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MANIPULATION OF THE IMMUNE SYSTEM TO INCREASE ANTI-TUMOR RESPONSE AGAINST OVEREXPRESSED HER-2/NEU PROTEIN

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By

Horacio Soto

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ABSTRACT

MANIPULATION OF THE IMMUNE SYSTEM TO INCREASE ANTI-TUMOR RESPONSE AGAINST OVEREXPRESSED HER-2/*NEU* PROTEIN

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Current treatments for cancer include surgery, radiation, and chemotherapy. Although these treatments have improved over the past two decades, prognosis for patients with malignancies remains poor. Immunotherapy appears to be a promising treatment option for cancer patients. In immunotherapy, a patient's immune system is used to elicit an immune response against specific antigens found on tumor cells. Dendritic cells (DC) as professional antigen-presenting cells (APC) play a vital role in activation of $CD8^+$ T_C cells via class I major histocompatobility complex (MHC). This interaction is crucial in developing a cytotoxic response against malignant cells.

The purpose of this study was to determine how effective DC would be in activating a $CD8^+$ T cell, via an MHC class I pathway, when pulsed with either soluble Her-2/*neu* or Her-2/*neu* bound to polystyrene beads. Additionally, the maturity of the DC were compared to determine how immature versus mature DC would effect activation. The use of mature DC pulsed with Her-2/*neu* coated beads resulted in increased antigen specific activation of $CD8^+$ T cell at much lower concentrations when compared to immature DC tested in the same manner. Activation using Her-2/*neu* coated beads was superior to soluble Her-2/*neu* or ovalbumin alone. This study illustrates the importance of DC maturity as well as the mode of antigen delivery required to elicit a specific cytotoxic immune response. The results of this study indicate a promising outlook in developing specific immunotherapies for malignancies and thus warrant further investigation.

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This thesis follows the format prescribed by *The Publication Manual of the American Psychological Association* and the Department of Biology.

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INTRODUCTION

Background

The American Cancer Society defines cancer as "a group of diseases characterized by uncontrolled growth and spread of abnormal cells" (American Cancer Society, 2009). Death may be an end result if the spread of abnormal cells is not controlled. Cancer may be caused by either external factors (tobacco, chemicals, radiation and infectious organisms) or internal factors (inherited mutations, hormones, immune conditions, and metabolism caused mutations), these factors may even act together or in series to promote carcinogenesis.

It is estimated that 1,479,350 new cancer cases will be diagnosed in 2009, of these 192,370 new cases of invasive breast cancer cases are expected to occur among US women (American Cancer Society, 2009). Breast cancer is the most commonly diagnosed cancer in women. The American Cancer Society has estimated that in 2009 breast cancer will account for 40,610 deaths, with 40,170 of these individuals being women, and 440 of them being men (American Cancer Society, 2009). The most current data indicates that the mortality in women as a result of breast cancer has dropped approximately 2.2% per year from 1999 to 2005 in all women, with the greatest decreases seen in women younger than 50 years of age. Despite of all the advancements in screening and treatment, breast cancer still remains the second leading cause of death in woman (American Cancer Society, 2009). The current five year relative survival rate for localized breast cancer, breast cancer that has not spread to surrounding tissue or organs is reported to be approximately 98%. In patients whose tumors have metastasized, the survival rate decreases dramatically to only 27% (American Cancer Society, 2009). The

survival rate for all breast cancer groups decreases past five years, with a ten year survival rate of 81%, compared to an overall breast cancer survival rate of 89% at five years (American Cancer Society, 2009).

The early onset of breast cancer can be detected by mammograms. Mammograms can usually detect abnormalities before they can be detected by either the woman or a health care provider. The American Cancer Society's screening guide lines recommends yearly mammograms starting at age 40. If the patient declines to obtain the yearly mammogram abnormalities and complications may develop. If cancer is left undiagnosed it can grow to the point where physical signs and symptoms can be observed. The signs and symptoms may include a breast lump, thickness, swelling, distortion, or tenderness; it is also common to see skin irritation or dimpling, nipple pain, scaliness, ulceration, retraction or spontaneous discharge (American Cancer Society, 2009). Breast pain has been observed in some cases, but usually it is due to other conditions and it is not generally related to breast cancer.

The risk of being diagnosed with breast cancer increases with age. The most important factors that increase the risk of this type of cancer in women include inherited genetic mutations, a family history of breast cancer, biopsy confirmed hyperplasia, especially atypical hyperplasia, and the tissue density of the breast (a mammographic measurement used to determine the amount of glandular tissue relative to fatty tissue within the breast) (American Cancer Society, 2009).

It has been reported that mutations, specifically mutations that occur on chromosome 17 are associated with HER-2/*neu* positive breast cancer. HER-2/*neu* positive breast cancers account for 20-30% of all reported cases and are linked to poor

prognosis because they do not respond to conventional treatment. The mutations that cause HER-2 overexpression occur on the long arm of chromosome 17 (17q21) which encodes for a 185 KDa cytoplasmic membrane glycoprotein that is involved in tyrosine kinase signal transduction for epithelial cell proliferation and known as either HER-2/*neu* (Human Epidermal Growth Factor Receptor 2/*neu*) proto-oncogene or c-erbB-2 (RefSeq NM_001005862) (Schippinger et al., 2004). The abnormal HER-2/*neu* gene contains additional exons at the 5' end and lacks an alternate 5' noncoding exon, when compared to the normal gene. HER-2/*neu* gene amplification or receptor overexpression has been reported in approximately 20-30% of human primary breast cancers and in over 90% of cases, HER-2/*neu* oncogene overexpression may be a result of HER-2/*neu* gene amplification (Schippinger et al., 2004; Wang, Saboorian, Frenkel, Hyman, Gokaslan, & Ashfaq, 2000). This amplification causes an increase in the number of gene copies in the cell, resulting in an increased concentration of the subsequent mRNA and eventually an increase in HER-2/*neu* oncoprotein. Evaluating the status of HER-2/*neu* has gained importance in the clinical assessment of patients with breast cancer, especially since HER-2/*neu* overexpression or amplification in node positive cases has been linked to poor prognosis, including short-ended disease free periods and decreased overall survival time, possibly due to a resistance to conventional therapy (Schippinger et al., 2004). Determining the status of HER-2/*neu* in patients with breast cancer enables healthcare providers to better predict the response of the patient to specific treatments and may guide the course of treatment the patient will receive.

Treatment for breast cancer may vary depending on tumor size and tumor characteristics. Treatments may include two or more methods in combination. Surgery is

one of the options healthcare providers and patients consider when treating breast cancer. The surgical options include procedures such as lumpectomy (removing only the tumor), mastectomy (removal of the entire affected breast), as well as excising some of the axillary lymph nodes, which are used to obtain precise information regarding the stage of the disease. Other treatments include radiation therapy, chemotherapy, and hormone therapy (American Cancer Society, 2009). Patients who are diagnosed with HER-2/*neu* positive breast cancer may be treated with the immunotherapeutic agent trastuzumab (Herceptin). Herceptin is a humanized anti-HER-2/*neu* monoclonal antibody which can be used not only for HER-2/*neu* positive breast cancer but also for reoccurring breast cancer or progression during chemotherapy (Selvarajan, Bay, Chng, & Tan, 2004).

Immunotherapy is a newer concept that is being considered as a treatment option against many different types of cancer. The basis of immunotherapy is stimulating the patient's immune system in order to initiate an attack against malignant tumor cells. An ideal immunotherapy for cancer would be tumor-specific, easy to construct, and safe when it is administered to patients. It would allow patients diagnosed with a tumor to be treated based on specific proteins that are expressed by the tumor, allowing for a site-specific cytotoxic response. Through the use of antigen presenting cells, specifically dendritic cells (DC), a cytotoxic response may be initiated against a specific tumor allowing for little to no undesirable side effects.

Dendritic cells are highly specialized professional antigen-presenting cells (APC) that have unique morphological and molecular properties enabling them to be efficient initiators and modulators of the immune system. They are found in all tissues and in the blood in extremely small numbers and they make up about less than 0.1% of all the

circulating leukocytes. Dendritic cells are capable of internalizing foreign matter by micropinocytosis and phagocytosis. Upon internalization of foreign matter dendritic cells can process antigen into short peptides that can be presented on either major histocompatability complex (MHC) I or II (Banchereau & Steinman, 1998).

The term Dendritic cell (DC) was proposed by Steinman and Cohn (1973), where they reported the morphology and the sites in which these cells could be found. The authors went into great detail in describing the cell's morphology: they describe them as having, "a large nucleus, refractile, contorted in shape, contain small nucleoli (usually two); abundant cytoplasm, processes that varied in length and width and the cells contain many large spherical mitochondria". Based on their findings Steinman and Cohn reported that in mice, the spleen was the most abundant source of dendritic cells, comprising approximately 1.0-1.5% of the total nucleated cell population. They also reported that primary lymphoid organs such as Payers patches and lymph nodes contained only a small number of dendritic cells, while the other primary lymphoid organs, bone marrow, intestine and thymus, did not contain dendritic cells.

In 1986, Bowers and Berkowitz conducted a study where they were able to determine that DC were not detectable in fresh preparations in bone marrow cultures. They did however notice that DC arise from bone marrow cultures after short term culturing, indicating that DC originate from bone marrow. They also observed that DC originated from a precursor cell within bone marrow, which had a number of distinguishable properties that did not resemble DC in any way. It is now known that a number of cells that comprise the immune system are made in the bone marrow from pluripotent hematopoietic stem cells through a process known as hematopoiesis. All

blood cells are derived from pluripotent hematopoietic stem cells, these cells can divide without differentiation to renew themselves, and they can also differentiate into a number of more mature cells, such as DC. The differentiation of pluripotent hematopoietic stem cells is regulated by cytokines (Thomas & Lipsky, 1996). Depending on the cytokine signal received, pluripotent hematopoietic cells in the bone marrow will differentiate into a variety of mature cell types. It has been shown that pluripotent hematopoietic stem cells differentiate into DC in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF), which has been reported to play an important role in the development and maturation of DC (Kampgen et al., 1994).

Dendritic cells are found in an "immature" and "mature" state. The "maturation" state is a functional term given to DC in order to describe them in a given moment and site that describes a preference for either the ability to process antigens or the ability to migrate and activate T cells. Subsequent data has shown that the majority of DC reside in primary lymphoid organs, such as the spleen and thymus, as well as in the skin and the mucosa. Once immature DC have encountered and internalized antigens they undergo changes ("maturation") including down regulation of protein, increased antigen processing capacity and ability to migrate to regional lymph nodes where antigenic-epitopes are presented to T lymphocytes (Inaba et al., 1992).

Studies have shown that the addition of granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-alpha (TNF-α) to a culture containing human hematopoietic progenitor cells, stimulated the growth of a subset of monocytes and cells that resembled DC, functionally and phenotypically. When the human hematopoietic progenitor cells were grown in the presence of GM-CSF alone,

there was an increase in the quantity of monocytes within the culture and no increase in DC proliferation was observed. The cultured DC grown in the presence of GM-CSF and TNF-α were found to be as active as other purified DC. This was a great success, in that it shows that human DC are derived from hematopoietic progenitor cells, and that these cells can also be used to conduct immunological studies (Koch, Heufler, Kampgen, Schneeweiss, Bock, Schuler, 1990). However, recent studies have shown that in mice, GM-CSF alone, is sufficient for the proliferation and differentiation of DC from their hematopoietic progenitor cell precursors (Thomas & Lipsky, 1996). Inaba et al. (1992) described a method in which mouse DC can be cultured from bone marrow; this method requires the depletion of lymphocytes, macrophages, and granulocytes and the culturing of the remaining cells in GM-CSF for seven days.

Dendritic cells have been recognized as the initiators and the modulators of the immune system. Dendritic cells are effective educators of B and T lymphocytes. Unlike B cells that can become activated by antigen complexing with their cell surface receptors, T cells require antigen to be processed and presented to them by antigen processing and presenting cells (Banchereau & Steinman, 1998). Dendritic cells are professional antigen processing and presenting cell and have a unique ability to initiate an immune response *in vivo* (Caux et al., 1996). *In vitro* incubation of DC with tumor lysates or transfection of genes encoding for tumor-associated antigen results in the generation of DC that activate T-cells. Dendritic cells express high levels of antigen-presenting molecules (MHC class I or II), which enables the internalization and amplification of a specific immune response to target antigens (Caux et al., 1996).

Dendritic cell vaccination experiments in animals have prevented growth of transplanted cancer cells and reduced the size and growth of established tumors (Mayordomo et al., 1995). In order for the immune system to attack cancer cells or tumors, antigens must first be presented via the MHC class I pathway to cytotoxic T cells (T_C cell) for activation (Diegel, Chen, Laus, Graddis, & Vidovic, 2003). Dendritic cells loaded with tumor proteins are believed to be capable of presenting antigens through both the MHC class I and class II pathways. The fact that dendritic cells can process exogenous antigens and present them in the MHC class I pathway, indicates that DC can carry out a process that has been termed cross-priming or cross-presentation. The presentation of antigens in the MHC class I pathway is key in activating cytotoxic T cells. It has been shown that only lymphoid dendritic cells cross-present antigens, and therefore these cells are essential for the initiating of a cytotoxic T lymphocytes (CTL) response (den Haan, Lehar, & Bevan, 2000). Therefore, isolation of dendritic cells from a lymphoid organ, and subsequent loading of exogenous antigen (i.e. tumor proteins) must be completed *in vitro* before they can cross-present and initiate the differentiation of naïve CD8 T cells into CTL.

Dendritic cells have many properties that enable them to have great surveillance capabilities. By introducing proteins to the immune system that are specific to either tumors or tumor cells it may be possible to initiate early surveillance against tumors or tumor cells that would normally be overlooked because in early tumorgenesis the cells are not recognized as foreign. Developing an effective way to introduce tumor associated antigens into DC and using their ability to cross-present and activate CTLs, a protective

and therapeutic anti-tumor treatment may be developed allowing for patients to have an additional treatment option to consider when dealing with the discovery of a tumor.

Experimental Design & Objectives

The purpose of this study was to determine the effect that peptide-loaded DC will have when co-cultured with a T cell hybridoma, B3Z, which is able to express β-galactosidase when activated via an MHC class I pathway. The DC were loaded with either soluble protein or protein attached to microspheres. Given that APC specifically DC, are believed to take up particulate matter more efficiently than soluble protein, we hypothesized that attaching the proteins to the microspheres allowed for a greater up-take of protein and thus a greater immune response. This immune response was measured by using a T cell hybridoma that is activated by SIINFEKL, an eight amino acid sequence from chicken ovalbumin. This sequence was incorporated into the amino acid sequence of a recombinant form of HER-2 protein, referred to as EVI.

MATERIALS and METHODS

Production of Colonies

Escherichia coli transfected with the plasmid vector pTriex (Novagen) into which EVI DNA had been cloned was obtained from Dendreon Corporation of Seattle, Washington. These cells produced a recombinant protein known as HER-2/*neu*. The acronym EVI stands for External - OVA – Internal, where the external and internal refers to the extracellular and intracellular portions of the HER-2/*neu* receptor. The OVA portion within EVI refers to SIINFEKL, amino acid residues 257-264 of chicken

ovalbumin which are recognized by the B3Z CD8+ T cell hybridoma. The recombinant protein was also engineered with a six-histidine tag, which was used to isolate the protein during the purification process. Using sterile technique, *E. coli* cells were streaked on a plate containing LB agar with chloramphenicol (34mg/ml) and carbenicillin (100mg/ml). The plate was incubated at 37°C for 48 hours. Archive plates were sealed in parafilm and stored at 4°C for future use.

Culture Cultivation

From the archive plate, a single isolated and viable bacterial colony was obtained on the end of a sterile toothpick and used to inoculate a 25 ml volume of LB (Luria-Bertani) broth with carbenicillin (100mg/ml) and chloramphenicol (34mg/ml). This "overnight" culture was placed on a shaker at 200 rpm in a 34^{o}C incubator until the solution was cloudy. From this culture, 1 ml was removed and added to a 2 L Erlenmeyer flask containing 500 ml LB broth with antibiotics, as previously described. A second flask was prepared using the same method to produce a total of 1 L of culture. Flasks were incubated at 34^{o}C while shaking at 200 rpm until cloudy. A spectrophotometer set to 600 nm absorbance was used to find the optical density (OD) of the broth. When the Abs_{600} reached 0.5, which is equal to 5 x 10^5 colony forming units/ml, 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to the culture and allowed to continue incubating for an additional two hours. These cultures were divided into 4-500 ml Nalgene centrifuge bottles and centrifuged using the Sorvall RC-5B Plus centrifuge at 10,000 g using the SLA-3000 rotor at 4°C. Pellets from 500 ml of broth were combined and stored at -20°C.

Isolation and Purification of Inclusion Bodies

The combined bacterial pellets were removed from the freezer and allowed to thaw. Cells were thoroughly resuspended using 40 ml Buffer A (50 mM Tris, 25% Sucrose, pH 8.0) per bottle. The cells were then lysed by addition of 100 ml of Buffer B (20 mM Tris, 100 mM NaCl, 1% Deoxycholate, 1% Triton X-100, 0.3mg/ml Lysozyme, pH 8.0). The suspension was shaken for 10 minutes at room temperature using horizontal shaker. Following the incubation, 0.2 µl/ml Benzonase and 1mM $MgCl_2$ were added to each bottle. The suspension was allowed to stir for an additional five minutes, a marked decrease in viscosity was noted. The samples were centrifuged at 20,000 g for 15 minutes at 4°C and supernatant was discarded. Pellets were resuspended in 25 ml of Buffer C (20 mM Tris, 100 mM NaCl, 1% Triton X-100) and additional Buffer C was added to bring the total volume to 200 ml per bottle.

Following centrifugation at 20,000 g for 10 minutes, 4°C, the supernatant was discarded. This resuspension-centrifugation step involving Buffer C was repeated three additional times. Upon completion of the centrifugation step, the pellets were resuspended in 200 ml per bottle of Base Buffer (20 mM Tris, 100 mM NaCl) and spun at 20,000 g for 10 minutes at 4°C, and the supernatant was discarded. The pellets were then resuspended in 25 ml Base Buffer and centrifuged at 20,000 g, 10 minutes, 4°C, after which the supernatant was removed and inclusion bodies were stored at -20°C.

Solubilization of Inclusion Bodies

Inclusion bodies samples were removed from -20°C and were allowed to thaw at room temperature. Once the samples had thawed they were resuspended in 20 ml

solubilization buffer (20 mM Tris, 6M Guanidine HCl, 0.5 M NaCl, 10 mM imidazole, pH 8.0) with 5mM β-mercaptoethanol. The pellets were vortexed vigorously to ensure resuspension of the pellet. Tubes were then incubated at room temperature for 55 minutes while shaking. Following incubation the tubes were centrifuged at 10000 x g, 10 minutes, 4°C. The solubilized inclusion bodies (supernatant) were saved, and passed through a 0.22 μm cellulose acetate filter and stored at -20°C.

FPLC Isolation of EVI Recombinant Protein

Recombinant protein was purified by passing over a 5 ml Hitrap chelating column (GE Healthcare) which was loaded with $NiSO_4$ (100mM) following the manufacturers protocol using FPLC. The pumps were washed with sterile H_2O, then separately washed: pump A was filled with solubilization buffer (20 mM Tris, 6M Guanidine HCl, 0.5 M NaCl, 10 mM imidazole, pH 8.0), and pump B was filled with elution buffer (20 mM Tris, 6 M Guanidine HCl, 0.5 M NaCl, 300 mM Imidazole, pH 8.0).

In order to ensure the column was free of unwanted protein a mock elution was performed using elution buffer. While the mock elution was underway, 10 ml of protein sample was injected via syringe into a 10 ml loop. The loading of protein to the loop was performed a second time in order to bring the total sample size loaded on the Ni-column to 20 ml. To prevent the loss of any protein the fraction collector was set to collect 10 ml per tube at the time the protein was injected into the column. The fractions were saved and labeled “flow thru”. After the second injection of protein into the loop, 20 ml of buffer was pumped through the column before the fraction collector was reset to 1 ml per

tube. The column was then flushed with solubilization buffer (20mM Tris, 6 M Guanidine HCl, 0.5 M NaCl, 5mM β-mercaptoethanol, 10 mM imidazole, pH 8.0), samples were saved from this elution and labeled 20 mM. The final rinse for the column was performed with elution buffer (20 mM Tris, 6 M Guanidine HCl, 0.5 M NaCl, 300 mM Imidazole, pH 8.0); these samples were collected, pooled, and labeled 300 mM. The samples were then combined based on the absorbance values (Fig. 1). Upon completion EDTA (2 μM) was added to the 300 mM sample. The samples were stored at -20°C. The purity of the sample was determined by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in NuPAGE® 4-12% Bis-Tris Pre-Cast gels (Invitrogen). A standard BCA assay was performed in order to determine the approximate concentration of the 300 mM sample. The standard curve was prepared by performing serial dilutions of 1mg/ml of bovine serum albumin (BSA) to phosphate buffered saline (PBS), (0 mg/ml BSA).

Refolding of Isolated Recombinant Protein

The 300 mM imidazole samples were removed from the -20°C and allowed to thaw completely. Just prior to refolding, DTT was added to 2 mM; the concentrations of the samples were determined using the extinction coefficient for HER-2 with the incorporated ovalbumin (SIINFEKL), using the Shimadzu spectrophotometer at 280 nm (extinction coefficient for HER-2 + SIINFEKL measured at 280 nm in water was 41715 M^{-1} cm^{-1}). The sample was then prepared as follows: 2μl of DTT were added to 500 μl of the protein sample; to this, 498 μl of buffer (20 mM Tris stock, 6 M Guanidine HCl, 0.5 M NaCl, and 2 mM EDTA) was added, and the entire solution was incubated at 37°C for 30 minutes. Following the incubation, additional buffer was added to bring the total

volume to 15 ml, and this solution was dialyzed using a Pierce Slide-a-Lyzer dialysis cassette with a MWCO of 10 kDa.

The protein sample was then dialyzed against renaturation buffer (100 mM Tris stock, 0.5 M L-arginine, 2 mM EDTA, pH 8.0, with 2.5 mM reduced glutathione and 0.5 mM oxidized glutathione added just prior to use) for 13 hours at 4°C with constant spinning to ensure circulation of buffer. This was replaced after the 13-hour period with fresh 4°C renaturation buffer, and allowed to stand for an additional 9 hours. Afterwards, the buffer was replaced with 1x PBS and left for 3 days at 4°C.

Forced Protein Adsorption on Polystyrene Microspheres

The polystyrene microspheres used in this study were purchased from Bangs Laboratories Inc. The manufacture's protocol for forced adsorption was followed. A 7 ml bead and adsorption buffer (PBS: 10.4 mM dibasic potassium phosphate and 1.8mM monobasic sodium phosphate) solution was prepared by combining 0.7 ml of beads and 6.3 ml of adsorption buffer. The pH of the solution being used for each individual protein was adjusted according to the iso-electric point (pI) for that specific protein (pI of EVI = 5.63 and pI of OVA= 5.19) used in the study. Based on the specifications and the information provided by the manufacturer the microsphere surface saturation was determined to be between 720 μg – 2400 μg of protein. The beads and the adsorption buffer were placed into a flat-bottom test tube and stirred for 10 minutes to ensure proper mixture of the solution. The desired amount of protein was added after 10 minutes of stirring and was allowed to continue stirring for an additional 30 minutes at room temperature. Upon completion of the 30 minute incubation, a sample was taken and

viewed under the microscope. If agglutination was seen, the sample was then incubated at 37^{o}C for an additional 30 minutes. If no agglutination was seen, the samples were then incubated at 4^{o}C overnight while stirring. The following day, the samples were removed from the stir plate, and the beads were allowed to settle for 24-48 hours. The supernatant was then tested by performing a BCA assay (Pierce) in order to determine the amount of free protein that was not attached to the microspheres.

Isolation of Dendritic Cells from a Secondary Lymphoid Organ

The experiments involving the use of vertebrate animals were all conducted with the approval of Northern Michigan Universities, Institutional Animal Care and Use Committee.

A female H2-K^{b} positive C57BL/6 mouse was euthanized by cervical dislocation, and the spleen was removed using aseptic technique. The spleen was placed in a Petri dish containing 10 ml of complete RPMI-1640 (RPMI-1640, supplemented with 100 ml FBS, 10 ml kanamycin, 10 ml nonessential amino acids, 10 ml sodium pyruvate, 10ml HEPES, 10 ml β-mercaptoethanol, sterile filtered using a 0.22 μM filter) . In a class II biosaftey cabinet, two sterilized, frosted end microscope slides were rinsed in sterile PBS and used to homogenize the spleen using the rough side of the frosted end of the slides. The grinding of the spleen was done over a Petri dish containing the 10 ml of complete RPMI-1640. The cells were collected and placed in a 15 ml centrifuge tube. The Petri dish and microscope slides were then rinsed with 5 ml of media which was added to the 15 ml centrifuge tube. The sample was centrifuged at room temperature for 5 minutes at 400 g. The media was then removed and the cell pellet was resuspended in 1 ml of red

blood cell lysing buffer (Sigma-Aldrich) per spleen, and incubated for 1 minute at room temperature. An additional 14 ml of media was then added and the sample was centrifuged as before. The cells were resuspended in 7 ml of complete RPMI-1640 per spleen and transferred to a 25 ml cell culture flask. The cells were then incubated at 37^{o}, 5% CO_2, for 2-4 hours. Once incubation was complete the media and non-adherent cells were removed by aspiration, and 10 ml of complete RPMI-1640 was added to the flask. The cells were harvested by using a 25 cm cell scraper and scraping the flask. The cells were collected and placed in a 15 ml centrifuge tube were they were centrifuged and resuspended in 5 ml of complete RPMI-1640 per spleen.

Preparation of Bone Marrow Derived Dendritic Cells

A plasmacytoma cell line (J558) which had been transfected with the murine GM-CSF gene cloned into a mammalian expression vector was allowed to thaw at room temperature under a sterilized class II biosaftey cabinet. The J558 cells (1 ml) were then transferred into a 15ml centrifuge tube using sterile technique with an additional 9 ml of IMDM media (Invitrogen Corporation, Carlsbad, CA). The tube was centrifuged for 5 minutes at 1250 rpm. The supernatant was aspirated and the wash steps were repeated two additional times. The cells were transferred into a 25 ml cell culture flask and 50 mg/ml (37.5 µl of antibiotic to 1 ml of IMDM 10% FBS) of Geneticin (Invitrogen Corporation, Carlsbad, CA) was added to the culture flask and incubated at 37^{o}C, 5% CO_2 for 48 hours. On day two the media was replaced using IMDM 10% FBS (Invitrogen Corporation, Carlsbad, CA) and 50 mg/ml of Geneticin, by centrifugation for 5 minutes at 1250 rpm, aspirating the old media and replacing it with the new media for an

additional 48 hours. Cells were observed and divided when necessary. On day six, the cells were collected by centrifugation as before and resuspended in 10ml of IMDM 10% FBS and placed into a new 25 cm^2 cell culture flask and incubated for an additional 48 hours. When the media was replaced, the supernatant was collected and stored at 4^oC to be used later as a source of GM-CSF necessary for the differentiation of DC from hematopoietic stem cells found in bone marrow.

Bone marrow was collected using sterile instruments and technique from female C57 BL/6 mice. The mice were euthanized by cervical dislocation. They were then transferred to the laboratory were they were wetted with 70% EtOH. Incisions were made to expose and remove both femurs and tibias. After removal of all muscle and connective tissue the bones were placed into 70% EtOH then washed twice in PBS. The bones were then transferred into a Petri dish which contained 10 ml RPMI 1640 media (Invitrogen Corporation, Carlsbad, CA). Using sterile scissors both ends of the femur and tibia were removed and a 25-gauge needle attached to a 3 ml syringe was inserted into either of the exposed ends and the bone marrow was flushed out with 2 ml of RPMI. This was repeated for each bone collected. The RPMI was collected in a 15 ml tube and the Petri dish was rinsed with an additional 5 ml of RPMI, which were also transferred into the 15 ml tube. The tube was centrifuged for 5 min at 1250 rpm. The supernatant was aspirated and 1 ml of red blood cell lysing buffer (Sigma chemical, St. Louis, MO) was used to resuspended the pellet and allowed to incubate for 1 minute before adding an additional 14 ml of RPMI 1640. The tube was then centrifuged as before and the pellet was resuspended in 5 ml of growth media (Growth media consisted of 1/3 J558 supernatant (collection described above), which contains the GM-CSF and 2/3 RPMI media + 10%

FBS). The samples were placed into 24 well plates and incubated at 37°C, 5% CO_2 for two days. Feeding of the cells began on day two, 75% of the media in each well was removed and replaced with fresh growth media. Feedings continued every two days. On day five, aggregates of DC were sub-cultured by removing them with a sterile Pasteur pipet and placing them in a new 24 well plate where the feeding continued every two days.

On day nine, the cells were observed using an inverted microscope. Upon confirmation that the cells were not contaminated, the following steps were conducted in the biosaftey cabinet. The loosely adherent cells were removed from the cell culture plate using a sterile Pasteur pipet and placed in a 15mL conical centrifuge tube. The wells were then rinsed with 1X trypsin solution and the plate was gently swirled for 1-2 minutes. RPMI 1640 media + 10% FBS was added to deactivate the trypsin and the cells were then collected and combined with the loosely adherent cells.

The ability of the DC to take up extracellular material by phagocytosis was determined by placing the cultured DC with uncoated and protein-coated polystyrene beads. This was conducted using varying amounts of beads: 0 µl (Sample 1), 5 µl (Sample 2), and 10 µl (Sample 3) of beads per 1 ml of DC. The phagocytosis assay was conducted by placing microscope cover slips into 24 well plates and adding DC along with polystyrene-beads. This culture was incubated in RPMI 1640 media + 10% FBS at 37°C, 5% CO_2 for 48 hours. After the incubation period was complete, the DC were examined at varying magnifications and digital photo documentation was used to record the results. The loaded cells were also tested in their ability to initiate an immune

response by activating T-cells via an MHC class I pathway, specifically by testing their ability to present SIINFEKL to B3Z/LacZ T cell hybridomas.

B3Z Cell Harvesting

T-cell hybridoma cells (B3Z/LacZ clone 28, generously provided by Dr. Nilabh Shastri, Department of Molecular and Cell Biology, University of California, Berkley) were obtained in a 1 ml frozen aliquot stored in cryovials. The vials were removed from liquid nitrogen and placed in 37°C water bath until just before completely thawed. They were then resuspended in 9 ml complete RPMI-1640 and centrifuged in tabletop centrifuge at 400 g for 5 minutes. The supernatant was removed, and cells were resuspended in 10 ml media. This solution was then placed into a Corning 25 cm^2 sterile cell culture flask and incubated at 37°C, 5% CO_2 overnight. Cells were monitored three times per week for growth and percentage confluency. When the cell density was approximately 90% confluent the cells were passed, if the cell density was less than 90% and the pH indicator in the media indicated that the media was becoming acidic the supernatant was aspirated and replaced with fresh complete RPMI media.

B3Z Assay

The B3Z assay was performed in a sterile 96 well plate. The well plate was prepared by serial dilutions of antigen (microspheres-EVI, microspheres-OVA, or microspheres-only) using PBS with dilutions from 10 μM to 0 μM. Harvested dendritic cells and B3Z cells were then added at volumes of 50 μl per well. The plate was incubated at 37°C, 5% CO_2 overnight. The assay also included the addition of Brefeldin

A (*Penicillium brefeldianum*) which blocks the function of the Golgi apparatus thus preventing the processing and presentation of antigen within the DC. This lactone antibiotic provides a unique opportunity to determine if the protein is being transported from the interior of the cell to the cells surface via the internal antigen presenting pathway and thus causing the activation of the B3Z cells. This will help diminish the likelihood that the activation seen is a direct effect of free protein in solution combining with the MHC molecules on the T cell surface as opposed to the protein being processed and presented by the DC.

B3Z cell activation was then determined by the amount of β-galactosidase produced by the B3Z cells. Luminescent β-gal detection kit II (BD-bioscience) was removed from 4°C and allowed to warm to room temperature. The 96 well plate was centrifuged at 400 g for 5 minutes. The media was removed and 200 µl of PBS was added to each well, the plate was centrifuged as before. The PBS wash was repeated twice before the addition of the 50 µl of M-PER (Mammalian Protein Extraction Reagent; Pierce) to each well. The plate was then incubated for 5 minutes at room temperature while gently shaking. Working solution for the luminescence assay was prepared (1:50, substrate to buffer) and added to the plate at 150 µl per well. The supernatant was then removed and placed into a sterile white bottom 96 well plate. The plate was placed in the GloRunner DXL plate reader (Turner Biosystems) and the luminescence was taken and saved as an Excel file by the GloRunner DXL computer program. The results were then graphed and saved for future reference.

An interference assay was also performed in order to determine what effect, if any, the beads would have on the assay. The assay was done by performing serial

dilutions of β-galactosidase from a concentration $2x10^6$ mg/ml to 0 mg/ml, beads were placed in the wells at concentrations equal to those of the B3Z assay and all other steps were the same as described above. All of the dilutions were done in PBS as well as the suspension of the beads. This was compared to beads alone as well as pure PBS.

RESULTS

Verification of Protein Isolation/Purification

The purification and isolation of the recombinant HER-2/*neu* protein was confirmed by using SDS-PAGE (Fig.2). The samples loaded onto the gel included the pre-column proteins and the fraction collected during the affinity chromatography procedure. The flow thru proteins were analyzed to make sure the protein of interest was bound to the column, 20 mM fraction was analyzed to make sure the protein of interest was bound tightly and the 300 mM elution fraction was analyzed to be certain that the protein of interest was eluted where expected. Additionally, the gel electrophoresis aided in determining the presence and the purity of the protein. Prior to electrophoresis an examination of the samples via a standard BCA assay (Pierce) was performed. This information was used in order to ensure the sample's protein content did not exceed the sensitivity of the detection method as specified by manufacturer's protocol (0.5 µg total protein per sample). The samples (no more than 25 µl / well) were loaded into the wells of the 4-12% Bis-Tris Pre-Cast gel. Each sample was loaded into two different wells at different volumes (10 µl and 20 µl). Staining of the gel was achieved using SimplyBlue SafeStain (Invitrogen). The staining procedure produced blue bands in the area where protein was present; the intensity of the band corresponds to the amount of protein present in each sample. The gel was then dried using DryEase® Mini-Gel Drying System (Invitrogen). The gel and two pieces of cellophane were soaked in Gel-Dry™ Drying (Invitrogen) solution loaded onto a drying rack and the gel was allowed to dry. The dried gel was stored at room temperature.

Upon examination, of the gel it was clear that the flow thru and the 20 mM imidazole samples contained a great deal of protein but contained very little of our HER-2 protein. A band at 58 kDa which corresponds to our protein of interest, HER-2, was present in the 300 mM imidazole sample. It also showed that there was little to no additional protein in the 300 mM sample, indicating that the sample was successfully isolated and purified. This data suggest that our protein was successfully isolated and the incorporated 6-histidine tail proved to be extremely useful in the isolation procedure.

Isolation / Differentiation of Dendritic Cells

Dendritic cells were obtained from either a mouse spleen or were derived from mouse bone marrow. The cells were cultured using two different protocols as described in the methods. The cells isolated from a secondary lymphoid organ were cultured for only a few hours and did not require an extend growth period, unlike the cells derived from bone marrow. The DC derived from bone marrow required the addition of Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and were cultured for nine days. There were some differences in the final results between the two procedures with the most noticeable difference being the number of cells that were produced.

Isolating DC from the spleen is a very efficient way to grow DC, however, the number of cells produced in this particular procedure was not nearly as great as that seen with the differentiation of bone marrow. Not only were the number of cells low when compared to the bone marrow derived DC, but their ability to take up particulate matter was not very impressive (Fig. 3) when compared to the uptake seen by bone marrow derived DC.

The DC isolated from bone marrow showed significant growth of what were believed to be immature DC. Images were obtained on day five once the cells were isolated in a new 24-well plate. Figure 4 shows images of cells that were isolated from bone marrow and it is clear that these cells do not share any phenotypic characteristics similar to those seen in mature DC. One distinctive example is that there is an apparent lack of cytoplasmic extensions, which is a key phenotypic feature seen in mature DC (Fig. 6).

On day nine the cells were once again viewed under a light microscope. An image was taken to compare the cell's appearance at day nine to that of cells viewed at day five. After observing the cells obtained in a variety of slides, it was clear that they all shared the same appearance as the representative cells shown in Figure 5. It should be noted that the cells did not appear to share any phenotypic characteristics of mature DC (Fig. 6). Thus, it may be assumed that the cells observed at day nine are similar, if not identical, to those observed at day five. The only visible difference between the two samples was an increase in the number of cells present at day nine (Fig. 5) versus day five (Fig. 4).

On day eleven, after culturing the cells for an additional two days with or without polystyrene beads, the cells were observed and photographed. Sample 1 (0 μL of beads per 1 mL of cells) resulted in cells differentiated into mature dendritic cells. This conclusion was reached due to the phenotypic characteristics of the cells that now appeared as mature dendritic cells including cytoplasmic extensions. These phenotypic changes were not present at day five or nine. Figure 6, shows the cytoplasmic membrane extensions which are a key morphological characteristic of a mature DC.

In sample 2 (5 μL of protein loaded beads per 1 mL of cells) the cells not only matured into mature DC, but also phagocytized the polystyrene beads that were present. These cells also appear to be active at phagocytosis, which is a functional feature of dendritic cells. In Figure 7, we can clearly identify the beads located within the cells, the beads within the cell are distinctive and are enclosed within the cells cytoplasmic membrane. It is also clear that the cells have the long cytoplasmic extensions, which indicates that the cells have differentiated from an immature state to a mature state.

In sample 3 (10 μL of protein loaded beads per 1 mL of cells) the cells also differentiated from an immature state to a mature state, which can be seen in figure 8. These cells were also able to phagocytize the polystyrene beads. However, it did not appear that these cells were able to phagocytize the same number of beads as the cells in sample 2. In comparing sample 3 to sample 2, a great deal of bead agglutination had occurred in sample 3 which did not occur in sample 2. Figure 8 clearly illustrates the differentiation of the cells, the phagocytosis of the beads by the mature DC, and the agglutination of the beads. The microsphere agglutination resulted in particles that were possibly too large for DC to phagocytize and thus fewer microspheres were available to be phagocytized. This may indicate a problem with the concentration of beads used. It was also observed that the cells in sample 3 did not differentiate to the extent that was observed in sample 2. The amount of cell differentiation observed in sample 3 was comparable to that seen in sample 1 (Fig. 6, 8). This could indicate that the cells require a certain threshold of particulate matter to phagocytize that was not present in sample 3 as a result of microsphere agglutination.

It was apparent that the DC prepared from bone marrow appeared to have a much greater ability in taking up particulate matter when compared to the DC isolated from the spleen. This was seen in each of the each of the samples, however, more particulate matter was up taken in sample 2 (5μL of protein loaded beads per 1mL of cells) than in sample 3 (10μL of protein loaded beads per 1mL of cells). However, one of the most interesting results was from the B3Z assay.

B3Z Assay

The B3Z assay provided a very useful and practical way to test the DC ability to initiate a cytotoxic response *in vitro*. The activation of the B3Z/Lac-Z cells, which were only able to be activated via the MHC class I pathway, resulting in the production of β-galactosidase provided a convenient way to measure activation of the T-cell hybridoma (Shastri & Gonzalez, 1993).

The first question to be addressed was what problem, if any, would be caused by the presence of the phagocytized microspheres while luminescence was being measured in the luminometer. To answer this question an interference assay was performed (Fig. 9). Using known concentrations of β-galatosidase and varying concentrations of beads in PBS as well as PBS alone any possible interference due to the beads was determined. The assay demonstrated that the beads did not cause any interference with the assay and also provided insight into the sensitivity of the assay (Fig. 9). Based on the results of this assay the lowest concentration of β-galatosidase detectable using this assay was approximately 1.82×10^{-7} μM. Once it was confirmed that the beads did not appear to

have a negative effect on the luminescence values, protein coated beads were cultured with DC derived from bone marrow as well as DC isolated from the spleen.

The bone marrow derived DC (BMDC) had an extraordinary ability to phagocytize the protein coated beads. The number of bone marrow derived DC appeared to be much greater in number when compared to the DC isolated from the spleen. However, the ability of BMDC to activate the B3Z cells following exposure to protein coated beads was only slightly improved when compared to DC isolated from the spleen which had been pulsed with soluble protein (Fig. 10). The data also demonstrated increased activation of B3Z cells, when BMDC were pulsed with protein coated beads versus soluble protein (Fig. 10). Activation of B3Z cells was greater when the BMDC were pulsed with protein coated beads than when they were pulsed with soluble protein: a lower concentration of protein coated beads achieved greater B3Z cell activation compared to soluble protein. The amount of activation appeared to drop at higher concentrations of protein in the BMDC. This was apparent in both of the samples that were pulsed with protein coated beads; the beads which possibly assisted in the increased activation of B3Z β-gal production could have also been harmful to the DC when higher concentrations of protein coated beads were used. In contrast we found that all the DC pulsed with soluble protein still had an increasing activation trend (Fig. 10). In the assays were the DC where pulsed specifically with EVI coated beads or OVA coated beads there was a great increase in the ability to activate the B3Z cells seen at lower concentrations however their ability to maintain the activation decreased at the highest concentration for EVI and at the two higher concentrations for OVA (Fig. 10). The data also demonstrate that the cells pulsed with EVI coated beads resulted in an overall activation almost

equally as great as that seen with the OVA coated beads. However, when the activation of the B3Z cells is compared to the concentration of the protein it was apparent that the EVI coated beads caused a greater response at lower concentrations, specifically at the 5 μM EVI concentration (Fig. 10).

The data clearly demonstrate that there was an apparent difference in the activation of the B3Z cells by BMDC pulsed with either EVI coated beads or OVA coated beads when compared to soluble protein. The difference in activation was even more apparent when using DC isolated from murine spleen (Fig.11-14). These DC were loaded with protein-coated beads and compared to soluble protein. Dendritic cells isolated from the spleen were not very aggressive in phagocytizing the protein-coated beads. Regardless of the cells decreased ability to phagocytize particulate matter their ability to activate the B3Z cells was far greater than the BMDC. This was especially true with the EVI coated beads and the OVA coated beads when compared to soluble protein (Fig. 12). It is interesting to note that protein coated beads resulted in activation of the B3Z cells at a much lower concentrations versus the higher concentrations required to activate the B3Z cells when the proteins were in their soluble form. Activation resulting by spleen derived DC loaded with OVA coated beads was seen at a concentration of 1.56 μM, by far one of the lowest concentrations that resulted in activation of B3Z cells seen during the course of this study while using OVA as activating agent. Typically, when DC were loaded with soluble OVA activation of B3Z cells was usually seen at concentrations of approximately 25 μM. The DC appeared to reach a maximum capacity in their ability to activate B3Z cells, as it was observed at the higher concentrations (25, 50 and 100 μM)

of the OVA-beads loaded DC, concentrations that there was no greater degree of stimulation (Fig. 11 & 13).

The spleen derived DC loaded with EVI coated beads resulted in an increased activation of the B3Z cells which was initiated by an EVI-bead concentration of 0.156 μM (Fig. 12 &14). It was consistently seen throughout the course of the project that the lowest concentration of EVI coated beads was sufficient to initiate activation of B3Z cells to a greater extent than soluble protein alone. The data clearly demonstrates how much more effective the EVI-bead combination was to activate the B3Z cells. When the activation of EVI-bead combination was compared to soluble EVI alone it is clear that it combining the EVI with the beads provides a way to activate B3Z cells with greater effectiveness. It was interesting to note that the activation of the B3Z cells with 10 μM of EVI-beads resulted in an activation of the B3Z cells which was approximately half that seen with 100 μM OVA-beads (Fig. 11 & 12). The data also demonstrated that the activation could possibly continue to rise but eventually, like the results seen with the DC loaded with OVA-beads combination, a plateau would likely occur, thus, indicating that there is a limiting factor that is occurring either with or within the cells (Fig. 13). Comparing mature spleen derived DC pulsed with soluble EVI and EVI coated beads and treated samples with Brefeldin A, provided data suggesting that the activation of B3Z cells was in fact due to DC processing and presenting antigen via MHC I pathway (Fig. 14). Thus, B3Z cell production of β-galactosidase could only be due to B3Z cells being activated by DC and not by free protein in solution combing with the MHC molecules on the B3Z cells surface.

DISCUSSION

It has been shown that dendritic cells are extremely effective at capturing, processing and presenting antigen. Dendritic cells incorporated into immunotherapies may provide a useful and novel treatment against specific cancers. Through the use of known tumor associated antigens, such as HER-2/*neu*, a specific therapy can be developed against tumors which overexpress a specific protein or proteins.

In developing this study it was imperative to integrate methods which would (1) produce a great deal of protein that could be isolated and purified, (2) a way to make the protein more antigenic, (3) determine if the dendritic cells were presenting the antigen via a class I MHC molecule. The isolation and purification of EVI was clearly achieved by the design of the protein. Incorporating the EVI-DNA into Rosetta *Escherichia coli* and allowing them to grow, produce and store the EVI-protein in inclusion bodies allowed for production of a large amount of protein which could be isolated and purified. The integral six histidine tail allowed for the protein to isolated using Ni-based affinity chromatography thus allowing for isolation of EVI with a great deal of specificity.

Attaching the protein to the polystyrene microspheres facilitated the increase in antigenicity. Pulsing DC with beads coated with EVI provided insight into exactly how many beads would be taken up, but it also allowed for a greater amount of protein to be introduced into the DC for processing. This in turn allowed for a direct comparison between immature and mature DC ability to take up particulate matter, something that could not have be accomplished without the polystyrene beads. It was very clear that the beads not only facilitated a greater amount of protein to be loaded into the DC but also

demonstrated that immature DC ability to take up particulate matter far exceed that seen with the mature DC.

Determining the presentation of the antigen was achieved by performing a B3Z assay. The assay incorporates DC loaded at varying concentrations and a T-cell hybridoma, the B3Z cell, which recognizes the SIINFEKL (amino acid residues 257-264 of chicken ovalbumin) epitope which was incorporated into the design of EVI. The activation of the B3Z cells was measured by the amount of β-galactosidase produced by the B3Z cells (Shastri & Gonzalez, 1993). The greater the activation the greater the amount of β-galactosidase produced. It was very interesting to observe that the mature DC which had been loaded with EVI coated beads were able to activate the B3Z cells to a greater extent when compared to mature DC loaded with soluble EVI and immature cells loaded with EVI coated beads. Given that SIINFEKL is found within ovalbumin, ovalbumin was used as a positive control in a soluble form as well as bound to polystyrene beads. The OVA coated beads were compared to soluble OVA which were pulsed onto immature and mature DC. When comparing the β-galactosidase production by B3Z cells, mature DC were able to produce levels that were detectable at far lower concentrations of OVA when OVA was bound to the polystyrene beads compared to immature DC. The immature DC were not as effective in demonstrating a difference in activating B3Z cells when pulsed with either soluble OVA or OVA coated beads. For mature DC it was clear that the OVA coated beads were able to elicit a greater activation of B3Z cells than soluble OVA alone. This data implied that mature DC provided superior activation of the B3Z cells when the DC were pulsed with OVA coated beads. While the immature DC were far more effective in the uptake of the OVA-albumin

coated beads, their ability to activate B3Z cells was mediocre at best when compared to the mature DC. Activation of B3Z cells by mature and immature DC loaded with soluble EVI and EVI coated beads was also tested. The data from these experiments appears to coincide with the results gathered from the OVA experiments. The activation of B3Z cells by mature DC was far greater when the DC were pulsed with EVI coated beads. Mature DC loaded with soluble EVI provided greater activation of B3Z cells than the immature cells loaded with either soluble EVI or EVI coated beads.

There may be a few possible explanations for the lack of B3Z cell activation by the immature DC. Given that immature DC were far greater at taking up particulate matter when compared to mature DC one might expect that immature DC would elicit a greater activation of B3Z cells, however, this was not the case. The fact that immature DC took up so many of the antigen coated beads could have posed a problem. The polystyrene beads are not biodegradable this may have caused an obstruction within cellular compartments of the DC, particularly within the Golgi apparatus. Causing a blockage within the Golgi apparatus would result in prevention of packaging and transportation of antigen epitopes to the cell's surface. In addition to the possible obstruction of the Golgi apparatus, immature DC display low amounts of MHC I and MHC II, specifically the class I MHC. This may have resulted in decreased activation of B3Z cells. It is essential to note that the results observed with the immature DC experiments provide vital insight into the importance of maturing DC prior to immunization. However, it is also imperative to mention that *in vivo* it is possible for DC to migrate to secondary lymphoid organs and undergo further maturation. Thus, despite

the recent *in vitro* observations we cannot dismiss immature DC as important initiators of an immune response.

The importance of maturing the DC was certainly confirmed by the *in vitro* studies. These studies demonstrated how effective mature DC were in initiating the activation of the B3Z T-cell hybridoma. In addition to maturing DC, it was also evident that pulsing mature DC with protein-coated beads resulted in increased activation of the B3Z cells possibly due to a greater amount of protein (antigen) entering the cell. The levels of β-galactosidase observed were not only much higher but they also detected antigen at much lower concentrations in the groups where the mature DC were pulsed with the OVA and EVI coated bead. This indicates that a greater amount of protein was actually being taken up and processed by the cells, when the protein was bound to the polystyrene beads. These results show that the antigenicity of the protein was greater when bound to the polystyrene beads than in its soluble form. It is important to note that the negative effect the beads had on the immature DC was not seen in the mature DC, possibly due to the lesser number of beads which were taken up by the mature DC. However, the toxic effects that were observed in the immature DC experiments may be overcome by binding antigens to bio-degradable beads.

In conclusion, the maturity of dendritic cells does appear to play a crucial role in developing an antigen specific immune response. In addition, the method of delivering the antigens to APC seems to be just as important. By binding antigens of interest to particulate matter, such as polystyrene beads or bio-degradable beads, the antigenicity seems to increase dramatically. This increase in antigenicity may in turn trigger an immune response against a tumor-associated antigen of interest, such as HER-2/*neu*. This

is just one example as to how manipulation of the immune system may be able to elicit a specific and possibly an extremely effective immune response against specific cell surface antigens which are over-expressed in certain malignancies, such as HER-2/*neu* positive breast cancer.

Immunotherapy is most certainly a novel concept which may be patient specific and may provide patients with an adjuvant treatment which may work in conjunction with the conventional therapy. Immunotherapy may have far less side effects when compared to the current standard of care; however, there are still many questions that need to be answered before each malignancy has it own specific and effective treatment.

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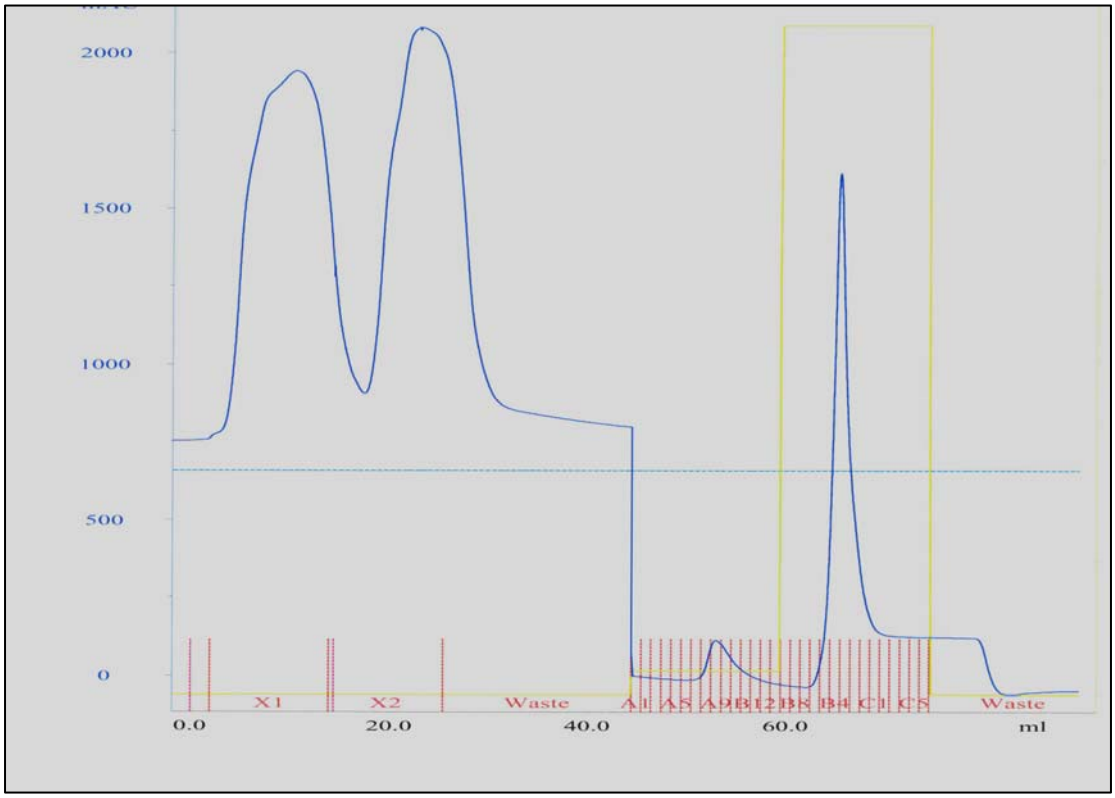

Figure 1: Representative affinity chromatography data indicating the results of a complete FPLC protein isolation. The first two peaks indicate the flow through, protein loaded onto the column that did not bind. The next peak indicates the 20 mM imidazole fraction which will remove the protein that is loosely adhered to the column. The final peak, within the yellow outline, indicates the elution of the protein of interest. Each of the red lines in the bottom of the figure indicates a 300 mM imidazole elution fraction that was collected. These fractions were then combined and the results are confirmed via SDS-PAGE.

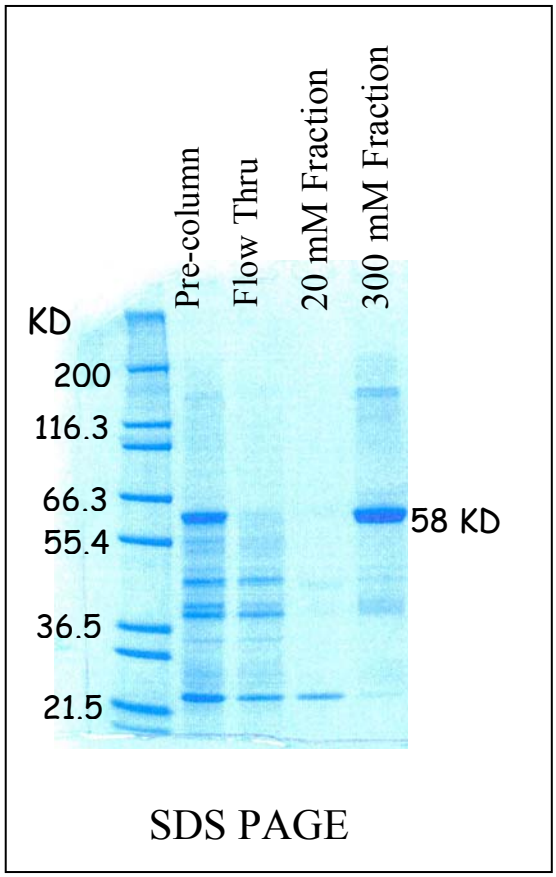

Figure 2: SDS-PAGE was used to confirm the presence of the protein of interest, EVI, within the 300 mM imidazole fraction. The gel demonstrates that the protein of interest was found within the pre-column fraction and only a small amount within the flow thru. There was no EVI detected within the 20mM fraction, however, a large amount of the 58 KD protein (EVI) was detected in the 300 mM fraction, thus confirming the results of the FPLC absorption spectra as well as indicating that we were able to purify our protein from nearly all other proteins observed in the pre-column sample.

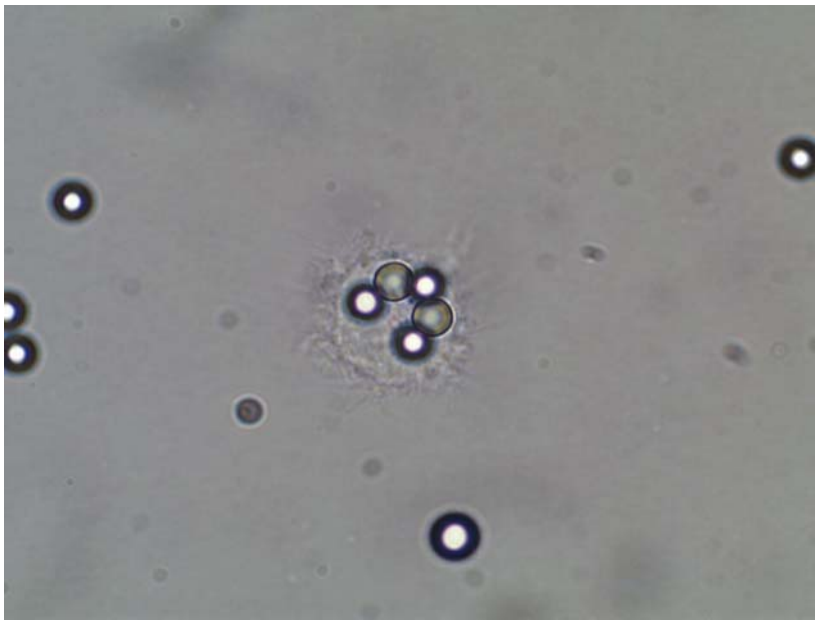

Figure 3: Image of a mature spleen-derived dendritic cell. The image was obtained at 1000x. The sample was obtained after a brief two culture preparation. This sample conatined 1 mL of spleen derived dendritic cells, with 5 μL of beads added to the sample. It is important to note the phagocytosis of polystyrene beads, which is a functional characteristic of dendritic cells. Spleen derived DC also display phenotypic characteristics which are similar to those seen in mature dendritic cells. This image demonstrates that cells are functional, however, the number of cells is much lower than what was seen when dendritic cells were generated from murine bone marrow.

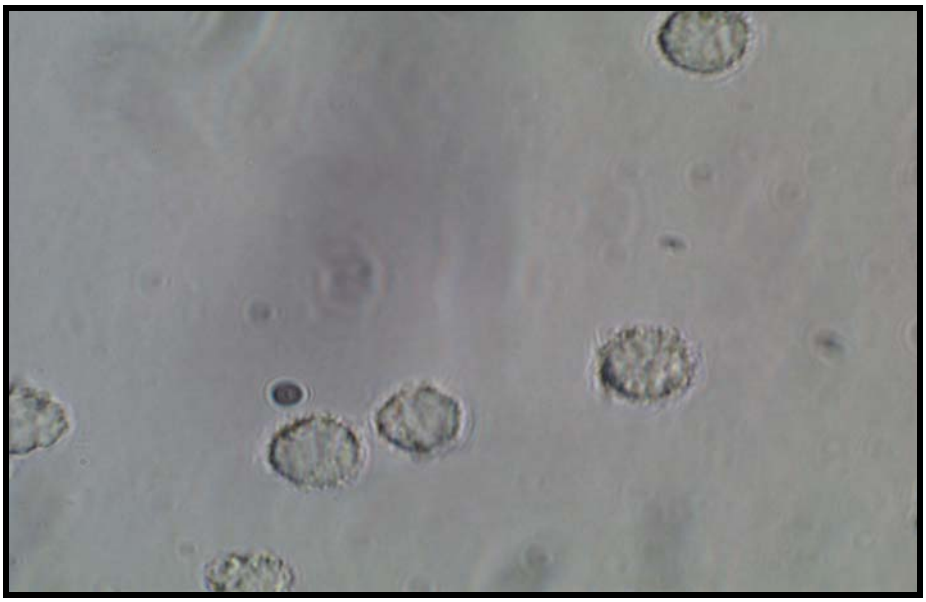

Figure 4: Image of immature bone marrow derived dendritic cells obtained at 400x. The sample was obtained after five day subculture preparation. It is important to note that the cells share no phenotypic characteristics similar to those seen in mature dendritic cells.

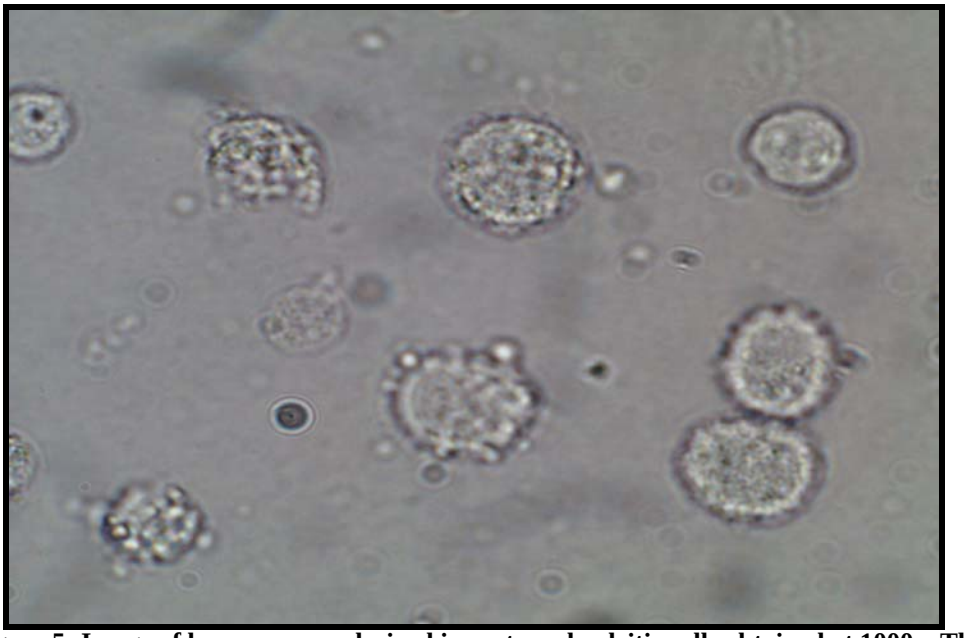

Figure 5: Image of bone marrow derived immature dendritic cells obtained at 1000x. The sample was obtained after a nine day subculture preparation. It is important to note that the cells have not developed phenotypic characteristics similar to those seen in mature dendritic cells.

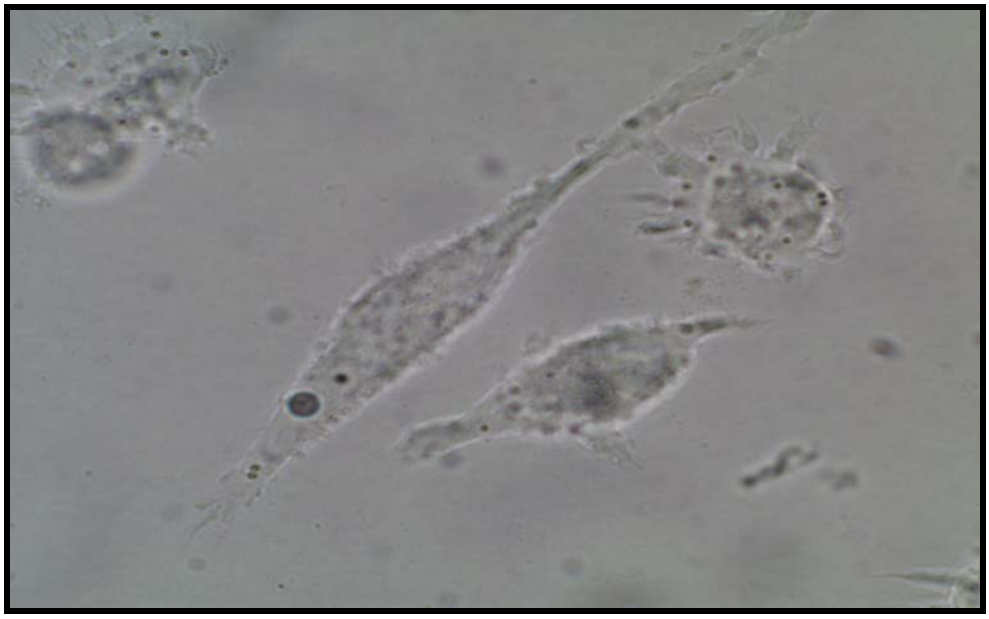

Figure 6: Image of bone marrow derived mature dendritic cells obtained at 1000x. The sample was obtained after the eleventh day subculture preparation, of 1mL of dendritic cells, with no beads added to the sample. The BMDC have now developed phenotypic characteristics similar to those seen in mature dendritic cells, which were not observed in the cells at day five or day nine.

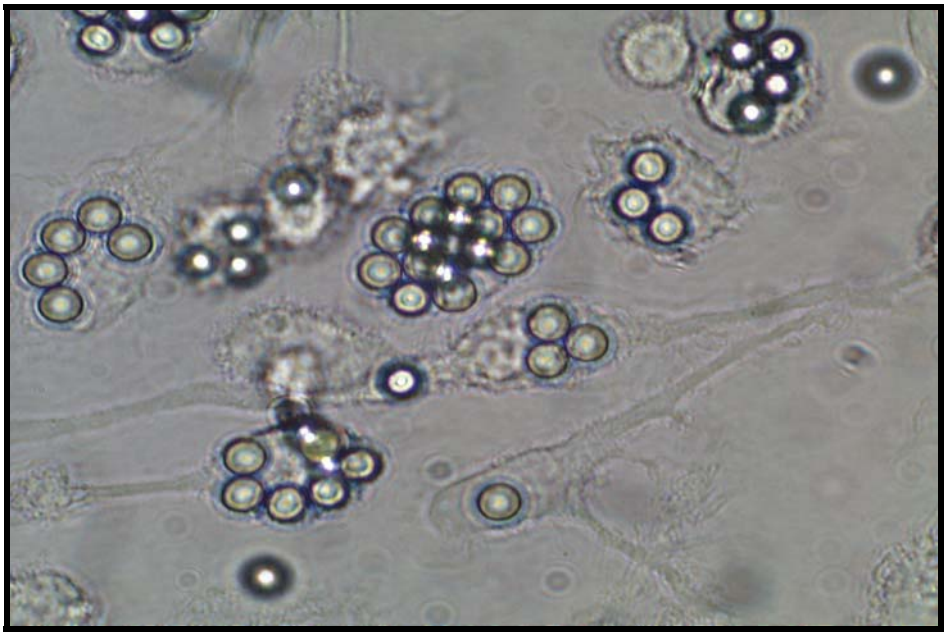

Figure 7: Image of mature dendritic cells obtained at 1000x. The sample was obtained after the eleventh day subculture preparation, of 1 mL of dendritic cells, with 5 μL of beads added to the sample. It is important to note the phagocytosis of polystyrene beads, which is a functional characteristic of dendritic cells, as well as, phenotypic characteristics which similar to those seen in mature dendritic cells. The phenotypic characteristics were not observed in the cells at day five or day nine.

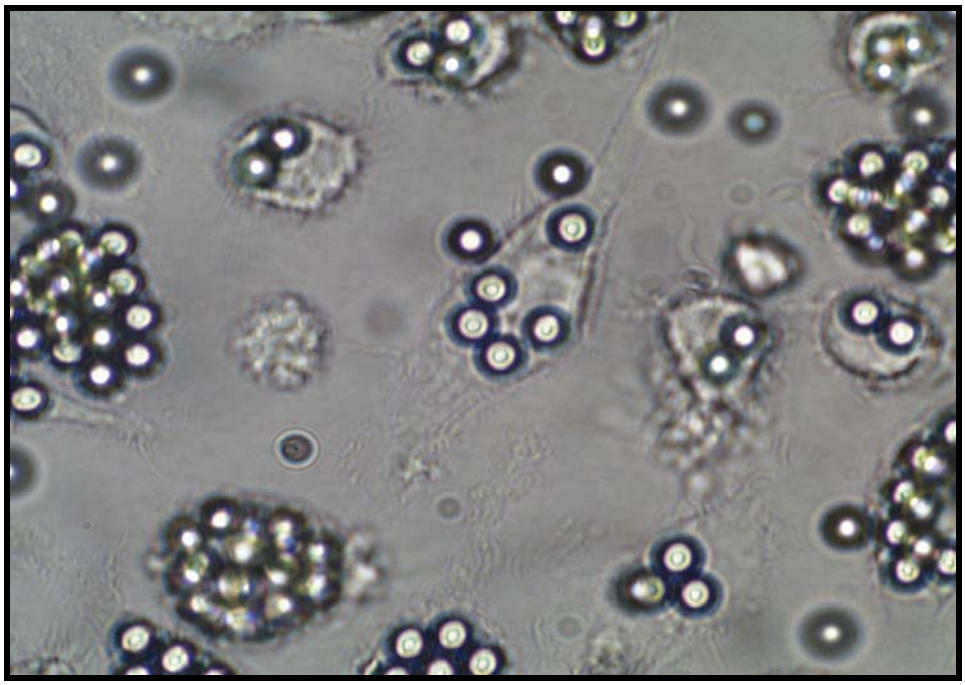

Figure 8: Image of mature Dendritic cells obtained at 1000x. The sample was obtained after the eleventh day subculture preparation, of 1 mL of dendritic cells, with 10 μL of beads added to the sample. It is important to note the phagocytosis of the polystyrene beads, which is a functional characteristic of dendritic cells, as well as phenotypic characteristics which are similar to those seen in mature dendritic cells. In this sample it must be noted that there was an increased amount of bead agglutination. The phenotypic characteristics were not observed in the cells at day five or day nine.

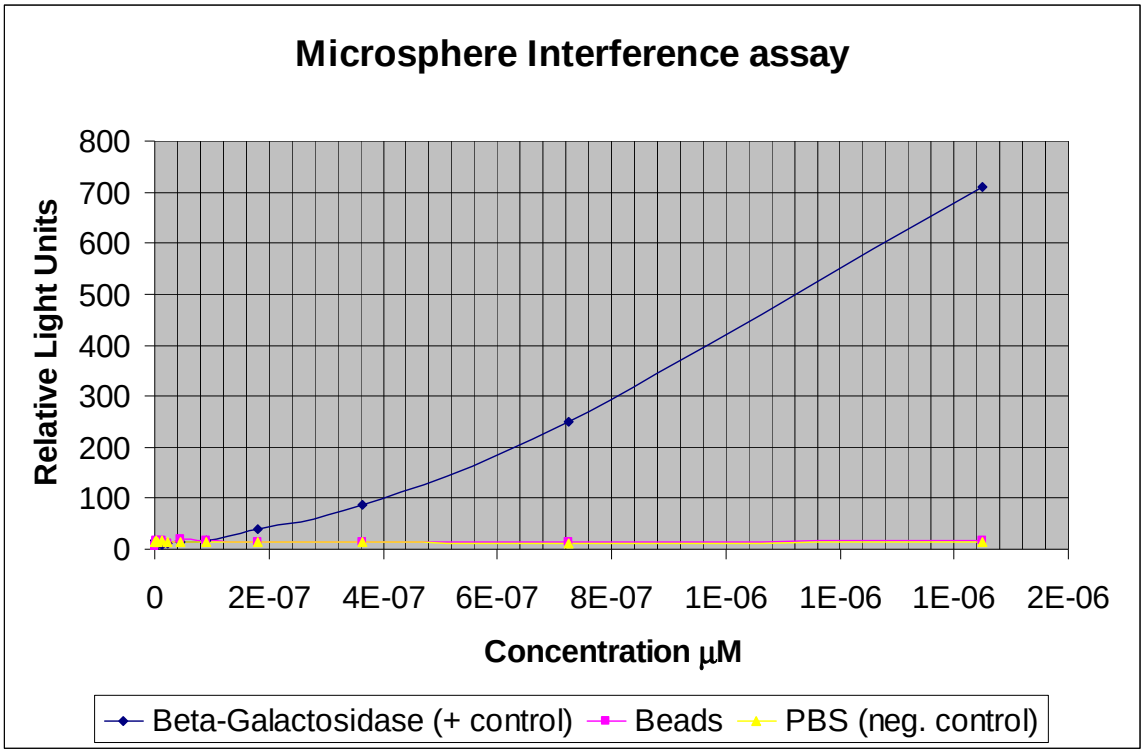

Figure 9: An interference assay was performed in order to determine if the polystyrene beads would have a negative effect when gathering data via a microplate luminometer. In addition to determining that the beads had the same effect as PBS, thus no negative effect on the luminescence, an assay detection range was also determined. This was of critical importance because it demonstrated the lowest amount of β-galactosidase that needed to be produced by the B3Z cells to be detected in our assay system.

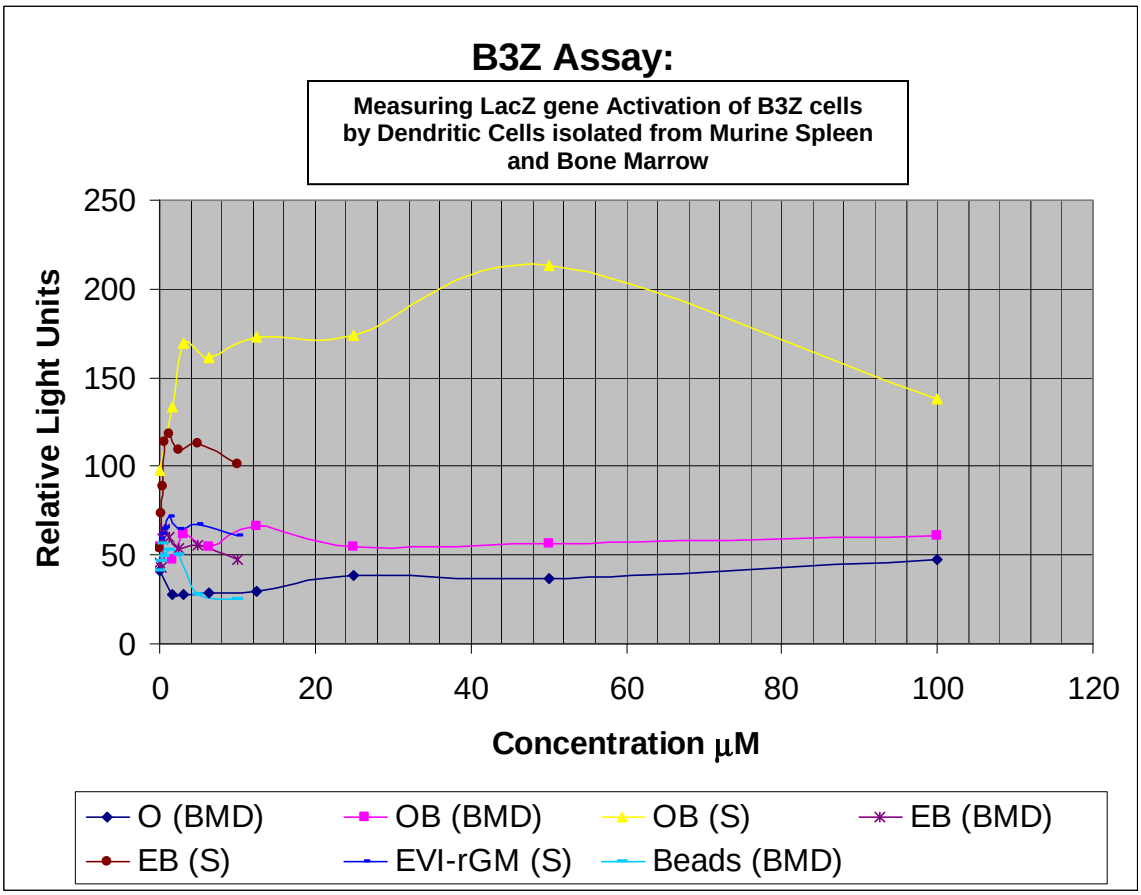

Figure 10: Comparing B3Z cell activation by bone marrow derived dendritic cells (BMD) and spleen derived dendritic cells (S). B3Z CD8$^+$ T-cell hybridoma clone specific for the OVA SIINFEKL peptide (amino acids 257-267) which express the LacZ gene under the control of the interleukin-2 promoter was used to determine activation via MHC-I by DC isolated from murine spleen or derived from murine bone marrow. A direct comparison of DC isolated from spleen to DC derived from bone marrow where the DC were loaded with OVA, OVA-coated beads, EVI, EVI-coated beads, beads without any protein, or pulsed with EVI-rGM. Activation of the B3Z cells was determined by measuring LacZ activity after incubating the cells with loaded DC for 12-24 hours. The data illustrates that DC isolated from spleen provided greater activation of T-cell when compared to DC derived from bone marrow, this activation was even more apparent when the protein was attached to the polystyrene beads.

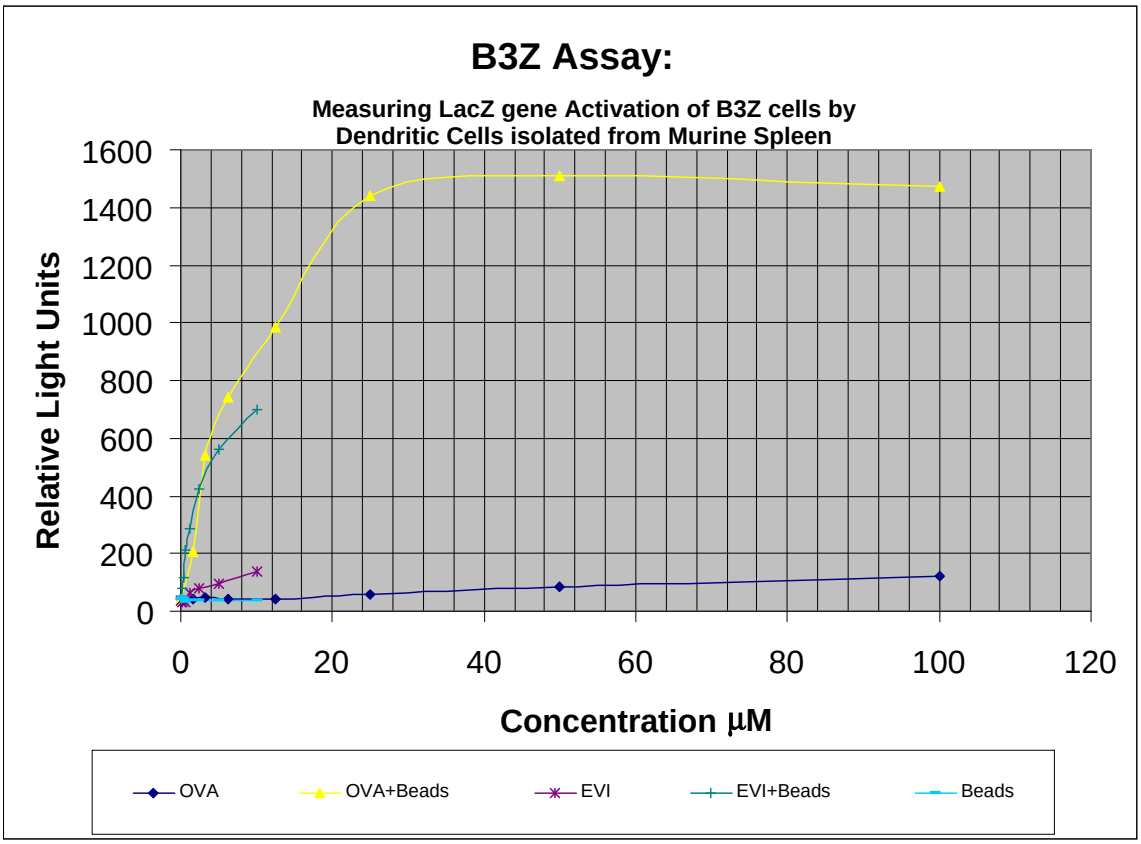

Figure 11: B3Z CD8$^+$ T-cell hybridoma clone specific for the OVA SIINFEKL peptide (amino acids 257-267) which express the LacZ gene under the control of the interleukin-2 promoter was used to determine activation via MHC-I by DC isolated from murine spleen. Dendritic cells were been loaded with OVA, OVA-coated beads, EVI, EVI-coated beads, or beads without any protein. Activation of the B3Z cells was determined by measuring LacZ activity after incubating the cells with loaded DC for 12-24 hours.

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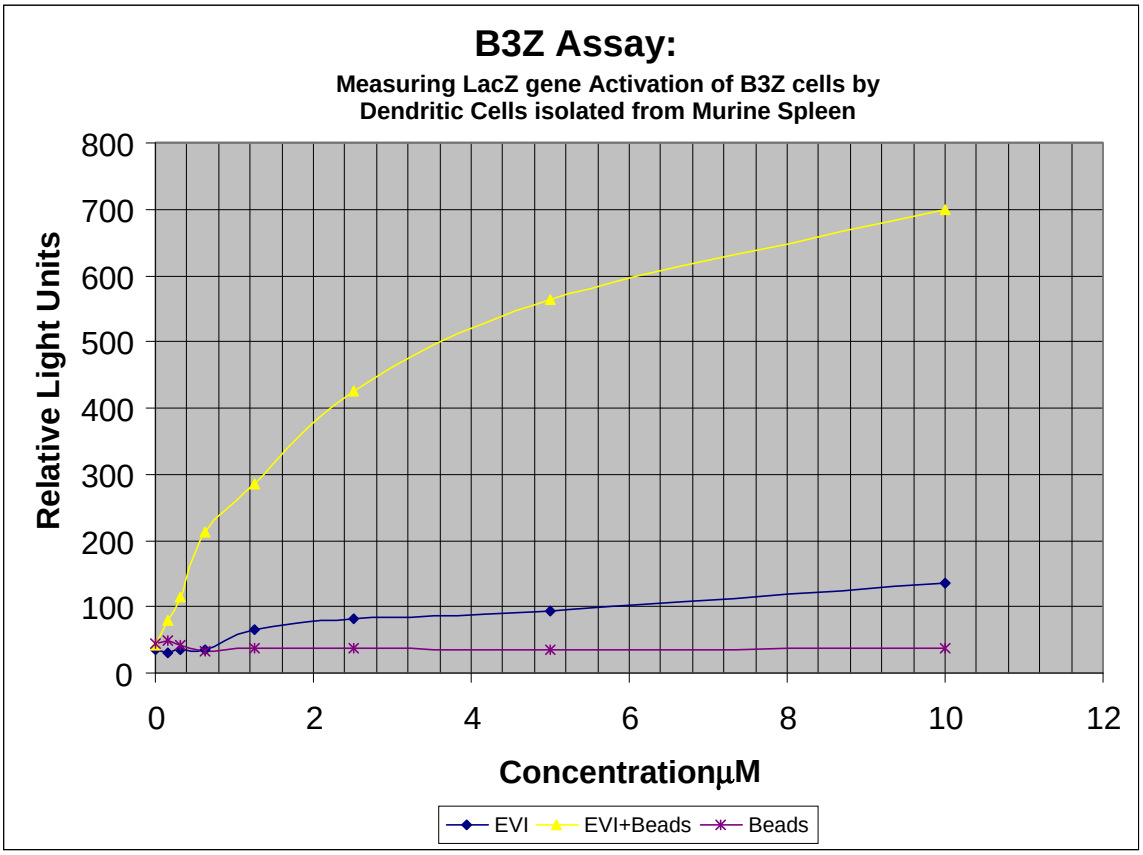

Figure 12: B3Z CD8+ T-cell hybridoma clone specific for the OVA SIINFEKL peptide (amino acids 257-267 of ovalbumin) which express the LacZ gene under the control for the interlukin-2 promoter was used to determine activation via MHC-I by DC isolated from murine spleen. Dendritic cells were loaded with EVI, or EVI-coated beads. Activation of the B3Z cells was determined by measuring the LacZ activity after incubating the cells with loaded DC for 12-24 hours.

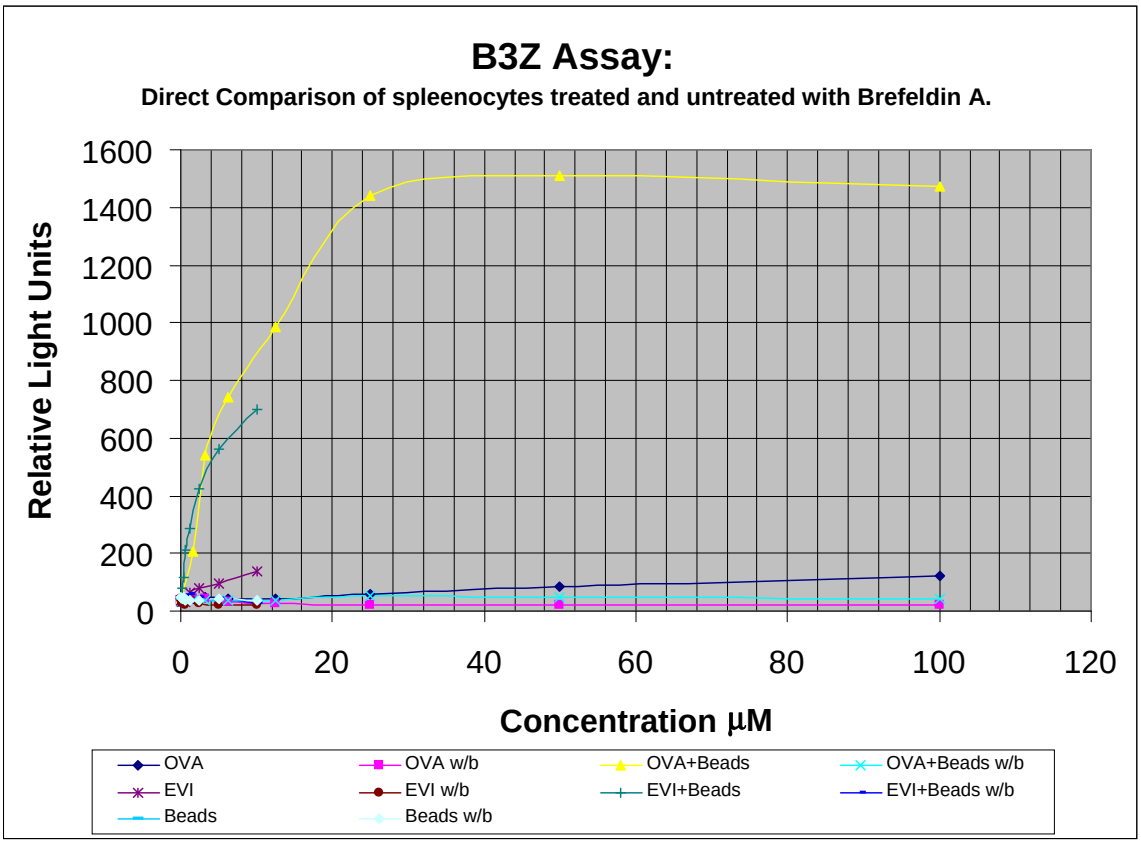

Figure 13: B3Z Assay Results Using DC isolated from murine spleen. Dendritic cells have been loaded with one of the indicated proteins, or the protein-coated beads. The cells were also treated with Brefeldin A (5 µg/ml) in order to confirm that the activation seen was a result of a protein presented via an MHC-I pathway and that the activation of the B3Z cells had not resulted from free floating protein.

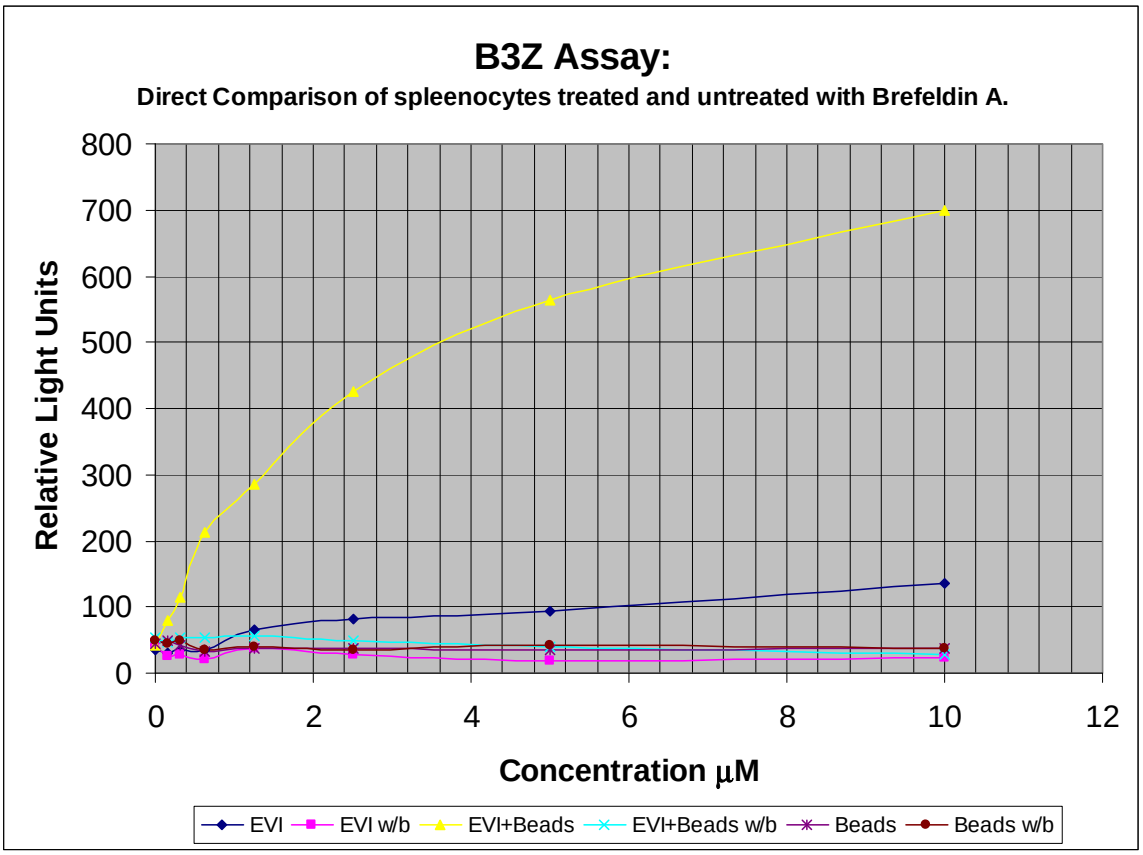

Figure 14: B3Z cell activation using DC isolated from murine spleen. Dendritic cells have been loaded with EVI, or EVI-coated beads. Activation of the B3Z cells was determined by the amount of β-galactosidase produced by the B3Z cells. The cells were also treated with Brefeldin A (5 μg/ml) in order to confirm that the activation seen was a result of protein presented via an MHC-I pathway by DC and that activation of the B3Z cells had not resulted from free floating protein binding with cell surface MHC on the B3Z cells.

APPENDIX A:

LETTER OF APPROVAL FOR THE USE OF VERTEBRATE ANIMALS IN RESEARCH FROM NORTHERN MICHIGAN UNIVERSITY'S DEAN OF GRADUATE STUDIES

Northern Michigan University

College of Graduate Studies
1401 Presque Isle Avenue
Marquette, MI 49855-5322
906-227-2300
FAX: 906-227-2315
Web site: www.nmu.edu

April 4, 2006

TO: Robert Winn
Biology Department

FROM: Cynthia A. Prosen, Ph.D.
Dean of Graduate Studies & Research

RE: **Application to use Vertebrate Animals**
Application # IACUC 040
Approval Period: April 4, 2006 – February 1, 2007

The Institutional Animal Care and Use Committee has approved your project to use vertebrate animals in research entitled "Manipulation of the Immune System for Anti-Tumor Response."

If you have any questions, please contact me.

ljh

cc: Biology Department