Table 3: * Mice in this group did not survive due to damaging EP conditions.
Summary

The experiments in this section demonstrated that phIL-15 delivery using EP promotes more IL-15 expression at 2.0mg/ml electroporated at 500V/cm. The EP condition 500V/cm with long pulses delivered more energy to the cells compared to 1300V/cm with short pulses. Both EP parameters maintained expression levels when delivering the plasmid with multiple treatments and spacing the treatments to promote cellular recuperation following each EP treatment. There were notable differences in human IL-15 compared to mouse IL-12 expression when delivery of plasmid using EP into different cell types, using different EP electrode applicators, and plasmid concentrations. It was observed that EP is an efficient way to introduce plasmids into cells. The next section explored the immunological effects obtained when using this delivery system to introduce phIL-15 plasmid into tumors.

Specific Aim 3

To identify key immune effector cells and cytokines that was directly or indirectly stimulated following intra-tumoral delivery of phIL-15 using EP

The goal of Aim 3, was to characterize the immune response that was stimulated by phIL-15 and pVax delivered with EP. The purpose was to identify the key immune effector cells and cytokines that were directly or indirectly stimulated by IL-15 protein production introduced by intra-tumoral delivery of phIL-15 using EP.

Immunohistochemistry assays were performed on zinc fixed and snap frozen tumor samples to identify the up-regulation of local CD8+ and CD4+ T cell responses to untreated tumors and compared to treated tumors. Tumors were harvested at 24h and 48h and after 1 Tx, as well as Day 7 and Day 11 after 3 Tx. The tumor sample
containing periphery of skin tissue was stained for T cell markers CD8 and CD4 and NK cells. No NK cells were detected on Day 1, 2, 7, or 11.

![Image](image_url)

**Figure 45: IHC Sections CD4+ and CD8+ T cells Staining Day 1.** 1Tx of mouse tumors collected after 24h and tested for specific CD4 and CD8 staining. 100x n=4 Bar= 50-200µm. Green staining is the methyl green counterstain, dark brown color is natural pigmentation of melanoma and the red staining is the specific antibody labeled CD4 or CD8 markers.

Tumor with skin samples were collected from the mouse, cryo-sectioned, fixed in acetone and then stored at -20°C until analyzed. Sections were stained separately for CD8 or CD4 T cell markers and compared to negative controls. The negative controls consisted of the secondary antibody excluding the addition of specific primary antibody and unstained cells were counterstained with methyl green.
B16.F10 cells were naturally pigmented and were observed as a dark brown staining indicative of tumor location in the section. All pictures were taken with an Olympus microscope camera at 100x Magnification. Scale bars were added to indicate specific size of tissue in each picture.

Figure 45, shows the up-regulation of T cells at 24h after one treatment, all groups showed the presence of CD4+ T cells (CD4) and select groups showed a small number of CD8+ T cells (CD8). Non-specific staining was not observed in negative controls exposed only to the secondary antibody. The T cells were observed mostly in the periphery of the tumor with only a few in the center of the tumor. The no treatment
tumor samples demonstrated the presence of some CD4 in the center, but most were located in the periphery of the tumor and no CD8 cells were observed in the no treatment group at 24h. In the EP alone saline+1300V/cm and saline+ 500V/cm groups there was an increased of CD4 around the periphery and the center. The saline+EP+1300V/cm group showed some CD4 present in the periphery, but did not show presence of CD8, the same CD4 results were detected in the saline+EP+500V/cm group, but there were few CD8 cells in the periphery. The pVax injection group showed low presence of CD4 and no CD8. Also, at 24h, the injection of phIL-15 showed few CD4 cells in the periphery similar to what was observed in the saline+EP+ groups. The phIL-15 injection group did demonstrate a pronounced presence of CD8 throughout the tumor and around the periphery. The pVax+1300V/cm and pVax+500V/cm groups showed very low presence of CD4 and no CD8 at 24h. The phIL-15+EP+1300V/cm showed detected low staining of CD4 and CD8 while phIL-15+500V/cm group showed much more CD4, but few CD8. The phIL-15+groups showed more CD4 and CD8 staining than control groups at 24h, but less CD8 cells than the phIL-15 injection group.

Results from tumor samples that were collected and sectioned at 48h after one treatment were observed (Figure 46). The no treatment group showed low presence of CD4 and no stimulation of CD8. The saline+1300V/cm group showed a slight increase of CD4 stimulation in the periphery and few in the center while there were a small amount of CD8 cells in the periphery. The saline+500V/cm group showed presence of CD4 in the periphery and in center, and there was low staining of CD8 detected toward the center of the tumor and periphery. The pVax injection group did not show CD4 or CD8, while the phIL-15 injection group showed the presence of CD4 in the periphery but not as much as saline+EP+ groups 1300V/cm or 500V/cm. No CD8 cells were detected in the phIL-15 injection group. No CD4 or CD8 cells were detected in the
pVax+EP+1300V/cm and 500V/cm groups. The phIL-15+EP+1300V/cm group showed low expression of CD4 similar to the phIL-15 injection group and most of the section was stained specifically for CD8 throughout the tumor and the periphery compared to other groups at 48h. The phIL-15+500V/cm did not show expression of CD4 or CD8.

Results of tumor staining for CD4 and CD8 on samples collected at Day 7, 12h after 3Tx protocol Days 0, 3, and 6 are shown in Figure 47. These sections were evaluated for possible CD4 and CD8 infiltrate. The no treatment tumor groups showed presence of more CD4 compared to 24h-48h and some CD8 cells were detected. The pVax injection group showed a few CD4 cells in the periphery and in the center, there were some CD8 cells detected in the periphery.

Figure 47: IHC Sections CD4+ and CD8+ T Cells Staining Day 7. 3Tx of mouse tumors collected on Day 7 and tested for specific CD4 and CD8 staining. 100x n=4 Bar= 50-200µm. Green staining is the methyl green counterstain, dark brown color is natural pigmentation of melanoma and the red staining is the specific antibody labeled CD4 or CD8 markers.
The phIL-15 injection group showed no CD4 or CD8 stimulation at Day 7. The pVax+1300V/cm and 500V/cm showed low presence of CD4 and no CD8, while the pIL-15+1300V/cm showed the most CD4 and CD8 cells in the periphery compared to the other groups. The highest number of CD4 and CD8 cells was detected in the periphery of the phIL-15+EP+ 500V/cm group.

Figure 48 shows representative tumor sections that were collected on Day 11, 5 days after the third treatment, the tumors were evaluated for infiltrating CD4+ and CD8+ T cells. The no treatment group showed no CD4 or CD8 stimulation. The pVax injection group showed low stimulation of CD4 and CD8 in the periphery and some infiltrate in the...
center on Day 11 compared to Day 7 samples. The pVax+EP+1300V/cm showed a low higher CD4 stimulation at the periphery compared to pVax injection additionally more CD8 stimulation in the periphery infiltrated into the center of the tumor compared to pVax injection. Comparing the tumor sizes between pVax injection and pVax+1300V/cm groups, demonstrated a reduction of tumor size in pVax+1300V/cm with the same optical magnification 100x. The damaging conditions of pVax+500V/cm group showed an extremely high infiltrate of both CD4 and CD8 at the periphery and infiltrating into the tumor. Both pVax+EP+ groups showed tumor sizes that were smaller compared to pVax injection groups. The phIL-15 injection showed more CD4 and CD8 stimulation around the periphery compared to pVax injection. The phIL-15+1300V/cm group showed more CD4 and CD8 stimulation around the periphery and infiltration into the center compared to IL-15 injection and a more pronounced infiltrate than pVax+1300V/cm group. The tumors in phIL-15+ groups had decreased in size more quickly by Day11 than pVax+EP+ groups. The phIL-15+ 500V/cm group had comparable CD4 and CD8 T cells in the periphery and infiltrate towards the center similar to the pVax+500V/cm group. It was very difficult to section these tumor samples due to scabbing and scarring of the tissue. During sample collection tumor cells were completely regressed in the phIL-15+500V/cm groups compared to pVax+EP+ 500V/cm group.

Additional experiments were performed on tumors treated with the 3Tx protocol to test for systemic IFNγ production. The purpose of the experiment was to analyze IFNγ production in treatment groups to explain IHC CD8 and CD4 infiltrate and regression data. ELISpot assay was used to analyze IFNγ production in splenocytes or PBMCs from mice that were treated with pVax injection, phIL-15 injection, pVax+EP+ or phIL-15+EP+ 1300V/cm or 500V/cm. The splenocytes and PBMCs were re-stimulated with tumor cells in culture and evaluated for specific expression of IFNγ (Figure 49). It was
expected that mice exposed to plasmid plus EP would generate IFNγ production.

General populations of cells were isolated from spleens and peripheral blood, not just CD8 specific T cells. Previous publications have reported that irradiated B16.F10 can stimulate splenocyte populations for IFNγ production\textsuperscript{164}. The splenocytes and PBMCs were prepped for ELISpot analysis one day after samples were collected. The assay consisted of 300,000 splenocytes and 200,000 PBMCs freshly collected on Day 11 and Day 14.

Initially Day 11 and Day 14, unstimulated cells were tested for IFNγ production without additional secondary B16.F10 stimulation. PBMCs did not show IFNγ production, were eliminated from analysis and all splenocytes tested at Day 11 did not show expression of IFNγ. At Day 14, splenocytes samples pVax and phIL-15+ 1300V/cm and 500V/cm showed some expression of IFNγ compared to pVax and phIL-15 injection groups. The pVax injection group n=3, served as a control because the IHC staining demonstrated that pVax injection group showed CD8 infiltrate on Day 7. phIL-15 injection n=3, served as a control because stimulation of CD8 infiltrate was observed on Days 1, 2, and 11 in IHC. Fresh, unstimulated splenocytes from pVax+1300V/cm mice showed a 2.1-fold increase on Day 14, n=3 compared to pVax injection group.

The pVax+500V/cm, phIL-15+1300V/cm and phIL-15+500V/cm showed less IFNγ spot forming cells than pVax+1300V/cm. Testing the same general population of splenocytes with B16.F10 plus recombinant IL-2 stimulation for 4 days there was a difference of INFγ production in treatment groups. Splenocytes from mice treated with pVax+1300V/cm showed a 2.5-fold increase compared to pVax injection and pVax+EP+500V/cm showed a 2.1-fold n=4 increase compared to pVax injection.
While IL-15+EP+1300V/cm showed a 1.3-fold increase and IL-15+EP+ 500V/cm showed a 1.4-fold n=4 compared to IL-15 injection alone. There was no real indication of differences between the pVax+EP+ and IL-15+EP+ groups for the production of systemic IFNγ. The ELISpot did not show an expression difference between the groups as was seen with IHC and regression studies. Splenocytes did demonstrate IFNγ production when stimulated with B16.F10 compared to unstimulated. This does suggest why there was CD8 and CD4 cellular infiltrate in plasmid+EP groups detected at Day 7 and Day 11 as demonstrated by IHC.

A multi-cytokine Luminex Bead Array (LBA) was performed to further characterize the immunological signals that are associated with the plasmid+EP delivery
system. Ten cytokines that are known to be stimulated by IL-15 were analyzed in tumor lysate and serum samples from many different treatment groups containing n=3 mice per group. Tumors were treated with 100µg (2.0mg/ml) of plasmid pVax or phIL-15 with or without EP. Tumor and serum samples were collected at 12h, 24h, and 48h after 1TX, on Day 3 (12h) after 2 Tx, on Day 6 (12h) after 3Tx, and Day 11, Day 14, Day 30 and Day 60 after 3Tx. The expression of the cytokines that were measured in this multiplex assay included IL-1β, IL-2, IL4, IL6, IL-10, GMCSF, MIP1β, TNFα, IL-12, and IFNγ. The cytokines were analyzed in tumor tissue samples and recorded as average total picograms (pg), while the serum levels were recorded as average picograms per milliliter (pg/ml). Each analyte was measure in each of the varied groups and then plotted together on a graph to characterize the type of cytokines up-regulated by IL-15, T cells, and NK cells. These cytokines were endogenously expressed in the mouse in response to pro-inflammatory signaling.

Figures 50-89 shows levels from 10 different cytokines expressed in tumor lysate and serum samples 12h, 24h, 48h, Day 3 and Day 6, after treatments. The 1Tx, 2Tx and 3Tx protocols were analyzed against controls tumor alone, pVax injection, pVax +1300V/cm and pVax+500V/cm collected at 24h for each analyte. Cytokine expression levels within each group was determined by calculating fold change compared to phIL-15 injection alone (Table 4-7, Appendix A Table A1-A6 detailed expression levels). A dotted or solid line superimposed on the graphs demonstrates increased cytokine stimulation compared to background levels expressed by the no treatment group. The controls no treatment, pVax injection, pVax+EP+1300V/cm and 500V/cm were collected at 24h, D11, D14, and naïve samples on D30 and D60 were used to compare stimulated vs unstimulated cytokine production between treatment groups. Table 4 and 6, describes fold change (fold) expression from tumor lysate for each analyte tested compared to
treatment groups and to the IL-15 injection only groups. Table 5 and 7 describe fold change for serum levels within the treatment groups compared to phIL-15 injection alone. Treatment groups, phIL-15+1300V/cm and 500V/cm, were also analyzed for overall cytokines expression compared to control pVax injection, pVax+1300V/cm and pVax+500V/cm.

**Table 4: Cytokines Evaluated in Luminex Bead Array Measured in Tumor Lysate.**
Fold change compared to phIL-15 injection group.

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>IL-1β</th>
<th>IL-2</th>
<th>IL-4</th>
<th>IL-6</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL15 1300 12h</td>
<td>4.21</td>
<td>4.32</td>
<td>1.47</td>
<td>7.66</td>
<td>0.93</td>
</tr>
<tr>
<td>IL15 500 12h</td>
<td>3.26</td>
<td>2.15</td>
<td>1.20</td>
<td>14.06</td>
<td>0.26</td>
</tr>
<tr>
<td>IL15 1300 24h</td>
<td>2.69</td>
<td>0.22</td>
<td>0.41</td>
<td>1.19</td>
<td>0.09</td>
</tr>
<tr>
<td>IL15 500 24h</td>
<td>2.61</td>
<td>0.40</td>
<td>0.65</td>
<td>6.61</td>
<td>0.43</td>
</tr>
<tr>
<td>IL15 1300 48h</td>
<td>1.65</td>
<td>0.32</td>
<td>0.99</td>
<td>0.38</td>
<td>0.59</td>
</tr>
<tr>
<td>IL15 500 48h</td>
<td>0.88</td>
<td>0.08</td>
<td>0.91</td>
<td>1.33</td>
<td>0.01</td>
</tr>
<tr>
<td>IL15 1300 D3</td>
<td>3.41</td>
<td>1.19</td>
<td>0.46</td>
<td>2.68</td>
<td>3.67</td>
</tr>
<tr>
<td>IL15 500 D3</td>
<td>3.71</td>
<td>4.25</td>
<td>1.11</td>
<td>2.30</td>
<td>5.08</td>
</tr>
<tr>
<td>IL15 1300 D6</td>
<td>1.65</td>
<td>0.13</td>
<td>0.38</td>
<td>1.14</td>
<td>0.01</td>
</tr>
<tr>
<td>IL15 500 D6</td>
<td>1.05</td>
<td>0.05</td>
<td>0.20</td>
<td>6.66</td>
<td>0.01</td>
</tr>
</tbody>
</table>

IL-1β is stimulated by stromal fibroblast in response to existing melanoma, mediating an inflammatory response, stimulating lymphocyte cell proliferation, differentiation and apoptosis\(^{165}\). The groups tested in tumor lysate and serum included backbone vector pVax injection, pVax+EP+ at 1300V/cm and 500V/cm, phIL-15+EP+ 1300V/cm and 500V/cm. Results shown in Figure 50 demonstrate that the no treatment tumor group, pVax injection and pVax+EP+500V/cm groups did not stimulate expression of IL-1β at 24h and expressed lower levels than treatment groups. The control group pVax+EP+1300V/cm showed a 10-fold increase compared to pVax injection. The tumor samples treated with phIL-15+EP+ 1300V/cm and 500V/cm were collected at 12h, 24h, 48h, after 1Tx showed an increase an IL-1β compared to controls and to pIL-15 injection. At 12h, hPL-15+1300V/cm showed a 4.2-fold increase and phIL-15+ 500V/cm showed a 3.3-fold increase of expression compared to IL-15 injection alone. At 24h phIL-
15+1300V/cm and 500V/cm had a 2.7-fold increase compared to IL-15 injection alone. At 48h there was a 1.7-fold increase in group phIL-15+1300V/cm compared to IL-15 injection alone, but there was no increase with phIL-15+500V/cm.

The stimulation of IL-1β expression was also measured after 2Tx, the first treatment was initially administered at day 0 and the second treatment was given on Day 3. The tumors were collected 12h after the last treatment. There was a 3.4-fold increase in IL-1β expression using phIL-15+1300V/cm and 3.7-fold increase using phIL-15+500V/cm EP conditions. The 2Tx protocol showed the highest level of IL-1β expression compared to the other treatment groups and time points at Day 3. The tumors were also treated three times on Day 0, Day 3 and Day 6. phIL-15 was delivered

Figure 50: Luminex Bead Array Measures IL-1β in Tumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
with EP and 12h after the last treatment tumors were collected and assessed for IL-1β stimulation. There was a 1.7-fold increase in the phIL-15+1300V/cm groups and 1.1-fold phIL15+500V/cm increase in expression as compared to injection of IL-15 alone. The 3Tx protocol expression levels were similar to the phIL-15+1300V/cm and phIL15+500V/cm of the 1Tx protocol at the 12h time point.

The serum samples from tumor treatment groups were collected and analyzed. Additional serum groups were added for Luminex analysis such as Day 11, Day 14, Day 30 and Day 60 collected after the 3Tx protocol 0, 3, and 6 (Table 5). Tumor samples and splenocytes for these groups were utilized for the previous IHC and ELISpot on Day 11, and 14. Samples were collected at Day 11, 5 days after the last treatment and on Day 14, 8 days after, these time points were chosen based on tumor regression data. The observations indicate that treated tumors regressed as early as 4-7 days and were

![Figure 51: LBA Measures IL-1β in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.](image-url)
completely regressed by 16-21 days compared to controls groups. The serum samples were analyzed to identify cytokine expression in the blood and characterize the systemic response to tumor treatments.

Table 5: Cytokines Evaluated in Luminex Bead Array Measured in Serum. Fold change compared to pHIL-15 injection group.

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>IL1β</th>
<th>IL2</th>
<th>IL4</th>
<th>IL6</th>
<th>IL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15 1300 12h</td>
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<td>0.09</td>
<td>1.04</td>
<td>0.39</td>
<td>0.42</td>
</tr>
<tr>
<td>IL-15 500 12h</td>
<td>0.03</td>
<td>0.66</td>
<td>1.00</td>
<td>0.87</td>
<td>0.41</td>
</tr>
<tr>
<td>IL-15 1300 24h</td>
<td>0.01</td>
<td>0.54</td>
<td>1.00</td>
<td>0.08</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-15 500 24h</td>
<td>0.08</td>
<td>0.57</td>
<td>1.00</td>
<td>0.39</td>
<td>2.72</td>
</tr>
<tr>
<td>IL-15 1300 48h</td>
<td>0.53</td>
<td>0.53</td>
<td>0.96</td>
<td>1.17</td>
<td>0.94</td>
</tr>
<tr>
<td>IL-15 500 48h</td>
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<td>0.66</td>
<td>0.96</td>
<td>10.20</td>
<td>1.19</td>
</tr>
<tr>
<td>IL-15 1300 D3</td>
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<td>0.90</td>
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</tr>
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<td>IL-15 500 D3</td>
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<td>43.77</td>
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<td>IL-15 1300 D6</td>
<td>1.00</td>
<td>0.47</td>
<td>1.00</td>
<td>7.63</td>
<td>0.24</td>
</tr>
<tr>
<td>IL-15 500 D6</td>
<td>27.41</td>
<td>0.49</td>
<td>1.56</td>
<td>135.90</td>
<td>3.72</td>
</tr>
<tr>
<td>IL-15 1300 D11</td>
<td>5.73</td>
<td>0.79</td>
<td>0.90</td>
<td>0.14</td>
<td>1.92</td>
</tr>
<tr>
<td>IL-15 500 D11</td>
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<td>1.33</td>
<td>0.67</td>
<td>0.82</td>
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<tr>
<td>IL-15 1300 D14</td>
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<td>1.04</td>
<td>0.62</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-15 500 D14</td>
<td>72.41</td>
<td>0.67</td>
<td>3.85</td>
<td>0.78</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Comparing cytokine expression levels in the tumor and serum showed mainly lower levels of cytokine expression in the serum than in the tumor. The expression levels detected in the serum were compared to control groups such as no treatment at early time points (similar to tumor sample), pVax injection on D11, Day 14, and naïve pooled samples for later time points Day 30, and Day 60. Results shown in Figures 51-53, demonstrate IL-1β expression from serum (Day 11 and Day 14) and late time points (Day 30 and Day 60). There was a slight up-regulation in the pVax+1300V/cm groups at
24h, Day 11, and Day 14, the pVax+500V/cm showed a slight increase at Day 11, but a more robust stimulation at Day 14, compared to the no treatment group and pVax injection. All treatment groups from showed low stimulation of expression at 48h, Day 3, Day 6, Day 14, Day 30 and Day 60. IL-2 enhances T cell and NK cell proliferation and IL-15 down regulates IL-2 functions and competes with IL-2 receptor binding\(^{77, 166}\). In tumor lysate the control groups showed low expression in the no tx, pVax injection, and pVax+500V/cm groups, while in the pVax+1300V/cm group there was a 7-fold increase of IL-2 expression compared to pVax injection (Figure 54).

![Figure 52: LBA Measures IL-1β in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection alone group. n=3. Error bars were calculated as standard deviation.](image)

Serum levels were much lower than tumor samples, comparing treatment groups to phIL-15 injection Table 5 shows increased levels of expression depicted by change fold. Complete expression values can be obtained from supplementary Tables A1-A6 located in the Appendices.
Expression of IL-2 was present in pVax+1300V/cm, similar to phIL-15 injection and phIL-15+1300V/cm at Day 3. At 12h, phIL-15+1300V/cm showed a 4.3-fold and phIL-15+500V/cm showed a 2.2-fold increase compared to IL-15 injection. IL-15 injection was stimulated higher than phIL-15+EP groups at 24h, 48h and Day 6. The 2Tx protocol 12h after Day 3 showed 1.2-fold increase in the phIL-15+1300V/cm group and 4.3-fold increase in phIL-15+500V/cm. The phIL-15+1300V/cm at 12h and phIL-15+500V/cm on Day 3 showed the highest expression of IL-2 in the tumor compared to all other groups.

Figure 53: LBA Measures IL-1β in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naive group. n=3.
Figure 54: Luminex Bead Array Measures IL-2 in Tumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 55 LBA Measures IL-2 in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
Figure 56: LBA Measures IL-2 in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection. n=3. Error bars were calculated as standard deviation.

Figure 57: LBA Measures IL-2 in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naïve group. n=3.
IL-2 expression levels were much lower in the serum compared to the tumor (Figures 55-57). At the early time points 1Tx, 2Tx and 3Tx there was very low expression in treatments than control groups. The pVax+1300V/cm 24h and IL-15 injection at 12h showed a slight stimulation of IL-2 expression compared to the no treatment group. The expression detected at later and late time points after 3Tx also demonstrated low IL-2 expression in mouse serum. On Day 11, there was a slight simulation in the pVax+500V/cm and phIL-15+500V/cm groups compared to pVax injection. On Day 14, the pVax+1300V/cm and pVax+500V/cm groups showed a slight stimulation in expression over pVax injection, and all other treatment groups showed low stimulation compared to controls. The expression of IL-2 at later time points after 3Tx showed slight stimulation of IL-2 in pVax+1300V/cm on Day 30 and Day 60 as well as phIL-15+1300V/cm on Day 30, and Day 60. The phIL-15+500V/cm did not stimulate IL-2 production at the later time points.

Figure 58: Luminex Bead Array Measures IL-4 in Tumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
IL-4 stimulates monocyte differentiation into immature dendritic cells and is an anti-inflammatory cytokine\textsuperscript{167}. As expected, IL-4 stimulation was low in the tumor lysate (Figure 58). The highest levels were observed with pVax+1300V/cm inducing a 2.5-fold increase compared to pVax injection. The groups at 12h had low detectable IL-4 expression, at 24h and Day 6. The phIL-15 injection groups demonstrated low levels compared to control pVax+1300V/cm at early time points, but higher than phIL-15+EP groups. At 48h all phIL-15+EP+ groups showed similarly low expression compared to the control groups pVax injection and pVax+500V/cm. The next highest expression of IL4 was detected on Day 3 in the phIL-15 injection and phIL-15+500V/cm groups compared to pVax+1300V/cm, but there was very small difference between the groups.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure59.png}
\caption{LBA Measures IL-4 in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.}
\end{figure}
**Figure 60:** LBA Measures IL-4 in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.

**Figure 61:** LBA Measures IL-4 in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naïve group. n=3.
Serum levels of IL-4 were low in all groups at early time points (Figures 59-61). There was a defined increase in IL-4 expression by pVax+500V/cm on Day 14, but no other groups at Day 11, Day 14, Day 30 or Day 60 stimulated expression.

IL-6 expression is stimulated by TILs and restores the NK activity and IFNγ stimulation\textsuperscript{168}. Tumor lysate from control groups showed IL-6 expression in pVax+1300V/cm and the highest expressing group was pVax+500V/cm measuring 66-fold increase of IL-6 production compared to pVax injection (Figure 62). Amongst the treatment groups at the 12h time point, phIL-15+1300V/cm showed a 7.7-fold increase and phIL-15+500V/cm showed a 14.6-fold increase compared to phIL-15 injection. At 24h, phIL-15+1300V/cm showed a 1.2-fold and phIL-15+500V/cm showed a 6.6-fold increase. At 48h, phIL-15+1300V/cm did not demonstrate an increase in IL-6 production, but there was a 1.3-fold increase by phIL-15+500V/cm.

Figure 62: Luminex Bead Array Measures IL-6 in Tumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
The 2Tx protocol showed a 2.68-fold increase in phIL-15+1300V/cm and 2.3-fold in phIL-15+500V/cm groups. The 3Tx protocol showed a 1.1-fold increase in phIL-15+1300V/cm and 6.7-fold increase in phIL-15+500V/cm groups, all compared to phIL-15 injection group.

IL-6 stimulation in the serum was similar to tumor lysate (Figures 63-65). Expression levels of IL-6 production in the treatment groups were less than control pVax+500V/cm at 24h showing >1000pg/ml of protein production. The following groups also showed IL6 stimulus, phIL-15 Injection 12h, 24h, phIL-15+1300V/cm 12h, 24h, Day 3 and Day6. The phIL-15+500V/cm group showed stimulation at 12h, 24h, 10.2-fold increase compared to phIL-15 at 48h, 43.8-fold at Day 3 and 135.9-fold at Day 6 (Table 5). At Day 11 and Day 14, there were lower levels of stimulation compared to earlier time points. On Day 11, the phIL-15 injection group was the only group that showed increased IL-6. The pVax+500V/cm group at Day 14 had IL-6 levels that were slightly greater than pVax Injection. From the groups measured at Day 30 and 60 the only groups that stimulated IL-6 were pVax+1300V/cm and phIL-15+1300V/cm at Day 30 and Day 60. The late time points showed much less IL6 expression than the earlier time points.
Figure 63: LBA Measures IL-6 in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 64: LBA Measures IL-6 in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.
IL-10 is a mediator between Th1 and Th2 and functions to regulate IL-15 production by macrophage, and suppresses TNFα and IFNγ by inhibiting IL-12 production\textsuperscript{169}. The stimulation of IL-10 expression in tumor lysate was enhanced in groups such as pVax+1300V/cm at 24h (Figure 66). The phIL-15+1300V/cm groups showed expression at Day 3, a 3.7-fold increase and phIL-15+500V/cm group showed a 5.1-fold increase. Again IL-15 injection showed an increase in IL-10 expression at 12h, 24h, 48h and Day 6 comparable to pVax+1300V/cm at 24h and similar to results found in IL-4 on a larger scale.

IL-10 expression in the serum was similar to expression in the tumor lysate (Figure 67-69). The groups that slightly stimulated IL-10 were pVax+500V/cm 24h, phIL-15 injection 12h, 24h, and Day 6. The phIL-15 +500V/cm group showed 18-fold higher expression than pVax+500V/cm Day 3 and 3.7-fold higher than phIL-15 injection on Day...
There was no stimulation of IL-10 at 12h, 24h, 48h and Day 11 in the treatment groups. On Day 14, the only groups that slightly stimulated IL-10 were pVax+500V/cm and phIL-15+500V/cm. At late time points Day 30 and Day 60 stimulation of IL-10 were low in the serum in groups pVax+1300V/cm, pVax+500V/cm, and phIL-15+1300V/cm at Day 30 compared to naïve groups, while no groups stimulated IL-10 at Day 60.

**Figure 66: Luminex Bead Array Measures IL-10 in Tumor Lysate 12h-Day 6.** 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
Figure 67: LBA IL-10 in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 68: LBA Measures IL-10 in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.
Additional cytokines were assayed using the Luminex bead as shown in Table 6.

Pro-inflammatory cytokine IL-12 functions to up-regulate IFNγ, through NK, and CD8+T cell response and promotes CTL effector function\textsuperscript{170}. The level of IL-12 was reduced in
the tumor lysate, the group that showed an increase in expression compared to controls were pVax +1300V/cm with a 2.4-fold compared to pVax injection (Figure 70).

![IL12p70](image)

**Figure 70: Luminex Bead Array Measures IL-12 in Tumor Lysate 12h-Day 6.** 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

The phIL-15 injection group had slightly increased IL-12 expression compared to treatment groups at 24h, 48h, Day 3 and Day 6, but there were low levels at 24h compared to control. There were small differences between controls and treatment groups for this cytokine at the tumor level.

Evaluation of IL-12 expression in the serum demonstrated that the treatment groups and control groups showed similar levels to expression levels found in the tumor (Figures 71-73). All samples expressed IL-12 lower than the no treatment group at the early time points. IL-12 expression in the serum were slightly higher at later time points than the early time points, the groups that demonstrated IL-12 stimulation were
pVax+500V/cm on Day 14, phIL-15+1300V/cm and 14.1-fold higher in phIL-15+500V/cm compared to phIL-15 injection on Day 14 (Table 7). At the late time points, IL-12 expression levels were low again and comparing groups there was a stimulation of expression in groups pVax+1300V/cm on Day 30 and Day 60, pVax+500V/cm on Day 60 and phIL-15+1300V/cm on Day 30, compared to naive groups.

**Figure 71: LBA Measures IL-12 in Serum 12h-Day 6.** 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
Pro-inflammatory IFNγ functions to further up-regulate T cells and CTL effector function\textsuperscript{167}. IFNγ was stimulated in the treatment groups compared to controls groups (Figure 74). The highest expression of IFNγ was demonstrated in tumor lysate in the following groups phIL-15+1300V/cm showed a 62.8-fold and phIL-15+500V/cm showed a 30.5-fold increase compared to phIL-15 injection at 12h. At 24h, phIL-15+500V/cm showed a 2.7-fold increase in expression compared to phIL-15 injection. At 48h, all groups were lower than controls groups. The 2Tx protocol demonstrated a 3.8-fold increase in expression at Day 3 group phIL-15+1300V/cm and 1.7-fold increase in phIL-15+500V/cm compared to injection. The 3Tx protocol showed a 1.1-fold increase phIL-15+1300V/cm at Day 6, but the phIL-15+500V/cm did not show an increase in expression.

Figure 72: LBA Measures IL-12 in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.
Table 7: Cytokines Evaluated in Luminex Bead Array Measured in serum.
Fold change compared to phIL-15 injection group.

<table>
<thead>
<tr>
<th>Fold Change</th>
<th>IL12p70</th>
<th>IFNγ</th>
<th>GMCSF</th>
<th>MIP1β</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL15 1300 12h</td>
<td>0.82</td>
<td>0.25</td>
<td>0.03</td>
<td>0.36</td>
<td>0.48</td>
</tr>
<tr>
<td>IL15 500 12h</td>
<td>0.32</td>
<td>0.81</td>
<td>0.35</td>
<td>0.78</td>
<td>0.47</td>
</tr>
<tr>
<td>IL15 1300 24h</td>
<td>0.62</td>
<td>0.03</td>
<td>0.13</td>
<td>0.61</td>
<td>0.06</td>
</tr>
<tr>
<td>IL15 500 24h</td>
<td>2.09</td>
<td>0.03</td>
<td>2.40</td>
<td>1.49</td>
<td>0.12</td>
</tr>
<tr>
<td>IL15 1300 48h</td>
<td>0.36</td>
<td>0.66</td>
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<td>0.43</td>
</tr>
<tr>
<td>IL15 500 48h</td>
<td>0.58</td>
<td>0.17</td>
<td>0.26</td>
<td>0.98</td>
<td>0.31</td>
</tr>
<tr>
<td>IL15 1300 D3</td>
<td>1.02</td>
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<td>1.00</td>
<td>2.64</td>
<td>3.12</td>
</tr>
<tr>
<td>IL15 500 D3</td>
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<td>7.21</td>
<td>1.51</td>
<td>4.71</td>
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</tr>
<tr>
<td>IL15 1300 D6</td>
<td>1.00</td>
<td>0.10</td>
<td>0.28</td>
<td>1.48</td>
<td>0.26</td>
</tr>
<tr>
<td>IL15 500 D6</td>
<td>2.25</td>
<td>3.60</td>
<td>0.39</td>
<td>2.33</td>
<td>0.26</td>
</tr>
<tr>
<td>IL15 1300 D11</td>
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<td>1.00</td>
<td>32.88</td>
<td>1.18</td>
<td>1.57</td>
</tr>
<tr>
<td>IL15 500 D11</td>
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<td>7.56</td>
<td>2.42</td>
<td>1.27</td>
</tr>
<tr>
<td>IL15 1300 D14</td>
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<td>1.55</td>
<td>0.28</td>
<td>0.84</td>
<td>3.21</td>
</tr>
<tr>
<td>IL15 500 D14</td>
<td>14.11</td>
<td>1.00</td>
<td>0.28</td>
<td>0.54</td>
<td>28.20</td>
</tr>
</tbody>
</table>

Figure 73: LBA Measures IL-12 in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naïve group. n=3.
Levels of IFNγ in the serum were much lower compared to tumor lysate (Figures 75-77). At early time points the groups that stimulated IFNγ in the serum were pVax+1300V/cm, pVax+500V/cm at 24h, phIL-15 injection at 12h, 24h and Day 6, phIL-15+1300V/cm at 12h, 48h, and 21.2-Fold compared to phIL-15 injection on Day 3, phIL-15+500V/cm at 12h, 7.2-Fold on Day 3 and Day 6 compared to controls and other treatment groups (Table 7). The stimulation of IFNγ was lower at later time points, there was no difference observed at Day 11 except for phIL-15+500V/cm on Day 14. Late time points showed very low stimulation in groups pVax+1300V/cm at Day 30, phIL-15+1300V/cm Day 30 and Day 60 and phIL-15+500V/cm at Day 30 compared to controls and treatment groups.
Figure 75: LBA Measures IFNγ in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 76: LBA Measures IFNγ in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3 Error bars were calculated as standard deviation.
Figure 78, evaluates expression of GM-CSF which promotes differentiation of monocytes into immature dendritic cells and is produced by stimulated macrophages and neutrophils\textsuperscript{167, 171}. The groups that stimulated GMCSF were pVax+1300V/cm at 24h, phIL-15 injection at Day3, and Day 6, phIL-15 1300V/cm at 12h, and Day 6, phIL-15+500V/cm at 12h, 48h, and Day 3 (Figure 78).

The 1Tx protocol at 12h showed an up-regulation of GM-CSF in the phIL-15+1300V/cm showing a 3-fold increase and phIL-15+500V/cm showed a 9.2-fold increase compared to phIL-15 injection, at 48h there was a 2.1-fold increase using the phIL-15+500V/cm compared to phIL-15 injection, but lower than controls. There was no increase of GM-CSF in the phIL-15+1300V/cm group and injection of phIL-15 showed higher expression of GM-CSF at 24h, Day 3 and Day 6 similar to IL-2, IL4, IL-10, and IL-12.
GM-SCF expression in serum was lower than tumor lysate (Figures 79-81). At the early time points control pVax+1300V/cm showed stimulation at 24h and levels were higher than the treatment groups. phIL-15 injection showed GM-CSF stimulation at 12h, 24h, 48h, and Day 6 compared to the treatment groups. The only group that reflected stimulation levels similar to controls was phIL-15+500V/cm at 24h. There was higher GM-CSF stimulation in the IL-15+1300V/cm group 32.9-fold compared to phIL-15 injection (Table 7) at Day 11 compared to phIL-15 injection and the other controls and at Day 14, the pVax+500V/cm seem to stimulate GM-CSF compared to the other controls and treatment groups. At the later time points the only group that stimulated very low expression levels was control pVax+1300V/cm at Day 30; there were no other groups that stimulated GM-CSF at Day 30 or Day 60.

![Figure 78: Luminex Bead Array Measures GM-CSF in Tumor Lysate12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.](image-url)
Figure 79: LBA Measures GM-CSF in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 80: LBA Measuring GM-CSF in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.
The chemokine MIP-1β (macrophage inflammatory protein) functions as a chemoattractant for tumoricidal monocytes\textsuperscript{172}. The controls demonstrated low expression levels in tumor lysate except pVax+1300V/cm showing a 50-fold increase compared to pVax injection (Figure 82). The 1Tx protocol demonstrated expression at 12h in the phIL-15+1300V/cm group showing a 1.3-fold increase and phIL-15+500V/cm showed a 1.2-fold increase and compared to the increased IL-15 injection group. At 24h, the phIL-15+1300V/cm showed a 6-fold increase and phIL-15+500V/cm showed a 6.3-fold increase. At 48h, the phIL-15+1300V/cm group showed a 3.6-fold increase and phIL-15+500V/cm showed a 4-fold increase compared to injection of phIL-15. The 2Tx protocol showed a 3.3-fold increase at Day 3, in the phIL-15+1300V/cm, but the phIL-15+500V/cm group did not show expression of MIP-1β. The 3Tx protocol on Day 6, demonstrated a 2.4-fold increase in the phIL-15+1300V/cm group and a 1.9-fold increase in the phIL-15+500V/cm group.
Expression of MIP-1β in serum was lower than tumor lysate. At the earlier time points, the groups that stimulated expression were phIL-15 injection 12h, 24h and slight stimulation at 48h and Day 6. The phIL-15+1300V/cm group showed stimulation at 12h, 24h, 48h, 2.6-fold compared to phIL-15 injection on Day 3 and Day 6, and phIL-15+500V/cm showed stimulation at 12h, 24h, 48h, 4.7-fold on Day 3 and Day 6 (Figures 83-85, Table 7). The treatment groups at 48h were equivalent to injection of phIL-15 injection group. At later time points there was no real difference in treatment groups compared to controls on Day 11. The groups that slightly stimulated MIP-1β at Day 14 were pVax+500V/cm, pVax+1300V/cm and phIL-15 injection. At late time points there was an increase in MIP-1β expression at Day 30 and 60. The groups that stimulated MIP-1β were pVax+1300V/cm on Day 30 and Day 60, phIL-15+1300 Day 30 and Day 60 and pIL-15+500 on Day 60 compared to controls and expression levels on Day 14.

Figure 82: Luminex Bead Array Measures MIP-1β inTumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
group that demonstrated the best MIP-1β expression was phIL-15+1300V/cm demonstrating a 4-fold increase compared to the naïve group on Day 30 and pVax+1300 showing a 12.5-fold increase at Day 60.

![Figure 83: LBA Measures MIP-1β in Serum 12h-Day 6.](image)

12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

![Figure 84: LBA Measures MIP-1β in Serum Day 11 and 14.](image)

3Tx Protocol, expression levels were compared to pVax injection group. n=3. Error bars were calculated as standard deviation.
TNFα functions to cause tumor necrosis, enhances NK activity and facilitates leukocyte adhesion and migration\textsuperscript{170, 172-173}. There was an increase in TNFα expression in control group pVax+1300V/cm with a 2.6-fold expression at 24h compared to the other control groups (Figure 86). At 12h phIL+1300V/cm and phIL-15+500V/cm groups showed a 1.6-fold increase, at 24h the phIL-15+1300V/cm did not express TNFα, but the phIL-15+500V/cm group showed a 1.1-fold increase compared to injection of phIL-15. At 48h, the phIL-15+1300V/cm and the phIL-15+500V/cm groups showed a 1.3-fold increase. The 2Tx protocol did not show expression in the phIL-15+1300V/cm group, while the phIL-15+500V/cm group showed a 1.1-fold increase in expression compared to phIL-15 injection. The 3Tx protocol did not show expression in the phIL-15+1300V/cm group, while the phIL-15+500V/cm showed a 1.3-fold increase of TNFα expression. The IL-15 injection group showed an increase in expression at 24h, Day 3 and Day 6. There

![Figure 85: LBA Measures MIP-1β in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naïve group. n=3.](image-url)
was small expression differences identified between treatment groups expressing TNFα in tumor lysate.

TNFα serum levels were slightly lower than tumor lysate. At early time points, the pVax+1300V/cm and pVax+500V/cm showed increased expression compared to pVax injection (Figures 87-89). The IL-15 injection group showed stimulation of expression higher than EP groups at 12h, 24h, and 48h, and was slightly stimulated at Day 3 and Day 6. The phIL-15+1300V/cm increased expression at 3.1-fold compared to phIL-15 injection on Day 3 and phIL-15+500V/cm showed an increase of expression 15.1-fold at Day 3 and 5.11-fold Day 6 (Table 7), but much lower levels than tumor lysate. At later time points, there was not much expression detected between treatment groups on Day 11, there was a prominent stimulation of TNFα in pVax+500V/cm at Day 14 and phIL-15+500V/cm at Day with a14.3-fold increase (Table 7). The levels experienced in the serum at Day 30 and Day 60 were much lower than the earlier time points. The groups pVax+1300V/cm and phIL-15+500V/cm slightly stimulated expression compared to the naïve group. The phIL-15+1300V/cm group stimulated slightly more expression on Day 30 and Day 60 compared to controls and other treatment groups.
Figure 86: Luminex Bead Array Measures TNFα in Tumor Lysate 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.

Figure 87 LBA Measures TNFα in Serum 12h-Day 6. 12h, 24h, 48h after 1Tx, Day 3 after 2Tx and Day 6 after 3Tx Protocol, expression levels were compared to no treatment group. n=3. Error bars were calculated as standard deviation.
Figure 88: LBA Measures TNFα in Serum Day 11 and 14. 3Tx Protocol, expression levels were compared to pVax Injection group. n=3. Error bars were calculated as standard deviation.

Figure 89: LBA Measures TNFα in Pooled Serum Day 30 and 60. 3Tx Protocol, expression levels were compared to naïve group. n=3.
Summary

Utilizing IHC, ELISpot and Luminex bead array assays characterized the expression of immune modulators present at different time points after tumor treatments. The three assays showed similar results and detailed the specific cytokines and T cells stimulated in the primary and secondary responses when delivering of pHIL-15 using EP. The assays also demonstrated the type of immune response cells and cytokines that were up-regulated to promote tumor regression and enhance mouse survival as observed in Aim 2. The delivery of plasmid plus electroporation stimulated an innate and adaptive response against melanoma and facilitated a long-term response to tumor recurrence.
DISCUSSION

Specific Aim 1

3DTumor Model

A unique biosynthetic 3D tumor model was generated to test EP delivery conditions and study cellular interactions. This large-scale, free floating 3D tumor model was used to elucidate EP parameters to enhance phIL-15 gene delivery into melanoma cell lines. Furthermore, the in vitro 3D tumor model was utilized to study the effects of EP on different cell types, and evaluate different EP conditions to improve delivery techniques in vivo. Utilizing the 3D tumor model, it demonstrated that similar levels of IL-15 expression patterns were obtained compared to the skin and tumor following in vivo delivery. Therefore, the goal was to use the EP conditions established in the 3D tumor model to develop a potential adjuvant system that would up-regulate IL-15 expression and induce an immune response against melanoma.

Generating a Stable 3D Model System

In Aim 1, the 3D model was used to obtain information about cellular behavior when subjecting cells to chemical and physical stimuli. The environment generated by the 3D scaffold encouraged melanoma cells to grow and develop into semi-normal, tumor cells populations. The objective was to understand how the 3D scaffold promoted an environment suitable for tumorigenesis and how this model could be used to optimize delivery methods for therapeutic interventions against cancer. Several matrices were evaluated to provide a framework that would support the growth of melanoma in a 3D construct (Figure 3-7). However, the initial matrices tested expanded melanoma growth
over many days, and were unable to provide a large enough structure for evaluating delivery applications. The aggregation of HaCaT cells forming a spheroid was able to support the proliferation and development of B16.F10 cells. This process was facilitated by the HARV’s horizontal rotation, laminar flow of fluid, hydrodynamic forces and passive oxygenation. It was these mechanical properties that provided a microenvironment suitable for cellular growth, encouraging cells to behave similar to those in nature\textsuperscript{44, 53, 56}. The HARV promoted aggregation of HaCaT into spheroids as quickly as 1 to 2 days, and measuring about 1 cm in diameter. The time period required for the scaffold to organize cells and support tumor mass formation exceeded the dimensions and time it took for cancer cells to develop on the bead, sponge and algi matrix scaffolding\textsuperscript{44, 174-175}.

\textit{Viability and Stability of Model}

The malleable, stable HaCaT construct mimicked the complex environment needed to integrate many small populations of melanoma cells throughout the scaffold thereby encouraging viable cellular interactions (Figure\textsuperscript{13-16, 21}), proliferation (Figure 19), cellular communication (Figure 18), expansion (Figure 17), differentiation (Figure 18, 22), and production of its own extracellular matrix (Figure 20). The adherent scaffolding was able to keep loose, fluid B16.F10 cells in close proximity to promote tumorigenesis and stimulate B16.F10 differentiation. Maturation was observed by the production of mature pigmented cells (Figure 13-14) similar to malignant cancer development\textsuperscript{176}.

Due to the strong adhesive properties of the HaCaT scaffold, diffusion of nutrients into the center of the construct was reduced. However, injection of the B16.F10 cells into the scaffold, caused an influx of the surrounding media and provided nutrients to the cells during proliferation. This injection technique enhanced cellular viability 4-6 days after inoculation. Viability of both HaCaT and B16.F10 cells remained greater than
80% because they received an even exchange of nutrients and oxygen while rotating in the bioreactor (Figure 15-16). Viability and stability was monitored over a 10-15 day period. The heavily pigmented cells located in the center of the scaffold were mostly viable (Figure 13).

The mechanism underlying the functionality of this model was determined by IHC analysis. The cellular makeup of the spheroid was determined to be a 3:1 ratio of HaCaT spheroids to B16.F10 cells. The HaCaT cells in the spheroid enhanced and regulated B16.F10 proliferation through keratinocyte’s adhesive proteins (Figure 15). IHC analysis showed evidence of cellular proliferation. This was indicated by variable nuclei staining of the B16.F10 and HaCaT cells. The varying shades of brown suggested that each cell was present at different stages of the cell cycle (Figure 19). Moreover, stable cellular proliferation, communication, signaling and survival properties were also observed by IHC staining. Immunofluorescence (IF) analysis demonstrated changes in B16.F10 cellular morphology by Day 6. The cells underwent elongation to form dendrite-like processes suggesting that the B16.F10 cells were interacting and communicating with adjacent B16.F10 and HaCaT cells in the spheroid environment (Figure 18). HaCaT cells in the 3D model have the same properties as naturally occurring keratinocytes and have the ability to mimic with similar cellular reactions. HaCaT cells regulated B16.F10 growth and survival, displayed organized growth patterns forming tight junctions with adjacent HaCaT cells and promoted cell-cell interaction of B16.F10 cells through their dendrite-like processes. The IHC showed a collective grouping of B16.F10 cells into smaller populations that readily integrated with HaCaT cells (Figure 17). The IF assay demonstrated that while HaCaT and B16.F10 cells resided in the spheroid, the cells would stack upon one another forming the walls of the scaffold (Figure 17). Interestingly, the tumors proliferated in the absence of the basement membrane and
matrix rigidity. The HaCaT and B16.F10 cells stimulated the production their own extracellular matrix while proliferating and developing in the 3D spheroid construct (Figure 20).

**Communication and Functional Properties**

The spheroid model was shown to be a functional system that supported the production of E-cad protein production by keratinocytes. These adherent proteins function to control the fast growing properties of melanoma during early stages of development (Figure 22). Western blot analysis was used to confirm the up-regulation of E-cad expression and thereby the functionality of the 3D system (Figure 22). At early stages of melanoma development, the keratinocytes expresses E-cad to support and control melanoma growth. As described in the literature, melanoma progresses into metastatic melanoma by decreasing E-cad production and increasing N-cadherin expression. This process, termed cadherin-switch, facilitates the escape of melanoma from keratinocyte control allowing melanoma to metastasize into deeper layers of the skin. The data suggests that, HaCaT+B16.F10 cells residing in the 3D model together up-regulated adherent properties of E-cad to ensure the proliferation and stability of tumor development. As expected, there was a definitive difference in E-cad expression levels between HaCaT+B16.F10 cells compared to HaCaT alone and HaCaT+SKMEL-5. The low levels of E-Cad expression observed in the SKMEL-5 cell lines were expected because previous reports in the literature indicate these cells have lower levels of E-Cad expression when interacting with keratinocytes in vivo.

There was a reduction in terminal differentiation of the HaCaT cells when injected with B16.F10 cells. The evidence was provided by the western blot analysis demonstrating a decrease in Cyto 8 production compared to HaCaT cells alone. This suggests that HaCaT differentiation was reduced to promote B16.F10 survival and
progression. The 3D tumor model demonstrated its functionality through the production of human E-Cad and Cyto 8 collected on Day 2 and Day 4 after melanoma inoculation into the HaCaT scaffold. The expression of Cyto 8 and E-cad proteins were verified by the presence of positive control MCF-7 cells\textsuperscript{183}. Furthermore, it is known that cyto 8 is up-regulated during late stage cancer differentiation and decreased during early time points\textsuperscript{184-185}. The slight decrease in cyto 8 by HaCaT+melanoma suggests that melanoma retards HaCaT terminal differentiation and helps to maintain the structural integrity of the spheroid by up-regulating adhesive protein E-Cad. The AE1/AE3 is a broad spectrum pan-cytokeratin antibody that measures all states of epithelial cell development at different states of differentiation\textsuperscript{186}. It can be inferred from the staining of HaCaT with AE1/AE3 in IF analysis cells possibly expanded in an undifferentiated state or possibly driven to a state of differentiation to allow continuous survival of B16.F10 cells, (Figure 15, 20-21). In IF staining, there was a strong fluorescent signal observed for HaCaT indicating that keratinocytes were undergoing proliferation and differentiation while a weak signal meant that cells were present at an undifferentiated stage (Figure 17-18). Further testing to determine the state of differentiation, must be performed to confirm observations.

\textit{Optimizing EP Conditions Using the 3D Tumor Model}

This large-scale, free-floating \textit{in vitro} 3D tumor model is a functional and stable model that allows us to test delivery of genes using varying EP conditions and parameters. The goal was to generate an \textit{in vitro} tumor model would withstand the electric fields of EP, transfect melanoma cells with phIL-15, to promote IL-15 protein expression and translate the delivery methods \textit{in vivo}. Differences in IL-15 and GFP expression levels were observed between B16.F10 and HaCaT as well as EP conditions 1300V/cm and 500V/cm. The differences in transfection efficiencies were confirmed by
immunofluorescence, flow cytometry and ELISA. As predicted, numerous melanoma cells injected into the spheroid were strongly transfected with GFP using tumor EP conditions 6PE 1300V/cm and 500V/cm compared to HaCaT spheroid electroporated with 4-needle and 4-PE conditions (Figure 23-24). One theory for this phenomenon is that loosely suspended B16.F10 cells injected into the spheroid are more likely to be transfected than the organized HaCaT spheroid. HaCaT cells express the adherent protein E-cad, which promotes tight aggregate formation in the spheroid and decreases EP transfection into cells\textsuperscript{42}. The literature imply that the electric fields (EF) fired in multiple directions may help overcome cellular barriers in a 3D structure\textsuperscript{28, 42, 160}. This information suggest that using electrodes that fire the electric field in multiple directions improved expression levels but it also depends on the cell type and its physiology.

\textit{Testing In Vitro Delivery Conditions with phIL-15}

It was determined that a 6PE firing an electric field (EF) in multiple-directions increased GFP and phIL-15 transfection compared to the 4-needle or 4-PE. The transfection was not only dependent on the electrode, but also the cell type because during the experiment it was observed that B16.F10 cells were transfected more readily than tightly bound HaCaT cells. Also, GFP and IL-15 expression was dependent on the EP conditions used, plasmid transfection increased with lower EF, 500V/cm with longer pulses 20ms compared to higher EF, 1300V/cm with shorter pulses 100\textmu s as determined by IF distribution (Figures 24-25), quantitative flow cytometry and ELISA analysis (Figure 26-28). The flow data showed a 7.3-fold significant increase in the GFP+500V/cm group (Figure 27).The ELISA analysis showed that there was a 10-fold increase of IL-15 expression at 24h and a 15-fold increase at 48h delivering phIL-15+500V/cm into the HaCaT+B16.F10 group (Figure 28). In this model, it was important to fire the EF over a large radius. The 6PE contains a 0.8cm-1cm diameter gap between
the electrode plates, lower EF and longer pulses were needed to ensure delivery of GFP and phIL-15 to many cells at one time and overcome this potential cellular barrier while avoiding cell death (Figures 27-28). This data shows that different EP conditions and electrode configurations were required to obtain the desired delivery and expression amongst different cell types and their physiological properties. We also observed that closely packed HaCaT cells contributed to lower levels of IL-15 and GFP protein expression compared to the B16.F10 cells that were injected into the spheroid prior to EP. Decrease in HaCaT transfection was possibly due to the presence of adhesive proteins that could cause cells to aggregate and become less permeable thereby requiring different EP electrodes/conditions to enhance DNA delivery.

Comparing phIL-15 Delivery Using the EP Conditions In Vitro vs In Vivo

The in vivo studies demonstrated that the 3D model was able to predict IL-15 expression patterns similar to in vitro. The expression levels between skin and tumor differed; tumor samples were easily transfected compared to skin cells. Plasmid delivery and protein expression was enhanced at 500V/cm compared to 1300V/cm (Figure 29). There was a 9.6-fold increase in expression using phIL-15+500V/cm and 7.1-fold increase using phIL-15+1300V/cm, similar to fold changes measured at 24h in the 3D tumor model. The amount of IL-15 expression differed between the in vitro 3D models and in vivo mouse models at different time points, but the overall trend in expression was the same between the groups. In vivo, IL-15 expression is regulated by many factors such as the plasmid promoter region, at the transcription and translation level, mRNA and protein short half-life, at the protein expression level, negative feedback inhibition mechanisms stimulated through paracrine and autocrine pathways, and decreased internal secretion controlled by the golgi. The advantage of the 3D model system is that investigators can study each pathway system independently from one another in culture, and compare to
cell signaling *in vivo*. In nature, the skin undergoes homeostatic changes and regeneration. It is necessary to stabilize the synthetic 3D model for long periods of time to study the effects of complex signaling. For example, using a matrix that does not interfere with 3D cellular expansion or migration, could help stabilize the model and allow for cells to mimic natural processes more closely for functional studies.

Schoop *et al.*, discussed the production of an organotypic co-culture system ranging 1-2 cm in size after a 2-week period that was used to study complex systems. This model was not a free floating construct, and was evaluated for potential skin transplantation not tumorigenesis. This 3D tumor model is a unique system with the potential to have multiple functions. The 3D model is large scale functional model useful for adapting EP electrodes and testing delivery techniques *in vitro*.

The 3D tumor model can be used to control experimental conditions for different experimental analysis. For example, this model would be useful for evaluating different cellular behaviors, isolating particular cell systems for analysis, increasing sample for assays and most importantly reduce live animal experiments. In these studies, the 3D model was used as a tool to predict how cells react to EP electric fields, stimulate gene expression, differentiate transfection efficiencies, determine protein expression levels, and optimize plasmid concentration. The goal was to verify that the plasmids worked optimally prior to moving delivery techniques *in vivo*. Specifically, the 3D system was used to analyze protein expression levels between different cell types and determine the best route for plasmid administration. These features were all important variables necessary for the development of an efficient delivery system for therapeutic purposes.
Specific Aim 2

Examining phIL-15 Plasmid

In Aim 2, EP delivery of phIL-15 was shown to stimulate an increase in primary and secondary immune responses against tumor antigens. Surprisingly, the delivery of pVax plasmid with EP also enhanced an immune response and promoted tumor regression as well. A restriction enzyme digestion was performed on the plasmids to ensure that the appropriate plasmids were utilized during EP experiments. The gel demonstrated that negative control pVax (back bone) plasmid was of appropriate size and absent of the IL-15 gene insert (Figure 30). This suggests that phIL-15 and pVax both had an adjuvant effect on tumor regression, cytokine production and T cell response stimulation. The literature suggests that DNA delivered by EP can stimulate pro-inflammatory effects when the DNA is internalized by cells. The inflammatory response occurs when unmethylated CpG oligonucleotides stimulate Toll like receptor 9-dependent pathways in plasmocytoid dendritic cells\textsuperscript{192-193}. On the other hand, TLR-9-independent pathways promote inflammation by B-form DNA stimulating IκB kinase protein, Tank binding Kinase 1 in both immune and non-immune cells\textsuperscript{193-194}. The stimulation of the immune response by pVax+EP delivery has not been completely established. The stimulation of IL-15 signaling improved the primary response and secondary response to challenge against B16.F10 compared to pVax stimulation. Tumor regression and challenge studies suggest that IL-15 protein production had a more specific effect on tumor regression and enhanced mouse survival compared to pVax plasmid delivery groups (Figure 44, Table 2-3). The low level of IL-15 production was reflected in expression studies and regression data. Low levels of IL-15 expression produced by transfected monocytes, dendritic cells and B16.F10 cells was possibly due
to a decreased hIL-15 binding to endogenous mouse IL-15Rα, decrease in transcription of IL-15 protein, or secretion pathways. Since IL-15 expression was reduced, there was a possible decrease in the long-term responses against melanoma antigens.

It has been suggested that the administration of exogenous IL-15 through an intra-peritoneal injection promotes short-lived memory CD8+T cells response and the exogenous administration of IL-7 enhances long-lived memory response\textsuperscript{195}. Short and long-lived memory response is dependent upon the survival of CD8+T cells\textsuperscript{195}. This is important because IL-15 and IL-7 share a common γ\textsubscript{c} receptor on NK and T cells\textsuperscript{195-196}. IL-15 mainly functions to maintain memory by activating anti-apoptotic signals to enhance CD8+ T cell survival\textsuperscript{197}. Some antigen specific CD8+ T cells from the initial expansion of effector cells are retained to establish long-term immunological memory response. In these studies, it is possible that the negative feedback mechanism of producing cells such as monocytes and DC, down regulated IL-15 production and reduced survival of CD8+T cells causing less IL7 stimulation during the contraction phase. The contraction phase is when most CD8+ T effector cells undergo cell death and few effector cells survive\textsuperscript{195}. It was also found that IL-15 inhibits immunosuppressive function of T-reg on naïve or memory CD8+T cells\textsuperscript{198}. These publications suggest that IL-15 is not only regulated at the innate immune response, but also at the memory response. This means that additional boosts of IL-15 delivery may need to be administered into the tumor and/or the muscle to enhance survival of CD8+T cells and reduce T-regulatory function.
Translating EP Conditions In Vivo to Further Test Delivery of phIL-15

In previous publications, it has been suggested that the benefit of using EP to deliver plasmid DNA for the therapeutic applications is that it enhances transfection efficacy and efficiency of specific cell types\textsuperscript{27}. Increased plasmid transfection into cells found in the target tissue allows for increased production of the gene encoded by the plasmid and thereby enhancing therapeutic efficacy\textsuperscript{27,36}. This was demonstrated in the \textit{in vivo} mouse IL-12 and human IL-15 data. While delivering phIL-15 with EP had minimal effect on increase IL-15 expression, the IL-12 studies clearly demonstrated the ability of EP plasmid uptake and IL-12 expression. EP enhanced the production of IL-12 compared to IL-15. The ability for EP to enhance IL-12 and not IL-15 production could be attributed to the highly regulated mechanisms associated with IL-15 expression and secretion. Also, the sequence homologies between mouse IL-15 and human IL-15 are 73\%\textsuperscript{79}, but there may not be enough similarities in functional properties to promoted IL-15 expression and enhanced IL-15 stimuli as expected.

\textit{Regulation of phIL-15}

There are many publications suggesting that IL-15 is maintained at low levels through post-transcriptional and translational mechanisms because IL-15 messenger RNA is ubiquitously expressed in many tissues\textsuperscript{77}. The overstimulation of IL-15 is known to cause many deleterious effects in the human body such as systemic lupus erythematoses, rheumatoid arthritis, Type I diabetes, hypertension, artherosclerosis, and muscle disease\textsuperscript{77}. Delivery of respective plasmids using EP increased IL-12 or IL-15 expression and maintained protein levels over time (Figures 32-43). Additionally, the IL-15 western blot analysis and the 3D tumor model demonstrated that IL-15 production
was reduced *in vivo* compared to mouse IL-12 expression data. This data suggests that the protein levels of IL-15 expression were tightly regulated at the tumor level. It has been shown that soluble IL-15 expression is difficult to detect in serum due to regulatory mechanisms at the transcriptional, translation and secretory stages\textsuperscript{101, 150,199}. Several groups have investigated methods to improve IL-15 protein expression and secretions\textsuperscript{149,200}. These publications report that optimized human IL-15, also known as ECRO-IL-15, plasmid transfected into human Rabdomyosarcoma cells increased IL-15 expression 87-fold higher than native pIL-15 and 5.7-fold higher than the long signal peptide (LSP) IL-15, while short signal peptide (SSP) was retained in the nucleus\textsuperscript{199}. A recent publication by Beragamaschi et al 2009, reported that IL-15 SSP was involved in IL-15 regulation\textsuperscript{201}. The experiments performed suggested that IL-15 SSP competed with IL-15 LSP for the binding of IL-15Rα possibly reducing IL-15 secretory pathway stimulation. A publication by Eisenman et al demonstrated that human IL-15 can be used in mouse models and that human IL-15 ligand does bind to mouse IL-15Rα, but activity may be hampered because optimal activity occurs with a mouse IL-15 ligand\textsuperscript{196}. This idea is possible because there are sequence homologies between mouse/human ligand and receptor proteins. Mouse IL-15Rα is 54% homologous to human IL-15Rα and their active site sushi domains are 85% homologous\textsuperscript{79}. The homology suggests that human optimized (opt) expressed IL-15 protein could bind the active sushi domain of mouse IL-15Rα, out compete the IL-15 SSP protein binding and promote IL-15 secretion. The other possibility is IL-15 SSP protein binding blocks opt-IL-15 protein and promotes the negative down-regulation of IL-15 production.
Bergamaschi et al., demonstrated that it is possible to up-regulate IL-15 secretion when delivering a duel expressing plasmid containing both IL-15 and IL-15Rα genes\textsuperscript{201}. The co-expression of both IL-15 and IL-15Rα in one plasmid would promote intracellular complex formation and stabilization persuading IL-15 ligand to bind the IL-15Rα with high affinity. The co-expressing plasmid would result in two active forms; 1) a membrane bound IL-15Rα that would promote autocrine IL-15 binding and trans-presentation to responding cells and 2) a secreted active form of soluble IL-15/IL-15Rα ready to stimulate NK and T cells\textsuperscript{201}. There is an abundance of literature reporting studies that have genetically altered the native IL-15 gene to enhance transcription, translation, expression, and secretion. The opt-IL-15 plasmid used in these studies was the optimized form, ligated into a vector containing constitutively active CMV promoter\textsuperscript{149,199-200} published in Kutzler et al., to enhance protein expression and ensure secretion of IL-15 plasmid from the transfected cells\textsuperscript{149}.

**Intra-tumoral Delivery of phIL-15**

The IL-15 expression levels obtained from intra-tumoral transfection were described in Aim 2. The tight regulation of IL-15 at the cellular level was overcome slightly by delivering the plasmid in high plasmid concentrations (100µg) with EP. western blot analysis demonstrated IL-15 protein expression at 24h, but expression was undetectable by 48h post-delivery (Figure 31). The studies conducted in the *in vitro* 3D tumor model, demonstrated that majority of the IL-15 protein produced was secreted into the supernatant, but there was still a detectable level of IL-15 protein in the spheroid homogenate (Figure 28). These experiments suggest that IL-15 expression occurs
earlier than 24h and that some IL-15 expression is retained intra-cellularly. Studies evaluating expression of IL-15 in vivo demonstrated that IL-15 expression was short-lived. The tumors in the mice were tested at early time periods 12h and 18h in addition to the 24h and 48h time points. As expected, IL-15 expression was detected as early as 12h in the phIL-15+1300V/cm and phIL-15+500V/cm groups. In the phIL-15+1300V/cm group expression peaked at 24h while in the phIL-15+500V/cm groups peaked at 12h. In both groups lower expression of IL-15 was seen by 48h (Figure 32). The same pattern of early expression was obtained from experiments testing the skin as a possible delivery tissue for transfection peri-tumorally to target tumor cells, but levels were much lower than direct tumor transfection (Figure 33). The IL-15 expression pattern detected in these studies is similar to that reported by Jalah et al. publication. The authors, compared expression levels between different engineered optimized IL-15 plasmids, including the opt-IL-15 plasmid used in this project. They described that a single hydrodynamic injection with the opt-IL-15 plasmids into mouse tail veins resulted in higher IL-15 expression detected in the serum at early time points. The expression of the IL-15 from the optimized plasmids steadily increased up to 24h, at which point IL-15 expression peaked and then steadily decreased overtime after 1TX. This study supports the IL-15 expression levels obtained from the 3D tumor model and in vivo mouse experiments conducted in this project.

Observations of the 3D tumor model and mIL-12 data; indicated that there was much lower transfection efficiency of IL-15 delivery to the skin than there was to the tumor. As discussed earlier, epidermal skin cells are keratinized cells containing mostly dead cells with tight adherent properties, while B16.F10 cells are mostly live viable cells with low adherent properties. Based on the skin and tumor experiments, the electric field
strength and blast radius encompasses more live tumor cells than viable dermal skin cells found below the epidermis. The difference in protein expression by different cells types depends on the stage of cell cycle, viability, and adherent properties\textsuperscript{42}. B16.F10 cells have less adherent properties, there more are viable cells and longer lived compared to keratinocytes. The keratinized skin cells undergo cell death as they move towards the epidermal layer\textsuperscript{202}. The cells near the basal, germinal level are less keratinized and have less adherent proteins than cells in the epidermal layer\textsuperscript{14} and are more likely to uptake plasmid DNA delivered by EP. These observations suggest that intra-dermal EP would demonstrate better transfection of cells near the basal layer compared to keratinized cells near the epidermis\textsuperscript{160}.

**Maintenance of phIL-15 Expression with Multiple Treatment Parameters**

The information gathered from the *in vitro* and *in vivo* experiments suggests that in order to obtain the highest level of IL-15 expression multiple evenly spaced treatments protocols should be administered to treat existing tumor cells. This project demonstrated that a series of EP plasmid deliveries could produce enough signal to stimulate the immune response and control the fast growth of aggressive B16.F10 cells. A 2Tx and 3Tx protocol administered phIL-15+ EP (1.0mg/ml) at early time points at 0, 1, 2, days did not stimulate robust IL-15 expression. It was inferred that delivery of phIL-15 by EP at such close, adjacent time points may not allow sufficient time for cellular immune response. For example, there should be sufficient lag time to allow innate immune cells like phagocytes and macrophages to clear away dying cells, APCs. to process antigens and recruit new monocytes from the blood to the delivery area that could respond to additional plamid+EP treatments. Other limiting factors that may have interfered with IL-15 expression during the early multiple treatment protocols was possibly due to 1)
enhanced destabilization of the plasma membrane from transfected cells by the applied electric fields causing some cell death and less time for clearance between the first treatment and the next treatment, and 2) also adjacent treatments did not allow enough time for IL-15 production to recruit cells to the area or IL-15 protein transpresentation to responding cells, which was possibly due to IL-15 negative feedback properties as well. Experiments using the 3D tumor model demonstrated that the plasmid GFP delivered with EP to melanoma cells was readily taken up the plasmids and expressed within 24h, but there was a slight decrease in viability 5-10%. The HaCaT were much hardier cells with viability close to 92-96% but transfection was much lower than B16.F10 cells (Figure 26-27). The results suggest that using different EP parameters would increase the level of HaCaT expression.

Increasing the time between the treatments for the 2Tx and 3Tx protocols delivering phIL-15+EP+ (2.0mg/ml) was explored on Days 0, 3, and 6. There was a significant 2-3-fold increase at Day 3, and 2-fold increase at Day 6 in both EP groups. The 3Tx protocol suggests that cellular turnover can occur as early as 72h after the first treatment. There was a slight, but significant increase in IL-15 expression at Day 3 and Day 6 compared to the 12h, 36h, 60h protocol, but not to the extent as predicted (Figure 42-43). The 3Tx protocol on Days 0, 3, and 6 was selected for further studies to monitor tumor regression and mouse survival. The decision was based on the observations from early tumor regression studies (Table 2). Fast growing tumors began to regress in plasmid EP treatments groups by Day 4 and 7, while untreated tumors and tumors injected with pVax or phIL-15 exponentially grew after Day 9 (Figure 44a-c). These studies suggested that the multiple treatment protocols using phIL-15+EP required some time to recruit new cells to the area. This was further suggested by the up-regulation of IL-1β cell proliferation cytokine, MIP-1β recruitment chemokine and TNFα tumor
necrosis detected in tumor lysate when cells were treated with IL-15+ 1300V/cm and phIL-15+500V/cm at 12h, 24h, Day 3, and Day 6. The highly regulated IL-15 protein expression and delivery to the mouse model demonstrated that human IL-15 can possibly bind to mouse IL-15Rα, may trans-present to receptive cells such as NK cells to promote IL-15 stimulated immunological activity in the mouse and can cause tumor regression.

Analyzing Tumor Regression and Mouse Survival with Delivery of phIL-15 and EP

The main goal of Aim 2 was to stimulate an immune response against tumor antigens and promote long-term responses to enhance mouse survival. Delivery of both phIL-15 and pVax using EP elicited a detectable immune response, but it was necessary to up-regulate a specific innate immune response and in turn stimulate down-stream signaling proteins to promote an adaptive immune response. The adaptive immune response would encourage T cells to recognize tumor antigens presented by dendritic cells and in turn mount a systemic response against B16.F10 cells. The mechanism by which IL-15 and EP stimulated tumor-specific response is not clear. Therefore experiments were designed to characterize the innate and adaptive immune responses that were initiated by IL-15/pVax+EP in tumors and prevented relapse of B16.F10 growth. The combination of IL-15 or pVax with EP acts as an adjuvant therapy demonstrating tumor regression, cytokine stimulation, and T cell stimulation.

The majority of the mice that had undergone tumor regression survived B16.F10 challenge. The best groups that responded with long term effects were the phIL-15+500V/cm group which contained 8 of 15 mice that had complete regression of primary tumor and had 75% (6 out of 8) of the mice that survived tumor-free after subsequent tumor challenge, the phIL-15+1300V/cm group contained 11 of 15 mice with
complete regression of primary tumor and 45% (5 out of 11) of the mice that survived tumor-free after subsequent challenge and the pVax+500V/cm group contained 11 of 15 mice with complete regression of primary tumor and 36% (4 out of 11) of the mice that survived tumor-free after subsequent challenge (Table 3). Although the pVax+500V/cm and phIL-15+1300V/cm both demonstrated similar results, it was the phIL-15+500V/cm group that stimulated enough of the innate and adaptive immune response 50 days after challenge to enhance a possible memory response\textsuperscript{203}. The lack of response in some mice may possibly be due to variability in tumor size or the metastatic properties of tumor cells that were injected deep into subcutaneous tissues. Perhaps, for the delivery of phIL-15, many more tumor cells must be subjected to electric fields to increase transfection area during each treatment and ensure delivery of IL-15 plasmid to increase expression levels that making up for regulatory processes of IL-15. The multiple treatment protocol allowed tumors to maintain IL-15 protein expression over time improving tumor regression and the mouse survival outcome (Figure 42). The regression and challenge data indicates that IL-15 delivery using EP may be an effective treatment regimen for melanoma. Evaluation of the data suggests that IL-15 can be used as a possible cytokine therapy against melanoma because of its ability to promote specific anti-tumor properties. Also, pVax delivery using EP demonstrates that the back bone delivered to melanoma can act as an adjuvant causing tumor regression and non-specific anti-tumor properties. In the regression data, there were a few mice in the phIL-15+1300V/cm group and all mice in the phIL-15+500V/cm group with pigmentation loss to the skin and hair due to EP effects (Table 2-3, Figure 44). Vitiligo occurs as a result of the TH1/INF\gamma or TH2/IL-4 balance between the pro-inflammatory and autoimmune stimulation\textsuperscript{204}. It is possible that inducing vitiligo with EP may also contribute to maintenance of immune response.
Specific Aim 3

*Characterizing Immune Response Delivery of pVax and phIL-15 Plus EP Using IHC*

Characterization of the immune response that stimulated tumor regression was further explored by IHC, ELISpot and bead array to explore the immune mechanisms that promoted regression and mouse survival. IHC demonstrated the presence of CD8+ and CD4+ T cell in response to different control and treatment groups (Figures 45-48). The saline+EP groups both caused stress to the surveying CD4+ T cells resident to the area compared to the no treatment group on Day 1 and 2. The saline+500V/cm stimulated many residential CD4+ T cells and a few CD8+ T cells. This signified that EP alone evoked an initial stimulatory effect on residential CD4+ and CD8+ T cells or cells already stimulated by melanoma. The phIL-15 injection group which expressed very low amounts of protein from tumors (described in Aim 2), promoted a few more CD4+ and CD8+ T cells compared to saline+500v/cm and pVax injection on Day 1 and less on Day 2. The pressurized injection of phIL-15 into the tumor could have possibly caused production of IL-15 expression and promoted the activation of residential CD4+ and CD8+ T cells. The phIL-15+500V/cm group also stimulated more of both CD4+ and CD8+ T cells activity compared to pVax+EP groups and phIL-15+1300V/cm on Day 1 and decreased stimulation of T cells by Day 2. Also on Day 2, phIL-15+1300V/cm group stimulated residential CD8+ T cells. This indicates the possibility that IL-15 had an effect on priming T cells against melanoma. The IL-15+EP groups containing higher electric field strengths with shorter pulses, such as the phIL-15+1300V/cm groups, stimulated initial CD8+T cells more quickly than phIL-15+500V/cm. On the other hand, IL-15+EP groups with lower field strengths and longer pulses stimulated CD4+T cells more quickly at early time points. On Day 7, phIL-15+1300V/cm and phIL-15+500V/cm showed more
CD8+T cells stimulation compared to the other control groups indicating the CD8+T cells were recruited by IL-15 to remove tumor antigens most likely through CTL effector activity. The response was more defined at Day 11 showing an up-regulation of expanded specific CD8+ and CD4+T cells in groups pVax+1300V/cm, pVax+500V/cm, phIL-15+1300V/cm and phIL-15+500V/cm. Also by Day 11, the tumor size had regressed significantly in these groups suggesting that CTLs were actively removing tumor cells. The increased stimulation of CD8+ T cells CTL response was possibly facilitated by increased CD4+ T cells helper cytokine activity and/or promotes CTL differentiation by APC through the help of antigen-stimulated CD4+ T cell166. These conclusions were made based on the fact that more CD4+T cells were present in phIL-15+1300V/cm group compared to pVax+1300V/cm. The lower amount of CD4+T cells in the pVax+1300V/cm group may explain why there was a relapse of tumors during the challenge. CD4+T cells provide help for antigen-specific CD8+T cells stimulation and to activate the destruction of melanoma by CTL effector cells166. In contrast to these observations, on Day 11, there were increased amounts of CD4+ T cells stimulated in the pVax+500V/cm groups than there were in phIL-15+500V/cm group. These results indicate that phIL-15+500V/cm stimulated an early response of CD4+ T cells at Day 1, the levels were beginning to diminish since tumors in the phIL-15+500V/cm groups were necrotic and only residual tumor cells were left behind for CTL and macrophage removal. Both phIL-15+500V/cm and pVax500V/cm+EP+ groups had sufficient numbers of CD8+ T cells present but the pVax+EP+ group had small tumors that were still intact compared to phIL-15+500V/cm by Day 11, based on tumor volume measurements. The ELISpot and bead array assays show up-regulation of IFNγ in plasmid+EP+ which agrees with increased CD4+T cell infiltration and CD8+T cells increase observed in IHC.
**ELISpot Assay Performed to Identify Presence of IFNγ Producing Cells**

To provide further evidence of possible stimulation of immune response up-regulation through IFNγ expression, an ELISpot assay was performed on splenocytes collected from plasmid+EP+ mouse groups. On Day 14, the ELISpot assay showed an increase in IFNγ production in all four pVax+EP+ and phIL-15+EP groups stimulated with B16.F10 and rIL-2. The ELISpot results complimented the IHC images showing the recruitment of primed CD8+ T cells that had homed into the tumor area at Day 11 and reduced tumor volume possibly through CTL and NK activity. Based on the literature, IL-15 binds to IL-15Rα expressed on APC and monocytes and trans-present IL-15 to responding CD8+ T cells and NK cells that express the popular βγc receptor complex\(^ {118}\). Trans-presentation and direct stimulation of responding cells depends on expression of IL-15Rα. It is known that CD8+ T cells express IL-15Rα while NK cells do not express IL-15Rα making trans-presentation essential for NK stimulation and further enhancement of CD8+ T cell activation\(^ {118,197,205}\). The bead array also demonstrated an up-regulation of IFNγ stimulation at 12h, 24h, Day 3 and Day 6 in the tumor from groups phIL-15+1300V/cm and phIL-15+500V/cm. Although serum levels of IFNγ levels decreased, expression was still detected at 24h in pVax+1300V/cm at 12h and 48h in phIL-15+1300V/cm, Day 3 and Day 6 in phIL-15+500V/cm and phIL-15+1300V/cm. The detection method used in this study to measure NK response, did not show NK stimulation, there was stimulation of both CD8+ T cells and IFNγ indicative of possible NK activity involvement since tumor regression began as quickly as Day 4 after 2Tx. The IHC, Luminex bead array and ELISpot data also suggests that IL-15 may stimulate NK secretion of IFNγ at early time points (12h-Day 6), and indirectly through up-regulation of IL-12 production by dendritic cells, monocytes or NK cells to promote additional CD8+ T cell CTL response and infiltrate\(^ {163}\) at later time points (Day 11-14) Also, since the bead
array identified low levels of IL-12 production by tumor cells and serum levels at early time points. The only groups that demonstrated an increase of IL-12 in the serum at Day 14 were pVax+500V/cm and hIL-15+500V/cm possibly indicating stimulation T cells expansion and T cell effector response. Heller et al., demonstrated that early IFNγ expression was detected in the serum on Day 4 after delivering 1Tx intra-dermal to mice in groups vector+EP+ control, pmIL-12 injection, pmIL-12+1500V/cm groups\textsuperscript{160}. Additionally, a publication from Lucas et al., demonstrated that intra-tumoral delivery of pmIL-12+1500V/cm treatment stimulated IFNγ in the tumor lysate at Day 5\textsuperscript{28}. These results indicated that plasmid delivered via EP were able to stimulate an innate immune response and an adaptive immune response that caused tumor regression and possibly explains long-term effects against melanoma. In this study, the delivery of phIL-15+EP+ possibly restored immune surveillance\textsuperscript{118} and controlled melanoma growth, since there were no visible metastatic melanoma lesions located in the lung, liver, lymph nodes or spleens from mice survived and were analyzed on Days 7, 11, 14, 30 and 60 (Samples from Figures 45-48 and Table 2).

**Comparing Differences in Plasmid Constructs pmIL-12 and phIL-15**

The primary tumor regression levels stimulated by plasmid+EP groups were similar to results detailed in Lucas et al.,\textsuperscript{28} for the delivery of pmIL-12. Comparing the results, there were similar primary tumor regression levels in the pmIL-12+1500V/cm group compared to phIL-15+1300V/cm group, but phIL-15+1300V/cm group was approximately 55% less effective against challenge compared to pmIL-12+1500V/cm group. Although, there was no correlation between pmIL-12 and phIL-15+500V/cm gene expression experiments, this group demonstrated the best long-term responses against challenge for the IL-15 experiments. The phIL-15+500V/cm group demonstrated the
highest IL-15 protein expression levels secreted from tumor cells in vitro and in vivo compared to phIL-15+1300V/cm. Challenged mice in 5000V/cm demonstrated 25% less effective than mIL-12. In IHC the phIL-15+500V/cm group showed an increased CD8+T cells, quickly removing tumor cells compared to the rate at which phIL-15+1300V/cm removed tumor cells by Day 11. Furthermore, 1300V/cm showed the best primary response causing tumor regression in more mice, possibly due to mild EP conditions causing less tissue damage compared to phIL-15+500V/cm. However, it was the phIL-15+500V/cm group that up-regulated long-term responses and possibly promoted CD8+T cell memory response against melanoma compared to phIL-15+1300V/cm and pVax 500V/cm.

Utilizing the Luminex Bead Array to Characterize Cytokines Stimulated by pVax and phIL-15+EP

In Aim 3, a 10-cytokine bead array assay was used to characterize the innate and adaptive immune responses stimulated by the plasmid+EP+ delivery system (Figures 50-89). Treatment groups and controls groups (no treatment and pVax injection) were analyzed using the bead array to characterize the immune response stimulated by phIL-15+EP and pVax+EP+. It was expected that non-specific innate immune cells (i.e. macrophage, NK cells, dendritic cells and monocytes) were present in the tumor and serum during normal surveillance. It was predicted after the delivery of phIL-15 using EP, the IL-15 protein would activate mature NK cells and the cytotoxicity of NK and T cells to target tumors. Macrophages would recognize and process antigens for presentation to receptive cells77,197,206. The receptive NK and T cells would then mount a specific response by secreting signaling cytokines and chemokines. These signals would induce proliferation and help prime CD4+T and CD8+T cells for expansion
to target melanoma. The specific stimulation of CD8+ T cells would then promote a long term response against recurrent tumor antigens\cite{77,197,206}. The purpose for utilizing the phIL-15+EP+ delivery system was to transfect the tumor cells and promote IL-15 expression to re-stimulate a tolerant system. The stimulated immune cells would enhance the concentration of cytokine signaling and effector cells signaling\cite{207} in the tumor location and promote melanoma elimination.

**Examining the Stimulation of Different Cytokines Up-regulated by the Innate and Adaptive Cellular Responses**

The expression of cytokines up-regulated by phIL-15+EP+ or pVax+EP+ delivery and involved in tumor regression, preventing tumor relapse, were analyzed by Luminex bead array. Macrophages are the first to respond to tumor cells. The macrophages engulf tumor cells, disrupt the cells and present the antigens to T lymphocytes\cite{206}. Macrophages produces a multitude of cytokines IL-1β, IL-6, IL-10, IL-12, IL-15, and TNFα that function to increase CD4+ helper T cells, additional macrophages, neutrophils, and dendritic cells populations. CD4+ helper T cells produce IL-2, IL-15, and IL-12 to stimulate NK and LAK cells. IL-12, IL-2, and IFNγ can then stimulate CD8+ T cells and CTLs. Also, helper T cells produce IL-2 to continuously stimulate proliferation and differentiation of CD4+ helper T cells, CD8+ T cells and CTLs. It is known that IL-15 does not stimulate IL-4, which promotes a Th2 response, but IL-10 production can be stimulated in Th1 and Th2 mediated responses\cite{166,206}. MIP-1β and IL-15 are chemokines that help recruit monocytes from blood to the tissue and into the stimulated area, while GM-CSF induces the production of granular cells, and maturation of macrophage and dendritic cells (DC) from bone marrow cells. Neutrophils stimulate macrophage and T
cells through TNFα, and macrophage stimulate neutrophils through IL-1 and TNFα. T cells stimulate macrophage through IL-1, IL-10, TNFα, IL-4 and IFNγ\textsuperscript{166, 208}.

Expression of IL-2 stimulates T cell proliferation, but can also compete for IL-2Rβγ binding with IL-15 ligand\textsuperscript{169, 173}. IL-2 can stimulate its own expression through autocrine factors to help promote T cell proliferation. It has also been suggested that IL-2 can promote T cell survival thereby enhancing memory response\textsuperscript{169, 173}. The goal of these studies was to enhance IL-15 production at early time points to activate NK cells, IFNγ and then stimulate IL-12 to increase systemic response against recurrent melanoma tumors. Macrophage inflammatory proteins are chemokines that have tumoricidal properties that drives Th1 responses\textsuperscript{172, 209}, IL-1 and TNFα facilitate leukocyte adhesion and migration\textsuperscript{172}. IL-15 promotes the production of GM-CSF, IFNγ, and TNFα from activated NK cells and synergizes with macrophage stimulation of IL-12 to enhance NK activity\textsuperscript{173}. This analysis helped characterize the signals activated in the tumor by delivery of IL-15 with EP, which amplified a local response and in turn promoted a systemic, long-term response against melanoma.

**Analysis of phIL-15 Injection Alone Group**

Tumor lysate was tested to identify local cytokine production in the tumor, while serum samples were tested to identify systemic cytokine production. The bead array results were analyzed for levels of cytokine production at different time points compared to the background levels of control groups no treatment, pVax injection or naïve samples. Each treatment group tumor lysate and serum samples will be analyzed for each cytokine in the sections below. The tumors injected with phIL-15 alone induced the production of different cytokines than the control groups such as IL-1β, IL-2, IL-10, and MIP-1β. These cytokines were mostly activated at early time points in the tumor. The
stimulation of these cytokines suggests that IL-15 protein activated macrophage proliferation, DC differentiation, T cells and NK cell proliferation and recruitment of neutrophils and monocytes to the area. Also IL-4, IL-12, IL-6, IFNγ, GM-CSF, and TNFα were mildly increased at early time points. Low stimulation of these cytokine suggests that IL-15 protein expression was short-lived, but was sufficient to activate CD4+ T cells surveillance. The reduced concentration of the cytokines suggested that CD8+ T cells or CTLs were not present. Based on IHC, phIL-15 injection did stimulate some CD4+ and CD8+ T cells in the tumor area, but the tumors were able to escape innate and cellular immune responses. The IHC data showed phIL-15 injection stimulated more surveillance cells than the no treatment group and pVax injection groups. Similar cytokine concentrations were detected in the serum of the phIL-15 group; all cytokines expressions were very low except for IL-1β, IL-2, IL-6, and MIP-1β at early time points. This suggests that IL-15 stimulated recruitment of lymphocytes from the blood to infiltrate the tumor and also stimulated monocyte, macrophage, T cell and NK cell proliferation, monocyte and macrophage differentiation and restored NK/IFNγ stress signals.

**Analysis pVax+1300V/cm Group**

Analyzing tumor lysate samples from the pVax+1300 V/cm group, demonstrated an up-regulation of cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, MIP-1β, TNFα, and IL-12. These cytokines were the same as those stimulated in phIL-15 injection group. In addition to stimulation of monocyte, macrophage, and T cell proliferation, DC differentiation, recruitment of tumor infiltrating lymphocytes (TILs), this group also stimulated tumor necrosis, enhanced restoration of NK and IFNγ signaling, some anti-inflammatory signals, and regulation of tumor infiltrating monocytes. In the serum the
cytokines that were stimulated included IL-1β, IL-10, and MIP-1β at early time points (12h, 24h, 48h, Day 3 and Day6) and IL-2, IL-6, GM-CSF, and MIP-1β at later time points (Day 11 and Day 14). The pVax+1300V/cm group also showed an increase in INFγ at Day 14, as detected by ELISpot. IHC demonstrated low levels of T cell infiltrate at early time points and these levels increased at later time points signifying that the response was delayed, but strong primary response, and a weak secondary response causing tumor recurrence. MIP-1β, TNFα and IL-12 seem to be the key cytokines stimulating tumor regression.

Analysis of pVax+500V/cm Group

The pVax+500V/cm group demonstrated up-regulation of the following cytokines in tumor lysate IL-6 and MIP-1β. This suggests that TILs, NK and IFNγ caused the CD4+ and CD8+T cells recruitment. In the serum the cytokines stimulated were IL-6, IL-10 IFNγ GM-SCF, and TNFα at early time points. IL-1β, IL2, IL4, IL6, GM-CSF, IL-10, IL-12, MIP-1β, and TNFα at later time points and IL-12 and MIP1β at late time points (Day 30 and Day 60). This indicates that pVax+500V/cm stimulated a robust primary response and secondary response as well as long-term effects due to the burning caused by the electric field. The extensive tissue damage stimulated macrophage and DCs to enhance a strong immune response to the wounds and promoted secondary response to antigens because of tumor ablation. Up-regulation of IL-4, GM-CSF, IL-6, MIP-1β, and TNFα indicated monocyte and DC differentiation, maturation, tumor necrosis and stimulation of TILs. There was an increase in IL-12 at late time points suggesting a secondary response and newly recruited T cells. This could explain the robust immune response at the tumor site and subsequently regression. Also, there was an increase of IFNγ observed by the ELISpot at Day 14, and IHC showed CD4+T and CD8+ T cells infiltrate
at Day 11, signifying that the pVax+500V/cm group did prime T cells for a strong secondary response and enhanced survival by 36%, and stimulated long-term responses to challenge. The tissue damage caused by EP simulated an increase in cytokine production causing a robust secondary response and T cell stimulation as seen in IHC at Day 11. IHC showed increased CD8+ and CD4+ T cell and tumor regression by Day 11. Tumor cells slowly regressed compared to phIL-15+500V/cm group that showed lots of tumor degradation.

**Analysis of phIL-15+1300V/cm Group**

The phIL-15+1300V/cm group demonstrated stimulation of the following cytokines in the tumor (some cytokines expressed increased levels and are designated by the word “high”), high IL-1β, IL-2, high IL-6, IL-10, GM-CSF, high MIP-1β, high TNFα, IL-12, and high IFNγ. In the serum the cytokines expressed were high IL-6, GM-CSF, MIP-1β, and TNFα at early time points. At later time points IL-12, IFNγ, GM-CSF, and MIP-1β were expressed and at late time points IL-12, MIP-1β, TNFα, and IFNγ were expressed. The ELISpot also demonstrated IFNγ production in splenocytes at Day 14. Since INFγ is found highly expressed at the level of the tumor this suggests that NK cells were present and promoting cytotoxic activity. The high concentrations of cytokines found in the tumor and in the serum suggest that these were the signals that promoted increased tumor regression and up-regulation of a secondary response. The enhanced CD8+ T cell response could have increased mouse survival to 45% and promoted long-term tumor rejection when challenged. IHC showed tumor regression and up-regulation of many CD4+ and CD8+ T cells. These results suggest the up-regulation of the innate immune response promoted increased cytokine production, and recruitment of primed T cells, CTL and NK cells through IFNγ expression. The IL-12, MIP-1β and TNFα
produced at the late time points could be the underlying cause of long-term responses against tumor challenge.

**Analysis of phIL-15+500V/cm Group**

The phIL-15+500V/cm group stimulated the following cytokines: high IL-1β, high IL-2, IL-4, high IL-6, IL-10, high GM-CSF, high MIP-1β, high TNFα, IL-12, and IFNγ. In the serum, the cytokines that were stimulated included IL-1β, high IL-6, high IL-10 at early time points, IL-1β, IL-10, IL-12, IFNγ, GM-CSF, high MIP-1β, and TNFα at late time points. IL-1β, high IL-12, IFNγ, high MIP-1β, and TNFα at late time points. The ELISpot also demonstrated stimulation of INFγ from treated splenocytes. Higher levels of cytokine stimulation in the tumor and in the serum contributed to tumor regression, mouse survival, and the best long-term response resulting in 75% of the mice remaining tumor-free after challenge. IHC showed a more dismantled residual tumor with unrecognizable tumor cell shapes and some scar tissue due to EP damage. There was an abundance of CD4+ and CD8+ T cells infiltrating the area. The only differences observed between the phIL-15+1300V/cm and phIL-15+500V/cm groups were increased levels of IL-2 and IL-4 in the tumor and increased levels of IL-10 and IL-6 in the serum that possibly contributed to CD8+ T cells survival and a potential memory response.

**Analyzing the Immune Response to Tumor Antigens**

NKs function as immunosurveillance cells. Mature NK cells are stimulated by IL-15 to promote anti-tumor effects. NK cells remove malignant cells through cytolytic properties and stimulate TNFα and IFNγ to promote an adaptive immune response. Although it is known that IL-15 causes an anti-tumor effect by NK and CD8+ T cells, studies show that when depleting NK and CD8+ T cells in an IL-15 transgenic mouse that anti-tumor mechanisms still occur. It is still unknown if it is a direct effect by IL-15
stimulation of cells involved in innate anti-tumor activity through the γc-chain signaling independently of NK and CD8. One theory is that IL-15 can stimulate macrophages directly\textsuperscript{6}. NK cells secrete the cytokines IFNγ, GM-CSF, and TNFα upon activation and require increased stimulation for cytolytic activity. IL-15 mediates NK cell proliferation through DC encounter\textsuperscript{170}. The activity of NK cells were not directly measured, but the cytokines associated with NK cells were measured and detected by the bead array, ELISpot and IHC. The phIL-15+1300V/cm and phIL-15+500V/cm showed an increase in TNFα, IFNγ, IL-12 and CD8+T cells.

IL-10 up-regulates IL-15 production by macrophages and over expression of IL-10 suppresses TNFα and IFNγ, which in turn inhibit IL-12 synthesis by mononuclear cells at early time points. The up-regulation of IL-15 would re-stimulate IFNγ and TNFα\textsuperscript{169}. IL-10 acts to regulate pro-inflammatory IL-15 production and anti-inflammatory effects on IFNγ and TNFα. The bead array demonstrated that phIL-15+1300V/cm and phIL-15+500V/cm increased TNFα, IFNγ, and IL-10.

\textit{In vivo} delivery using EP can stimulate many of the same cytokine and promote differentiation of DC. \textit{In vitro}, studies have demonstrated that monocytes differentiate into immature DC stimulated by GM-CSF and IL-4, and that differentiation can also occur through GM-CSF and IL-15. DCs mature in the presence of TNFα, IL-1β and IL-6 expressed together, and additional expression through intracellular toll like receptor 7/8 stimulated IFNγ and TNFα\textsuperscript{167,210}. GM-CSF stimulates neutrophils and IL-15 stimulates IL-1β by neutrophils\textsuperscript{171}.

ELISA demonstrated an increase in IL-15and the multi-plex bead array showed comparable stimulation of different cytokines responding in the tumor lysate. The cytokines expressed in the tumor stimulated by phIL-15+EP+ suggested that monocytes matured to DCs and migrated to peripheral lymph nodes. In the peripheral lymph nodes
they differentiated, matured into APCs and up-regulated Th1 IL-12, which in turn promoted T cell synapse leading to T cell expansion\textsuperscript{167}. IL-12 stimulates IFN\(\gamma\) by T cells and NK cells. phIL-15+EP+ groups showed an increase in IL-12, IFN\(\gamma\), IL-1\(\beta\), IL-2, MIP-1\(\beta\), GM-CSF, IL-6 and TNF\(\alpha\) indicating the therapy may have induced APC proliferation, differentiation and T cells expansion.

**Comparing Recombinant Protein Experiments and Gene Delivery Treatments**

Lewko *et al.*, demonstrates that recombinant IL-15 protein influences TILs or TDAC outgrowth to eliminate primary culture melanoma cells by Day 7. This shows that recombinant IL-15 was able to cause tumor elimination by Day 7 in culture\textsuperscript{211}. Similarly, in the IHC experiments there was an increase in tumor regression by Day 7 and there was possibly newly infiltrating T cells migrating to tumor site by Day 11 in the phIL-15+EP+ groups. It is possible that the low levels of IL-2 were detected because IL-15 competed for binding of the IL-2 receptor due to high affinity binding capabilities\textsuperscript{211}. In the bead array assay, both serum and tumor cells showed low IL-2 expression in phIL-15+EP+ groups. Some IL-2 was possibly stimulated through its own autocrine pathway\textsuperscript{211-212}. Additionally, it is suggested in the literature that human IL-15 binds mouse IL-15R\(\alpha\) up-regulates mature NK cells and acts as a growth factor for new T cells development by Days 7-11\textsuperscript{76,196-197}. The experiments similarly stimulate immune responses as the recombinant protein experiments suggesting that the gene delivery system is enhancing immune responses against melanoma.

**Tumor Evasion and Importance of Gene Therapy**

One of the major reasons tumors escape anti-tumor control is because they lose MHC class I expression rendering tumors unrecognizable by CTLs due to self tolerance. NK cells can recognize MHCI deficient cells, but the overgrowth of tumors escape lysis\textsuperscript{213}. IL-15 is known to enhance NK cell recruitment and development, it has been
suggested that this cytokine would be beneficial to use as a therapy against MHCI deficient melanoma cells\textsuperscript{213-214}. The purpose would be to effectively stimulate an immune response against poorly immunogenic B16.F10 using a cytokine treatment to enhance tumor regression and survival\textsuperscript{215-216}. Previous studies indicated that neither inactivated B16.F1 or short term pre-incubation with IFNα (B16Fα) introduced into existing disease like melanoma were able to enhance mouse survival\textsuperscript{215}. Although, inactivated and co-inoculation of active B16.F1 cells with IFNα or a long-term exposure to IFNα enhanced mouse survival\textsuperscript{215}. Intraperitoneal (IP) vaccination of B16F1 pre-incubated with IFNα recombinant proteins stimulated an immune response with long term effects, but did not up-regulate long term protective response against B16.F1. The mice had an established tumor at a distant site from inoculation. When treated IP or subcutaneously 3 days after inoculation with B16Fα 53% of mice showed a complete response, surviving 90 days after treatment but no challenge experiments were performed\textsuperscript{215}. An IL-15 transgenic mouse study demonstrated importance of IL-15's ability to stimulate CD8+T cell (CTL) response against inoculation of B16.F10 cells compared to non transgenic mice. The authors suggested that early production of IFNγ by NK cells developed a CTL response against B16.F10 in transgenic mice by over-expressing IL-15. The transgenic mouse enhanced CTL and inhibited tumor establishment, but was not protective against tumor growth\textsuperscript{217}. Some researchers have switched to direct delivery of cytokine therapy using EP to transfect plasmids encoding therapeutic genes directly into existing tumors to enhance tumor regression, mouse survival and long-term protective response.

**IL-15 Used as an Adjuvant**

Previous publications suggest that IL-15 is a useful adjuvant against existing tumors as long as there are pre-existing populations of tumor specific CD8+T cells. They
also suggest that IL-15 has the ability to stimulate preexisting CD8+ T cells through tumor specific CD8+T recall responses\textsuperscript{218}. This was demonstrated in the IHC results showing small presence of T cells at early time points Day 1 and Day 2 in phIL-15+EP+ groups, and a definite increase in T cells by Day 11. There was a robust CD4+ and CD8+T cell population specifically targeting tumor cells and causing regression. The serum levels show an increase in IL-6, MIP-1β, and TNFα causing tumor infiltrate at early time points 12h, 24h, 48h, Day 3, Day 6 and at the tumor level and there was definitely an up-regulation of cellular infiltrate from the blood because IL-1β, IL-6, MIP-1β, IL-12 and IFNγ were recruited to the area by 12h, 24h, 48h, Day 3 and Day6 and at later time points.

It is difficult to measure levels of IL-15 in the serum because expression is highly regulated. Studies have shown that the IL-15 leader sequences must be replaced to enhance protein production in transfected tumor cells to promote tumorigenicity\textsuperscript{214}. This study discusses the importance of CD8+ T cell anti-tumor activity when CD8+T cells are knocked down. IL-15 transfected tumors were injected into CD8+ T cell knockout mice. Initially the mice rejected tumor growth, but over time tumor cells eventually escaped immune effector activity and enlarged rapidly\textsuperscript{214}. Targeting tumors with EP is a beneficial approach because the delivery system allows targeting of specific tissue, efficient transfection tumor cells efficiently allows transient transfection and recruits innate and adaptive immune response to the electroporated area\textsuperscript{26}.

\textit{EP is an Adjuvant Delivery System that Stimulates an Inflammatory Response}

The effect of plasmid delivery using EP stimulated an inflammatory immune response, caused cellular infiltrate, cytokine stimulation and recruitment of T cells for primary response\textsuperscript{219}. EP enhancement of the inflammatory response proved to be beneficial for increased gene delivery to recruited cells during multiple EP treatment
protocols. The more cells that are recruited to the tumor area the more cells that are transfected, results in a more potent induction of protein stimulus and tumor regression as seen in pVax compared to phIL-15 delivery with EP. The difference is that IL-15 was able to stimulate long-term and protective immune response at higher levels than pVax thereby demonstrating specific immune responses induced by IL-15 protein. As mentioned in publications, plasmid+EP+ induces more antigen uptake and cross presentation. T cells are primed by antigens in the T cell zone of draining lymph nodes\textsuperscript{219}. This was indicated during experiments on Day 7, Day 11 and Day 14 in IHC, increased cellular infiltrate on those days indicated that new T cells were being primed and recruited to remove tumor antigens and causing tumor regression. The combination of EP and plasmid acts as an adjuvant to promote immune stimulus. This gene delivery system works synergistically to enhance immune effector cells, stimulating anti-tumor signaling and promoting prophylactic responses\textsuperscript{219}.

\textit{Intra-tumoral Delivery Stimulates Local Response}

Intratumoral EP delivery of IL-15 and IL-6 plasmids into canine melanoma caused tumor regression by up-regulating tumor infiltrating lymphocytes (TILs) in progressing cancer cells. The TILs stimulated high concentrations of IL-6 expression suppressed TGF\textbeta{} activity, and restored NK and IFN\gamma{} activity. Transfection of IL-15 restored NK activity and T cells as well as other growth factors to enhance anti-tumor effects\textsuperscript{168, 220}. IL-15 may require the combination of other cytokines as an additive response against tumor antigens to produce stronger primary and protective immune responses. In addition to protein stimulus, EP itself serves as an adjuvant to enhance immunostimulatory signals by the electrical impulses causing stress and damage to stimulated cells in the area\textsuperscript{221}. As observed in the IHC experiments on Day 11, the 500V/cm group stimulated a robust adaptive immune response recruiting more T cells.
and enhancing IL-15 expression to stimulated protective response. pVax and EP showed similar results of immune infiltrate, but showed less protective response against antigen challenge. The key is to recruit DC, CD4+T cells locally and to stimulate CD8+ CTL response.

Comparing Electroporation Parameters

Dayball et al. illustrated the importance of EP effects in mice using low voltage electric fields with long pulse durations and also demonstrated the importance of stimulating CD4+T cells with EP plasmid delivery\textsuperscript{221}. The experiments in this study compared delivery of pCMVβ luciferase plasmid into the muscle of MHC class II deficient mice using a 2 needle EP applicator at different pulse lengths with same electric field strengths. They tested 200V/cm at 20ms and 200V/cm at 50ms and found that both EP conditions increased luciferase expression. The 200V/cm with 50ms pulse duration EP condition was able to elicit a cellular response in MHC Class II deficient mice while 20ms did not. The 20ms did elicit a cellular response in wild type mice. The 50ms pulses were able to overcome the MHC class II requirement eliciting a CD8+T cell response and bypassing CD4+T cell activation. These observations suggest that MHC Class II presentation to CD4+T cells was required for CD8+T cell expansion\textsuperscript{221}. This paper also suggests that EP activity enhances its own adjuvant properties for eliciting a cellular immune response. The experiments also detected a cellular immune response with delivery of pVax+1300V/cm and pVax500V/cm. There was an up-regulation in CD4+T cell stimulation in the EP groups compared to Injection alone and no treatment groups as seen in IHC. Poorly immunogenic such as B16.F10 do have CD4+T cells present and can help stimulate and recruit CD8+ T cells to the tumor area and enhance CTL activity\textsuperscript{218}. The rapid tumor regression observed by Day 14 was due to the phIL-15+1300V/cm and phIL-15+500V/cm groups, which enhanced both CD4+ and CD8+T
cells response to promote rapid tumor regression, improve mouse survival and possibly support a long-term immune response against B16.F10 challenge. This indicates in this mouse model the presence of CD4+T cell stimulation is important for up-regulating CD8+T cells. Additionally, the stimulation of CD8+ T cells was maintained by the presence of TNFα, IFNγ, and IL-12 in the tumor, serum and circulating lymphocytes.

**EP Stimulates an Adaptive Immune Response**

EP itself helps break tolerance for immune cells that do not recognize evading tumor antigens\(^{222}\). The stress and damage caused by EP at 500V/cm does stimulate cells to undergo apoptosis and promotes macrophage phagocytosis to removing tumor cells. Macrophages release of cytokines to recruits APCs and stimulates residential CD4+T cells to present and stimulate CD8+T cells causing an up-regulation of CTL cytotoxicity activity and apoptosis by TNFα\(^{222}\). The data shows that TNFα is stimulated at the tumor and serum levels causing tumor necrosis in EP groups as early as Day 3 and Day 6. It is important to cause tumor necrosis, apoptosis and CTL response not only at the tumor levels but systemically\(^{222}\). Delivery of IL-15 at early time points may have stimulated NK activity which amplified the signal stimulating IFNγ production and activation of primed T cells. The process of eliminating subcutaneous melanoma has been previously demonstrated by Lucas *et al.*, This publication showed that pmIL-12 delivered using EP into the tumor increased IFNγ, CD4+ and CD8+Tcells promoting a 80% response against primary tumors and 100% protective response against challenge\(^{28}\). These results were comparable to the results from the IL-15 experiments presented here which showed a 73.3%-79% response (Table 2) to primary tumors and 75%response to challenge (Table 3).
Restimulation of Immune Surveillance with phIL-15 Delivery and EP

Tumors resemble normal tissue, derived from self cells and they maintain normal non-self tolerance to the immune response. Tumor antigens are identified by the following characteristics: antigen mutations, germ-line silenced genes, over expressed tumor antigens, oncogenic viral antigens and tissue-specific differentiated antigen\textsuperscript{222}. The goal of a therapeutic approach, like the one presented in Aim 2, is to establish an immune response using a system that will remove cells expressing tumor antigens. The goal is to protect the body from tumor recurrence and re-stimulate effector function to tolerant cells, inducing sufficient concentration of immune cells in the tumor area that will recognize tumor antigens and cause extensive lymphocytic infiltration of cells to tumor stroma\textsuperscript{222}. EP is an ideal way to introduce genes of interest like IL-15 into tumors without invasive surgeries or dangerous side effects. EP delivers genes to cells and does cause stress and damage to cells, depending on the cell type and EP parameters. The phIL-15+EP+ multiple treatment protocols helped to enhance short-term IL-15 protein expression and, maintain IL-15 expression over time in a controlled manner and prevents toxic effects due to over expression of protein. Most importantly the multiple treatment protocol recruits immune cells to the area causing tumor regression. IL-15 is highly regulated; this regulation may help or hinder cytokine therapies. IL-15 stimulates a controlled amount of cytokine production that may reduce the survival of CD8+T cell memory and limit short term/long term memory expansion in the mice. Based on the experimental results IL-15 combined with EP can potentially serve as an adjuvant therapy to potentially treat existing disease such as melanoma.
FUTURE DIRECTIONS

3D Tumor Model

The 3D tumor model is an adaptable construct that can be modified to fit different experimental interests such as wound healing, tissue regeneration, transplantation, cloning, drug delivery techniques and genetic manipulations\(^4^2,18^7,19^0,22^3\). The 3D model can also be used to study different cellular behaviors when subjected to salt, pH, protein concentrations, ligand/receptor concentration, temperature, adhesive properties, and oxygen solubility\(^4^3\). This 3D model can help in elucidating the differences in cellular properties when grown in a 3D environment or as a monolayer of cells in culture, in the hopes of discovering new information about cells grown \textit{in vitro}. Also, this model can be utilized to determine how tumor cells react in the presence of other stimuli that can promote or inhibit tumor development. For example, this model can aid investigators study proteins that are present during tumorigenesis such as FGF, interleukin 8, and VEGF\(^4^3-4^4,5^1-5^3,5^6,22^4\). Moreover, the 3D model would be a useful tool to understand how tumor cells react in the presence or absence of immune cells and compare to reactions with wild type, knockout, transgenic, NUDE or SCID mice models\(^5^3\). Future studies could be adaptable to clinical therapies such as plastic surgery, organ transplantation, species specific diseases, and toxicity of drugs\(^4^3,5^2\).

To further improve the model, the HaCaT spheroids could be reinforced by using different concentrations of growth factors, using a network of collagen type I and IV or introducing a heterogeneous population of cells such as fibroblasts to stimulate cell communication\(^4^3\). Fibroblasts have many signaling properties useful for skin and tumor
development. The heterogeneous population of cells could help in the study of the expansion, growth cycles and development of B16.F10 in the absence of a basement membrane and stroma. Introduction of an engineered-linker could help facilitate drug delivery or enhance nutrient exchange to cells residing in the center of the model. To further test functionality of the 3D tumor model and explore the activity of E to N cadherin-switch, angiogenic STAT3/5 pathways and VEGF production to study metastatic transformation is also possible in vitro. Additionally, the 3D model could be used to study specific gene expression, mutations, and cell signaling such as autocrine loops that regulate tumorigenesis. The 3D model is a highly reproducible system that could be used in many studies such as to optimizing EP delivery conditions before moving to in vivo animal studies and thereby reducing the use of animals for experimentation. The information gathered thus far from the 3D model indicated it can provide a wide range of information and has the potential as an adaptable model to mimic cellular in vivo processes.

**phIL-15 Delivery +EP Enhance Immune Stimuli**

The delivery of IL-15 using EP is a promising approach that could be an effective gene therapy option for the treatment of melanoma. Additional pre-clinical experiments should be performed to ensure its functionality. Based on experiments detailed in Aim 2, non-responsive, abnormally shaped tumors or deep tumors could escape immune control between Day 9 and Day 12. One option to avoid the pitfall is to increase the number of treatment regimens of IL-15, 2 or 3 separate treatments additional to the 3Tx protocol using low voltage and long pulse durations.

The delivery of phIL-15 using EP conditions at 500V/cm 20ms provided the strongest immune response against existing B16.F10, but was the most damaging. The
damage actually assisted in recruiting more cells to the area for plasmid up-take as seen in pVax+500V/cm group. To reduce damage and maintain delivery efficacy, additional EP conditions should be explored with the goal of stimulating IL-15-mediated tumor regression, but reducing tissue damage. Varying the delivery conditions could possibly promote different cellular responses to EP stimulus. One suggestion for future studies is to deliver the IL-15 with 500V/cm 20ms on the first treatment and then on the second and third treatment use milder conditions such as 804V/cm 400µs or 300V/cm-400V/cm with 20-50ms. The end goal of this therapeutic approach is to stimulate a strong initial immune response with IL-15 and then maintain expressions levels of the protein to promote stable immune cell recruitment. The expression data demonstrated that the highest levels of IL-15 protein were recorded at the earliest time points 12h or 24h and then decreased at 24h or 48h depending on the EP conditions. Each plasmid reacts differently to EP stimuli. The 3D model could be used to provide information about expression levels for optimization of IL-15 expression prior to moving farther with in vivo studies. For IL-15 experiments, it seemed as though IL-15 expression reached a threshold with the strongest EP condition 500V/cm and did not express higher levels with additional boosts. This suggests that inherent tight regulatory mechanisms that control IL-15 protein levels inhibited further expression.

Different Cytokine Combination Delivery Systems

It would also be important to evaluate different cytokine combinations that could complement IL-15 signaling and produce a synergistic response. Some suggested combinations are IL-15 plus IL-15Rα to enhance the availability of the high affinity IL-15Rα for IL-15 when co-expressed in the same cells when either bound to the membrane or secreted as an active, soluble form for trans-presentation to receptive
Another option is to use plasmid IL-12 encoding the mouse IL-12 delivered by EP increased expression, and based on previous studies, enhanced immune response\textsuperscript{28}. The combination of IL-15 and IL-12 could possibly promote tumor regression, CD8+T cell survival and memory responses. Researchers have demonstrated that some CD8+ memory T cells are long lived while others are short lived\textsuperscript{195}. To enhance memory, researchers combined IL-15 and IL-7 because IL-15 stimulates the survival of CD8+T cells and the short-lived memory, while IL-7 stimulates the long lived memory precursor CD8+T cells. The combination of IL-15, IL-7 and possibly IL-12 may enhance the innate, adaptive and memory responses resulting in a robust response against melanoma. The timing of delivery, plasmid concentrations and routes of delivery will be important to optimize for future immunological studies. The future of the immunogene therapy field may be in these combination delivery systems, to enhance multiple signaling pathways to mount a protective response against recurring tumors.
LIST OF REFERENCES


31. Ugen KE, Kutzler MA, Marrero B, Westover J, Coppola D, Weiner DB, Heller R, Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-


APPENDIX A: CYTOKINE EXPRESSION TABLES
Table A1: Cytokine expression from Multiplex Luminex Array testing tumor lysate measuring Average Total pg and Standard Deviation (SD).

<table>
<thead>
<tr>
<th>Tumor lysate</th>
<th>Avg Total pg</th>
<th>IL-1β SD</th>
<th>IL-10 SD</th>
<th>IL-2 SD</th>
<th>IL-6 SD</th>
<th>IL-4 SD</th>
<th>IL-12 SD</th>
<th>GM-CSF SD</th>
<th>MIF-1p SD</th>
<th>TNFα SD</th>
<th>IFNγ SD</th>
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<td>No Tx 24 hours</td>
<td>4.96 5.69 48.19 33.22 1.45 0.12 100.99 115.49 59.30 55.18 49.96 70.62 106.03 53.52 8.08 0.67 1.60 0.13 71.10 110.7</td>
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<tr>
<td>pVax Injection 24 hours</td>
<td>2.48 0.74 29.33 7.07 2.23 0.67 92.32 64.12 9.01 10.82 2.48 0.74 11.35 14.56 12.48 3.74 2.48 0.74 24.76 31.77</td>
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<tr>
<td>pVax 1300V/cm 24 hours</td>
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<td>pVax 500V/cm 24 hours</td>
<td>4.66 3.83 55.49 38.61 1.84 1.03 6611.97 6007.86 15.96 16.70 43.26 32.00 218.50 74.96 10.31 5.78 2.06 1.15 63.24 40.28</td>
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<tr>
<td>IL-15 injection 12 hours</td>
<td>26.06 18.36 29.18 17.30 1.00 0.37 284.64 53.65 71.72 103.30 19.09 22.75 1379.68 1129.79 7.90 2.84 1.11 0.41 4.97 4.99</td>
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<td>IL-15 1200V/cm 12 hours</td>
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<td>IL-15 500V/cm 24 hours</td>
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### Table A2: Cytokine expression from Multiplex Luminex Array testing tumor lysate measuring Average Total pg and Standard Deviation (SD).

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<th>IL-3 SD</th>
<th>IL-4 SD</th>
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<th>GM-CSF SD</th>
<th>MIP-1α SD</th>
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**Table A5: Cytokine expression from Multiplex Luminex Array testing serum, measuring Average pg/mL and Standard Deviation (SD).**
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ABOUT THE AUTHOR

Bernadette Marrero obtained her undergraduate degrees from the University of South Florida during 2002-2003, double-majoring in Biology and Chemistry. Her motivation and enthusiasm for science and research lead her to seek a graduate degree in the Medical Sciences. She was accepted into the Multidisciplinary Biomedical Science Ph.D. program at USF in 2004. She studied gene delivery methods to target cancer as a potential immunotherapy and she generated an in vitro 3D tumor model with the help of her advisor. She was awarded a Ruth L. Kirschstein Fellowship through the National Institutes of Health on 2007 to fund her graduate school stipend and research costs. She also obtained many travel grants to offset travel costs for the opportunity to present her research at national and international conferences. She hopes to continue her research interest in cancer therapy.