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Influence of Supramammary Lymph Node Extract on Cell Growth In Vitro

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INFLUENCE OF SUPRAMAMMARY LYMPH NODE EXTRACT ON CELL GROWTH *IN VITRO*

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ABSTRACT

The bovine supramammary lymph node, located on the dorso-caudal surface of the udder, is a protein rich organ that is discarded on a regular basis during slaughter. Experiments were conducted to evaluate whether or not the lymph node extract could be used as a substitute for bovine growth serum in cell media. Two different preparations of lymph node extract were made and tested. Three different cell lines (MDA-MB-435, MAC-T, and 1C6) were used in assays to evaluate the lymph node extract as a supplement. The first extract yielded a protein concentration of 3.0 mg/ml (heat inactivated) and the second extract contained 27 mg/ml (heat inactivated). MTT and CyQuant assays were used to determine cell viability and proliferation, respectively, in the presence of lymph node extract. Both heat inactivated preparations supported cell growth as well as or better than BGS following a two day serum starvation phase in culture. The second lymph node preparation provided a stimulatory effect following serum starvation on all three cell lines at 10 and 20% supplementation. Direct culture of cells into 10% lymph node extract did not support cell growth as expected. However, gradual adaptation process with lymph node supplementation into media maintained cell growth up to a full 10% extract. Once cells were trypsinized and re-seeded with the 10% lymph node extract they were unable to re-adhere, leaving them detached, and eventually appeared dead. Analysis of DNA revealed any apparent cell death visually observed was not due to apoptosis. The stimulatory effect at 10 and 20%, illustrated by the CyQuant

assays, may provide explanation as to why adherence was not achieved in culture. These higher protein concentrations may present an over abundance of mitogenic protein/factors resulting in over-stimulation of cells. Lowering the protein concentration, or fractionating the extract, could possibly provide a better environment for cells to grow and adhere in culture. In conclusion, substitution of BGS at 10% dramatically stimulates cells but does not maintain cell growth *in vitro*. Further research may prove lower concentrations can sustain cells in culture by lowering mitogenic protein/factors to sub-stimulatory concentrations.

DEDICATION

I would like to dedicate this thesis to my parents. Without their undying support I would not be where I am today.

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TABLE OF CONTENTS

Table of Contents (Continued) Page

LIST OF FIGURES

List of Figures (Continued)

1. LITERATURE REVIEW

Cell Culture and Serum Supplementation

The *in vitro* growth and expansion of animal and plant cells is termed cell culture. Although the methodology advanced during the first half of the twentieth century, animal cell culture became imperative in the 1940's and 1950's when polio epidemics became prominent and the need for mammalian cell-based vaccine production was required (Chaudry, 2004). Through cell culture, large amounts of the poliovirus were grown, and the polio vaccine was developed and became one of the first products of cultured animal cells. Later, during the 1970's, the technique for monoclonal antibody production was developed, and cell culture then became a more widely practiced methodology (Kohler and Milstein, 1976). Cell culture has been and is still a very important scientific research tool today for tissue construction, product testing and embryo propagation. Hence, it is a vital component of both research and industrial laboratories.

In order to support the growth of cells in culture the proper nutrient components must be included in cell media. Amino acids, antibiotics, vitamins, as well as a source of growth factors, are essential for cell survival in culture. Additional factors may be added to media depending on the type of cell being cultured. An important ingredient for almost all cell lines is serum. Bovine derived sera are commonly used in cell media because of the relatively easy access to large volumes of blood at abattoirs. Bovine growth serum, fetal calf and bovine sera, and bovine serum albumin are all used in cell culture. Different growth factors and carrier proteins in bovine serum support and

maintain cell growth. Fetal bovine serum contains many important proteins that contribute to nurturing the growth of cells. For example, adult pigment epithelial cells were treated with three different lots of fetal bovine serum, and then evaluated to determine whether a difference in cell growth rates would be observed among lots. Although one lot stimulated a higher cell growth rate, the amount of high abundance proteins did not discriminate between lots (Zheng *et al.*, 2006).

Major proteins identified in the serum were albumin, transforming growth factor-beta (TGF- β), insulin like growth factor binding protein (IGF-BP), and insulin like growth factor-II (IGF-II) (Zheng *et al.*, 2006). IGF-BP-III as well as IGF-II and IGF-I have also been found in bovine serum albumin preparations (Twigg *et al.*, 2000). IGF-I and -II have been identified in both adult and fetal bovine sera (Honegger and Humbel, 1986). Albumin also plays an important role in serum. As a carrier protein it has been suggested to carry cholesterol and fatty acids for membrane synthesis of cells (Washburn *et al.*, 1978). Additionally, bovine serum contains a complex assortment of different substances, some of which are still undetermined.

Due to potential interference by certain components of bovine sera on human tissue cultures, research has been conducted to replace animal-based serum with human serum albumin (HSA). Schneider *et al*. (2003) found that human serum albumin enhanced insulin production and cell viability of encapsulated rat islets over a period of 3 weeks. As a potential substitute for FBS, a study was conducted with HSA added to C2C12 myoblast cells and baby hamster kidney (BHK) cells. HSA did not support viability of BHK cells; however they found that HSA could be used with the C2C12

cells, but only for a limited time before protein secretion decreased (De Castro, et *al*., 2006).

Serum free media are also used in cell culture; although most cell lines require the addition of purified components obtained from serum. There are also cell lines that thrive in serum free media. Specifically, Kambe *et al.* (2000) found that fetal liver cells stimulated with recombinant human stem cell factor (HSCF) and cultured in a serum free medium produced more mast cells than fetal liver cells treated with media containing serum. In this case, serum adversely affected cell development induced by HCSF and serum free media allowed for greater expansion of mast cells under the influence of HSCF. Human corneal endothelial cells have also been shown to grow without bovine serum present in the medium (Bednarz *et al.*, 2001). Although some cell lines are capable of growth *in vitro* without serum supplementation, there remain a large number of important cell lines that require this critical supplement for survival in culture.

Much of what is understood about serum supplementation is anecdotal, but many cell lines in culture still require the growth supporting effect of bovine serum. Most cells in an organism rarely come in direct contact with blood plasma; however the use of serum to grow cells has not been overly questioned. Scientists assume cells need serum and initially culture cells with serum. Although different serum replacement supplements are commercially available, serum remains in high demand as an essential ingredient in media for many cell lines in culture today.

Whether cell culture supplementation includes serum or substitutes, a further consideration is cost for cell propagation and maintenance. Five hundred ml of heat inactivated Hyclone BGS costs $96 (Fisher Scientific). The same volume of bovine

based serum products, replacements for FBS, can cost up to $109 (Fisher Scientific). Because of the high cost of these supplements, other replacements as well as serum free media have been introduced into cell culture. A supplemented calf serum, Omni Serum, has been demonstrated to support growth of cells used to isolate herpes simplex virus in culture (Johnston and Siegel, 1990). Omni Serum was able to be used at lower concentrations than FBS and cell performance was not different. This replacement is commercially available (Advanced Biotechnologies Inc., Columbia Md.) and costs 31% less than FBS. Conditioned media is also available for use in cell culture. *Leishmania infantum chagasi* was grown in conditioned media that was obtained from cultures of McCoy cells grown in Eagle's medium supplemented with 5% FCS. After the McCoy cells grew in the medium, it was considered "conditioned" because of the excretion of metabolites that are beneficial for the growth and survival of the parasite (Noqueira and Galati, 2006).

Although alternatives to the different sera are available, different cell lines require different factors that may not be obtained from sources other than serum. The quest for less expensive serum replacements will continue. An optimal replacement would include an abundance of growth promoting factors that can support numerous cell types in culture. Considering the presence of interstitial fluid around cells in the tissue environment, the use of a protein-rich tissue extract may offer an alternate to serum. Interstitial fluid drains from tissues to enter the lymphatics and become the major fluid component of lymph. As lymph fluid moves through the lymphatics and lymph nodes, this fluid becomes enriched with cells and lymph factors (immunoglobulins, complement proteins, cytokines, chemokines, and growth and differentiation factors) prior to the

lymph mixing with the blood. Therefore, recovered tissue extracts from lymph nodes should possess a rich mixture of factors that may support the growth of cells, especially in culture.

Composition of lymph node

The lymph node is an immunological organ with an abundance of cells such as dendritic cells, macrophages, T cells, and B cells. The signaling molecules these cells produce, along with different growth factors potentially present in the lymph node, yield the lymph node as a protein-rich source. Lymph nodes are positioned along the lymphatic vessels throughout the body where lymph passes through them and transports fluid, cells and foreign antigens; the latter requiring processing in order to induce immune responses in the lymph node. Antigen presenting cells (primarily dendritic cells) are picked up from sub-epithelial sites by lymph, which is derived from interstitial fluid, and delivered to the lymph nodes (Willard-Mack, 2006).

The lymph node itself is described as having three compartments: the cortex, paracortex and medulla. Each compartment has a role in the overall function of the lymph node. The basic functional unit of the lymph node is the lymphoid lobule, which spans all three compartments. The number of lobules depends on the size of the lymph node; the amount can range from only one to many. Arranged side by side, the lobules contain follicles in the cortex, deep cortical units in the paracortex, and medullary cords in medulla. The follicles contain B cells, which when stimulated proliferate within the follicles to form germinal centers, ultimately transforming the primary follicle to a

secondary follicle. T cells localize in the paracortex and also proliferate in this area when stimulated by antigen-major histocompatibility complex presentation by an antigen presenting cell. The deep cortical unit is a passage for lymphocytes going back and forth from the T- and B-cell areas. Once B cells are stimulated and produce plasma cells through affinity maturation in germinal centers, the plasma cells move into the medullary cords in order to mature and secrete antibodies which are then released into lymph (Willard-Mack, 2006). In order to communicate with neighboring cells many of the immunological cells produce and secrete signaling molecules that perform a variety of growth and differentiation promoting functions. Examples include TGF-β and -α; IL-1, -2, -3, -4, -5, -6, -8, -9, -10, -12, and -13; and interferons.

The framework of the lymph node consists of a reticular meshwork. This meshwork is composed of fibroblastic reticular cells (FRC) and their reticular fibers. The meshwork serves as a migration site for the lymphocytes to adhere to for movement throughout the lymph node. The FRC are coated with migration ligands (fibronectin) on their surfaces which assist in the lymphocyte migration. Furthermore, a specific type of macrophage, termed 'sinus histiocyte', attaches onto the meshwork and "catches" different lymph components such as bacteria, cell debris, red blood cells, and injected carbon (Willard-Mack, 2006). The fibroblastic reticular cells also secrete fibers which are composed of collagens type I, III and IV; elastin; entactin; fibronectin; laminin-1; tenascin; vitronectin; and heparin sulfate (Willard-Mack, 2006).

An understanding of the structure and function of the lymph node allows for a better understanding of the general cellular components residing within a lymph node environment. Although all of the proteins in the lymph node have not been characterized,

many have been isolated and identified. It has been suggested that all proteins found in plasma are found in lower concentrations in lymph. This is pertinent considering it has been suggested that the lymph node itself contributes a small volume of high protein lymph to the efferent lymph (Quin and Shannon, 1977). Many different growth factors may be present in the lymph node. Lacasse *et al*. (1996), found that all of the IGF-BP's that are present in serum are also in lymph fluid, but have lower binding activities. Other growth factors such as TGF-β may also be present in lymph nodes. TGF-β is produced by activated T-helper 1 cells and Natural Killer cells. TGF-β has proliferative effects on a variety of mesenchymal and epithelial cells. A study by Zou *et al*. (1996) detected genes strongly expressed that coded for different cytokines in lymphoid tissues after infection with Simian Immunodeficiency virus. Proinflammatory cytokines included IL-6, IL-1β, and TNF-α. IFN-γ, IL-13, IL-4, IL-12, IL-10 and chemokines MIP-1α, MIP-1β, MCP-1, and RANTES were also expressed (Zou *et al*. 1996). The different cytokines present in the lymph node stimulate proliferation, differentiation, antibody production, and activation of a variety of cells. Cytokines provide a signaling system for the immune system to inter-cellularly communicate and properly function.

Supramammary lymph node

The bovine supramammary lymph nodes are located on the dorso-caudal surface of the udder (Bradley *et al*., 2001). The number and size of the supramammary lymph nodes varies, with a frequent configuration of one on each side of the udder. Age however, as well as mastitis history, can dictate the size of the supramammary lymph

node, with older cows and cows with a history of mastitis tending to have larger lymph nodes (Bradley *et al*., 2001). Lymph node anatomy examined ultrasonographically by Bradley et *al*. (2001) was consistent with the appearance described for other lymph nodes in cattle as well as humans.

Infections such as bovine mastitis can cause significant damage to the udder tissue and affect milk production in the cow. In order to elicit a quick recovery from these types of infections a rapid immune response is necessary. The supramammary lymph nodes are the first lymphoid organs that come in contact with antigens during bacterial invasions of the mammary gland (Watson and Davies, 1985). These lymph nodes, along with a localized inflammatory response, are crucial to the defense against antigens in the udder, and play an imperative role in protecting the mammary gland.

Although all of the supramammary lymph node components have not been characterized, the lymph which passes through this lymph node has been studied. Watson and Davies (1985) obtained results, from ewes immunized in the hind limb, indicating that most leukocytes in efferent mammary lymph are derived from the supramammary lymph nodes. Also, the general components listed in the previous section are maintained throughout all lymph nodes, thereby giving a general understanding of the cells and proteins which would constitute the supramammary lymph node.

A study analyzing the flow and content of afferent lymph found that there were major differences between the afferent lymph and blood plasma of cows (Gorweit *et al*., 1993). Specifically, the lymph contained up to ten times less protein, albumin, and globulin than plasma. Calcium levels in lymph were half that compared to plasma. Gorweit *et al*. (1993) found that glucose concentrations were similar between afferent

mammary lymph and plasma; however, afferent lymph contained 17 times less cholesterol than blood plasma and half as much triglyceride (Gorweit *et al*., 1993). A study by Waller *et al*. (2003) examined cytokines in afferent and efferent lymph of the supramammary lymph node after intra-mammary infusion with *Escherichia coli* endotoxin which causes mastitis in cows. Neutrophils, IL-8, and TNF-α were found in both efferent and afferent lymph as soon as 2 hours after infusion. IL-8 plays a primary role in the recruitment of neutrophils into the bovine mammary gland; therefore, the presence of IL-8 in the lymph from the supramammary lymph node is important in the defense against infection of the gland.

Naïve lymphocytes are recruited to lymph nodes through adhesion molecules. Peripheral node addressin (PNad) and vascular cell adhesion molecule (VCAM-1) adhesion molecules were found in the supramammary lymph node. PNad was detected in the lymph node at four different physiological stages in the cow: pregnant, colostral, lactation, and involution (Hodgkinson *et al*., 2007). PNad was not found in the mammary alveolar tissues, suggesting that it is involved in recruitment of lymphocytes to the supramammary lymph node. Leukocytes form a bridge while binding to PNad via P-selectin, which binds carbohydrate ligands on the leukocytes (Walzog and Gaehtgens, 2000). This binding aids in the recruitment of cells to the lymph node. VCAM-1 was found in the mammary tissues as well as the lymph node (Hodgkinson *et al*., 2007). VCAM-1 functions by mediating leukocyte-endothelial interactions by binding to the $\alpha_4/ß_1$ and the α_4/β_7-integrins as well as to the $ß_2$-integrins (Walzog and Gaehtgens, 2000).

The bovine mammary gland may also be an important determiner of components inside the lymph node since draining lymphatics would carry mammary tissue fluids in

the lymph. A study in which autologous lymphocytes were infused into the bovine mammary gland found that the lymphocytes were able to migrate into the supramammary lymph node (Kimura *et al*., 2005). The mammary gland is composed of several different cell types: epithelial cells, myoepithelial cells, fibroblast cells, adipocytes, endothelial cells, and plasma cells (Lacasse *et al*., 1996). Growth factors and other proteins produced and secreted by these cells could drain into the supramammary lymph node. IGF-I and prostaglandin E2 (PGE2) are two prominent factors found in the mammary gland and are also present in the lymph from the supramammary lymph node (Lacasse *et al*., 1996). Epidermal growth factor, TGF-α and -β are also important effectors of mammary growth and present in the mammary gland (Plaut, 1993).

The position of the supramammary lymph node provides a rapid immune response to antigens in the udder. Sordillo *et al*. (1995) stimulated monocytes from supramammary lymph nodes and peripheral blood of mid- to late- lactating and periparturient cows with lipopolysaccharide. Monocytes isolated from lymph nodes during the periparturient period had significantly higher levels of TNF-α compared to those from peripheral blood (Sordillo *et al*., 1995). Ewes vaccinated against *S. aureus* in the hind limb (directly primed' supramammary nodes) (before challenge) had significantly higher outputs of IgM and IgG2 containing cells in lymph as well as higher concentrations of IgG1 and IgG2 antibodies compared to controls, brisket immunized, and lymphadenectomised ewes (Watson and Davies 1985). Opdebeeck and Norcross (1984) also found that high levels of antibody can be maintained in milk if cows are vaccinated in the region of the supramammary lymph node with Freund's incomplete adjuvant along with staphylococcal and streptococcal antigens. The theme among these

studies was that the closer the antigen is delivered to the area of the lymph node; the better the immune response that can be produced. This provides some insight as to why the lymph node is positioned as it is in the mammary gland.

2. INTRODUCTION

The bovine supramammary lymph node located on the dorso-caudal surface of the udder (Bradley *et al*., 2001) is discarded during meat processing of cows. This protein-rich lymphoid organ is easily recovered during processing when the udder is removed. The apparent source of bioactive proteins in the lymph node led to the idea of substituting a lymph node extract for serum in cell culture media. In order to do this, the lymph node must have the appropriate growth factors to support growth of a variety of cell lines.

Lacasse *et al.* (1996) reported mitogenic activity of bovine mammary gland lymph on bovine mammary epithelial cells (MAC-T) and mammary fibroblasts. PGE2, which has been shown to stimulate proliferation of rodent mammary epithelial cells, was high in bovine lymph, and when more PGE2 was added to the lymph mitogenic activity increased (Lacasse *et al*., 1996).

A study also using MAC-T cells tested the mitogenic effects of mammary tissue extracts from prepubertal heifers raised to high- or low-rate of gain (Berry *et al*., 2003). The mammary tissue extracts from high-fed heifers stimulated less mitogenic activity compared to the mammary tissue extracts from low-fed heifers. Berry *et al*. (2003) also extracted tissue from ovariectomized (OVX) heifers and found there were no differences in proliferative responses of MAC-Ts between OVX or control mammary tissue extracts. Weber *et al*. (1999) showed that mammary extracts from prepubertal heifers stimulated mammary epithelial cell proliferation much more than heifer serum or IGF-I alone. The results from these studies collectively show that the bovine mammary gland contains

growth factors for mammary epithelium. IGF-I as well as TGF-α are produced in the mammary gland and stimulate DNA synthesis of mammary epithelial cells (Weber *et al*., 1999). IGF-II is also found in the mammary tissue of ewe lambs. In order to assess how much influence IGF-I has on cell proliferation, recombinant human insulin like growth factor binding protein-3 (rhIGFBP-3), which would bind to IGF-I, was added to serum and extracts, and it ultimately inhibited one third of the DNA synthesis produced by serum or extracts (Weber *et al*., 1999). Clearly, IGF-I is an important growth factor for mammary epithelial cells. Weber *et al*. (2000) also tested mammary tissue extracts on bovine primary mammary epithelial cells and investigated whether or not exogenous growth hormone (GH) or feeding level influenced mitogenic activity. Five percent mammary extract stimulated [^{3}H] thymidine incorporation into DNA of more cells than 10% fetal bovine serum or 100 ng/ml of IGF-I. In concurrence with Berry *et al*. (2003) high-fed compared to low-fed heifer mammary extracts inhibited [^{3}H] thymidine incorporation into cultured mammary epithelial cells. On the other hand, when GH was administered, high-fed compared to low-fed heifers had an increased [^{3}H] thymidine incorporation into mammary epithelial cells in response to mammary tissue extracts. Growth hormone treated high-fed heifers also had increased tissue IGF-I levels (Weber *et al*., 2000).

Bovine mammary epithelial cells (MAC-T) were used in the present study as well. A human breast cancer cell line (MDA-MB-435) and a hybridoma line (1C6) were also used. All three cell types had a common characteristic of adherence in culture. The MAC-T cells were used because of their bovine origin in the mammary gland, as well as their general characteristic as an epithelial cell. The MDA-MB-435 cancer cells were

used for a couple of reasons. As a mammary epithelial cell line they served as a human comparison to the bovine MAC-T cells. They were also used to determine the response of a cancer line to the extract. The 1C6 hybridoma cells were used because of their immunological capacity as well as their general difference in morphology from the MDA-MB-435 and MAC-T cells. Because 1C6 cells are a fusion hybridoma of a myeloma cell and a B cell, components in the lymph node which are naturally present to communicate with B cells, should theoretically stimulate these cells. The 1C6 cells do not possess the spreading characteristic that the other two lines present, but these cells do adhere and maintain a rounded morphology in culture. In order to analyze whether or not the lymph node extract could be used as a substitute in cell culture media these cell lines were used. These cells were chosen not only for their individual characteristics, but for the diversity of cell origin and apparent dependence on either the mammary gland or lymph node environment.

Rather than using mammary tissue or lymph, as previous studies have tested, we chose to use only the supramammary lymph node. As an immunological organ this lymph node is a rich source of a variety of leukocytes, cytokines, and potential growth factors. Unlike the mammary tissue, the supramammary lymph node is involved in the capturing and processing of antigens and may be void of infections that may be present in the udder. This allows assurance that toxins will not be introduced to the cells the extract is tested on in culture. Along with the possible mitogenic factors in the lymph node, the recovery and economical aspects were also factors in this study. At this time the udder along with the supramammary lymph nodes are thrown away as waste during slaughter. The lymph nodes are very easy to extract allowing one to obtain a large quantity of

material for research and potential commercial use. This project is an initial attempt to understand the effects of the bovine supramammary lymph node extract on cell growth as a potential cell culture supplement.

3. MATERIALS AND METHODS

Cell Culture

Three different established cell lines were used for this study. A human breast cancer epithelial cell line (MDA-MB- 435) was obtained from Tissue Culture Shared Resource at Georgetown University, Lombardi Cancer Center. Hybridoma cells (1C6) were created in Dr. Tom Scott's lab by fusion of spleen cells of immunized Balb/c mice with SP2/0 myeloma cells. Bovine mammary epithelial cells (MAC-T) obtained from Dr. Steve Ellis were also used (Huynh *et al*., 1991).

MDA-MB- 435 cells were thawed and initially cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% bovine growth serum, 1% penicillin/streptomycin, 1% amino acids, 11mg sodium pyruvate, and 4.0mM L-glutamine. The MAC-T cells were cultured in DMEM containing 10% bovine growth serum, 1% penicillin/streptomycin, and 4.0mM L-glutamine. The 1C6 cells were cultured with DMEM containing 10% bovine calf serum, 1% penicillin/streptomycin, 11mg sodium pyruvate, 238mg HEPES, 100 ml 2-mercaptoethanol, and 4.0mM L-glutamine. All cells were cultured in 75 cm^2 flasks and incubated at 37ºC in a humidified, water-jacketed CO_2 (5%) incubator.

Lymph Node Preparation

Supramammary lymph nodes, collected at random, of both beef and dairy cows were obtained from the Brown Packing facility in Gaffney, South Carolina. The isolated lymph nodes were prepared according to one of two methods.

Lymph Node Preparation #1:

Initially lymph nodes were trimmed of fat with scissors and homogenized in a food-grade blender with 10 ml of PBS for every five lymph nodes. The extract was then centrifuged for 30 min at 26,500 × *g*. The supernatant was recovered and centrifuged again for 30 min at 32,500 × *g*. Twenty ml of PBS were added to every 10 ml of extract and filter sterilized with serum acrodisc (0.2µm) syringe filter. The extract was then heat inactivated at 60ºC for 60 min. This lymph node preparation yielded a protein concentration of 3.0 mg/ml.

Lymph Node Preparation #2:

Lymph nodes were trimmed of fat with scissors and processed through a Hobart meat grinder until moderately homogenous. The homogenate was placed in freezer bags at -80ºC for two days. The frozen lymph node homogenate was then crushed into small pieces under liquid nitrogen in a mortar with pestle and lyophilized for approximately seven days in a Virtis freeze dryer (SP Industries Inc, Warminster, PA) . The homogenate was then ground into a fine powder using a small food processor. Five grams of the powder was weighed and mixed with PBS in a centrifuge tube to reach a total weight of 50 grams. The solution incubated at room temperature for 20 min and was then centrifuged for 15 min at 739 × *g*. The supernatant was removed and heat

inactivated at 60ºC for 60 min. The solution was then centrifuged for 30 min at 7,000 × g and filter sterilized (0.2 µm) with a Nalgene bottle top filter into a sterile container. This preparation yielded a protein concentration of 27.0 mg/ml.

Protein Concentration

Protein concentration of the lymph node homogenate was determined using the Warburg-Christian protein assay (Warburg and Christian, 1942). The homogenate was diluted with nanopure water to a 1:10 dilution. One hundred microliters of the diluted heat inactivated lymph node homogenate was added to a cuvette and read with an Eppendorf Biophotometer spectrophotometer. Readings at A_{260} as well as A_{280} were incorporated into the Warburg-Christian equation ($C= 1.55 \times A280 - 0.76 \times A260$). The product of the equation was then multiplied by a dilution factor of 10 in order to obtain the protein concentration.

MTT Assay

The MTT cell viability assay was used to assess the cellular activity of MDA-MB-435 breast cancer epithelial cells exposed to lymph node extract supplementation in culture medium. MTT [(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), Sigma-Aldrich, St. Louis, MO] is a tetrazolium salt that undergoes reduction (yellow to purple color change) in viable cells with active mitochondrial electron transport systems. Cells were trypsinized with HyQ® Trypsin (0.25%) and centrifuged

for 5 min at 400 × *g*. The supernatant was removed and the cells were re-suspended in approximately 2 ml of medium. Cells were counted and added to a 96-well plate with 10,000 cells/well and held overnight in a CO_2 incubator to adhere. The next day all media was removed and the wells were rinsed two times with 100µl sterile PBS. The appropriate media were then added to the wells without serum and incubated for 2 days for the starvation period of the assay. Lymph node and BGS media were made at different percentages with DMEM. 0, 1, 2, 2.5, 5, 10 and 20% media were added to the plate at 100µl/well. Zero percent medium was made without serum or lymph node extract. Lymph node preparation #1 was used for the MTT assay. In order to maintain an equal protein concentration across treatments, BGS and non-heat inactivated lymph node extract were diluted with PBS to the concentration of the heat inactivated lymph node extract (3.0 mg/ml). For the 1% media, 1 ml of 3.0 mg/ml BGS and lymph node extract (.03 mg/ml) was added to 99 ml of MD-MBA-435 media with the appropriate components. For 2%: 2 ml was added to 98 ml (.06 mg/ml), 2.5%: 2.5 ml was added to 97.5 ml (.075 mg/ml), 5%: 5 ml was added to 95 ml (.15 mg/ml), 10%: 10 ml was added to 90 ml (.3 mg/ml), and for 20%: 20 ml was added to 80 ml (.6 mg/ml). Four days later 50µl of MTT was added to each well four hours prior to end of incubation. The media with MTT was then removed and 150µl of DMSO was added to each well. The plate was placed on a shaker for approximately 15 minutes. Absorbance was obtained at wavelengths 570 and 650 nm for each well. The O.D. by difference was recorded for each well.

CyQuant Assay

The CyQuant assay is used to determine the degree of DNA synthesis and cellular proliferation through direct DNA staining with the CyQuant GR fluorescent dye. CyQuant GR dye and 20x cell lysis buffer were purchased from Invitrogen (Carlsbad, Cal). Serum starvation assay was run according to the protocol of MTT assay until addition of CyQuant GR dye. Lymph node preparations #1 and #2 were used in the CyQuant assay. MDA-MB-435 cells were treated with both preparations in two separate CyQuant assays, and MAC-T and 1C6 cells were treated with only lymph node preparation #2. Lymph node preparation #2 yielded the following protein concentrations: 1%: .27 mg/ml, 2%: .54 mg/ml, 5%: 1.35 mg/ml, 10%: 2.7 mg/ml, 20%: 5.4 mg/ml. After 3 days with the different treatments the plate was inverted and blotted. The CyQuant GR dye/lysis buffer contained 1.2 ml of 20x lysis buffer, 22.8 ml nuclease free distilled water, and 60µl of CyQuant GR dye. Two hundred µl of dye/lysis buffer were added to all wells. Plates were mixed gently and incubated for 2 to 5 min with covers to protect from light. Plates were read on a BioTek Synergy HT plate reader at excitation of 480 and emission of 520 nm. Fluorescence intensity of each well was recorded.

Cell Growth Assays

To grow cells directly in lymph node media a direct suspension method (adherence growth assay) as well as a modified adaptation method were used. Lymph node extract from lymph node preparation #2 was used for the cell growth assays. Both

assays used 10% lymph node medium with 1X DMEM as well as other medium components appropriate for the specific cell line in culture. Bovine growth serum was diluted with PBS in order to maintain all treatments at an equal protein concentration. A protein concentration of 2.7 mg/ml (10% of 27 mg/ml lymph node extract stock) was maintained throughout the assays. Diluted serum media was used as a positive control as well as a 0% serum/lymph node medium as a negative control.

Adherence growth assay:

Cells were cultured to 100% confluency in regular media containing 10% serum (BGS or BCS). Cells were then trypsinized and re-suspended in 2 ml of serum/lymph node-free medium. Once cells were counted they were seeded at 10,000 cells/well in 24-well plates with appropriate treatment media. Each treatment covered 12 wells. Five hundred µl of treatment medium was added to each well. Cells adhered overnight and were photographed once daily for 6 days using an inverted microscope fitted with a digital camera. Plates were properly positioned on the microscope stage and pictures were taken with the 40X objective.

Adaptation growth assay:

Cells were cultured to nearly 100% confluency in standard medium containing non-diluted 10% serum (approximately 6.6 mg/ml of BGS or BCS) in a 25 cm^2 flask as well as a six well plate. Once confluent, all media were removed and 25% of 2.7mg/ml lymph node media was added along with 75% regular media with BGS. Once medium was pulled off of the well of the six-well plate, Azure II dye was added and photos were taken. Photos of the cells were also taken without Azure II dye staining. Day 1 represents confluency, Day 2 is 25% lymph node media, Day 3 is 50% lymph node

media, Day 4 is 75% lymph node media, and Day 5 is 100% lymph node media. For the MDA-MB-435 cells as well as the MAC-T cells trypsin was added on Day 5 and cells were re-seeded into a new flask. 1C6 cells were scraped and re-seeded into a new flask. Cells were incubated overnight to adhere. This was done to evaluate the "adapted" cells ability to re-adhere with only lymph node medium in culture. Photos were taken of the newly seeded flasks without Azure II dye. Cell growth in the flasks and plates was assessed through visual analysis. Detachment of cells resulting in floating cells, cellular debris, and standard attachment and spreading out, were characteristics of cells observed in the cultures.

Apoptosis Assay

To determine if cell death was due to apoptosis during the growth assays, DNA extraction and gel electrophoresis were performed on 4 differently treated groups of MDA-MB-435 cells. Cells were plated 100,000 cells/well on 6 well plates. The first group went through a starvation period of 2 days before lymph node medium (2.7 mg/ml) was added. The second group was plated and allowed one day to adhere before lymph node medium was added. The third group contained cells that were adapted to lymph node medium and were plated the day of lymph node media addition. The fourth group of cells was plated the day of lymph node medium addition. Cells were trypsinized, scraped, and removed from wells at 0, 2, 4, 8, and 24 h time points post addition of lymph node medium. Cells were centrifuged at 13,000 x *g* for 5 min. DNA extraction was performed at each time point following the DNeasy Qiagen protocol. Pellets were

re-suspended in 200µl PBS and 20µl of proteinase K (600 mAU/ml) as well as 4µl of RNase A (7,000mAU/ml) were added. Two hundred µl of buffer AL (lysis buffer) was added and cells were incubated at 56°C for 10 min. Ethanol was added and sample was vortexed. The entire mixture was added to DNeasy Mini spin columns and centrifuged twice at 6000 x *g*. Buffer was added and solution was centrifuged for 3 min at 20,000 x *g*. One hundred µl of Buffer AE was added to columns and centrifuged twice for 1 min at 6000 x *g* for the elution of DNA.

DNA content for each sample was determined with a spectrophotometer in order to obtain DNA concentrations. The lowest concentration obtained dictated the addition of 100 ng of DNA per lane in an agarose gel. One kilobase (kb) and 100 base pair (bp) ladders were added at 6µl/well (ProMega benchtop ladders) in assigned lanes of each gel. One percent agarose gel with 0.5X TBE running buffer was used. Samples were loaded and the gel ran for 1 h at 126 volts.

Protein Gel

BGS as well as lymph node preparation #2 were run on a SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) 12% Tris/HCl gel. Samples were denatured with SDS PAGE sample buffer (100 mM Tris, 2% SDS, 5% ß-mercaptoethanol, and 15% glycerol) and boiled for 5 minutes. Five µl of each sample were loaded on a 30 µg/ml basis with GelCode® Blue Stain Reagent (Pierce Biotechnology, Inc., Rockford, IL) along with 2 Biorad Precision Plus Protein Dual Color

Standards (Bio-Rad Laboratories, Inc., Hercules, CA). The gel was run at 120 volts for 1 h.

Statistical analysis

MTT and CyQuant assay data were analyzed using the SAS program (Statistical Analysis System, Cary, NC). The MTT and CyQuant experiments were completely random designs with factorial arrangements of treatments. The main effects were media supplements (BGS vs. LN), and concentrations of supplements, with the interaction being media supplement by concentration of supplement. The experimental model was as follows: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$ where, μ= overall mean, $\alpha_i = i^{th}$ level of the media supplement, $\beta_j = j^{th}$ concentration of the media supplement, $(\alpha\beta)_{ij}$ = the interaction of the i^{th} level of media supplement with the j^{th} concentration of supplement, and eijk = random error. Variability between treatments was determined through an ANOVA procedure using PROC GLM with an α level of 0.05. Differences between treatment means were further separated by least-significant differences (LSD).

4. RESULTS

MTT assay with MDA-MB-435 cells

MDA-MB-435 cells underwent a serum starvation period of 2 days and a medium re-supplementation treatment period of 4 days. All media re-supplementation treatments were based on an equal protein basis (3.0 mg/ml), and MTT was added on the fourth day of treatment to assess all cell viability. Results in Figure 1 are expressed as stimulation indices (S.I. = O.D. supplemented media/O.D. 0% supplemented media) of cell viability assessed through MTT reduction. A stimulation index above 1 is representative of cell viability greater than the control group (0% serum or lymph node extract). Figure 1 shows that heat inactivated lymph node extract supports significantly greater cell viability at 10 and 20% supplementation (.3 and .6 mg/ml protein) than observed for both BGS and non-heat inactivated lymph node extract ($p<.0001$). 20% heat inactivated lymph node extract in fact supports cell viability even greater than it does at 10%. The heat inactivated extract is the only treatment with a S.I. above 1 at every supplement concentration. As low as 1% (.03 mg/ml) heat inactivated lymph node extract can support cell viability better than the traditional BGS supplementation at the same percentage. 1% heat inactivated lymph node extract also supports viability better than 10% and 20% BGS. BGS does support cell viability at 2, 2.5 and 5% supplementation, with marginal support at 10%. Non-heat inactivated lymph node extract does not sustain cell viability at any concentration. Non-heat inactivated lymph node extract inhibits cell viability as evidenced by the much lower S.I. values compared to the control point of 0%

supplementation (dashed line).

CyQuant assay with MDA-MB- 435, MAC-T, and 1C6 cells

MDA-MB-435 cells underwent the same serum starvation assay as with the MTT assay. CyQuant assays were run with both 3.0 and 27 mg/ml lymph node extracts. The results are expressed as a proliferation index (P.I. = fluorescence intensity of supplemented media/fluorescence intensity of 0% supplemented media). A proliferation index above 1 (indicated by the dashed line) is indicative of cell proliferation greater than the control group (0% serum or lymph node extract). Figure 2 represents proliferation of MDA-MB-435 cells with lymph node preparation #1 (3.0 mg/ml). This figure shows that the heat inactivated extract supports proliferation better than both non-heat inactivated lymph node extract and BGS at all percentages of supplementation. Bovine growth serum best supports cell proliferation at 2.5% (.075 mg/ml protein), followed by a decline in proliferation from 5 to 20%. One percent heat inactivated lymph node extract supports cell proliferation better than 5, 10, and 20% supplementation with BGS. In concurrence with Figure 1, a lower protein concentration of .03 mg/ml (1%) heat inactivated lymph node extract supports cells better than the standard BGS supplement at the highest three protein concentrations tested. Non-heat inactivated lymph node extract does not support cell proliferation at any percentage as evidenced by the apparent inhibitory level of cell proliferation demonstrated in Figure 2.

The CyQuant assays with lymph node preparation #2 did not include non-heat inactivated lymph node extract because of the evident inhibitory effects observed with

preparation #1. MDA-MB-435, MAC-T, and 1C6 cells were cultured with lymph node preparation #2. Figure 3 illustrates the effects of lymph node preparation #2 (27 mg/ml) lymph node extract on MDA-MB-435 cells. The proliferation of cells cultured with BGS and heat inactivated lymph node extract is significantly different ($P<.0001$) at all percentages except 1% supplementation (.27 mg/ml protein). BGS maintains cell proliferation at or slightly above the 0% control from 1-5%, with a slight drop at 10 and 20% supplementation (2.7 and 5.4 mg/ml protein). The heat inactivated lymph node extract maintains cell proliferation below the 0% control from 1-5% supplementation; however, MDA-MB-435 cells exhibit a dramatic increase in proliferation with heat inactivated lymph node extract at 10 and 20% supplementation. This latter effect of heat inactivated lymph node extract resulted in a significant supplementation by percent supplementation interaction ($P<.0001$).

MAC-T cell proliferation is significantly different between BGS and heat inactivated lymph node extract cultured cells at 1, 10, and 20% supplementations (Fig 4). BGS maintains cell growth at or slightly above the 0% control throughout all percent supplementations. Heat inactivated lymph node extract supports cell proliferation slightly above the 0% control from 1- 5% supplementation. Similar to the results with the MDA-MB-435 cells, MAC-T cells have a dramatic increase in proliferation at 10 and 20% supplementation leading to a significant interaction effect ($P<0.0001$).

1C6 cell proliferation is significantly different between BGS and heat inactivated lymph node extract at all percent supplementations ($P<0.0001$) (Fig 5). BGS maintains cell proliferation slightly below the 0% control at 1, 2, 5, and 10% supplementations, and right at the 0% control level at 20%. Heat inactivated lymph node extract maintains cell

proliferation at or above the 0% control at 1, 2, and 5% supplementations. In agreement with MDA-MB-435 and MAC-T cells, 1C6 cells also have a dramatic increase in proliferation at 10 and 20% supplementation and a significant interaction for supplementation source and percent supplementation ($P \leq 0.001$).

Adherence growth assays

MDA-MB-435 cells as well as MAC-T and 1C6 cells were cultured in respective treatment media. Media without serum or lymph node extract were used as negative controls (0%). Media with 10% bovine growth serum diluted to an equal protein concentration (2.7 mg/ml) were positive controls (BGS). Media were also made with 10% lymph node extract (LN). The photos were visually assessed to rate cell growth and adhesion. The adherence assay was also attempted using the 1C6 cells, however, the cells failed to grow in any treatment. Photos were not taken due to inadequate visibility of cells. On Day 1, with 0% supplementation, MAC-T cells did not appear to be actively proliferating, but did illustrate minimal adherence and spreading (Fig 6). Day 1 with BGS, MAC-T cells illustrated normal spreading, unlike cells with the LN which did not adhere or spread and did not have a healthy appearance (Fig 6). Days 2 and 3 continue the trend observed on Day 1 with the MAC-T cells and respective treatments (Fig 7 and 8). By Day 4 the LN treated cells had crenated membranes and cell debris was present (Fig 9). On Day 4 the MAC-T cells with BGS continued to grow to confluency, and the 0% serum cells were not proliferating or spreading out (Fig 9). Days 5 and 6 illustrated similar results as Day 4, with confluency (BGS), cell debris and apparent death (LN), and

a halt in proliferation (0%) (Fig 10 and 11). The cell membranes of the lymph node treated cells appeared picnotic compared to BGS and 0% supplemented cells at any time point. The MDA-MB-435 cells showed a trend similar to the MAC-T cells with all treatments. Day 1 is difficult to decipher because of the difference in magnification (100X vs. 400X) which was done to capture the amount of cells rather than specific morphology at that point (Fig 12). On Day 2 MDA-MB-435 cells showed 0% and BGS supplemented cultures had cells beginning to spread out (0% very minimally), with LN cells already presenting shriveled, crenated membranes and unadhered cells (Fig 13). Day 3 illustrates similar results as Day 2 for LN and 0% cells, with increased confluency of BGS supplemented cells (Fig 14). On days 4 through 6 MDA-MB-435 cells showed proliferation of cells with BGS, rounded clustered cells (halted growth) with 0% supplementation, and cell debris along with apparent cellular death with LN (Fig 15-17).

Adaptation growth assays

Photos were taken of Azure II dyed MDA-MB-435, MAC-T, and 1C6 cells in 6-well plates (Fig 18, 20, and 22) as well as non-dyed cells in 25cm² flasks (Fig 19, 21, and 23). Day 1 for all cell lines represents confluency without any LN media. Day 2 represents 25% of 10% LN media addition, Day 3: 50% of LN media addition, Day 4: 75% LN media addition, Day 5: 100% of 10% LN medium. Day 6, with the different cell lines in the flasks, represents cells after trypsinization or scraping. MAC-T cells in the plate and flask (up to Day 5) maintained confluency and normal morphology (Fig 18 and 19: i-v). On Day 6, after trypsinization, the cells did not re-adhere and cell debris is

present (Fig 19vi). The MDA-MB-435 cells followed the same trend as the MAC-T cells with confluency in the plate and flask up to Day 5 (Fig 20 and 21: i-v). Day 6, after trypsinization, did not show any adherence of cells, and the few floating cells appeared dead (Fig 21vi). Cell debris was not as prevalent as it was with the MAC-T cells. 1C6 cells did not maintain confluency like the MDA-MB-435 and MAC-T cells. Days 1 and 2 cells are confluent, but with 50% lymph node media addition on Day 3, 1C6 cells seem to have a change in morphology as well as a decrease in confluency (Fig 22 and 23). The cells are rounding up by Day 3 and all have a picnotic membrane appearance. 1C6 cells continue to change morphology on Days 4 and 5 with a significant decrease in confluency on Day 5 with the full 10% lymph node extract in the medium (Fig 22 and 23). When 1C6 cells were scraped and re-seeded, these cells were unable to adhere. Few remained floating and appeared dead (Fig 22vi).

Adaptation seemed to have been acquired with the MDA-MB-435 and MAC-T cells considering there were no apparent dead cells on Day 5 (Fig 18v-21v), but once cells were trypsinized and re-seeded they were unable to adhere and ultimately did not survive even following an adaptation phase (Fig 19 and 21vi). 1C6 cells illustrated a lack of adaptation by Day 3 (Fig 22 and 23iii).

Apoptosis Assay

Apoptosis can be determined on a DNA gel by the appearance of 200 bp multiples of DNA fragments. Figure 24 (0 and 2 h time points), Figure 25 (4 and 8 h time points), and Figure 26 (24 h time point) are photographs of 1% agarose gels run with DNA extracted from MDA-MB-435 cells under different conditions. Cells starved for two days and cultured with 10% lymph node extract (2.7 mg/ml) (Lanes 2 and 8), cells adhered overnight and cultured with 10% lymph node extract (Lanes 3 and 9), lymph node extract adapted cells cultured directly into 10% lymph node extract (Lanes 4 and 10), and cells cultured directly into 10% lymph node extract (Lanes 5 and 11). Figure 23 is only one time point (24 h); therefore the same conditions apply but only for lanes 2 through 5. DNA standards are run in Lanes 1(1kb) and Lane 6(100bp). Results obtained from gels at all time points do not indicate apoptosis occurred; however, it was apparent from the culture adaptation experiment that cells were not surviving under particular supplementation and treatment conditions.

Protein Gel

Figure 27 illustrates protein bands from both BGS and lymph node preparation #2 extract. Lanes 1 and 4 represent protein standards. BGS (Lane 2) has very heavy staining bands at 50 kD and above, which is most likely immunoglobulin heavy chains and albumin. Lymph node extract (Lane 3) has a less intense albumin band, as well as an

array of bands spanning the length of the standards. There are obvious differences in protein composition between BGS and the lymph node extract based on this protein gel.

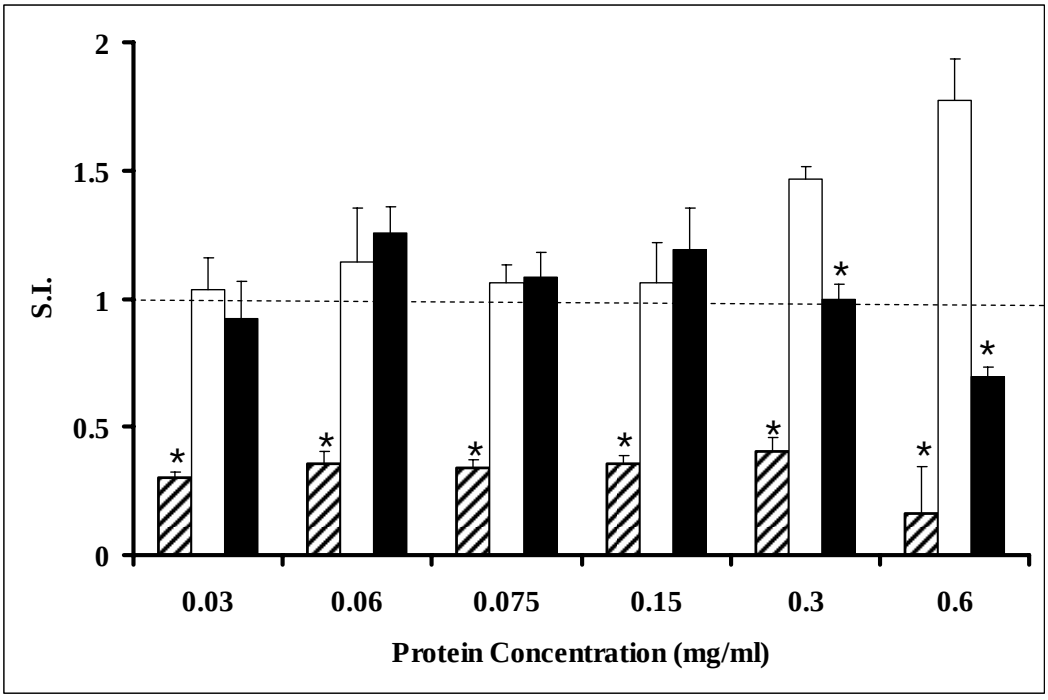

Figure 1. Stimulation index of heat-inactivated lymph node extract (LN) (white), non- heat inactivated LN (striped), and BGS (black) on MDA-MB-435 cells after a serum starvation assay (MTT). *Mean S.I. significantly different ($P \leq 0.05$) from respective mean for heat inactivated LN.

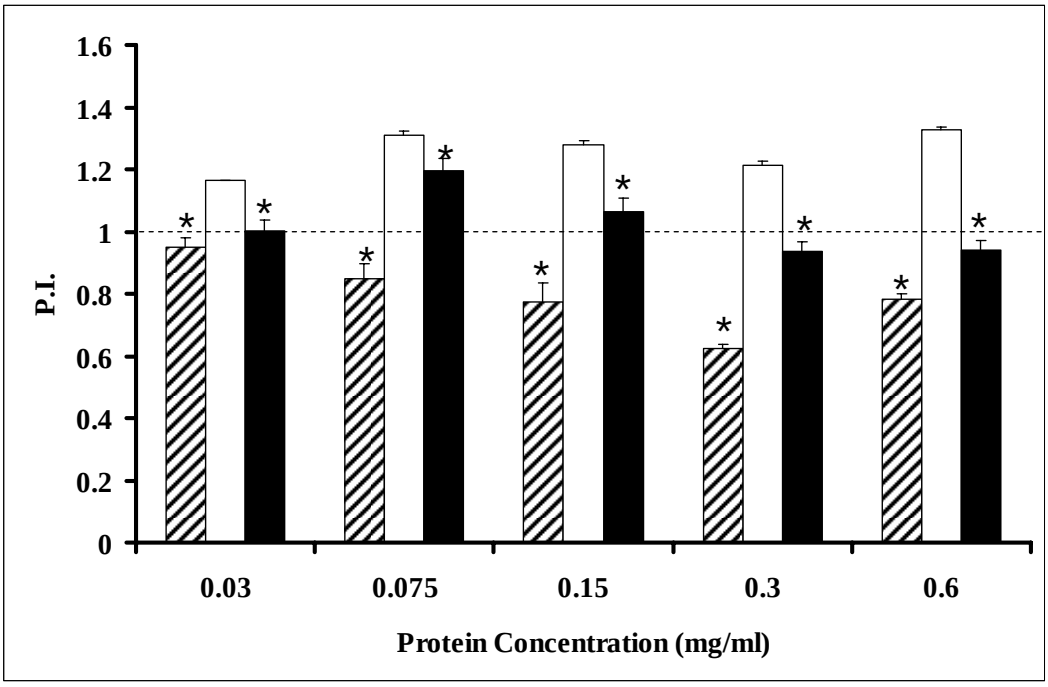

Figure 2. Proliferation index of MDA-MB-435 cells with heat-inactivated lymph node extract (LN) (white), non-heat inactivated LN (striped), and BGS (black). Cells assessed through CyQuant assay. *Mean P.I. significantly different ($P \leq 0.05$) from respective mean for heat inactivated LN.

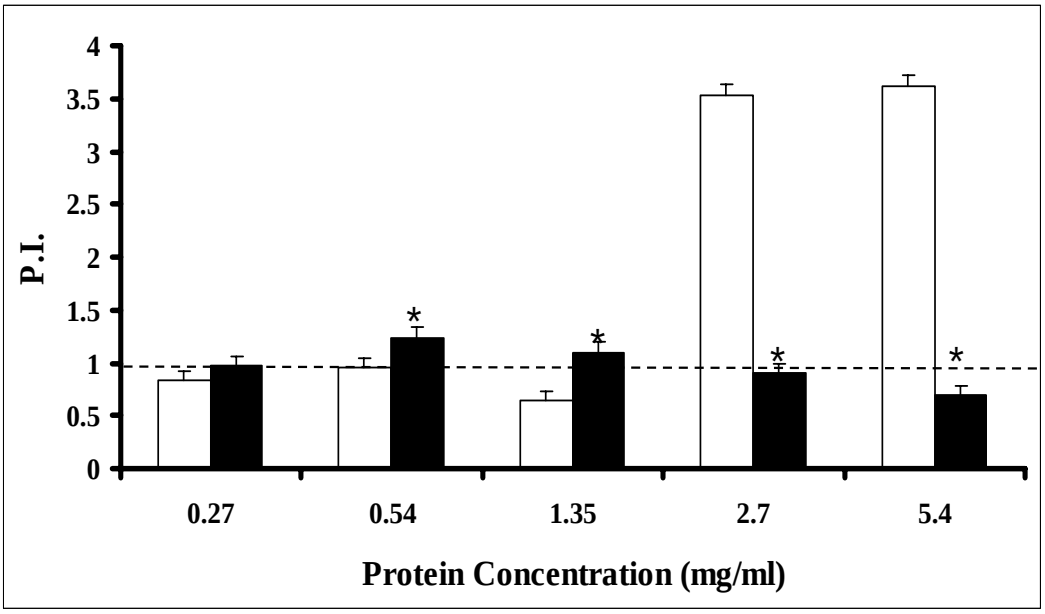

Figure 3. Proliferation index of MDA-MB-435 cells with heat-inactivated lymph node extract (LN) (white) and BGS (black). Cells assessed through CyQuant assay. *Mean P.I. significantly different ($P \leq 0.05$) from respective mean for heat inactivated LN.

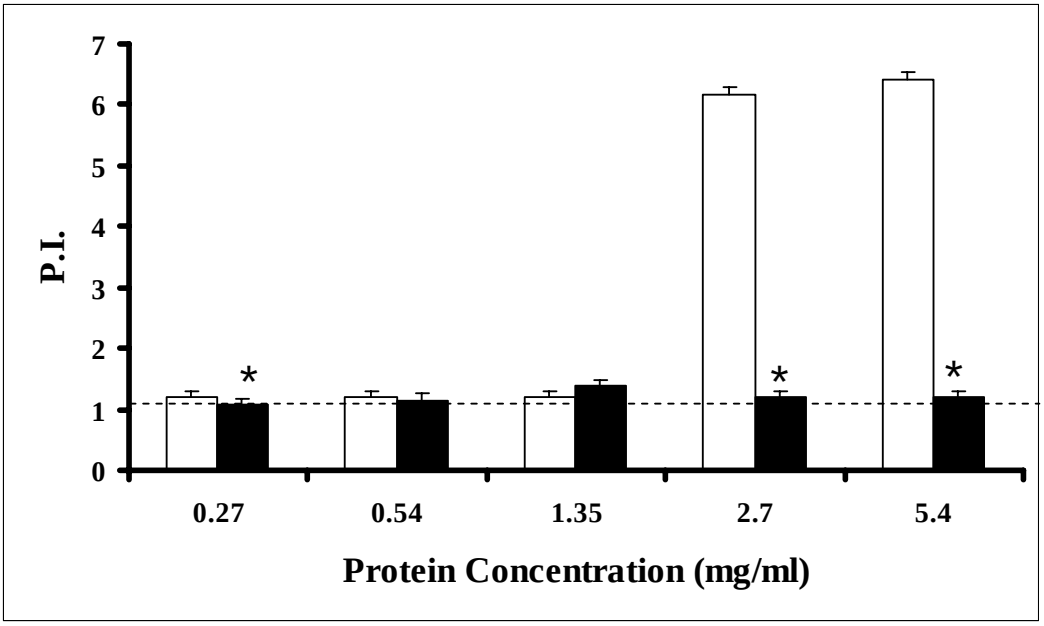

Figure 4. Proliferation index of MAC-T cells with heat-inactivated lymph node extract (LN) (white) and BGS (black). Cells assessed through CyQuant assay. *Mean P.I. significantly different ($P \leq 0.05$) from respective mean for heat inactivated LN.

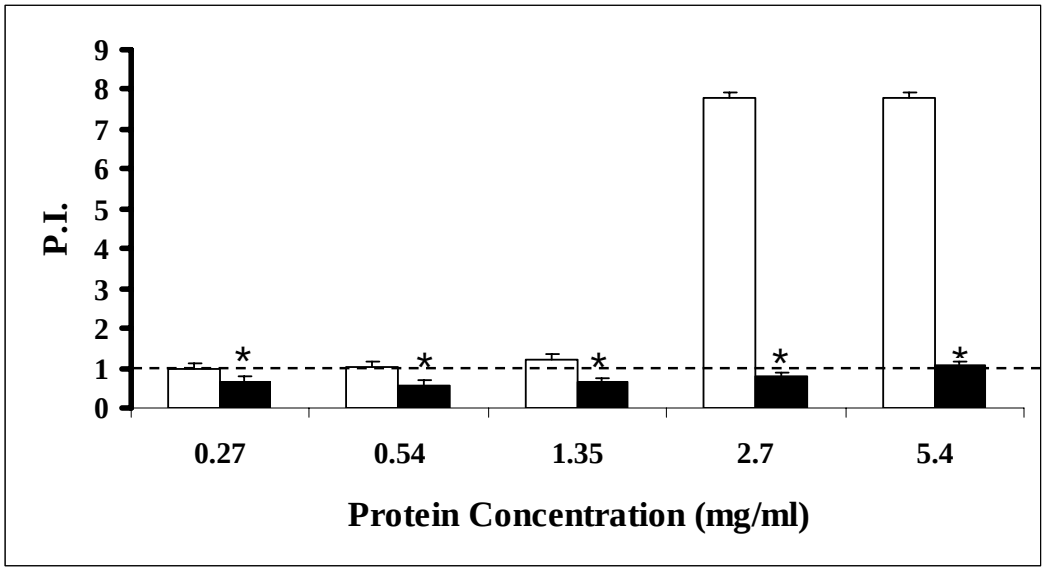

Figure 5. Proliferation index of 1C6 cells with heat-inactivated lymph node extract (LN) (white) and BGS (black). Cells assessed through CyQuant assay. *Mean P.I. significantly different ($P \leq 0.05$) from respective mean for heat inactivated LN.

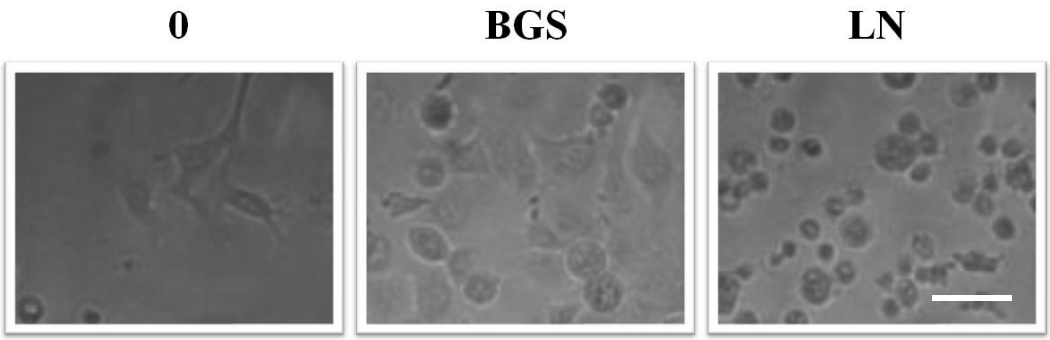

Figure 6. Photomicrographs of 1 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

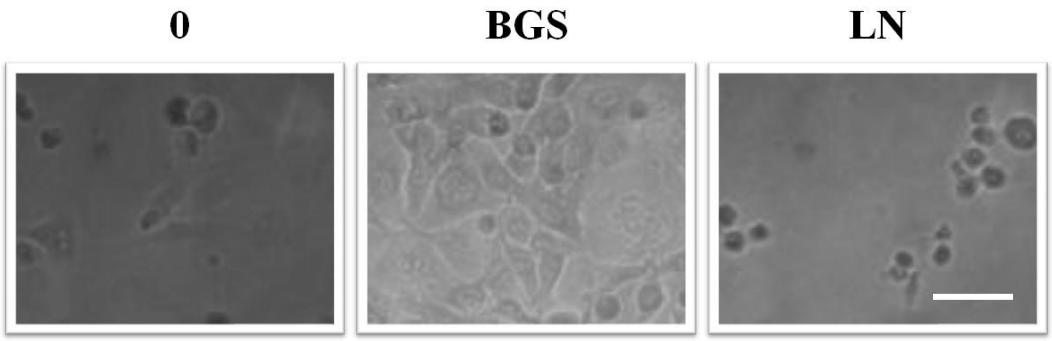

Figure7. Photomicrographs of 2 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

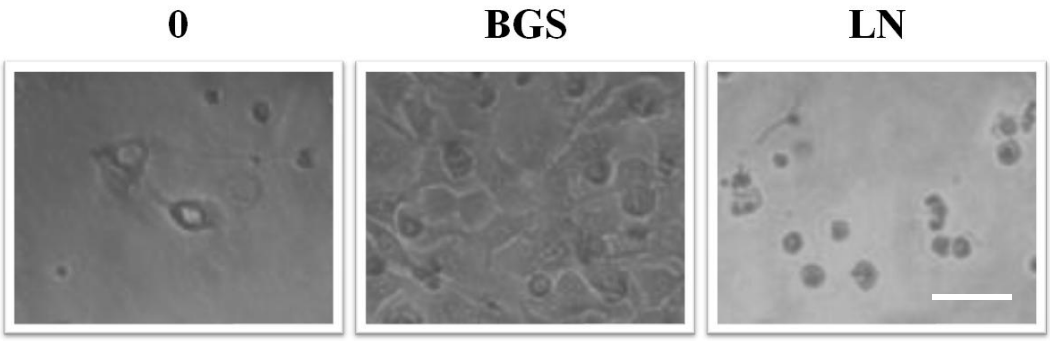

Figure 8. Photomicrographs of 3 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

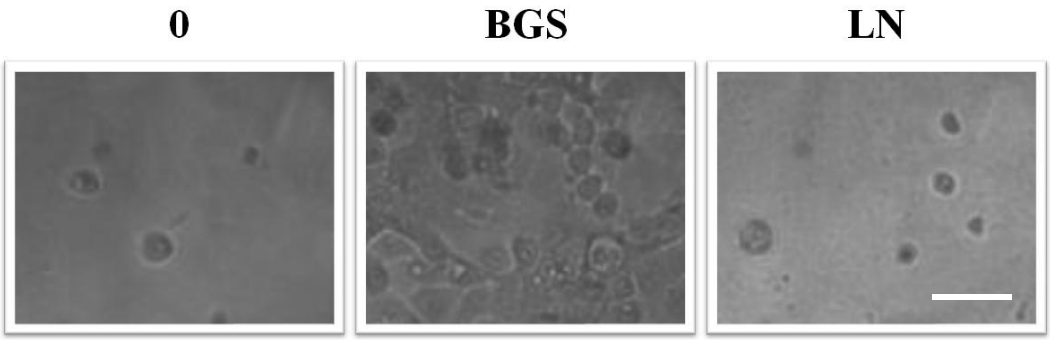

Figure 9. Photomicrographs of 4 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm

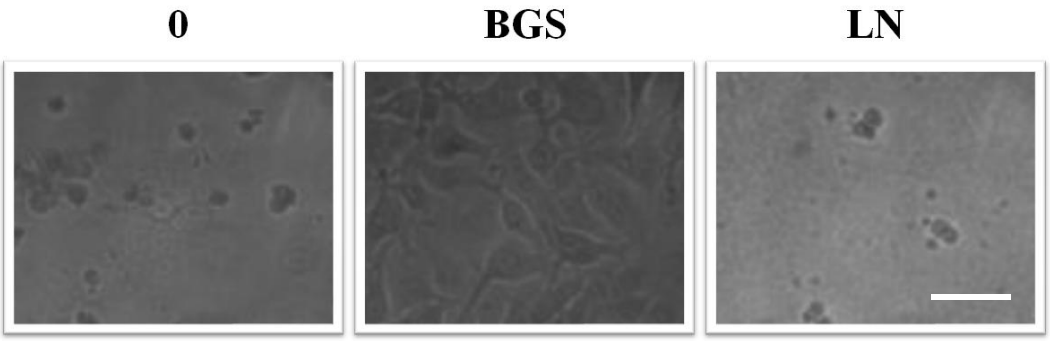

Figure 10. Photomicrographs of 5 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

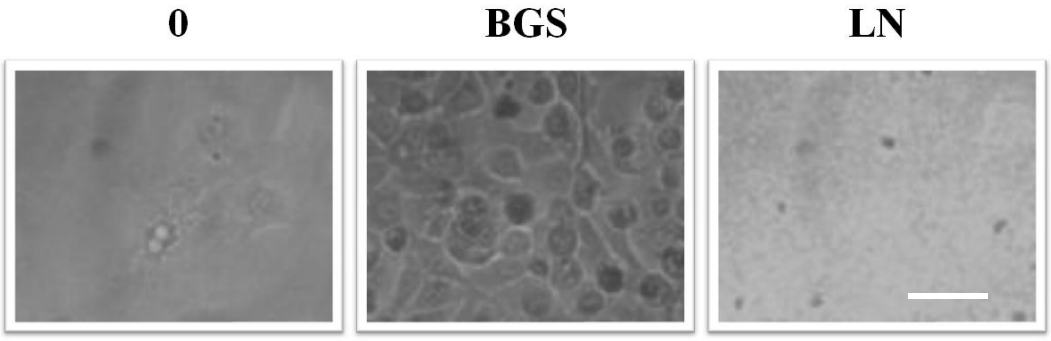

Figure 11. Photomicrographs of 6 day cultures of MAC-T cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

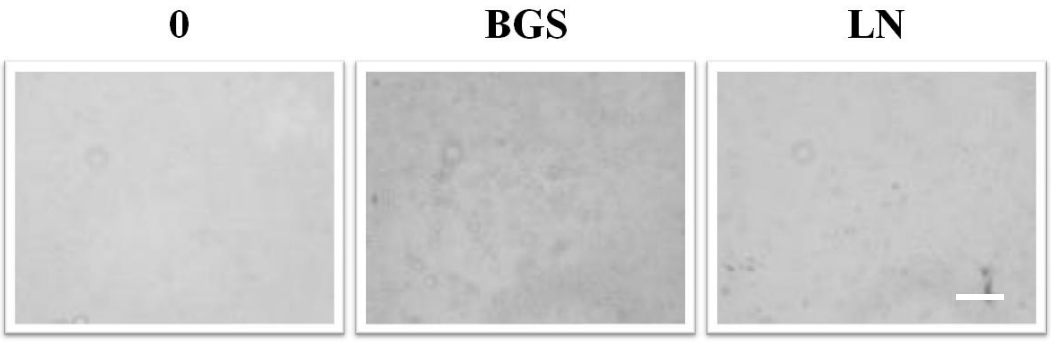

Figure 12. Photomicrographs of 1 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 50 μm.

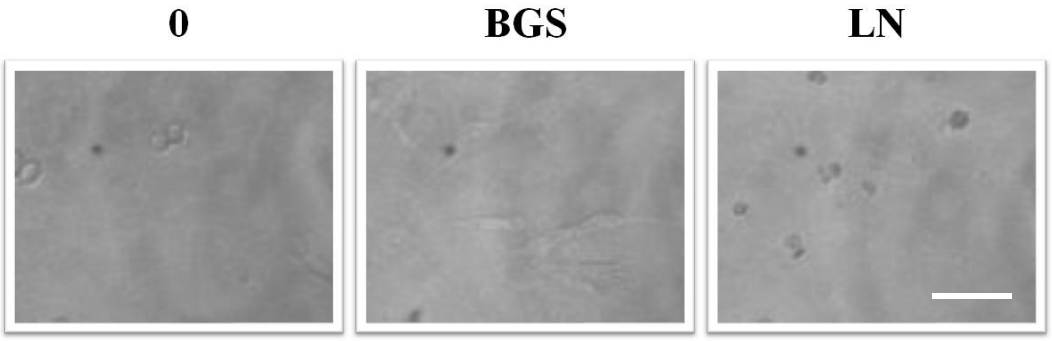

Figure 13. Photomicrographs of 2 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

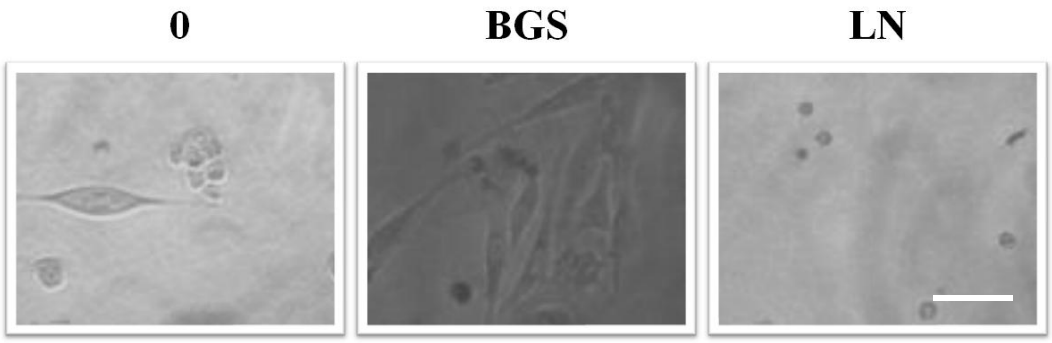

Figure 14. Photomicrographs of 3 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm

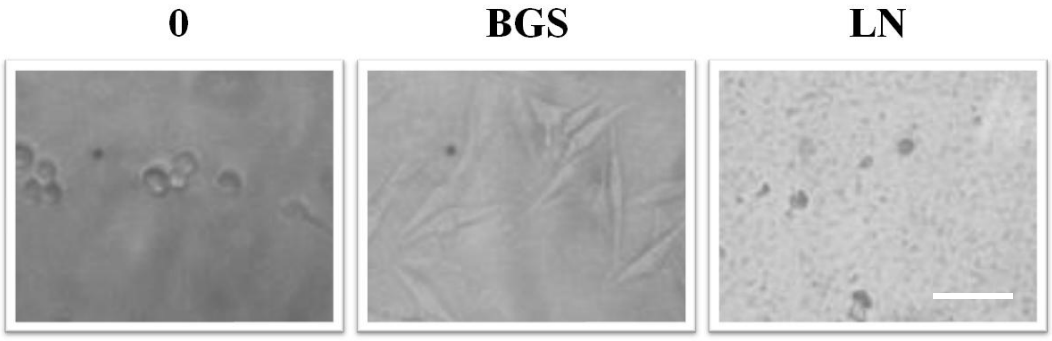

Figure 15. Photomicrographs of 4 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

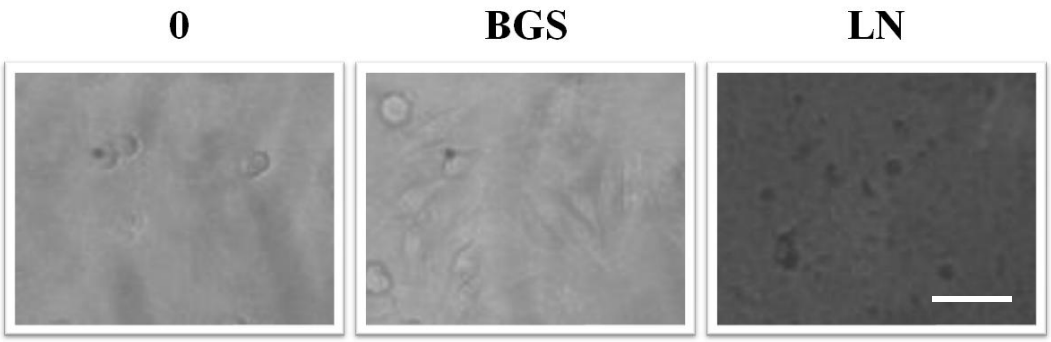

Figure 16. Photomicrographs of 5 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm

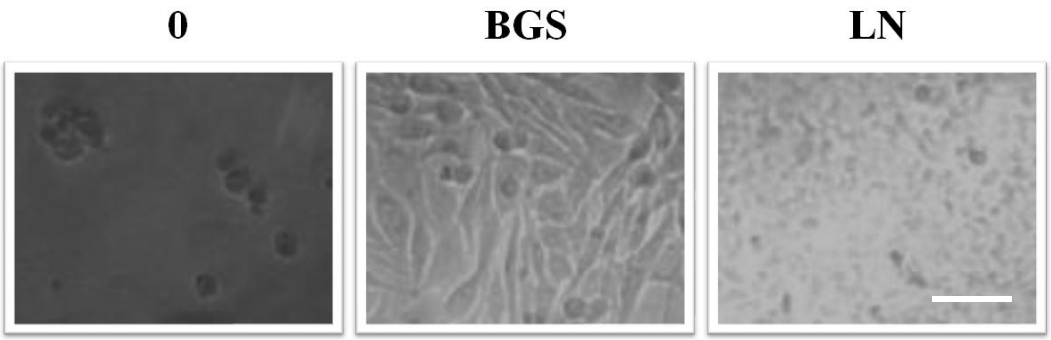

Figure 17. Photomicrographs of 6 day cultures of MDA-MB-435 cells cultured in media supplemented with 0% (0), 10% bovine growth serum (BGS), or 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

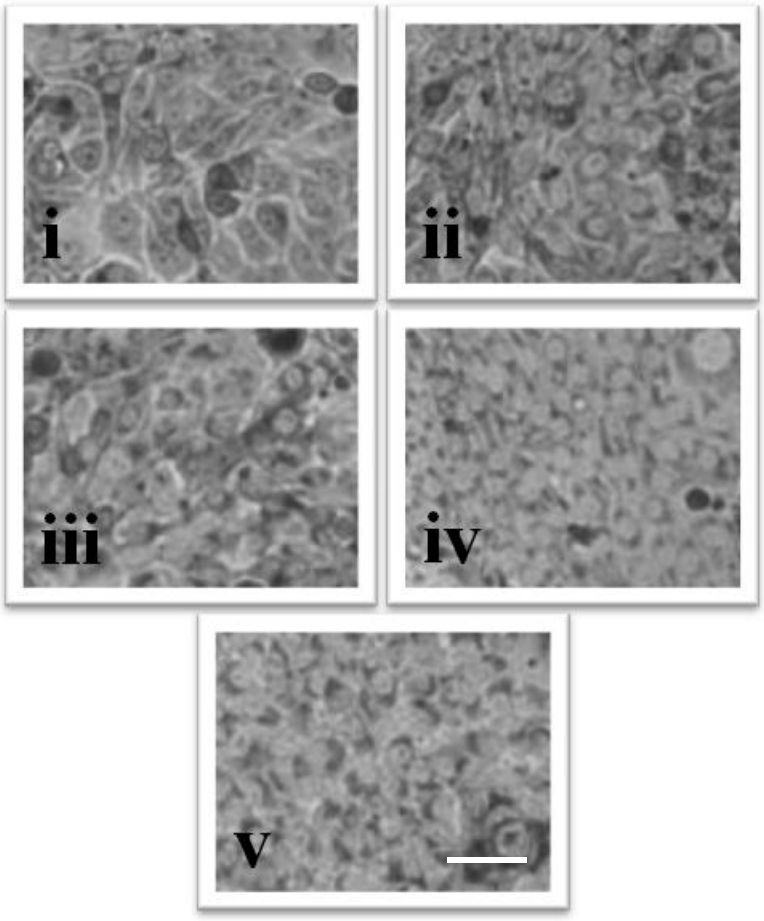

Figure 18. Photomicrographs over a 5 day period with cultures of MAC-T cells cultured in a 6 well plate with media supplemented with10% heat-inactivated lymph node extract (LN). White line = 20 μm.

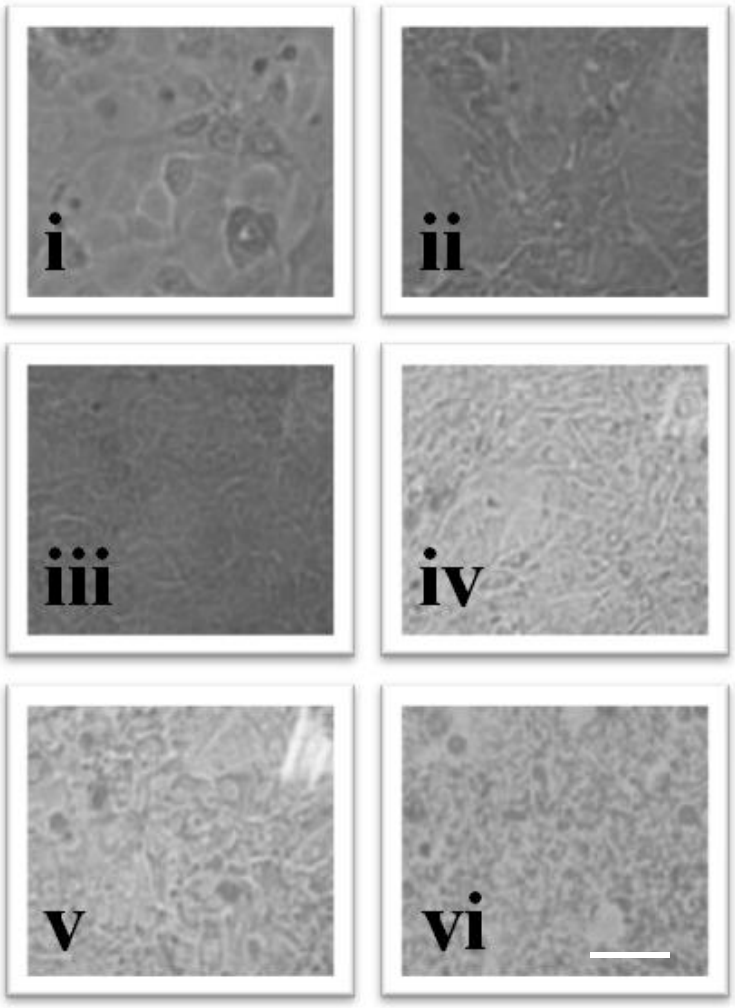

Figure 19. Photomicrographs over a 6 day period with cultures of MAC-T cells cultured in a flask with media supplemented with10% heat-inactivated lymph node extract (LN). Day 6 represents trypsinization. White line = 20 μm..

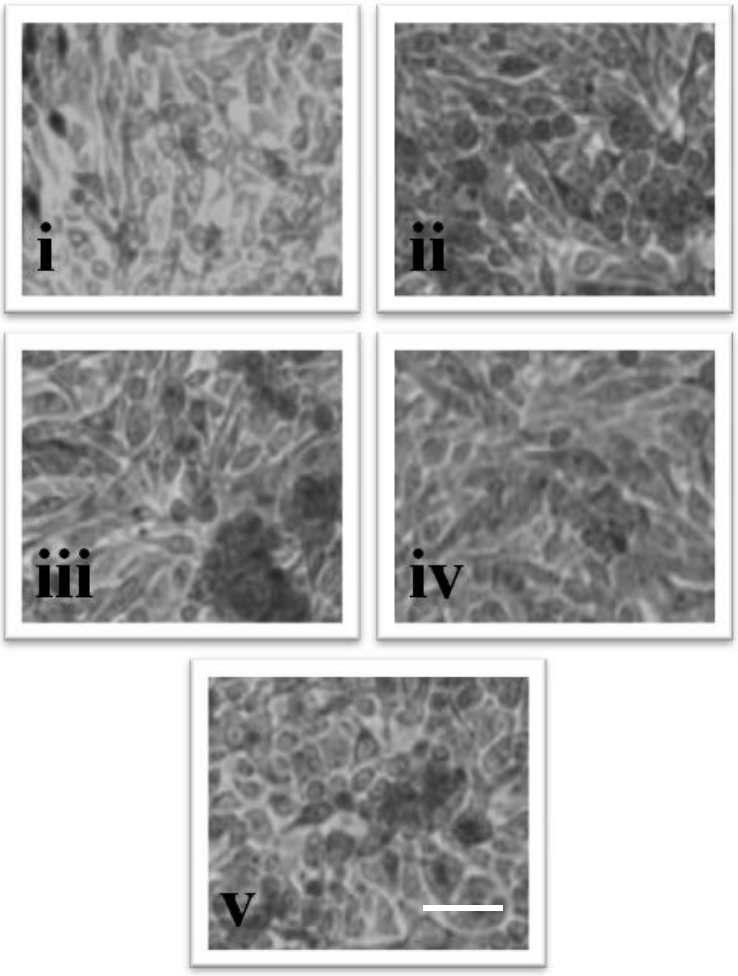

Figure 20. Photomicrographs over a 5 day period with cultures of MDA-MB-435 cells cultured in a 6 well plate with media supplemented with10% heat-inactivated lymph node extract (LN). White line = 20 μm.

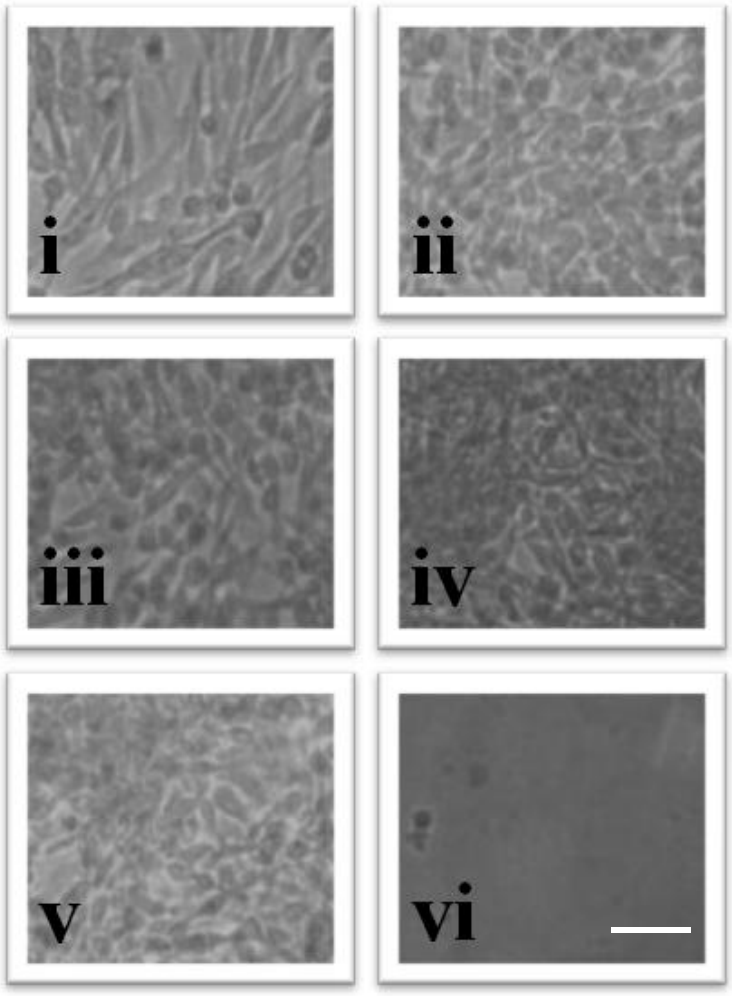

Figure 21. Photomicrographs over a 6 day period with cultures of MDA-MB-435 cells cultured in a flask with media supplemented with 10% heat-inactivated lymph node extract (LN). Day 6 represents cells after trypsinization. White line = 20 μm.

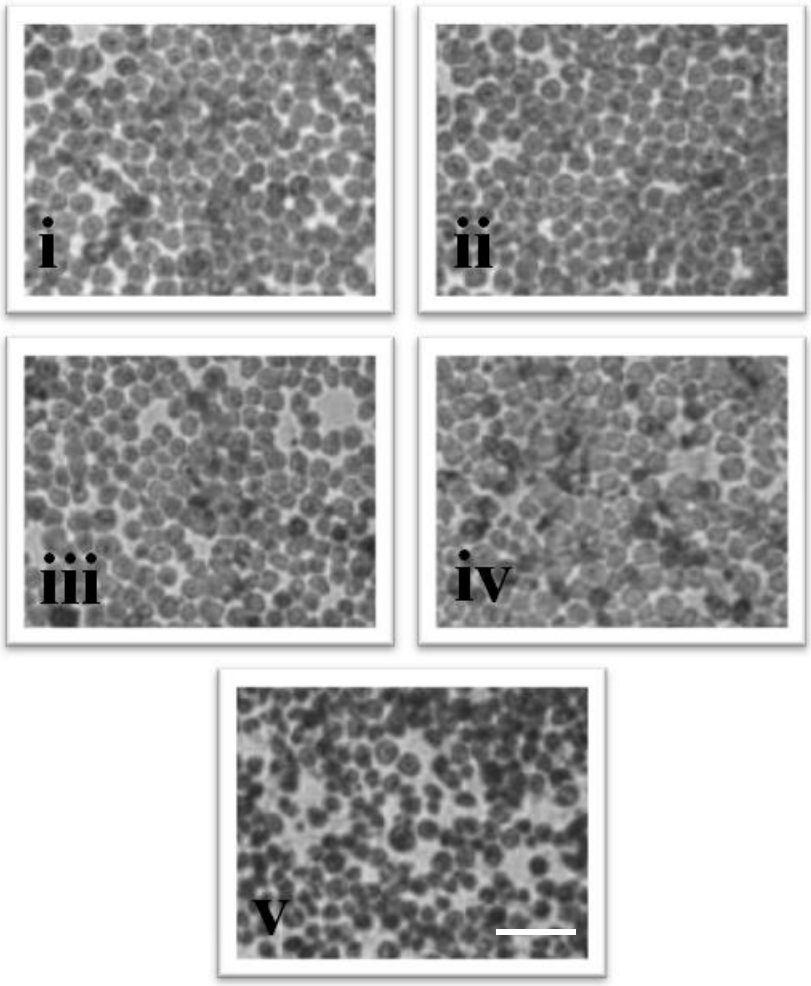

Figure 22. Photomicrographs over a 5 day period with cultures of 1C6 cells cultured in a 6 well plate with media supplemented with 10% heat-inactivated lymph node extract (LN). White line = 20 μm.

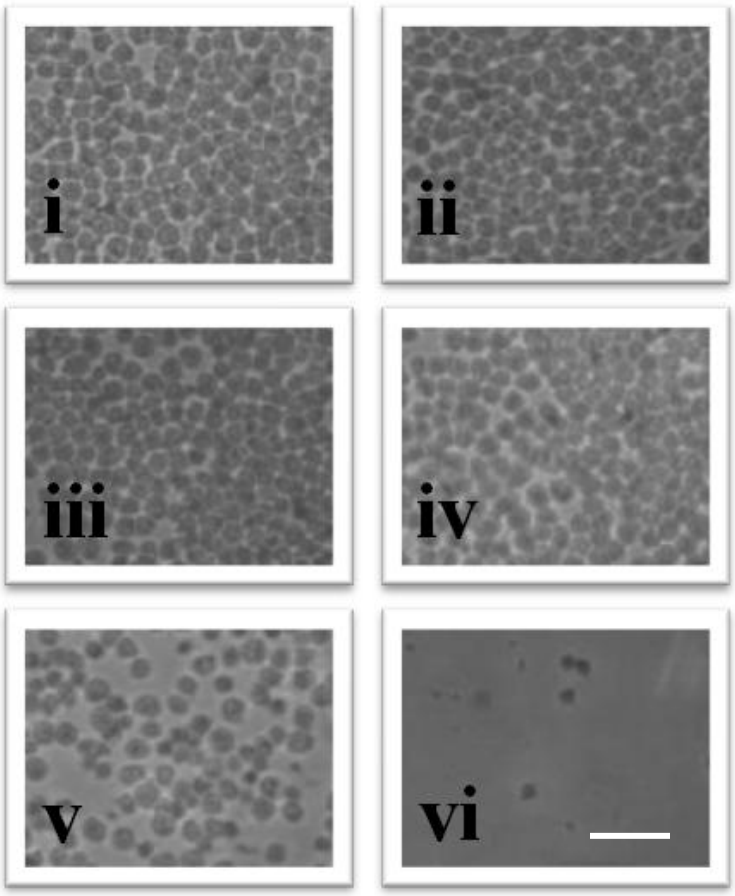

Figure 23. Photomicrographs over a 6 day period with cultures of 1C6 cells cultured in a flask with media supplemented with10% heat-inactivated lymph node extract (LN). Day 6 represents trypsinization. White line = 20 μm.

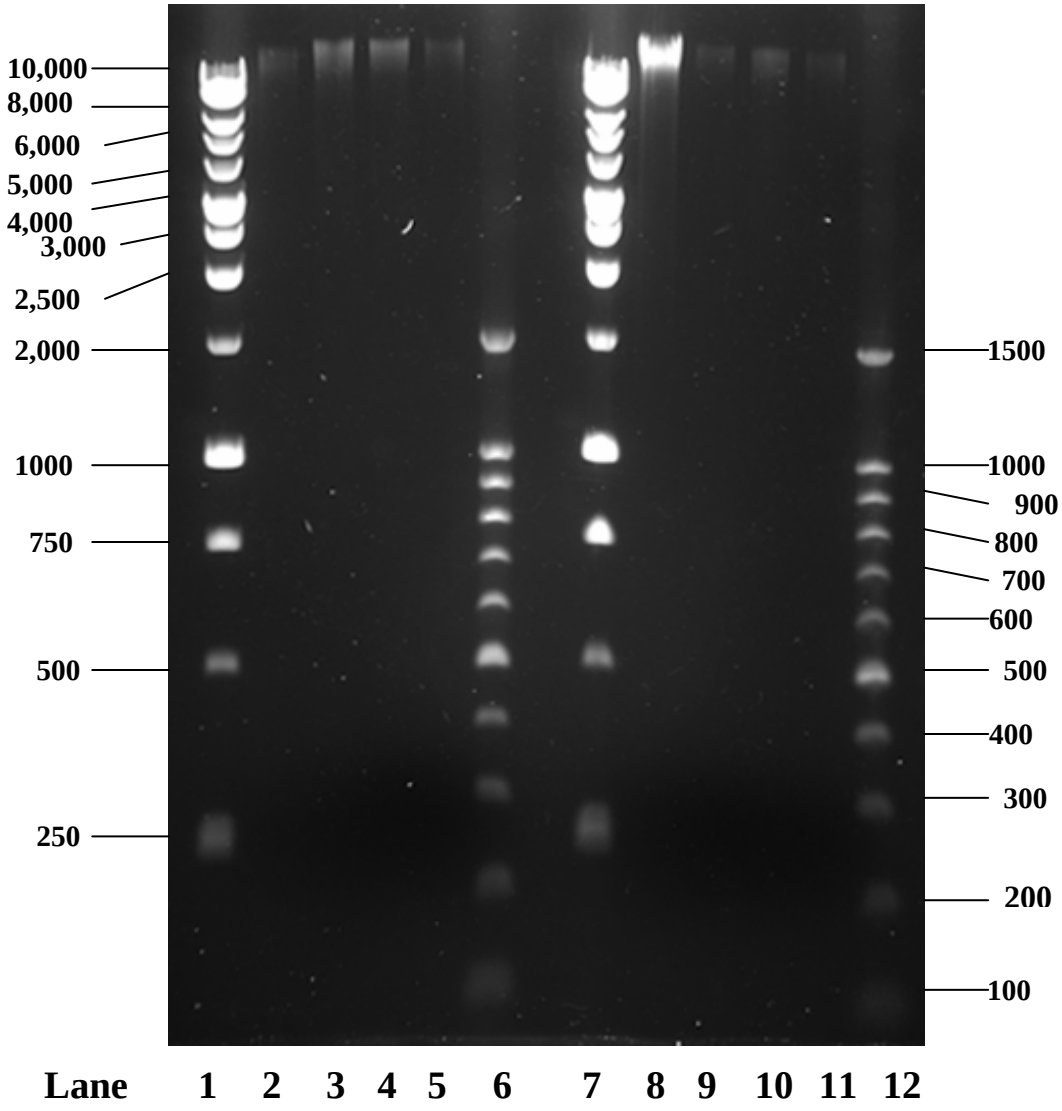

Figure 24. DNA extracts from 0 and 2 h time points run on a 1% agarose gel. Lanes 1 and 7: 1Kb ladder, Lanes 6 and 12: 100 bp ladder, Lanes 2 and 8: Cells starved for two days and cultured with 10% lymph node extract, lanes 3 and 9: Cells adhered overnight and cultured with 10% lymph node extract, Lanes 4 and 10: Lymph node extract adapted cells cultured directly into 10% lymph node extract, Lanes 5 and 11: Cells cultured directly into 10% lymph node extract.

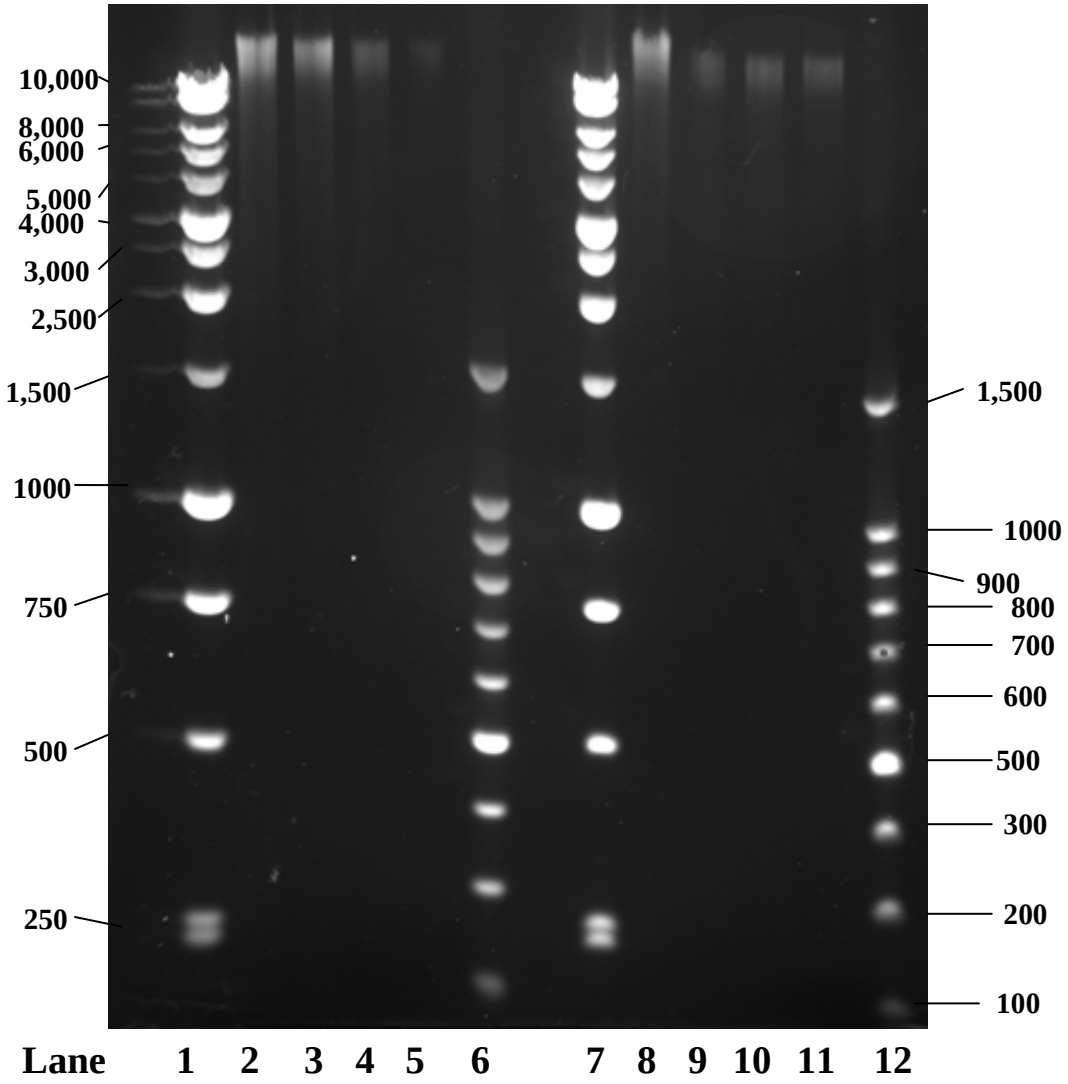

Figure 25. DNA extracts from 4 and 8 h time points run on a 1% agarose gel. Lanes 1 and 7: 1 Kb ladder, lanes 2 and 8: Cells starved for two days and cultured with 10% lymph node extract, Lanes 3 and 9: Cells adhered overnight and cultured with 10% lymph node extract, Lanes 4 and 10: Lymph node extract adapted cells cultured directly into 10% lymph node extract, Lanes 5 and 11: Cells cultured directly into 10% lymph node extract, lanes 6 and 12: 100 bp ladder.

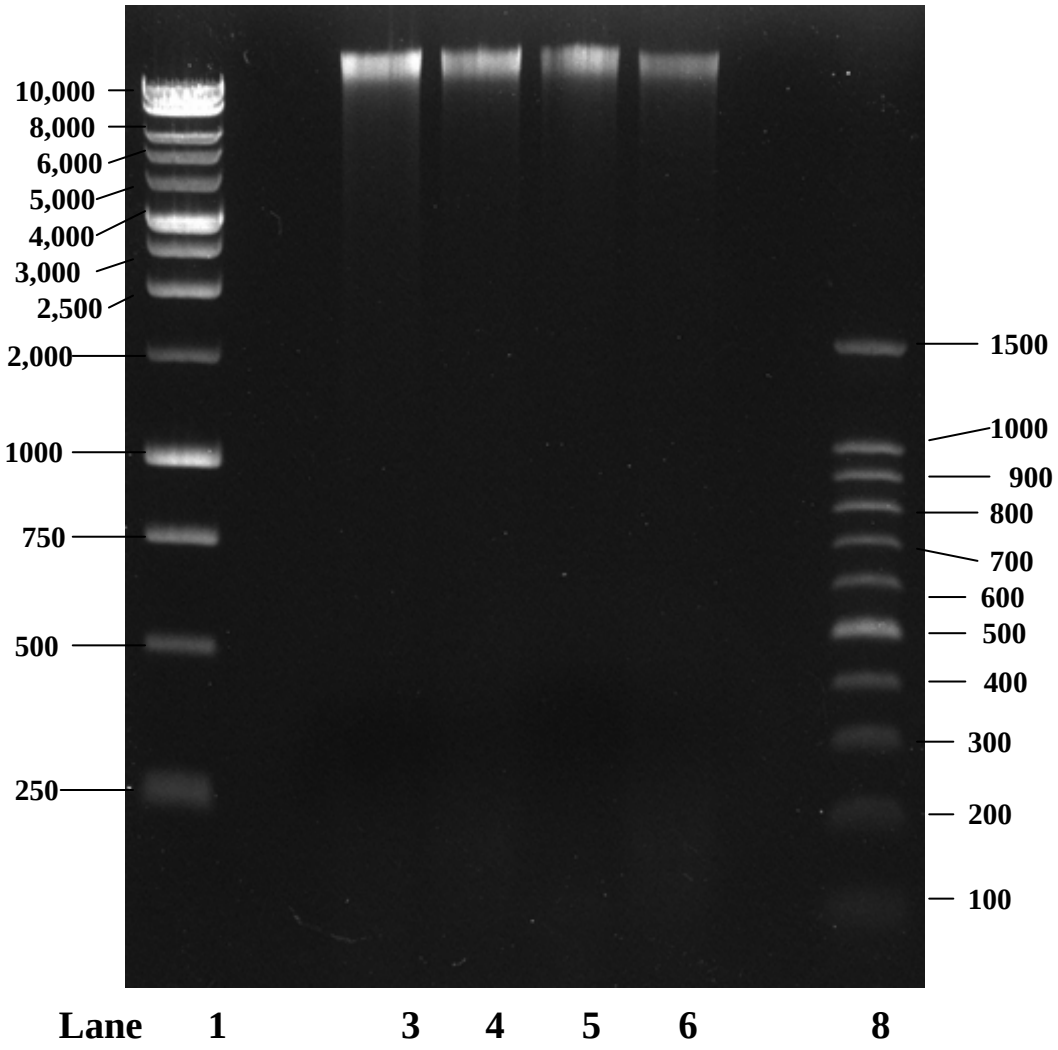

Figure 26. DNA extracts from 24 h time point run on a 1% agarose gel. Lane 1: 1Kb ladder, Lane 3: Cells starved for two days and cultured with 10% lymph node extract, Lane 4:Cells adhered overnight and cultured with 10% lymph node extract, Lane 5: Lymph node extract adapted cells cultured directly into 10% lymph node extract, Lane 6: Cells cultured directly into 10% lymph node extract, Lane 8: 100 bp ladder.

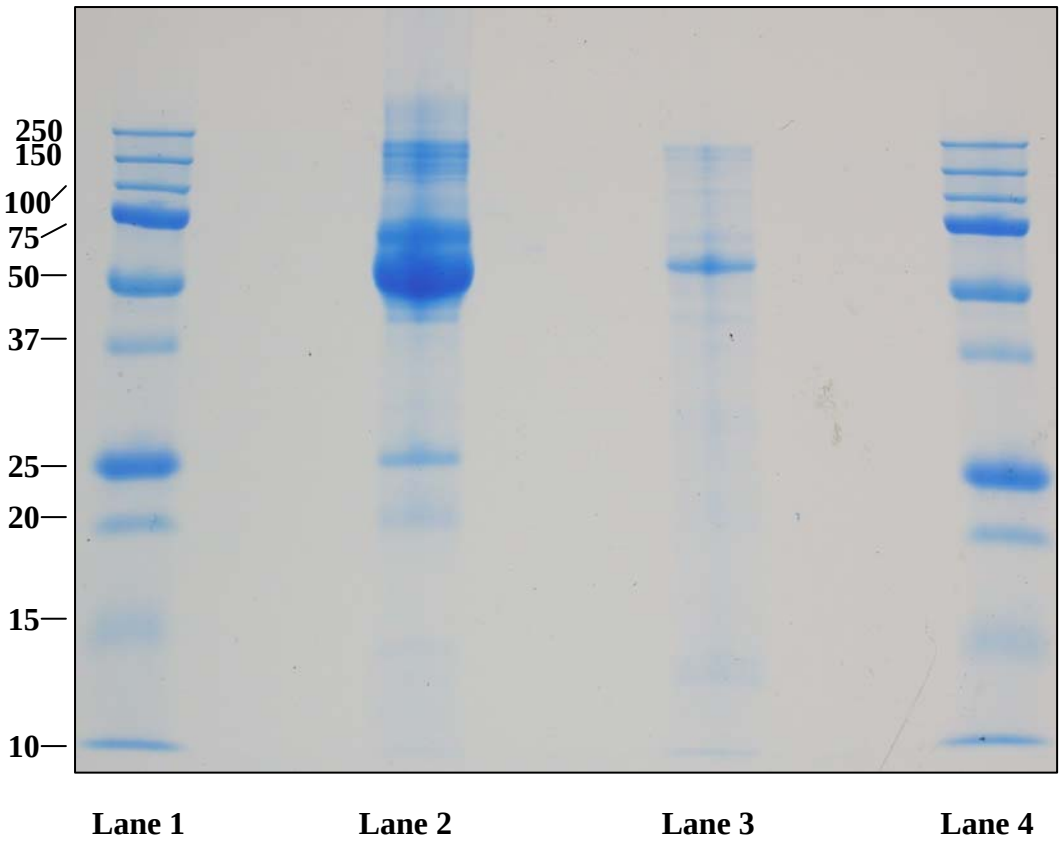

Figure 27. SDS PAGE analysis of BGS and lymph node extract from lymph node preparation #2. Lanes 1 and 4: Protein standards, Lane 2: BGS, Lane 3: Lymph node extract.

5. DISCUSSION

The question of whether or not substitution of the standard bovine sera supplementation with lymph node extract can support and maintain the growth of three different cell lines was addressed with a set of experiments. The MTT assay was used in order to determine cell viability, where MTT is reduced to purple formazan in the mitochondria of living cells (Mosmann, 1983). Essentially, the assay is a measurement of active mitochondrial reductase enzymes. The CyQuant assay directly measures the cellular DNA content. The CyQuant dye binds to the nucleic acids of the lysed cells, and fluorescence intensity of the dye is read (Jones *et al*., 2001). This assesses cell proliferation and cell numbers. Both of these reagents were used in serum starvation assays to assess the potential growth enhancing effects of the lymph node extract in culture media.

In the present assays the cells were starved over a period of 2 days, and the lymph node extract was then added for 3-4 additional days of culture. MTT results are presented as a stimulation index in order to determine cell viability above the negative control (0% supplementation). Lymph node preparation #1 (3.0 mg/ml) was used for the MTT assay. The non-heat inactivated lymph node extract did not support cell viability at any percent supplementation, and actually appeared to inhibit the cells. This inhibition could be due to complement proteins in the extract that can be inactivated with heat, which was done with a matched extract. BGS and other bovine sera used in cell culture

are routinely heat inactivated to remove complement, which is a heat sensitive factor (Carroll, 2004). The complement could be lysing the cells, thereby contributing to decreased viability. Complement factors can be released from macrophages stimulated by IL-6, TNF-α and interferon-γ (Carroll, 2004); all of which are found in the lymph node environment (Willard-Mack, 2006).

The significant support of cell viability at 10 and 20% with the heat inactivated extract was far beyond the support of BGS or non-heat inactivated extract at any percentage. The heat inactivated lymph node extract supported cell viability more than the 0% control at each percent supplementation, whereas BGS only supported viability above the 0% control at 2, 2.5, and 5% supplementation (with marginal support at 10%) (Figure 1). The drop in viability at 20% with BGS is difficult to understand considering at this supplementation the protein concentration would be the most concentrated. However, twenty percent in this assay was still significantly lower than what would traditionally be in cell culture media supplemented with BGS which can be as high as 66 mg/ml (20% of 66mg/ml: 13.2 mg/ml vs. 20% of 3.0 mg/ml: .6 mg/ml).

Similar to the MDA-MB-435 cell MTT results with lymph node preparation #1, the heat inactivated lymph node extract supports cell proliferation above the control at each percent supplementation (Figure 2). Although the best support is at 20% supplementation with the heat inactivated extract, 1-10% supplements were not significantly lower than the 0% control, suggesting that a difference in .57 mg/ml of protein (.6 mg/ml 20%- .03 mg/ml 1%) did not result in a dramatic change in proliferation. BGS supported cell growth best at 2.5% supplementation and was followed by a decline in proliferation with serum supplementation from 5 to 20%. Again, this is

difficult to comprehend based upon the standard protein concentration of BGS. Non-heat inactivated extract, in concurrence with the MTT results, did not support proliferation at any percent supplementation.

Lymph node preparation #2 was used for CyQuant assays with MDA-MB-435, MAC-T, and 1C6 cells. This preparation yielded a much higher protein concentration (27 mg/ml) compared to lymph node preparation #1 (3.0 mg/ml). The increase in protein content (due to the more refined method of preparation) was the deciding factor for which extract to continue using in the assays. The process involved in making lymph node extract #2 is also a more efficient system that may be used if this product was to be mass produced. MAC-T and 1C6 cells were introduced with this extract in order to obtain results from a variety of cell lines. MAC-T bovine mammary epithelial cells are a comparable cell line to the MDA-MB-435 human breast epithelial cell line. A hybridoma cell line (1C6) was introduced because of its immunological nature (B cell fused with a myeloma cell) which should respond to factors present in a lymph node.

All three cell lines exhibited a dramatic increase in cell proliferation at 10 and 20% lymph node extract supplementation. MAC-T cell numbers increased to proliferation indices of greater than 6 but less than 7, whereas MDA-MB-435 cells increased to greater than 3.5 but less than 4.0. 1C6 cells reach the highest proliferation indices (greater than 7 but less than 8) compared to MDA-MB-435 and MAC-T cells. The MDA-MB-435 cells are characterized as being more aggressive compared to the MAC-T and 1C6 cells, therefore stimulating their already accelerated growth rate did not illustrate as dramatic a difference. All three cell lines with #2 lymph node preparation reached higher proliferation indices compared to MDA-MB-435 CyQuant data with

lymph node preparation #1 (greater than 1.2 but less than 1.4) (Fig 2). This would be due to the significant difference in protein concentration and composition of the two lymph node preparations. With lymph node preparation #1 going from 5 to 10% is only a .15 mg/ml difference in protein, and 10 to 20% is only a .3 mg/ml difference. Comparing this to lymph node preparation #2, 5 to 10% is a 1.35 mg/ml difference, and 10-20% is a 2.7 mg/ml difference. The cell maintenance with BGS was similar, supporting all cell lines below or slightly above the 0% control. BGS had an expected maintenance effect on all three cell lines, stabilizing cells between a 1.0-1.5 stimulation index. Heat-inactivated lymph node extract provided a type(s) of stimulatory or mitogenic effect, which resulted in an accelerated growth rate for the cells. This is dramatically illustrated with lymph node preparation #2 at 10 and 20% with the three different cell lines (Fig 3, 4, and 5). This accelerated effect was not as apparent with lymph node preparation #1. The difference in protein composition offers a credible explanation for the differences in results produced by the different preparations.

The apparent accelerated effect the heat inactivated lymph node extract has on the cells may be due to a number of growth promoting factors present in the extract. Figure 27 illustrates the protein differences between BGS and the lymph node extract. A variety of cytokines (IL-6, IL-13, IL-4, IL-12, IL-10) are present in lymph nodes and may provide the proliferative effect seen with the cells (Zou *et al.* 1996). IGF's and PGE2 have been detected in the lymph draining the supramammary lymph node and may also be contributing to the cell growth (Lacasse *et al*., 1996). Although growth factors are present in BGS, the amount may not be sufficient enough to provide a stimulatory response, rather only provide maintenance of cell growth. IGF-I and II have both been

identified in BGS (Honegger and Humbel, 1986). There may be similarities in protein content between bovine serum and lymph node extract, but the actual concentrations of these growth promoting factors may define stimulation versus maintenance.

The ability to culture cells in the lymph node supplemented media over an extended period of time without BGS supplementation was the eventual objective in deciding whether or not to use the extract as a substitute in cell media. Two different growth assays were used to determine cell survival in culture.

The results of the adherence growth assays do not show promise for the substitution of 10% or greater lymph node extract in culture media. Cell lines did not adhere and appeared to be dying within the first day in culture. The adherence growth assay was not a gradual addition of the new media over time, rather an abrupt change. This overwhelming new condition may have been too different for the cells to adapt to all at once. A less aggressive approach of introducing the lymph node extract in the media was attempted in the adaptive growth assay.

The adaptive growth assay was performed with all three cell lines (MDA-MB-435, 1C6, and MAC-T). Different methods of adaptation were attempted, following Invitrogen protocol (2003) for adapting cells to serum-free media. Due to the cells inability to re-adhere once trypsinized with the lymph node extract, a new method was developed. Rather than continuing to passage cells with different percent supplementations of the lymph node extract, cells remained adhered and were not passaged until 100% adaptation had been achieved. As the lymph node extract was gradually added to the media with BGS still present in reduced percentages the cells appeared to adapt fairly well. The 1C6 cells did not thrive as well as the MAC-T or

MDA-M-435 cells and began to round up at day 3 (Fig 22 and 23iii). The morphology of the 1C6 cells changed, with a loss of their round full membranes and a more crenated membrane appearance. When all three lines were trypsinized or scraped and re-seeded into a new flask they were unable to adhere and appeared to die. The inability to adhere may be due to the 'stimulation overload' presented by the lymph node extract. Cells, such as human corneal epithelial cells, have been shown to grow without serum present in medium (Bednarz et al., 2001). If cells can maintain growth without serum, the addition of lymph node extract might provide a greater amount of growth promoting factors that would favor excessive mitogenesis rather than maintenance of steady-state growth. Cells exposed to the lymph node extract are being stimulated with more and different protein than the cells are normally accustomed to in BGS. The abundance of proteins in the extract may be offering too many mitogenic signals at one time for the cells to respond with adherence. Weber *et al*. (1999) observed mitogenic activity with bovine mammary extracts on bovine mammary epithelial cells. Cell growth with the mammary extract was 2 times higher than the growth with IGF-I alone. This potent mitogenic response was not seen with IGF-I or FBS alone. IGF-I supported cell growth significantly better than basal medium (0%), even at 3 μg/ml, implying that low concentrations of growth factors can support/sustain cell growth. While the high percent supplementations with the lymph node extract are mitogenic, lower percent supplementations might sustain cells in culture with sub-mitogenic concentrations of growth factors.

Apoptosis is defined as 'programmed cell death' (Glinsky, 1997). Cells can die through this process or cell death can occur nonspecifically through necrosis. The

agarose gel was run to determine if apoptosis had occurred in cells cultured in the presence of the lymph node extract under different circumstances. The cell growth assays, as well as the MTT and CyQuant serum starvation assays, suggest that if the cells were adhered before the extract was added; the cells would survive. Cells evaluated by the apoptosis assay that were given time to adhere were expected to survive. Once the lymph node media was added, as early as 2 h, the starved cells had a notably different appearance compared to the non-starved adhered cells. The starved cells maintained their standard spread-out 'healthy' glow, whereas the non-starved cells began to round up and displayed unhealthy membranes with dark granules present. The cells directly suspended in the lymph node extract (adapted and non-adapted) did not adhere at any point, did not have round full membranes, and eventually appeared dead. Although apoptosis was not detected there was definitely a change in appearance and apparent health of the cells in culture.

6. CONCLUSIONS AND FUTURE WORK

Combining results from all experiments has allowed for a degree of insight into understanding the effects of the lymph node extract on cells. The CyQuant data with all three cell lines illustrated a stimulatory response with 10 and 20% supplementation observed with the serum starvation assays. From these results 10% supplementation was chosen to culture the cells in order to obtain visual results of cell growth. Twenty percent could have been used, but 10% was chosen because of the standard use of 10% BGS in cell culture, and also because of the minimal difference in effect between 10 and 20%. Directly culturing cells in lymph node extract did not result in cell adherence, potentially due to 'over-stimulation' by the extract which favored proliferation of cells rather than promoting adherence for cell maintenance. The extreme stimulatory response seen in the CyQuant data helps support this conclusion. Adding lymph node extract gradually to the cells once they had already adhered illustrated results seen from the CyQuant data, with MDA-MB-435 and MAC-T cells proliferating to the extent of overgrowing the space in the wells/flasks. 1C6 cells may not have responded as well because of their specific cell type and morphology. These cells do not spread out or adhere as tightly as the MDA-MB-435 or MAC-T cells. They also do not share the characteristic of an epithelial cell with the MDA-MB-435 or MAC-T cells. Once the cells were trypsinized they were unable to adhere, agreeing with results obtained from the adherence growth assay. Bovine serum is a widely used growth supplement in cell culture. Bovine growth serum

can cost as much as $96 for 500 ml (Fisher Scientific), and for labs conducting large amounts of cell culture the expense of BGS can get extremely high. The ability to substitute serum with something more affordable and easy to obtain that supports cells as well, if not better than serum, would be optimum.

Potential research experiments with the bovine supramammary lymph node extract are countless. This study was an initial attempt to understand the potential use of the extract in culture. Although cells cannot be cultured and passaged with 10% heat inactivated lymph node extract they may thrive at a lower concentration. The stimulatory effect the extract has at 10 and 20% may be too potent for maintaining cells in culture. Attempts to culture cells in the extract at lower concentrations are required to fairly assess the usefulness of the extract. If a lower concentration successfully sustains cell growth this could be advantageous in the areas of cost and preparation. Selected fractions of the extract may also offer different results. In order to rid the extract of factors that may inhibit growth, fractionation of the extract should be performed and tested on different cell lines. Once different fractions are isolated, cell growth characterization under the influence of these proteins may be performed.

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