UNDERSTANDING THE ETIOLOGY OF INFLAMMATORY BREAST CANCER

by

Lauren M. Shuman

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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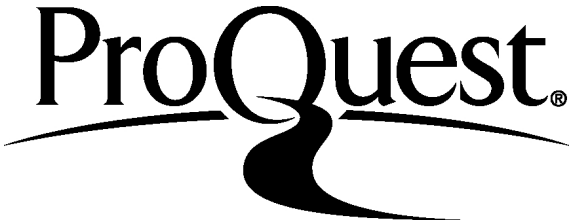

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With this, I am happy to present my hard work and research.

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ABSTRACT

Inflammatory breast cancer (IBC) is a particularly aggressive form of breast cancer that accounts for at least 1 to 6% of all breast cancer cases in the US each year and carries a poor prognosis, with a 5 year disease-free survival rate of less than 45%. IBC is usually diagnosed at a younger age compared to noninflammatory breast cancer and is more prevalent in African American women. Clinical features include erythema, edema, peau d'aurange appearance, and skin thickening. Pathologically, it typically does not result in a palpable tumor mass and tumor emboli are often found to have invaded the dermal lymphatic vessels. This invasion of the lymphatics is thought to be a driving factor of the rapid onset of metastasis as patients are diagnosed at stage IIIB or IV within six months of their first symptoms. It is clear that this disease warrants new methods of treatment and prevention.

This study aims to determine the involvement of Toll-like receptor 4 (TLR4) expression and polymorphisms, as well as infectious agents in the development of IBC. It is hypothesized that 1) alterations in TLR4 signaling impair responsiveness to infection and contribute to the development of IBC, and 2) an infectious agent is responsible for the development of IBC. Understanding the cause of IBC is of utmost importance as little is known about it, yet the possibility of it being infectious dramatically increases the

need for research to develop preventative methods, such as a vaccine, as well as more successful treatment options.

It has been found that TLR4 is highly overexpressed in IBC compared to nonIBC patients. TLR4 is activated by agents such as Gram-negative bacteria and some viruses. Correspondingly, the occurrence of IBC in clusters as well as seasonally suggests the involvement of an environmental agent. Immunohistochemical staining showed TLR4 was highly expressed in tumor cells as well as the epidermis, suggesting TLR4 activation. The effect of TLR4 expression on the IBC phenotype was studied by creating a TLR4 knockout IBC cell line. This knockout resulted in a decreased number of viable cells and thus may indicate TLR4 signaling is involved in promoting cell proliferation and could be a new target for treatment. In addition, two polymorphisms involved in infectious diseases were also screened for by PCR, neither were found to be common in IBC patients. In fact, the presence of polymorphisms was less than what would be expected and therefore may have a preventative effect.

For the detection of an infectious agent, a PCR procedure was developed to prevent false positive results, a common problem with bacterial screening. This was used to screen for bacterial and MMTV DNA present in cell lines. Bacterial screening of cell lines resulted in inconclusive results due to contamination, however, MMTV screening detected viral DNA in the SUM149 IBC cell line. Isolation of a bacterial agent was also attempted by culturing IBC cells, though no bacterial growth was obtained. However, these methods can be applied to patient samples which will provide more insight in identifying an infectious agent in the development of IBC.

Chapter 1

INTRODUCTION

1.1 Breast Anatomy and Physiology

The anatomy of the breast is primarily determined during embryonic development. The growth of the breast tissue begins with the replication of ectodermal cells at the chest which form an initial mammary bud. The bud will grow inwards and begin to subdivide and lengthen, forming hollow ducts that connect the nipple to developing lobes and lobules. Simultaneously, the mesenchyme gives rise to a network of fatty tissue, connective tissue, nervous tissue, and blood and lymphatic vessels that form the stroma. The further development of the female breast at puberty is largely induced by estrogen and progesterone. Estrogen secreted from the ovaries stimulates the growth of fat and connective tissue as well as further elongation and layering of the ducts. Progesterone is also released from the ovaries and stimulates further growth of the lobules and alveoli. At around age 14, the fully developed breast contains about 15 to 20 lobes each containing 20 to 40 lobules, and each lobule containing about 10 to 100 alveoli comprised of the secretory milk-

producing cells (Figure 1.1).[1,2] The lobes are comprised of three layers of cells: luminal lobular or ductal epithelial cells, myoepithelial cells, and basal cells (Figure 1.2).[3] During pregnancy, the lobular secretory cells become fully differentiated for lactation which is induced by oxytocin in response to a nursing child. Lactation is facilitated by the myoepithelial cells which contract to eject milk through the ducts to be released at the nipple.[1,3] At around age 40, the breast tissue begins to deteriorate causing involution of the glands.[1]

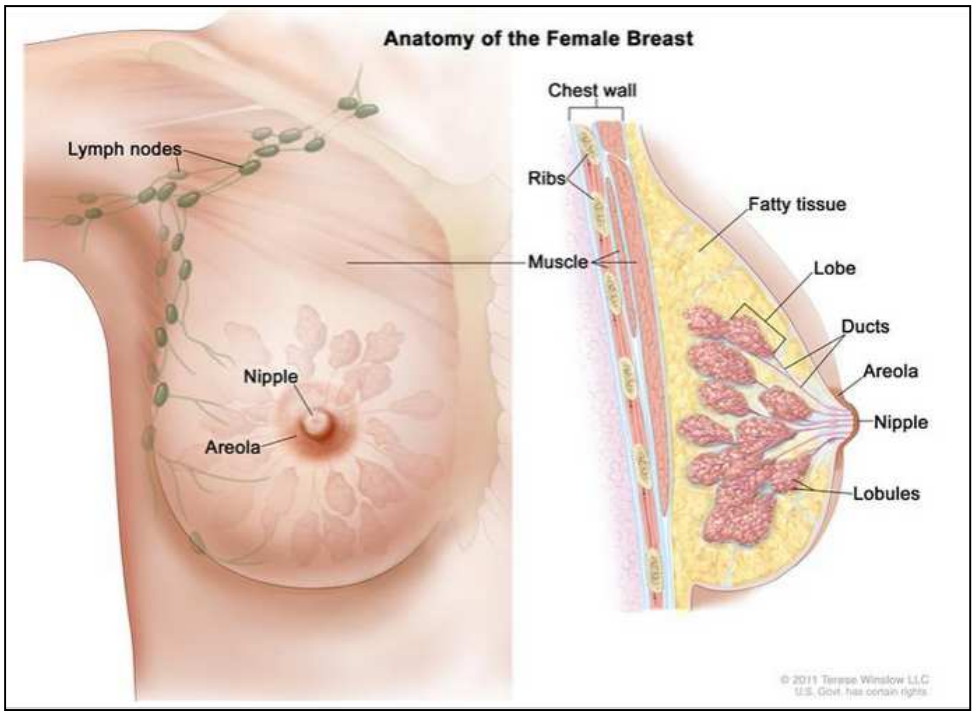

Figure 1.1. **A diagram of the fully developed female breast.** After puberty, the female breast contains a network of lobes, lobules, and ducts for the production of milk upon pregnancy. Credit: For the National Cancer Institute © 2011 Terese Winslow LLC, U.S. Govt. has certain rights. Reprinted from www.cancer.gov with permission.[4]

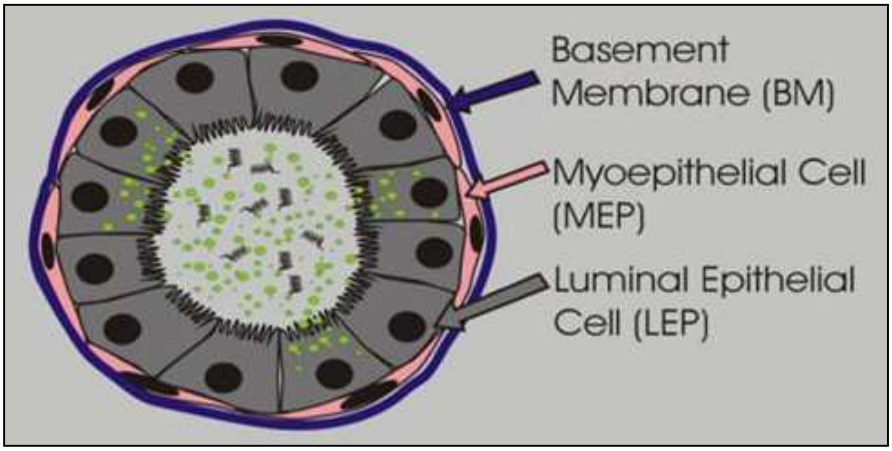

Figure 1.2. **Cross-section illustrating the basic cellular structure of breast tissue.** Lobes are composed of luminal epithelial cells, either lobular or ductal, surrounded by basal cells of the basement membrane and myoepithelial cells that facilitate the ejection of milk from the lobules to the nipple. Reprinted from "Myoepithelial cells: good fences make good neighbors" with permission.[3]

1.2 Breast Cancer

Breast cancer can generally be described as a group of malignant breast cells that grow uncontrollably and may gain the ability to invade surrounding tissues or metastasize to other organs of the body. About 12% of women in the United States will be diagnosed with breast cancer, making it the second most common cancer in women. It is also the second highest cause of cancer deaths in women. Risk factors for developing breast cancer include age, genetic mutations such as mutations in BRCA1 and BRCA2 genes, family history of breast cancer, early age of menarche, and being overweight, among others. As shown in Figure 1.3, the main symptom of breast cancer is the presence of a new lump, typically a hard, painless mass, although pain,

tenderness, swelling, and other skin changes may also occur. Diagnosis of breast cancer may be made after the appearance of symptoms or during a yearly screening upon reaching 40 years of age. Diagnosis is typically completed using mammography, but additional methods such as ultrasound and magnetic resonance imaging (MRI) may also be used if a suspicious area is detected.[5]

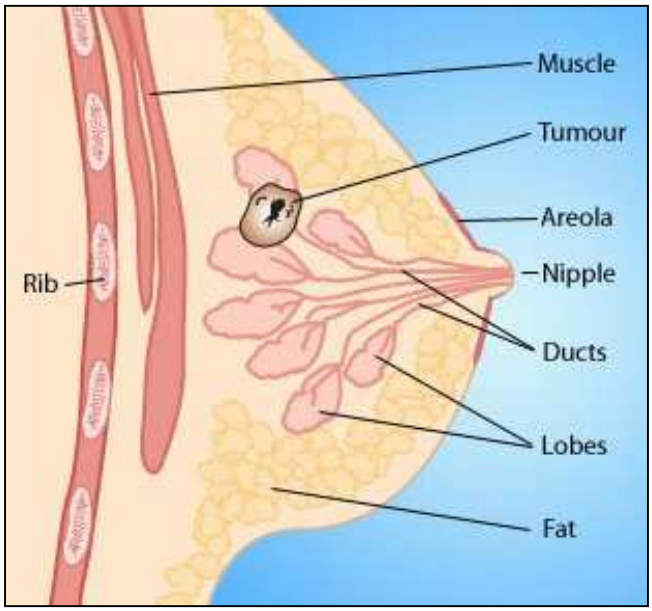

Figure 1.3. **An illustration of the common presentation of a breast tumor.** Breast cancer typically develops as a hard, painless lump in the breast. Adapted from http://www.abc.net.au/health/.[6]

If diagnosed, the extent of the cancer can be determined by screening for metastases using methods such as a chest x-ray, bone scan, computed tomography (CT) scan, MRI, positron emission tomography (PET) scan, and ultrasound. Results can then be interpreted to determine the stage of the

cancer.[5] The stage is commonly determined based on three characteristics: the size of the tumor described using a range from 0-4 (T), the extent of spread to the lymph nodes from 0-3 (N), and the presence of metastasis described as 0 or 1 (M).[7] Based on this system, referred to as the TNM system, the cancer is classified into one of four stages. Usually higher stage numbers are associated with more aggressive cancer and poorer survival rates (Table 1.1).[5,7]

Stage	T	N	M
0	Carcinoma *in situ*	N0	M0
I	T1	N0	M0
II	T1 T2 T3	N1 N0 or N1 N0	M0 M0 M0
III	T3 T4 Any T	N1 or N2 N0, N1, or N2 N3	M0 M0 M0
IV	Any T	Any N	M1

Table 1.1. **Breast cancer stages categorized using the TNM system.** Adapted from cancerstaging.org.[7]

After diagnosis, there are several other characteristics of the cancer that are determined that will help identify the best course of treatment. This is done using a biopsy of the tumor to classify the type of cancer. Breast cancer may be classified in several ways; first, they will be classified by type. Ductal

carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) are tumors that are derived from either the ductal or lobular epithelial cells, respectively, and have not invaded into the surrounding tissue. DCIS is more common than LCIS and both are very treatable, but may increase the chance of developing an invasive cancer. Other types are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) which again are tumors that have developed from ductal or lobular epithelial cells, but have broken through the basement membrane and spread to surrounding tissues. IDC is the most common breast cancer, but both IDC and ILC are treated similarly. Other less common types include tubular, medullary, mucinous, papillary, and inflammatory breast carcinomas.[5,8]

Breast cancer may also be classified based the presence of hormone receptors. Estrogen receptors (ER) and progesterone receptors (PR) are normally expressed by breast cells and may also be expressed by the cancer cells. Therefore, breast cancer may be classified as ER- and PR-positive or negative. Human epidermal growth factor receptor 2 (HER2) is another receptor that may be overexpressed due to the increased number of copies of the gene and is therefore classified as HER2-positive. HER2-positive tumors tend to be more aggressive than HER2-negative (normal expression) tumors. These receptor statuses often get grouped together to describe the tumor; for example, a cancer may be classified as triple negative, indicating that the tumor does not express any of the three receptors.[5,9]

A third method of classification is based on patterns of gene expression that may determine the cell type from which the tumor was derived. Luminal A

and B types are ER-positive and express genes similar to what is expressed by the normal luminal cells. Luminal B cancers tend to grow faster and have a poorer prognosis than luminal A. Basal-like tumors are usually triple negative and have a gene expression similar to basal cells, but are usually more aggressive and less responsive to treatment. Lastly, the HER2 type is characterized by HER2 overexpression and tends to have a poorer prognosis, although targeted treatment has been successful. Most breast cancers are of the luminal subtype.[5,9]

Based on the classification of the tumor, the appropriate treatment course can be administered. Most women will undergo surgery to remove the primary tumor and the type of surgery ranges from breast-conserving surgery to radical mastectomy and possibly lymph node surgery depending on the stage. Some patients may receive radiation therapy after surgery to help prevent recurrence, or it may be used to target metastases. Adjuvant chemotherapy may also be given after surgery to help prevent recurrence, or may be given as an neoadjuvant therapy in cases of locally advanced cancer to shrink the tumor prior to surgery. These standard chemotherapies include using drugs such as Docetaxel and Paclitaxel. Hormone therapy can be given to those who have an ER- and/or PR-positive cancer using drugs such as Tamoxifen and aromatase inhibitors which are often given as an adjuvant therapy. Finally, targeted therapies such as drugs that target HER2 (like Trastuzumab and Lapatinib) can be combined with chemotherapy in patients whose cancer is HER2-positive, and tend to have milder side effects.[5,9,10]

1.3 Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is arguably the most aggressive and deadly form of epithelial breast cancer of ductal origin and is notably distinct from other locally advanced breast cancers (LABC).[11] IBC comprises at least 1 to 6% of all breast cancer cases in the US, and up to 20% of breast cancers globally, with incidence rates reportedly increasing.[12-15] IBC affects younger women compared to noninflammatory breast cancers (nonIBC) with a median age of 57 years compared to 62 for nonIBCs.[16] In the United States, IBC incidence rates are higher in African American women compared to Caucasian women, and African American women are more frequently diagnosed at a younger age.[12] In addition, IBC comprises a higher percentage of breast cancers in North African countries such as Tunisia.[17] Women with IBC carry a very poor prognosis with a 5-year disease-free survival rate of less than 40%, much lower than that of other breast cancers with a survival rate of about 85%.[5,18,19] The poor prognosis is largely due to its rapid progression and metastasis, its high recurrence rate, and its often misdiagnosis delaying proper treatment.[18,20] Some risk factors of IBC are also notably different from those of nonIBC. While an early age of first pregnancy is generally considered protective against breast cancer, this does not seem to be the case for IBC. Also, obesity was found to be a potential risk factor for the development of premenopausal IBC, but not for other premenopausal breast cancers. Other risk factors may include younger age at menarche, extended periods of breast feeding, and lower socioeconomic status.[17]

IBC was first distinguished from other breast diseases by Drs. Lee and Tannenbaum who characterized IBC as a phenotypically distinct form of LABC with clinical features including erythema, edema, nipple retraction, dimpling of the skin described as a "peau d'aurange" appearance, and skin thickening.[21] Other symptoms can include itching, warmth, pain or tenderness, and swollen axillary lymph nodes.[22] As shown in Figure 1.4, symptoms of IBC will vary from patient to patient, some patients' symptoms may initially be very subtle and other patients may have more severe symptoms. Regardless, these symptoms will progress rapidly within weeks or months, or even occur overnight.[23] In fact, IBC is distinguished from other forms of LABC using the diagnostic criteria that symptoms must have developed within six months or less, accompanied with redness covering at least one third of the breast.[24,25] The name "inflammatory breast cancer" was given as a description of these clinical features, but implies the involvement of an inflammatory process when actually the presence of inflammatory infiltrate or symptoms such as fever and leukocytosis are rarely seen.[24,26] These symptoms are also cause for the high frequency of misdiagnosis of IBC as mastitis, Paget's disease, or other breast diseases.[24]

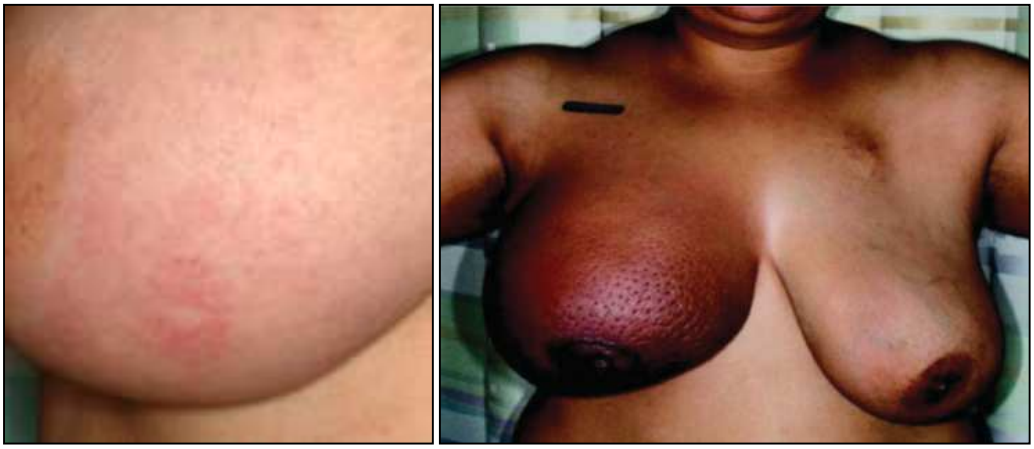

Figure 1.4. **Clinical symptoms of inflammatory breast cancer.** Classic symptoms of IBC include redness, swelling, and dimpling of the breast. The severity of symptoms may vary from patient to patient. Left image adapted from http://www2.mdanderson.org/cancerwise/.[27] Right image reprinted from "Inflammatory Breast Cancer: The Disease, the Biology, the Treatment." with permission.[24]

IBC is also pathologically distinct in that it typically does not develop as a palpable tumor mass, rather it spreads diffusely through the stroma.[24] This makes diagnosis of IBC by physical examination and mammography difficult and therefore makes additional imaging methods such as MRI necessary if IBC is suspected. Additionally, tumor emboli are often found to have invaded the dermal lymphatic vessels.[24,28] This invasion of the lymphatics is thought to be a driving factor of the rapid metastasis of the disease as patients are diagnosed with a stage IIIB, IIIC, or IV cancer.[20,28-30] Consequently, IBC has been classified as T4d by the TNM system, indicating the tumor has invaded the skin or chest wall and is distinct from other LABCs.[31] Most patients present with axillary lymph node involvement and some will already have distant

metastases to various organs including the lungs, liver, brain, and bone.[32,33] IBC is a ductal carcinoma and may be classified into the same categories as nonIBC, however it is found that most cases are either basal-like or HER2-positive. Other characteristics include a high proliferation rate, high grade, and overexpression of several proteins including epidermal growth factor receptor (EGFR), RhoC, caveolin-1 and -2, and E-cadherin.[11,34,35] Because of the aggressiveness of IBC, patients endure a grueling and rigorous treatment course compared to nonIBC. The current treatment strategy often used involves an initial neoadjuvant chemotherapy, followed by a modified radical mastectomy, then radiation therapy, and additional systemic therapy if necessary.[25] Despite these extensive measures, the prognosis remains low and it is clear that new treatment and possible prevention methods are warranted.

1.4 Infectious Agents and Cancer: Evidence for the Possible Involvement of an Infectious Agent in Inflammatory Breast Cancer

Infectious agents such as bacteria and viruses are known to be associated with the development of several cancers. For example, *Helicobacter pylori* is known to cause gastric cancer, and in some cases, gastric mucosa-associated lymphoid tissue (MALT) lymphoma. *Campylobacter jejuni* is suggested to be involved in another form of lymphoma called immunoproliferative small intestinal disease (IPSID).[36] Gastric cancer, MALT lymphoma, and IPSID can be treated using antibiotics.[37,38] Viruses have a larger impact on cancer development as they may be part of the cause of up

to 15% of cancers worldwide. These viruses include Epstein-Barr virus, human papilloma virus, hepatitis B and C viruses, human herpes virus-8, and human T lymphotrophic virus type 1, as well as other less studied viruses suggested to be associated with cancer.[39] Some of these have vaccines that can be administered to those at risk.

The involvement of an infectious agent in the etiology of IBC is a relatively new idea with an increasing amount of suggestive evidence. For example, there are many reports of clusters of IBC patients diagnosed in the same area and at around the same time. The first IBC cluster to catch the eye of the IBC research community was a cluster in California in which 3 women in an office of 24 were diagnosed with IBC within 10 months.[40] Another cluster was later observed in Texas which included 15 patients diagnosed over 13 years, which averages about 1 patient every 10 months.[41] Additional anecdotal evidence includes a husband and wife cluster in Michigan and small clusters in West Chester and Landenberg, PA, among others. A new tool that has helped identify clusters, including the aforementioned cluster in Texas, is "Terry's Map," which is an interactive Google map of IBC patients around the world, though mostly documents IBC patients in the United States (https://www.theibcnetwork.org/terrys-ibc-map/).[41,42] This map has facilitated the identification of IBC clusters and shows that clusters occur both in urban and rural areas, thus dismissing the idea that clusters are just a result of high population density. Rather this suggests that an environmental agent (or more specifically, an infectious agent) of that particular area may be involved in the development of IBC. Additionally, recent evidence reveals a seasonal pattern

of IBC onset in the northeastern region of the United States that shows more IBC cases are diagnosed in warmer months and fewer cases are diagnosed in the winter.[43] This further supports the hypothesis that an infectious agent is at least partially responsible for the development of IBC. More specifically, it is thought that an infectious agent may be acting as a trigger which promotes the rapid onset of symptoms and metastatic nature seen in IBC.

In addition to the patterns observed in the United States, the prevalence of IBC in African countries as previously mentioned also eludes to infectious agent involvement. This includes countries such as Algeria, Tunisia, Egypt, and Morocco. As also previously mentioned, IBC comprises a larger percentage of breast cancers in north Africa with some populations approaching a percentage of 15%.[44,45] Some studies have found rural residence to be associated specifically with IBC compared to nonIBC in African countries, although it is noted that rural areas also tend to be associated with lower socioeconomic status.[17] Therefore it is thought that the rural and/or lower socioeconomic areas may allow an infectious agent to have a greater impact in these countries compared to the United States. The idea that an infectious agent is responsible for the etiology of IBC is particularly important because little is known about IBC, yet it could be spreading without any knowledge of how to control or treat it. If an organism can be identified, then a vaccine can be developed and administered to those at risk. This would not only be greatly beneficial to the at-risk population of the United States, but even more so for those of north African countries.

Addressing the issue of identifying an infectious agent that may be contributing to the onset of IBC can be a daunting task as there is an enormous number of infectious agents. Therefore, for this investigation we have narrowed down the list of considered organisms based on: 1) their ability to be intracellular, as antibiotics targeted towards extracellular bacteria that would be given as a result of misdiagnosis have no effect on IBC; 2) previous evidence of infectious agents suggested to be involved in cancer, including IBC; and 3) the recognition of the organism by Toll-like receptor 4 (see Section 1.5). One such organism is *Bartonella henselae* which is a Gram-negative bacteria that has been detected in IBC tissue by DNA analysis.[46] In addition, *Bartonella sp*. infection has been found to mimic symptoms similar to IBC.[47,48] Another organism is *Brucella sp.* which are also Gram-negative bacteria that have been shown to cause some medulloblastomas.[49] This organism is considered due to the tendency of IBC to metastasize to the brain. A third organism considered is the mouse mammary tumor virus (MMTV). MMTV is known to cause murine breast cancers and it has since been suggested to cause human breast cancers as well.[50,51] MMTV-like sequences and particles have also been detected more abundantly in sporadic breast cancers, including IBC, compared to familial and other breast cancers. This proposed homologous virus has been termed human mammary tumor virus (HMTV).[52] It is with these organisms in mind that we have designed our approaches to determine the involvement of an infectious agent in IBC.

Overall, it is hypothesized that an intracellular bacteria or virus is responsible for the development of IBC. This study aims to test this hypothesis

by 1) determining if an intracellular bacteria is involved by PCR screening and bacterial culture of cell lines, and 2) determine if MMTV/HMTV is involved by PCR screening of cell lines.

1.5 Toll-like Receptor 4 Expression and Polymorphisms

As mentioned previously, IBC is characterized by the differential expression of several unique proteins. A study previously conducted in this lab by Lehman, *et al*. analyzed the expression of genes associated with the PI3K/Akt signaling pathway and showed several additional proteins that are overexpressed in IBC compared to nonIBC.[19] The most highly overexpressed protein in IBC was found to be TLR4 (Figure 1.5).

Toll-like receptor 4 (TLR4) is a member of the mammalian Toll-like receptor (TLR) family of type 1 transmembrane proteins which recognize microbe-associated molecular patterns (MAMPs) and activate the appropriate innate immune response for a particular infection. TLR4 most notably recognizes lipopolysaccharides (LPS) present in the cell wall of Gram-negative bacteria, but also recognizes other ligands such as viral proteins, including envelope proteins of MMTV. As illustrated in Figure 1.6, the activation of the TLR4 pathway begins with the binding of LPS-binding protein (LBP) to LPS. LBP will transfer components of LPS to CD14 which will then bind with a TLR4-MD2 complex. This complex will then dimerize with another TLR4 complex which initiates the first of the two signaling pathways regulated by

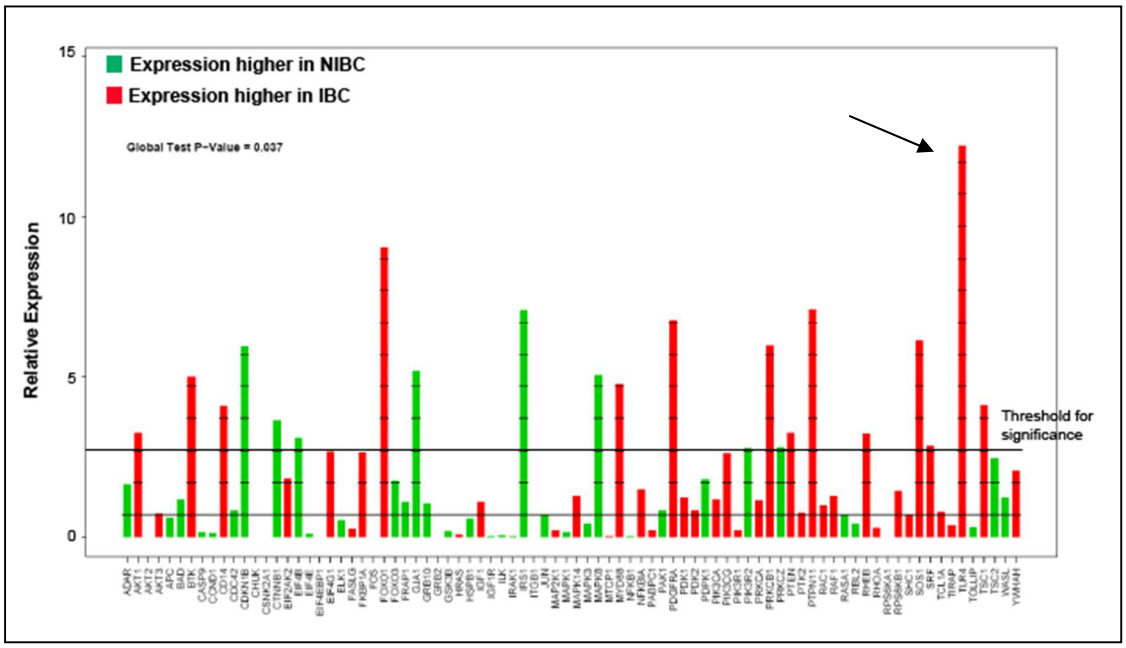

Figure 1.5. **A PCR array comparing the differential expression of a set of genes in IBC compared to nonIBC**. The most highly overexpressed protein in IBC is TLR4 (arrow). Reprinted with permission from "Regulation of Inflammatory Breast Cancer Cell Invasion through Akt1/PKBα Phosphorylation of RhoC GTPase."[19]

TLR4. This first pathway activates the primary inflammatory response which utilizes the MyD88-dependent pathway in which MyD88 is recruited to the cytosolic Toll-interleukin-1 receptor homology (TIR) domain of TLR4. This

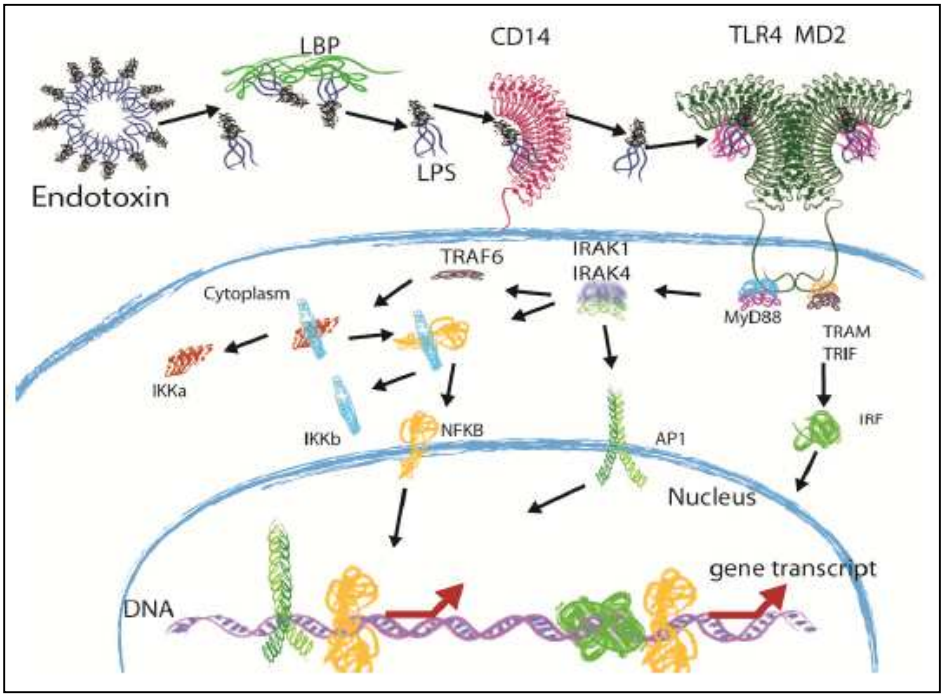

Figure 1.6. **The Toll-like receptor 4 pathway.** Pictured are the two TLR4 pathways that are activated by LPS which induces a primary inflammatory response via MyD88, and NFκB and AP1 activation, as well as a secondary regulation of innate immunity via TRIF and TRAM which leads to transcription of IFN. Reprinted with permission from "Recognition of LPS by TLR4: Potential for Anti-Inflammatory Therapies."[53]

allows MyD88 to bind with IRAK which in turn binds to TRAF6. TRAF6 activates the MAPK pathway for translocation of NF-κB to the nucleus. Simultaneously, AP1 transcription factors are activated so both AP1 proteins and NFκB initiate transcription of various proinflammatory cytokines including IL-6 and TNFα. The second pathway is initiated after endocytosis of the TLR4 complex which allows a second set of adaptor proteins, TRIF and TRAM, to bind and activate the transcription factor IRF. IRF induces the expression of Type 1 Interferon (IFN) which regulates an innate immune response. Thus,

TLR4 activates both an initial inflammatory response and a secondary innate immune response.[54-57]

TLR4 is expressed on the surface of leukocytes such as macrophages, mast cells, and dendritic cells, as well as epithelial cells such as gastric and renal epithelial cells, and endothelial cells. In the breast, TLR4 is expressed by macrophages and dendritic cells, as well as lobular and ductal epithelial cells.[58] TLR4 expression is increased in response to activation.[54,59,60] Therefore, it is hypothesized that TLR4 overexpression seen in IBC may be due to its activation by an intracellular bacteria or virus. This hypothesis is further supported by the environmental evidence as previously discussed. TLR4 expression in IBC was visualized by immunohistochemical staining to determine specifically which cells in the breast tissue express TLR4. Furthermore, to determine the effect that TLR4 expression may have on the IBC phenotype, TLR4 knockout IBC cells were created. It is hypothesized that TLR4 knockout IBC cells will lose their ability to form emboli.

In addition, other aspects of TLR4 signaling may also be altered and involved in the development of IBC. One such alteration being considered is TLR4 polymorphisms. Two TLR4 single nucleotide polymorphisms (SNPs) resulting in amino acid changes, D299G and T399I, have been shown to decrease host responsiveness to LPS.[61] These polymorphisms occur in the extracellular domain and are thought to prevent binding of TLR4 with its co-receptors MD2 or CD14, or complex dimerization, thereby preventing proper signaling of the immune response.[62] These polymorphisms have been found associated with infectious diseases, Gram-negative sepsis, and gastric cancer

caused by *Helicobacter pylori* infection.[63,64] These polymorphisms may occur individually or cosegregationally and the prevalence of these different haplotypes varies across different populations.[65] For example, as shown in Table 1.2, the D299G haplotype is more prevalent in African populations where IBC incidence is high. The cosegregation of the polymorphisms is more prevalent in Caucasian populations in which IBC incidence is relatively intermediate. And these polymorphisms have not been found in Asian populations in which IBC incidence is very rare. Therefore it is hypothesized that these polymorphisms may increase the risk of developing IBC and their frequency in IBC patients was evaluated.

In summary, it is hypothesized that alterations in TLR4 signaling impair responsiveness to infection, leading to the development of IBC. This study aims to 1) analyze TLR4 expression in IBC by immunohistochemical staining and by creating a TLR4 knockout IBC cell line, and 2) determine if TLR4 polymorphisms are present in IBC patient samples by PCR screening.

	TLR4 Polymorphism	Incidence of IBC
African	Mostly D299G	High
Caucasian	Mostly D299G/T399I	Intermediate
Asian	Not found	Rare

Table 1.2. **Distribution of TLR4 polymorphic haplotypes and incidence of IBC in African, Caucasian, and Asian populations**. Adapted from "TLR4 polymorphisms, infectious disease, and evolutionary pressure during migration of modern humans."[65]

Chapter 2

EXPERIMENTAL METHODS

2.1 Cell Culture

Experiments required the use of multiple cell lines including the inflammatory breast cancer cell line SUM149, breast cancer lines GILM2 and MCF7, and the mammary epithelial cell line MCF10A. SUM149 cells were grown in Ham's F-12 (Mediatech, Inc., Manassas, VA) with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1%Pen-Strep (Mediatech, Inc.), 1% antibiotic/antimycotic (Mediatech, Inc.), 1% L-glutamine (Mediatech, Inc.), 10 µg/mL insulin (Sigma-Aldrich, St. Louis, MO), 5.5 µg/mL transferrin (Sigma-Aldrich), 6.7 ng/mL selenium (Sigma-Aldrich), and 1µg/mL hydrocortisone (Sigma-Aldrich). GILM2 and MCF7 cells were grown in DMEM (Mediatech, Inc.) with 10% FBS and 1% Pen-Strep. MCF10A were grown in DMEM/Ham's F-12 50:50 medium (Mediatech, Inc.) with 5% FBS,1% Pen-Strep, 50 µg/mL bovine pituitary extract (BPE) (Life Technologies, Grand Island, NY), 0.5 µg/mL hydrocortisone, 20 ng/mL human epidermal growth factor (Life Technologies), 10 µg/mL insulin, and 100 ng/mL cholera toxin (Sigma-Aldrich). All cell lines were maintained at 37 °C in a humidified chamber with 5% CO_2.

2.2 Immunohistochemical (IHC) Staining

Paraffin sections were deparaffinized and hydrated. Antigen retrieval was then performed by bringing the slides to boiling in 10 mM sodium citrate buffer (pH 6.0), maintaining them at sub-boil for 10 minutes, and then allowed to cool for 30 minutes. Sections were then incubated with 3% hydrogen peroxide for 15 minutes and washed in a buffer bath of Tris-buffered saline with 0.1% Tween (TBST) for 5 minutes. Sections were then blocked using a fish skin gelatin buffer composed of 5% goat serum 1% bovine serum albumin (BSA), 0.1% fish skin gelatin, 0.1% Triton-X 100, 0.05% sodium azide, and 0.05% Tween in phosphate buffered saline (PBS) for 1 hour. Following the block, sections were incubated with rabbit anti-TLR4 primary antibody (Novus Biologicals, LLC, Littleton, CO) at a ratio of 1:100 for 30 minutes and washed in TBST for 10 minutes. Sections were then incubated with horseradish peroxidase (HRP) conjugated secondary antibody (EnVision+ System, Dako, Agilent Technologies, Denmark) for 30 minutes per manufacturer's instructions. Sections were washed in TBST for 5 minutes then incubated with 3,3'-Diaminobenzidine (DAB) (Becton-Dickinson, Franklin Lakes, New Jersey) until brown precipitate was formed, up to 15 minutes. Sections were then counterstained with methyl green for 10 minutes, dehydrated and cleared, then mounted with Permount. Images were obtained using the NIS-Elements system (Nikon Instruments, Inc.)

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2.3 Polymerase Chain Reactions (PCRs)

PCR screening was conducted to detect TLR4 polymorphisms, bacterial 16S rDNA, and MMTV DNA in cell lines and patient samples. TLR4 polymorphism screening was performed following a previously established allele-specific PCR and restriction fragment length polymorphism (RFLP) analysis by Lorenz *et al*. with some modifications.[66] Screening of cell lines used the published forward primers and modified reverse primers to create a smaller product in anticipation of applying the protocol to patient samples that were known to be difficult to PCR. The primers used for identification of the TLR4 D299G SNP were forward primer 5'-GATTAGCATACTTAGACTACTACCTC(G)CATG-3' and reverse primer 5'-GTTAACTAATTCTAAATGTTGCCATCC-3' for a product of 152 bp. The primers used for the determination of the TLR4 T399I SNP were forward primer 5'-GGTTGCTGTTCTCAAAGTGATTTTGGGA(C)GAA-3' and reverse primer 5'-TGAAAACTCACTCATTTGTTTCAA-3' for a product of 159 bp. The nucleotides in parentheses were substituted with the subsequent underlined nucleotide as described previously.[66,67] This creates PCR products with specific restriction enzyme recognition sites such that a restriction enzyme digest with the corresponding enzyme will produce two different size PCR products if the polymorphic allele is present.[66,67] D299G and T399I polymorphic alleles will digest to product sizes of 122bp and 126 bp, respectively.

The reactions included 50 ng DNA, 0.5 µL of 10µM of each primer, 0.75 µL of 10mM dNTP mix (Thermo Scientific), 0.5 µL DFS Taq DNA Polymerase

(Boca Scientific, Inc.), 2.5 µL 10x complete buffer (Boca Scientific, Inc.), and DNA-free water (Qiagen) was used to bring the reactions to a total of 25 µL. The PCR was conducted using the following cycling conditions: Initial 94 °C for 5 minutes; 40 cycles of 94 °C for 1 minute, annealing temperature for 1 minute, 72 °C for 1 minute; and a final elongation at 72 °C for 5 minutes, and held at 4 °C. The annealing temperature for D299G and T399I PCRs were 56 °C and 57 °C, respectively. One µL of 0.1 ng/µL of a polymorphic positive control template synthesized by Integrated DNA Technologies, Inc. was included for a positive control. DNA-free water was used as a negative control. Ten µL of the PCR products were then digested with 1 µL of the appropriate enzyme: NcoI with 2 µL of Buffer 3 (New England BioLabs, Inc.) for the D299G digest, and HinfI with 2 µL of Buffer 4 (New England BioLabs, Inc.) for the T399I digest. 17 µL of water was used to make a total 30 µL reaction. PCR and enzyme digestion products were analyzed by electrophoresis using a 2% agarose gel and 1x TAE buffer, visualized using ethidium bromide and the Syngene G:Box imaging system.

TLR4 polymorphism screening for patient samples was adapted from the previous procedure. Forty Algerian IBC patient DNA samples isolated from paraffin sections were used.[68] Due to the poor DNA quality, the previous procedure was adapted to optimize results. A nested PCR was designed such that the first PCR for D299G screening used the forward primer 5'-GACCATTGAAGAATTCCGATTA-3' and reverse primer 5'-AGTTAACTAATTCTAAATGTTGCCATC-3' for a product of 170 bp. The T399I screening used forward primer 5'-GAAATGGCTTGAGTTTCAAAGG-3' and

reverse primer 5’-GGAATACTGAAAACTCACTCATTTG-3’ for a product of 186 bp. PCR was conducted as previously mentioned using annealing temperatures of 53 °C and 58 °C, respectively. Based on the results of the first PCR, 1 to 7 µL of the first PCR product was used for the second PCR using the primers in the first procedure. Also added was an internal control using an HBV template synthesized from Integrated DNA Technologies, Inc. and primers modified from Cuestas, et al.[69] The template was modified to include one digestion site for each NcoI and HinfI to control for the digestion as well. Forward primer was 5'-GGTTTAAATGTATACCCAAAGACAA-3' and reverse primer was 5'-GAACATGGAGAACATCACATCAG-3' for a product of 676 bp. One µL of 0.1 ng/µL of positive control template and 0.5 µL of each primer were added to the reactions.

Bacterial 16S rDNA screening was conducted using the same PCR cycling settings. Two sets of primers were used: one set consisting of forward primer 5’-CCTACGGGAGGCAGCAG-3’ and reverse primer 5’-ATTACCGCGGCTGCTGG-3’ for a product of 193 bp and a second set consisting of forward primer 5’-GCGTGGGGAGCAAACAGGATTAG-3’ and reverse primer 5’-GCCCCCGTCAATTIATTTGAGTTT-3’ for a product of 162 bp.[70,71] *Brucella sp*. genomic DNA was used as a positive control and DNA-free water was used as a negative control. Reactions were prepared under strict conditions. All PCR reagents and supplies used were DNA-free and reactions were made in a sterile PCR hood (Mystaire, Inc.). All supplies were decontaminated using RNase Away (Molecular Bio-Products, Inc.) for RNase and DNA removal.

MMTV PCR screening was also conducted using the same cycling settings. A nested PCR was conducted using the primer sets 1N and 4, and 2N and 3N as previously described for products of 687 bp and 255 bp, respectively.[51] The annealing temperature was 57 °C for both reactions. 0.1 ng/μL of a positive control MMTV template synthesized by Integrated DNA Technologies, Inc. was used as a positive control and DNA-free water was used as a negative control.

2.4 Creating a TLR4 Knockout Cell Line Using TALENs

Plasmids for TALENs generation were constructed to target the TLR4 gene by Bryan Strouse and Pawel Bialk of Delaware State University. Two sets of these plasmids were designed to target the TLR4 gene in two different locations to determine which may be more efficient at creating a knockout. The constructs used for each set allow for GFP and blasticidin selection (Figure 2.1).

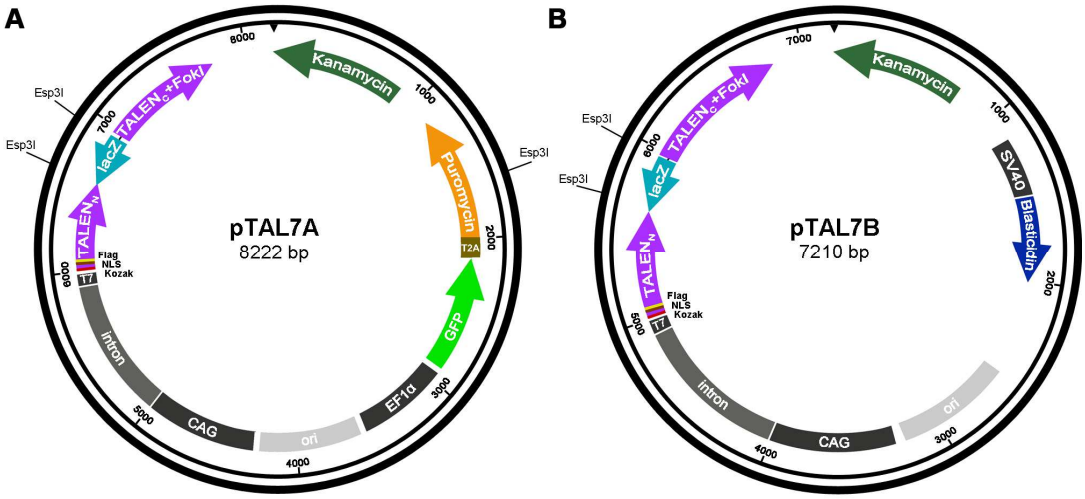

Figure 2.1. **Plasmid constructs used for generation of TALENs targeted towards the TLR4 gene**. Reprinted with permission from "A modified TALEN-based system for robust generation of knock-out human pluripotent stem cell lines and disease models".[72]

2 million SUM149 cells were seeded in a 60mm plate 48 hours before transfection. Cells were transfected with 0.5 µg of each plasmid using X-tremeGENE HP DNA Transfection Reagent (Roche) at a ratio of 3:1 as per manufacturer's instructions. 48 to 72 hours after transfection, GFP-positive cells were sorted and allowed to expand. Cells were selected using 15 µg/mL blasticidin. Clones were to be expanded and screened for successful knockouts by PCR and Western blotting. Knockouts were to be used to determine if the knockout prevents emboli formation. However, following selection the cells were apoptotic and thus the experiment was ended.

2.5 Bacterial Culture from SUM149 Cells

SUM149 cells were grown to confluency in 100mm plates. Cells were harvested and lysed in PBS using a tissue grinder. Cells were plated in triplicate either at a 1:1 or 4:1 ratio of cell culture to agar plates. 2 negative controls were included: 1 of the PBS used to wash and lyse the cells, and 1 of the cell culture media. Plates were incubated at 37°C for up to 20 days with periodic observation.

Chapter 3

RESULTS

3.1 Toll-like Receptor 4 Expression

TLR4 overexpression of IBC was visualized by IHC staining. SUM149 emboli grown *in vitro* were first stained and TLR4 was found highly expressed throughout the cell membranes (Figure 3.1). This staining was then applied to IBC patient samples. 19 samples from a set of 5 IBC patients were stained. TLR4 expression was seen in tumor cells of all patients. TLR4 was found moderately to heavily expressed in tumor cells of 4 patients, and lightly expressed in tumor cells of 1 patient. 2 patients showed TLR4 expression in alveolar epithelial cells. 2 patients for which skin punch biopsy samples were available showed expression of TLR4 in emboli cells and in the epidermis (Figure 3.2). 1 patient showed nuclear localization of TLR4.

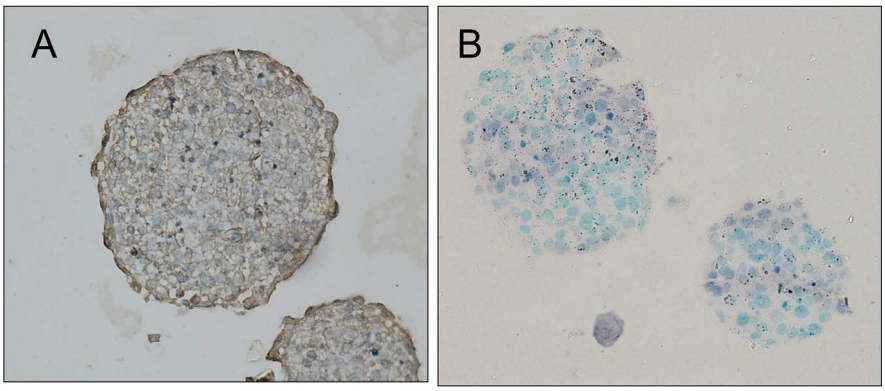

Figure 3.1. **TLR4 expression of SUM149 emboli grown *in vitro***. TLR4 is highly expressed throughout the cell membranes of IBC emboli grown in culture. A. TLR4. B. IgG negative control. 100x total magnification.

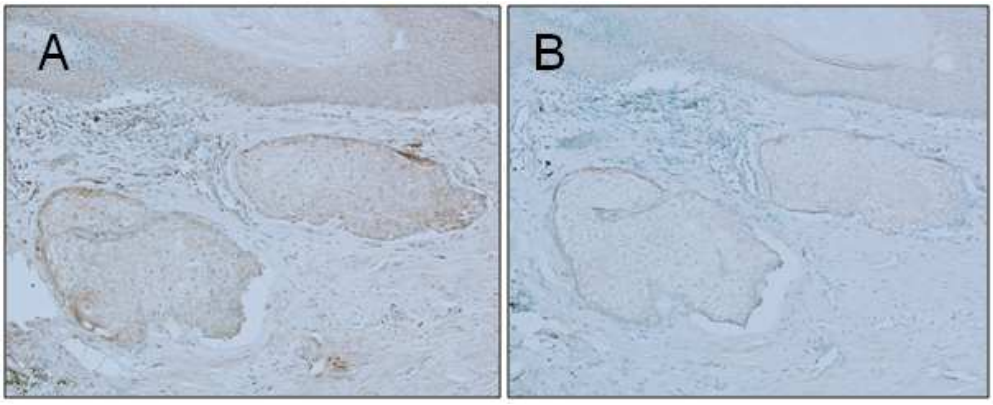

Figure 3.2. **A representative image of TLR4 expression in IBC patient samples**. TLR4 was found to be expressed in tumor cells as well as the epidermis of IBC patients. A. TLR4. B. IgG negative control. 100x total magnification.

To study the effect that TLR4 overexpression may have on the IBC phenotype, SUM149 TLR4 knockouts were created using TALENs. Two sets of plasmids targeting the TLR4 gene in two different locations were designed. SUM149 cells were successfully transfected with each set and sorted by GFP

expression. They were also selected using blasticidin. Initially, the cells were proliferating and began forming colonies as expected. However, then the population of living cells gradually decreased until there were no longer any viable cells able to be cultured. This occurred over the course of 1 month.

3.2 Toll-like Receptor 4 Polymorphism Screening

Toll-like receptor 4 (TLR4) polymorphisms D299G and T399I were screened using a previously established restriction fragment length polymorphism (RFLP) PCR procedure with modifications.[66] A group of cell lines were screened including SUM149, MCF7, GILM2, and MCF10A (Figure 3.3). No polymorphisms were detected in these cell lines. This screening was then applied to a set of 40 Algerian IBC patient samples. Due to difficulty acquiring PCR product using the protocol applied to the cell lines, the procedure was adjusted to create a nested PCR and included an internal control. This allowed for successful amplification to be used for the subsequent digest. Figure 3.4 is a representative image of the completed screening for the D299G polymorphism on a set of the Algerian samples. This image shows 2 of 18 patients were positive for this polymorphism. The remainder of the 40 samples were then completed, but no other samples were positive. Then the screening for the T399I polymorphism was completed on these samples. Only 1 patient was possibly positive for this polymorphism (Figure 3.5).

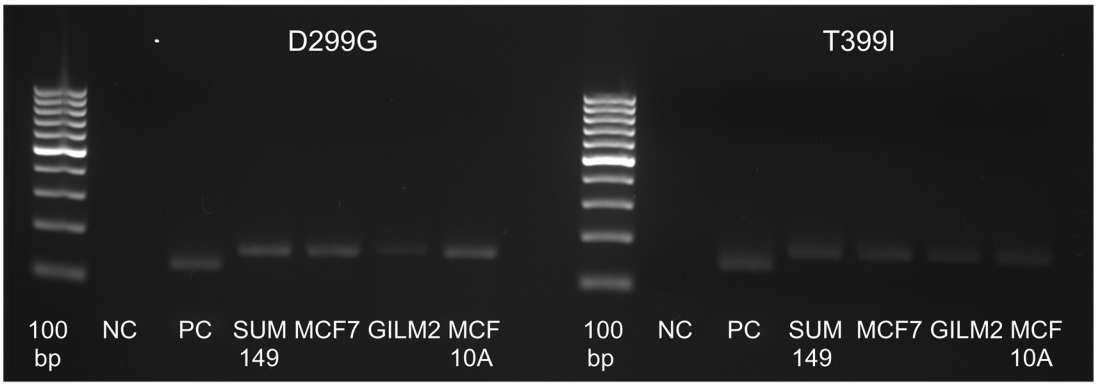

Figure 3.3. **RFLP-PCR screening of TLR4 D299G and T399I polymorphisms of cell lines**. This image shows screening of both polymorphisms on a group of cell lines. The digested positive control indicates the size of the polymorphic allele. None of the cell lines were found to have either polymorphism. NC, negative control. PC, positive control.

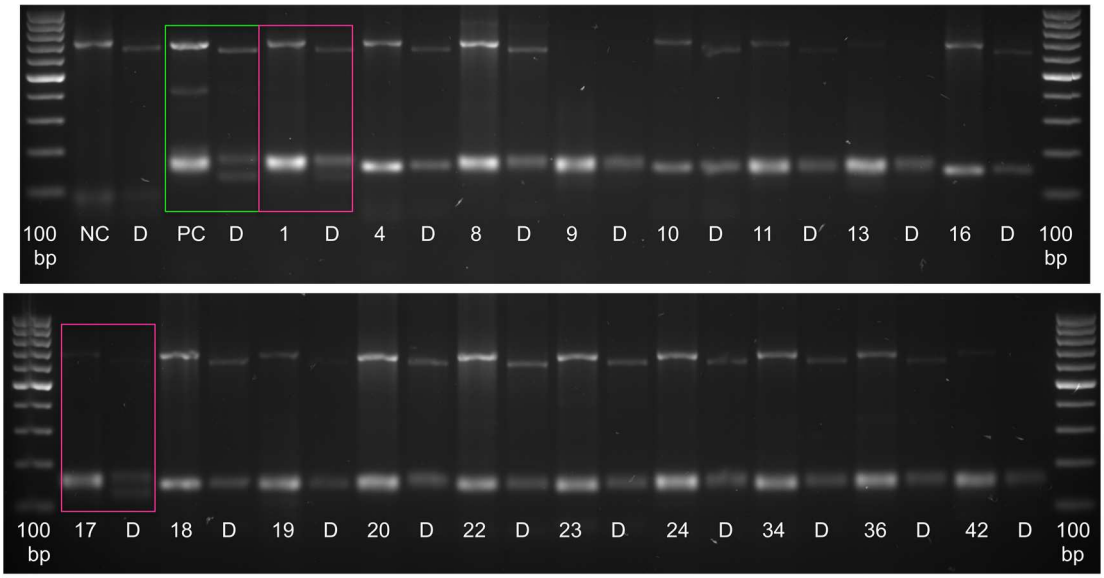

Figure 3.4. **TLR4 D299G polymorphism RFLP-PCR screening of Algerian IBC patient samples**. A representative image of the completed RFLP-PCR screening of Algerian IBC patient samples. Both undigested and digested (D) products were shown for easy comparison. The larger product around 700bp represents the internal control. Outlined by the pink boxes are 2 positive patients for this polymorphism out of the 18 shown here. The remaining samples were screened and no other patients were positive, resulting in only 2 of 40 positive. Numbers are in reference to the patient sample. NC, negative control. PC, positive control.

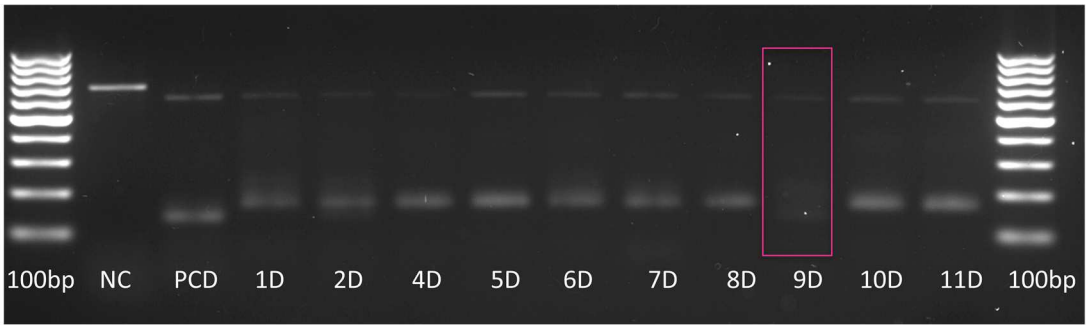

Figure 3.5. **TLR4 T399I polymorphism RFLP-PCR screening of Algerian IBC patient samples.** A representative image of the completed RFLP-PCR screening of Algerian IBC patient samples. The undigested negative control and digested positive control were shown for comparison. The larger product around 700bp represents the internal control. Outlined by the pink box is a patient positive for this polymorphism. Only one of the 40 patients was possibly positive for this polymorphism. Numbers are in reference to the patient sample. NC, negative control. PC positive control.

3.3 16s rDNA and MMTV PCR Screening of Cell Lines

To eliminate microbial DNA contamination and the occurrence of false-positives, 16S rDNA and MMTV PCR screening was optimized. Only DNA-free PCR reagents and supplies were used. PCR reactions were prepared in a sterile PCR hood and all instruments and surfaces were decontaminated with RNase Away. Appropriate negative and positive controls were optimized for two sets of 16S primers and for nested MMTV PCR primers (Figure 3.6).

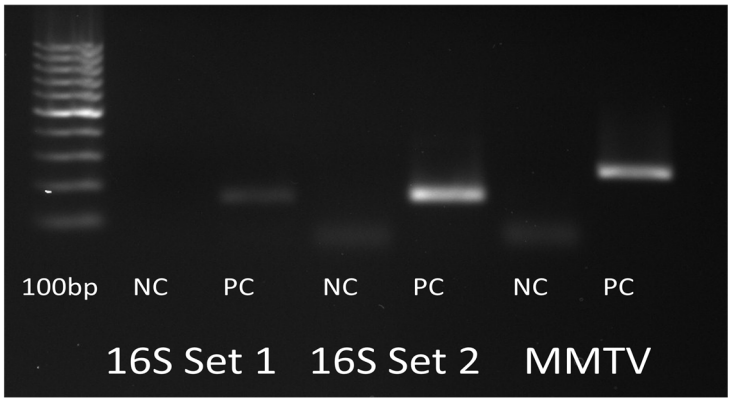

Figure 3.6. **Optimized PCR reactions for the screening of 16S rDNA and MMTV DNA.** Two primer sets for 16S screening and a nested PCR for MMTV screening were optimized for appropriate positive and negative controls.

Once PCR reactions were optimized, 16S screening was conducted on a group of cell lines. However, as seen in Figure 3.7, contamination is still present in the samples and thus PCR screening of cell lines in culture is inconclusive. MMTV screening of the cell lines was also performed and an initial screening showed a faint band present in SUM149 cells that is not present in the other cell lines (Figure 3.8). This screening was then repeated in triplicate and bands were seen in 3 SUM149 samples and 2 MCF7 samples (Figure 3.9). Bands were excised for sequencing.

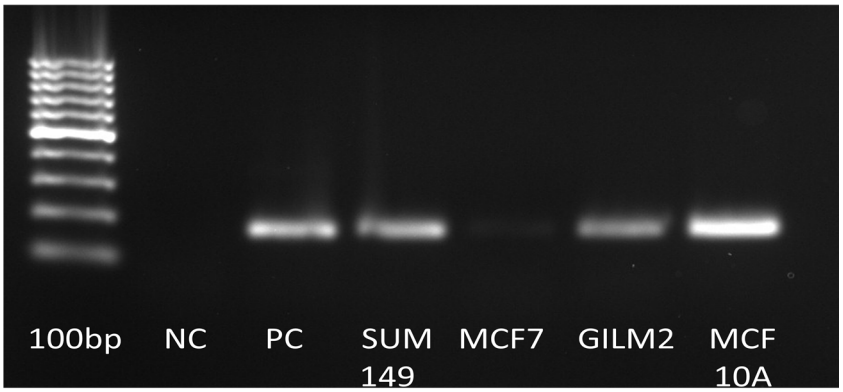

Figure 3.7. **16S rDNA PCR screening of cell lines**. 16s screening showed contamination of the cell line samples and thus results are inconclusive. NC, negative control. PC, positive control.

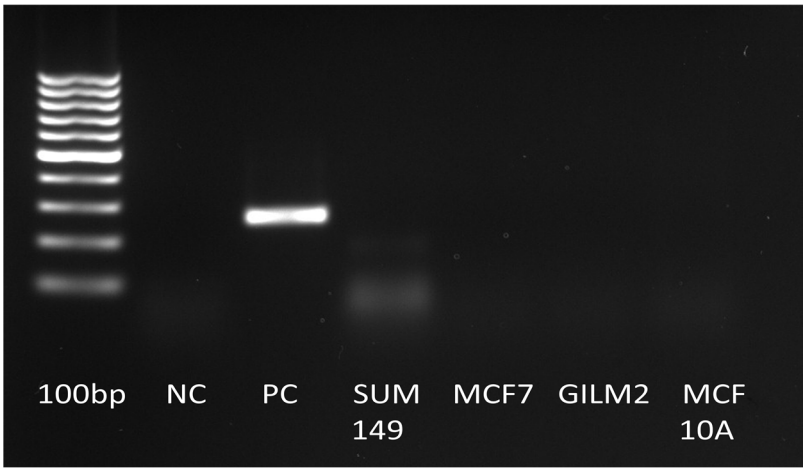

Figure 3.8. **MMTV screening of cell lines**. An initial MMTV screening of cell lines revealed a faint band present in the SUM149's that is not present in the other cell lines. NC, negative control. PC, positive control.

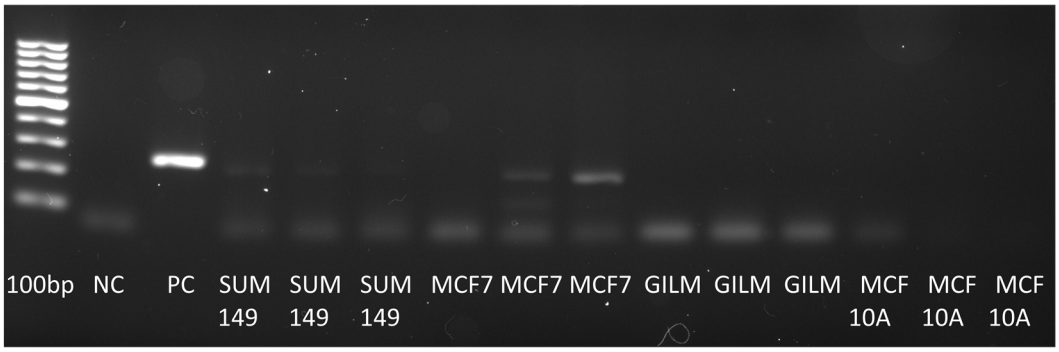

Figure 3.9. **MMTV screening of triplicate cell line samples**. Triplicate MMTV screening shows 3 SUM149 samples and 2 MCF7 samples contain PCR product. NC, negative control. PC, positive control.

3.4 Bacterial Culture from the IBC SUM149 Cell Line

Isolation of an intracellular bacteria from SUM149 cells was attempted by harvesting and lysing cells, and plating the lysate on chocolate agar plates. A ratio of 1:1 and 4:1 of plates of SUM149 cells to agar plate were conducted in triplicate alongside two negative controls of cell culture media and PBS. 20 days post-inoculation, no bacterial growth was seen (Figure 3.10).

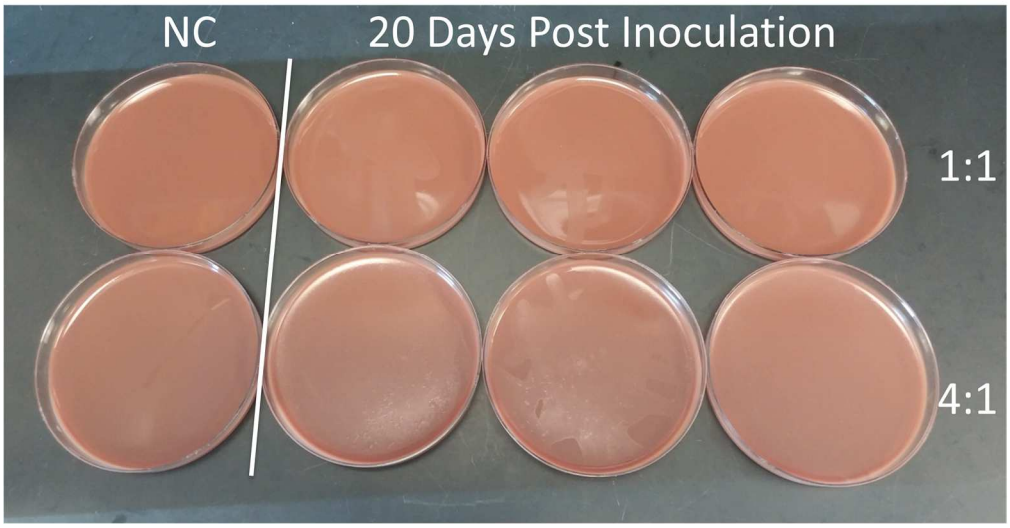

Figure 3.10. **Bacterial culture of SUM149 cells**. SUM149 cells were lysed and plated on chocolate agar and allowed to incubate for up to 20 days. Triplicate plates of 1:1 and 4:1 plates of SUM149 cells to agar plate were conducted. No growth from the SUM149's was seen. NC, negative control.

Chapter 4

DISCUSSION

4.1 Toll-like Receptor 4 Expression and Polymorphisms in Inflammatory Breast Cancer

As previously discussed and shown in Figure 1.6, TLR4 is highly expressed in IBC compared to nonIBC patients tissue. Therefore, IHC staining was used to determine TLR4 localization in IBC tissue. An initial staining of IBC emboli grown *in vitro* showed high expression of TLR4 throughout the cell membranes (Figure 3.1). This staining was then applied to IBC patient samples in which TLR4 was expressed by IBC tumor cells, consistent with the *in vitro* staining (Figure 3.2). As TLR4 is normally expressed by epithelial cells, it was not surprising to see expression in alveolar epithelial cells and epidermal cells. However, whether this expression is higher than that of normal breast tissue is not able to be discerned due to lack of normal breast/epidermal tissue for comparison. Future experiments should indicate the location of which the skin punch was obtained, as well as if it was in an area of edema or erythema. This should be compared to normal breast skin punches of the same area, if obtainable. It is interesting that 1 patient showed TLR4 localized to the nucleus. Nuclear localization of TLR4 is not well reported, but colocalization of TLR4 with LPS has been seen in lung

inflammation.[73] Perhaps TLR4 was mislocalized to the nucleus after activation, but the underlying mechanisms are unclear. It is also interesting that the patients had different levels of TLR4 expression. It may be beneficial to determine if TLR4 expression is associated with symptom severity or progression at the time of tissue collection. Increased TLR4 expression over a period of time may also indicate the misregulation of a negative regulator, as TLR4 normally would decrease back to normal levels.[54,60] Thus further research analyzing gene expression of components of the TLR4 pathway is suggested such as using RNA-Seq or microarrays.

To understand the effect that TLR4 expression may have on the IBC phenotype, a knock out cell line was developed. The IBC SUM149 cell line was used because it was established from a primary, triple negative IBC patient tumor of epithelial origin, and the molecular profile is much like that seen in majority of patient cases.[74] SUM149 TLR4 knockouts were created using two different TALENs targeted towards different areas of the gene. These were to be screened for the most effective knockout. Clones were to be expanded and used to determine the effect the knockout has on the cells ability to form emboli. This assay was chosen because emboli are highly characteristic of IBC and thought to be a driving force of metastasis. In addition, only IBC cell lines have the ability to form emboli and can therefore be compared to nonIBC cell lines.[75] It was expected that the knockout cells would lose the ability to form emboli, thus suggesting TLR4 is involved in the IBC phenotype. Furthermore, TLR4 expression may be contributing to the phenotype by promoting invasion and metastasis. The TLR4 pathway has

been found to activate Akt in murine macrophages.[76] A similar effect may be occurring in IBC which would activate RhoC by Akt, increasing motility.

However, after selection of the TLR4 knockouts, the number of viable IBC cells began to gradually decrease until no more living cells were able to be cultured. This could have been due to the stress of the procedure, but considering that the cells initially were successfully proliferating, this may not have been the case. It is possible that TLR4 signaling is involved in IBC cell proliferation, and this could in part be through Akt3 which has previously been found to be important in IBC cell survival.[19] LPS-induced TLR4 signaling has been shown to be involved in hepatocellular carcinoma proliferation, and knocking down TLR4 expression in other breast cancers has been shown to decrease proliferation.[77,78] Creating TLR4 knockouts of SUM149 cells should be repeated to determine if the same results are obtained. In addition, this could be compared to treating SUM149's with a TLR4 antagonist. There are several compounds currently being researched that target TLR4 in conditions such as colitis and chronic inflammation, and these may be potential candidates for IBC treatment.[79]

In addition, MCF10A cells which are a normal mammary epithelial cell line that is also triple negative, can also be used to determine the effect TLR4 expression has on the IBC phenotype. TLR4 knockouts can be created following the same procedure as the SUM149's. Then a TLR4 overexpressing plasmid can be introduced to mimic what is seen in IBC and the pathway can be activated using LPS. These cells can be tested for their ability to form emboli. It was hypothesized that these cells may gain, or partially, gain the

ability to form emboli, thus further supporting the role of TLR4 in the IBC phenotype. However, based on what was seen with the TLR4 IBC knockouts, additional assays analyzing proliferation such as an MTT assays could also be performed. It could also be beneficial to measure gene expression differences by RNA-Seq or microarray of these cells compared to SUM149 cells to help identify other alterations that may be driving IBC.

In addition to TLR4 expression, the possibility of TLR4 polymorphisms involved in IBC was evaluated. D299G and T399I polymorphisms are known to cause host hyporesponsiveness to LPS and therefore it was hypothesized that these polymorphisms may put patients at risk for IBC if exposed to an infectious agent. The D299G and T399I polymorphisms were screened for in a group of cell lines as well as patient samples. RFLP-PCR procedures that were previously published were modified for these experiments. The group of cell lines included the SUM149 and MCF10A lines for reasons as previously described. Also included were MCF7, a noninflammatory cell line, but derived from metastatic breast cancer cells and ER/PR positive; and GILM2, a noninflammatory breast cancer line that is also triple negative. Neither polymorphism was found in any of the screened cell lines (Figure 3.3). Screening was then performed on a set of 40 Algerian patient samples. Due to difficulty in acquiring PCR product for the RFLP digest, the procedure was modified to create a nested PCR including an internal control. This allowed successful completion of the screening. However, only 2 of 40 patients were positive for the D299G polymorphic allele (Figure 3.4). Then completion of the

T399I screening showed that only 1 of 40 patients were positive for this polymorphism (Figure 3.5).

Considering that neither of these polymorphisms were prevalent in these patients and the occurrence was lower than what would be expected normally in an African population, it is possible that these polymorphisms may be preventative against IBC. For example, the D299G polymorphism is present in 10-18% of the African population,[65] however only 5% of the IBC patients were found to be positive for this polymorphism. It has been found that this polymorphism can be protective against infectious diseases such as malaria,[65] so perhaps this polymorphism has a protective role in IBC as well. Although it is also noted that this is using a relatively small sample and therefore a large sample size may be more informative. This could also be analyzed using TALENs to create polymorphic MCF10A cell lines and see how these cells respond to infection. However, this still leaves the question as to why IBC is more common in African Americans and women of north African countries. There are many other alterations in TLR4 and the TLR4 signaling pathway that could impair a patient's ability to respond to an infectious exposure that may be prevalent in these groups, and thus further research would be warranted. Alterations in TLR4 signaling could contribute to the clinical inflammatory symptoms of IBC, but also explain why typical infectious responses such as fever are absent in IBC. This would provide a link between TLR4 expression and infectious agent involvement in IBC.

4.2 Infectious Agents in Inflammatory Breast Cancer

Increasing evidence for the involvement of an infectious agent in the development of IBC supports the need for an in depth study to determine its significance. Geographic and seasonal evidence along with TLR4 overexpression in IBC suggests the involvement of an intracellular bacteria or virus. In addition, symptoms such as the inflammatory appearance, warmth, and skin thickening suggest some type of immune response. It is hypothesized that potential organisms can include *Brucella sp*., *Bartonella sp*., or MMTV. While these organisms are considered in particular, it is acknowledged that it may be different agents that may be responsible. Additional viruses that have been found to be associated with IBC include human cytomegalovirus, Epstein-Barr virus, and human papillomavirus.[80-82] It is also possible that it may not be one agent in particular. The activation of TLR4 by any organism may act as a trigger of IBC progression for patients at risk.

However, if an infectious agent is involved, it raises the question of why the organism would be localized to the breast area. *Bartonella* infection can present as a local infection like from a scratch and so could possibly be localized at the breast.[83] In addition, *Bartonella* infection has been described as mimicking breast cancer, including IBC, and *Bartonella* DNA has been found in IBC samples.[46-48] *Brucella* however is rarely found in breast tissue, but has been reported.[84,85] It would be beneficial to determine if IBC patients were recently pregnant or breast feeding which can result in immunodeficiency and may make the patient susceptible to a breast infection. This could also account for why an early age of pregnancy seems to be protective against

nonIBC, but not IBC. This could hold true for younger patients, however, given that the median age of diagnosis is 57, immunodeficiency could also in some cases be related to age. The mechanism of how these organisms would infect breast epithelial cells specifically would also be of interest. MMTV uses transferrin receptor 1 (TfR1) which is highly expressed by breast epithelial cells during puberty and pregnancy to bind to and enter the cells.[86] It is possible that *Bartonella* and *Brucella* may be using a similar mechanism. In addition, perhaps the overexpression of caveolins seen in IBC could also be involved as many microorganisms are able to use caveolae and lipid rafts to evade degradation in lysosomes.[87]

To address the hypothesis that an infectious agent is responsible for the development of IBC, a reliable method of accurately detecting an intracellular bacteria or virus needed to be established. 16s rDNA PCR screening is a method that allows the presence of any bacterial species to be detected, making it a valuable tool. However, there is a high risk of contamination of the reaction with microbial DNA, especially bacterial, leading to false positive results. PCR reagents such as Taq polymerase are produced using *E. coli* and therefore carry high risk of contaminating DNA. Therefore strict measures to ensure the reactions remain free from contaminating DNA were used including using certified DNA-free PCR reagents and controlling the environment in which the reactions were prepared such as using a PCR hood and decontaminating surfaces and equipment with RNase Away. Using such measures allowed for the optimization of accurate controls for 16S as well as MMTV screening (Figure 3.6).

Once a reliable protocol was established, the screens were applied to cell line DNA samples. The cell lines used were SUM149, MCF7, GILM2, and MCF10A for reasons previously explained. Figure 3.7 shows a band present in all of the cell lines which indicates contamination of the DNA samples. To determine if these bands may have been a result of *Mycoplasma* contamination, *Mycoplasma* testing was conducted, but all samples were negative. However, DNA contamination could have occurred at any point during the cell culture process despite taking extra measures to use DNA-free materials when possible. Thus, using this method on cell lines is likely to lead to unreliable results, but should be more informative using patient samples. Patient blood samples collected and sent to the lab without any prior handling so that they can be properly managed to avoid contamination should give more accurate results. Samples such as the paraffin embedded Algerian samples are not optimal as processing of tissue would likely introduce contaminating DNA. Alternatively, it is possible these bands are true positive results of an infectious agent in these cell lines. All of these lines have been cultured together which may have allowed the spread of an organism from the SUM149s to the other lines. It would be interesting to screen cell lines cultured in different labs that would not have been exposed to SUM149 cells.

In addition to 16S PCR screening, MMTV PCR screening was also conducted on the cell lines. An initial screen of the cell lines showed viral DNA present in the SUM149 cell line that was not present in the other cell lines (Figure 3.8). Follow-up screens in triplicate found viral DNA in 3 of the SUM149 and 2 of the MCF7 samples (Figure 3.9). Though the bands are

slightly smaller in size to the positive control, the positive control is a pure MMTV sequence and thus a human variant would not necessarily be the same size. Presence of viral DNA in both SUM149 and MCF7 samples could be due to several reasons. It is possible IBC cells could contain the same or similar virus as nonIBC cells. It is also possible that there may be contaminating DNA as discussed with the 16S sequencing and again using patient samples would be more reliable. SUM149 cells and patient blood samples could also be used to isolate possible MMTV/HMTV viral particles using an established protocol by Melana, *et al*.[88] The bands have been isolated for sequencing.

As an additional experiment to determine if an intracellular bacteria may be present in the SUM149 cells, the cells were lysed and the lysate was plated onto chocolate agar plates (Figure 3.10). Chocolate agar was used because it supports the growth of various fastidious organisms including *Bartonella sp*. While no growth was seen after 20 days of incubation, it does not necessarily rule out the presence of an intracellular bacteria in IBC. The SUM149 cells have been in culture for a long time and may no longer carry the bacteria if initially present. Different media and culture conditions, such as a microaerophilic environment of 7-10% oxygen, may also be applied to facilitate the growth of organisms not supported by chocolate agar or aerobic environments such as *Bartonella sp*. These different culture conditions can then be applied to blood and tissue culture from IBC patients. Additional experiments that could be conducted can include treating the SUM149's with antibiotics targeted towards intracellular bacteria. Gene expression changes of treated cells versus non-treated cells could then be compared. These treated

cells could also be tested for their ability to form emboli and analyzed for changes in proliferation or invasion.

Collectively, these methods will be useful in determining the involvement of an infectious agent in IBC. This would provide a link to TLR4 overexpression and thus aid in the understanding of IBC progression. More importantly, it would provide answers as to how to prevent and treat this disease that is potentially infectious yet we have no knowledge of how to control it. This would also lead to the production of a vaccine that would not only impact the women in the United States, but even more so in north African countries that are desperately in need.

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Appendix A

PCR CONTROL TEMPLATES

A.1 TLR4 D299G T399I polymorphic positive control template

TGCAATTTGACCATTGAAGAATTCCGATTAGCATACTTAGACTACTACCTC
GATGGTATTATTGACTTATTTAATTGTTTGACAAATGTTTCTTCATTTTCCC
TGGTGAGTGTGACTATTGAAAGGGTAAAAGACTTTTCTTATAATTTCGGAT
GGCAACATTTAGAATTAGTTAACTGTAAATTTGGACAGTTTCCCACATTGA
AACTCAAATCTCTCAAAAGGCTTACTTTCACTTCCAACAAAGGTGGGAAT
GCTTTTTCAGAAGTTGATCTACCAAGCCTTGAGTTTCTAGATCTCAGTAGA
AATGGCTTGAGTTTCAAAGGTTGCTGTTCTCAAAGTGATTTTGGGATAATC
AGCCTAAAGTATTTAGATCTGAGCTTCAATGGTGTTATTACCATGAGTTCA
AACTTCTTGGGCTTAGAACAACTAGAACATCTGGATTTCCAGCATTCCAAT
TTGAAACAAATGAGTGAGTTTTCAGTATTCCTATCACTCAG

A.2 HBV Internal control template

TCTCCTCGAGGACTGGGGACCCTGCGCCGAACATGGAGAACATCACATC
AGAATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGA
CAAGAATCCTCACAATACCGCAAAGTCTAAACTCGTGGTGGACTTCTCTC
AATTTTCTAGGGGGAGCACCCGTGTGTCTTGGCCAAAATTCGCAGTCCC
CAACCTCCAATCACTCACCAACCTCCTGTCCTCCAATTTGTCCTGGTTATC
GCTGGATGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTAT
GCCTCATCTTCTTATTGGTTCTTCTGGATTATCAAGGTATGTTGCCCGTTT
GTCCTCTAATTCCAGGATCCACAACAACCAGTACGGGACCCTGCAAAACC
TGCACAACTCCTGCTCAAGGCAACTCTATGTTTCCCTCATGTTGCTGTAC
AAAACCTACGGATGGAAATTGCACCTGTATTCCCATCCCATCATCTTGGG
CTTTCGCAAAATACCTATGGGAGTGGGCCTCAGTCCGTTTCTCTTGGCTC
AGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCTTTCCCCCACTGT
TTGGCTTTCAGCTATATGGATGATGTGGTACTGGGGGCCAAGTCCCATG
GCCTTTATACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATACATTTAAA
CCCTAACAAAACAAAGAGATGGGGTTACTCCC

A.3 MMTV positive control template

TAAAAAAGAAGTTGCCCCCCAAATATCCTCACTGCCAGATCGCCTTTAAG
AAGGACGCCTTCTGGGAGGGAGACGAGTCTGCTCCTCCACGGTGGTTG
CCTTGCGCCTTCCCTGACCAGGGGGTGAGTTTTTCTCCAAAAGGGGCCC
TTGGGTTACTTTGGGATTTCTCCCTTCCCTCGCCTAGTGTAGATCAGTCA
GATCAGATTAAAAGCAAAAAGAATCTATTTGGAAATTATACTCCCCCTGTC
AATAAAGAGGTTCATCGATGGTATGAAGCAGGATGGGTAGAACCTACTTG
GTTCTGGGAAAATTCTCCTAAGGATCCCAATGATAGAGATTTCACTGCAC
TAGTCCCCCATACAGAATTGTTTCGCTTAGTCGCAGCCTCAAGACATCTT
ATTCTCAAAAGGCCAGGATTTCAAGAACATGAGATGATTCCTACATCTGC
CTGTGTTACTTACCCTTATGCCATATTATTAGGATTACCTCAGTTAATAGA
TATAGAGAAAAGAGGATCTACTTTTCATATTTCCTGTTCTTCTTGTAGATT
GACTAATTGTTTAGACTCTTCTGCCTACGACTATGCAGCGATCATAGTCAA
GAGGCCGCCATATGTGCTGCTACCTGTAGATATTGGTGATGAACCATGGT
TTGATGATTCTGCCATTCAAACCTTTAGGTATGCCACAGATTTAATTCGAG
CCAAGCGATTCGTCGCAGCCATTATCCTGGGCATATCTGC

Appendix B

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Dr. Bissell asked me to respond to your request. She gives her permission, as requested, under the assumption that you will give full citation for the image.

Your request is below:

> I am a graduate student at the University of Delaware and I would like to request permissions to reprint an image from the paper "Myoepithelial cells: Good fences make good neighbors" in my Master's Thesis. The figure I am referring to is Figure 1 and I would like to reprint the diagram at the top of the figure, if permissions are granted. I look forward to hearing from you.

Thank you,
Irene Kuhn
Bissell Group

Lauren Shuman <lmshuman@udel.edu> 10:43 PM (14 hours ago)

to Massimo.Cristo.

Hello Dr. Cristofanilli,

I am a graduate student working with Dr. van Golen at the University of Delaware and I would like to request permission to reprint an image from the paper "Inflammatory Breast Cancer: The Disease, the Biology, the Treatment" in my Master's Thesis. The image I am referring to is Figure 2B and I will of course include a full citation. I look forward to hearing from you.

Thank you for your time,

Massimo Cristofanilli 8:20 AM (5 hours ago)

to me

Dear Dr. Shuman,

Permission granted.

Best regards

Massimo Cristofanilli, MD, FACP
Professor of Medical Oncology
Deputy Director of Translational Research
Director of the Jefferson Breast Center and Clinical Program
Thomas Jefferson University-Kimmel Cancer Center
1025 Walnut St-Suite 700
Philadelphia, PA 19107
Tel:215-503-5098
Fax: 215-503-3408
Email: Massimo.Cristofanilli@jefferson.edu

License Number	3594830887983
License date	Mar 23, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	CA: Cancer Journal for Clinicians
Licensed Content Title	Inflammatory Breast Cancer: The Disease, the Biology, the Treatment
Licensed Content Author	Fredika M. Robertson,Melissa Bondy,Wei Yang,Hideko Yamauchi,Shannon Wiggins,Samira Kamrudin,Savitri Krishnamurthy,Huong Le-Petross,Luc Bidaut,Audrey N. Player,Sanford H. Barsky,Wendy A. Woodward,Thomas Buchholz,Anthony Lucci,Naoto Ueno,Massimo Cristofanilli
Licensed Content Date	Oct 20, 2010
Licensed Content Pages	25
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 2B
Will you be translating?	No
Title of your thesis / dissertation	Understanding the Etiology of Inflammatory Breast Cancer
Expected completion date	Apr 2015
Expected size (number of pages)	70
Requestor Location	Lauren Shuman 2402 Stone Gate Blvd. ELKTON, MD 21921 United States Attn: Lauren Shuman
Billing Type	Invoice
Billing address	Lauren Shuman 2402 Stone Gate Blvd. ELKTON, MD 21921 United States Attn: Lauren Shuman
Total	0.00 USD

Lauren Shuman <lmshuman@udel.edu> Mar 20 (2 days ago)

to J.vanStrijp

Hello,

I am a graduate student at the University of Delaware and I would like to ask permission to reprint an image in my Master's Thesis. The image I am referring to is from "Recognition of LPS by TLR4: Potential for Anti-Inflammatory Therapies" and is Figure 2 on the TLR4 signaling pathway. A full citation would be included. I look forward to hearing from you.

Thank you,

Strijp, J.A.G. van 11:31 AM (8 hours ago)

to me

Lauren
You have my permission

I would be interested in a pdf-copy from your Master Thesis when finished
Greetings
Jos

Prof. Jos A G van Strijp, PhD / Medical Microbiology
UMC Utrecht / Utrecht / the Netherlands/
0031 88 7556528 / j.vanstrijp@umcutrecht.nl

Lauren Shuman <lmshuman@udel.edu> 8:25 PM (14 hours ago)

to boris.greber

Hello,

I am a graduate student at the University of Delaware and I would like to ask permission to reprint an image in my Master's Thesis. The image I am referring to is from the paper "A modified TALEN-based system for robust generation of knock-out human pluripotent stem cell lines and disease models." It is Figure S1 showing the pTAL7 plasmids. It will include a full citation. I look forward to hearing from you.

Thank you,

Boris Greber 4:38 AM (6 hours ago)

to me

Dear Lauren,

yes, sure, please go ahead...

Best wishes,
Boris

Boris Greber, PhD
Max Planck Institute for Molecular Biomedicine
Human Stem Cell Pluripotency Laboratory
Von-Esmarch-Straße 54
48149 Münster, Germany

+49 (0)251 8346906 (office)
+49 (0)251 8346834 (lab)

License Number	3610891501082
License date	Apr 16, 2015
Licensed content publisher	American Association for Cancer Research
Licensed content publication	Molecular Cancer Research
Licensed content title	Regulation of Inflammatory Breast Cancer Cell Invasion through Akt1/PKBα Phosphorylation of RhoC GTPase
Licensed content author	Heather L. Lehman, Steven J. Van Laere, Cynthia M. van Golen et al.
Licensed content date	October 1, 2012
Volume number	10
Issue number	10
Type of Use	Thesis/Dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Will you be translating?	no
Circulation	2
Territory of distribution	North America
Title of your thesis / dissertation	Understanding the Etiology of Inflammatory Breast Cancer
Expected completion date	Apr 2015
Estimated size (number of pages)	70
Total	0.00 USD

Appendix C

IRBS AND CONSENT FORMS

Form Version 03/20/2009

GEORGE WASHINGTON UNIVERSITY & MEDICAL CENTER
OFFICE OF HUMAN RESEARCH • INSTITUTIONAL REVIEW BOARD
ohrirb@gwumc.edu • PHONE: 202.994.2715

MODIFICATION REQUEST FORM

IRB#	IRB# 030105 ER	STUDY EXPIRATION DATE:	
CLASSIFY THIS STUDY:	Other (specify):		

PROTOCOL TITLE AND SPONSOR:
TITLE: The establishment of an inflammatory Breast Cancer Registry and Biospecimen Repository
SPONSOR: Department

PRINCIPAL INVESTIGATOR INFORMATION				
LAST NAME:	Levine	FIRST NAME:	Paul	Degree: MD
CITI TRAINING COMPLETED:	Biomedical Course			
DEPARTMENT:	Epidemiology and Biostatistics			SCHOOL: SPHHS
ADDRESS:	Ross Hall, 2300 I St. NW, Suite 118, Washington DC 20037			
PHONE:	202-994-4582	EMAIL: sphphl@gwumc.edu		

MODIFICATION CLASSIFICATION:	
IRB approved protocol/research consent form:	**IRB approved enrollment/recruitment documents:**
☐ *Change design/methodology*	☒ *Administrative/Clerical changes*
☐ *Change in eligibility criteria*	☐ *Editorial/clarification*
☐ *Change in number of subjects*	☐ *New safety information for enrolled subjects*
☐ *Change in number of groups*	☐ *Other, describe:*
☐ *Changes to the research consent form*	
☒ *Other, describe:* **Add Research asst. and collaborator**	

1.	Summary of modification(s) requested: (required): **Addition of new staff: Michael Hagler (Research Asst) and Ken Van Golen at University of Delaware (Study Collaborator).** **No need to modify the Informed Consent as consents have already been signed and we are only collecting tissue samples.**
2.	Justification for the modification (s): **NEW STAFF**
3.	Is the requested modification major or minor? **Minor Modification**
4.	List all attached documents. Include versions and dates: **N/A**

Signature of PI:	Date:

THIS BOX FOR OHR USE ONLY! OHR TRANS#

_____

Should all subjects be reconsented: _____ YES / _____ NO/ _____ NA

Date Reviewed by **Full Committee or IRB Chair/Designee:** ___ / ___ / ____

Final IRB Approval:

Chair/IRB Designee: _____ Signature: _____ Date: ___ / ___ / ___

Title of this Tissue Bank
Inflammatory Breast Cancer Tissue Bank

Description
You are invited to take part in this tissue bank. The information in this form is meant to help you decide whether or not to take part. If you have any questions, please ask. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

Please read and sign this consent form, then send to:

Dr. Paul Levine, College of Public Health
c/o Department of Epidemiology
984395 Nebraska Medical Center
Omaha NE 68198-4395

Why are you being asked to participate in this tissue bank?
You are being asked to be a part of this tissue bank because you are an adult (19 years old or older) who has been diagnosed with inflammatory breast cancer (IBC), a rare but aggressive form of breast cancer. It often causes the breast to swell and affects the skin of the breast so that the breast looks inflamed.

What is the reason for creating this tissue bank?
The purpose for creating this tissue bank is to collect and store tissue and associated clinical information for use in future research studies on IBC and its possible causes.

What will be done and what material will be collected for storage in the tissue bank?
Material to be collected

With your consent, we will collect a sample of the breast cancer tissue that was collected when you were diagnosed with IBC. Additionally, we will interview you and collaborate with your physician to collect general information about you, your medical history, your breast cancer diagnosis, and possible environmental exposures.

What will be done

We will also analyze your tissue sample for evidence of factors that may have contributed to inflammatory breast cancer. The tissue samples will be sent to collaborating laboratories at the University of Nebraska Medical Center (and possibly Baylor, in the future) for testing.

The interview will be done by the researcher, Dr. Paul Levine, over the phone from his private office.

What are the possible risks of participating in this tissue bank?
There are few risks of participating in this tissue bank. There is a risk of emotional upset or discomfort in discussing your medical history. The researcher will conduct interviews when you are ready to answer questions, and you can refuse to answer questions or stop your participation in this study at any time. Since your samples will have identifying health information associated with them, there is a risk for loss of confidentiality.

What are the possible benefits to you?
You are not expected to get any benefit from allowing your samples to be stored in the tissue bank.

What are the possible benefits to other people?
Society may benefit from the research based on this tissue bank by gaining more information regarding possible exposures that can contribute to inflammatory breast cancer. This may help doctors identify inflammatory breast cancer more quickly and more precisely by knowing what can cause inflammatory breast cancer to develop.

What are the alternatives to participating in this tissue bank?
Instead of allowing your samples to be stored in this tissue bank you can choose not to participate.

What will participating in this tissue bank cost you?
There is no cost to you to you to participate in this tissue bank.

Will you be paid for participating in this tissue bank?
You will not be paid to participate in this tissue bank. In the event that any commercial products are developed from your donated tissue, there are no plans to share any revenues with you from those commercial products. It is policy that all donated tissue is the property of the Organization.

Who is paying for this tissue bank?

This tissue bank is being paid for by the Department of Epidemiology at the University of Nebraska Medical Center.

What should you do if you are injured or have a medical problem as a result of participating in this tissue bank?
Your welfare is the main concern of every member of the tissue bank team. If you are injured or have a medical problem as a result of participating in this tissue bank, you should immediately contact one of the people listed at the end of this consent form.

How will samples and information about you be protected?
All necessary steps will be taken to protect your privacy and the confidentiality of your samples.

You have rights regarding the privacy of your medical information collected for this tissue bank. This medical information called "protected health information" (PHI). PHI used in this tissue bank, may include, results of physical exams, blood tests, x-rays, as well as the results of other diagnostic and medical procedures, as well as your medical history. Your tissue bank data and medical records will be maintained in a secure manner.

Who will be able to use my samples for research?
Your samples may be studied by the Investigators listed on this protocol, such as Dr. Paul Levine, whose research proposals have obtained approval from the Institutional Review Board at the University of Nebraska Medical Center will be able to access this tissue bank for research. Your samples may also be studied in the future by other researchers here at UNMC.

Who will have access to information about you?
By signing this consent form, you are allowing the tissue bank team to have access to your PHI. The tissue bank team includes the individuals listed on this consent form and other personnel involved in this tissue bank at the Institution.

Your PHI will be used only for the purpose(s) described in the section "What is the reason for creating this tissue bank?"

You are also allowing the research tissue bank team to share your PHI, as necessary, with other people or groups listed below:

- The UNMC Institutional Review Board (IRB)
- Institutional officials designated by the UNMC IRB
- Federal law requires that your information may be shared with these groups:

- The HHS Office of Human Research Protections (OHRP)
- The HIPAA Privacy Rule requires the following groups to protect your PHI:
  - The Eppley Cancer Center Scientific Review Committee (SRC)

You are authorizing us to use and disclose your PHI for as long as the tissue bank is storing your sample(s).

What will happen if you decide not participate this tissue bank?
You can decide not to participate in this tissue bank. Deciding not to participate will not affect your medical care, if applicable, or your relationship with the tissue bank team or the Institution. Your doctor will still take care of you and you will not lose any benefits to which you are entitled.

What will happen if you decide to stop participating once you start?
You can stop participating in this tissue bank (withdraw) at any time. Should you wish to stop participating in the tissue bank, you must contact one of these individuals in writing requesting that your samples and associated information be removed from the tissue bank and no longer used for future research purposes.

Dr. Paul Levine, College of Public Health
c/o Department of Epidemiology
984395 Nebraska Medical Center
Omaha NE 68198-4395

Will you be given any important information during the tissue bank?
You will be informed promptly if the tissue bank team gets any new information that may affect whether you would want to continue participating in the tissue bank.

What should you do if you have any questions about the tissue bank?
You have been given a copy of "What Do I Need to Know Before Being in a Research Study?" If you have any questions at any time about this tissue bank, you should contact the Principal Investigator or any of the tissue bank personnel listed on this consent form or any other documents that you have been given.

What are your rights as a participant in this tissue bank?
You have rights as a participant in this tissue bank. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights or complaints about the tissue bank, you can contact any of the following:

- The investigator or other tissue bank personnel

- Institutional Review Board (IRB)
  - Telephone: (402) 559-6463.
  - Email: IRBORA@unmc.edu
  - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-6941
  - Email: unmcrsa@unmc.edu

Documentation of informed consent

You are freely making a decision whether to participate in the tissue bank. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.
- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to participate in the tissue bank.
- If you have any questions, you have been directed to talk to one of the individuals listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

Signature of Participant: _____

Date: _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the participant. In my judgment, the participant possesses the legal capacity to give informed consent to participate in this tissue bank and is voluntarily and knowingly giving informed consent to participate.

Signature of Person Obtaining Consent: _____

Date: _____

Authorized Tissue Bank Personnel

Principal

Levine, Paul

alt email: paulhlevine@earthlink.net

phone: 402-559-4248

alt #: 301-469-7394

degree: MD

Secondary
Paul, Ladan
alt email: Ladan.Paul@gmail.com
alt #: 703-593-7979
degree: MD

What Do I Need To Know Before Being In A Research Study?

You have been invited to be in a **research study**. Research studies are also called "research surveys", "research questionnaires" or "scientific protocols." **Research** is an organized plan designed to get new knowledge about health, disease, behaviors, attitudes and interactions of, among and between individuals, groups and cultures. The people who are in the research are called **research subjects**. The **investigator** is the person who is running the research study. You will get information from the investigator and the research team, and then you will be asked to give your **consent** to be in the research.

This sheet will help you think of questions to ask the investigator or his/her staff. You should know all these answers before you decide about being in the research.

What is the **purpose** of the research? Why is the investigator doing the research?

What are the **risks** of the research? What bad things could happen?

What are the possible **benefits** of the research? How might this help me?

How is the research different than what will happen if I m not in the research?

Will being in the research **cost** me anything extra?

Do I have to be in this research study? How will it affect my status at the institution if I say **no**?

Can I **stop** being in the research once I ve started? How?

Who will look at my **records**?

How do I reach the investigator if I have more **questions**?

Who do I call if I have questions about being a **research subject**?

Make sure all your questions are answered before you decide whether or not to be in this research.

Academic Research & Services Building 3000 / 985830 Nebraska Medical Center / Omaha NE 68198-5830
402-559-6463 / FAX 402-559-3300 / Email: irbora@unmc.edu / http://www.unmc.edu/irb

THE RIGHTS OF RESEARCH SUBJECTS
AS A RESEARCH SUBJECT YOU HAVE THE RIGHT

to be told everything you need to know about the research before you are asked to decide whether or not to take part in the research study. The research will be explained to you in a way that assures you understand enough to decide whether or not to take part.

to freely decide whether or not to take part in the research.

to decide not to be in the research, or to stop participating in the research at any time. This will not affect your medical care or your relationship with the investigator or the Nebraska Medical Center. Your doctor will still take care of you.

to ask questions about the research at any time. The investigator will answer your questions honestly and completely.

to know that your safety and welfare will always come first. The investigator will display the highest possible degree of skill and care throughout this research. Any risks or discomforts will be minimized as much as possible.

to privacy and confidentiality. The investigator will treat information about you carefully, and will respect your privacy.

... to keep all the legal rights you have now. You are not giving up any of your legal rights by taking part in this research study.

to be treated with dignity and respect at all times

The Institutional Review Board is responsible for assuring that your rights and welfare are protected. If you have any questions about your rights, contact the Institutional Review Board at (402) 559-6463.

Academic Research & Services Building 3000 / 987830 Nebraska Medical Center / Omaha NE 68198-7830
402-559-6463 / FAX 402-559-3300 / Email: irbora@unmc.edu / http://www.unmc.edu/irb

UNM HEALTH SCIENCES CENTER

Human Research Review Committee
Human Research Protections Office

February 16, 2015

Eric Prossnitz, PhD
EProssnitz@salud.unm.edu

Dear Dr. Prossnitz:

On 2/16/2015, the HRRC reviewed the following submission:

Type of Review:	Modification and Continuing Review
Title of Study:	Molecular and Viral Characteristics of Inflammatory and Breast Cancer
Investigator:	Eric Prossnitz, PhD
Study ID:	11-050
Submission ID:	MODCR00001139
Funding:	None
Grant ID:	None
IND, IDE, or HDE:	None
Submission Summary:	Modification and Continuing Review #4 for Study 11-050 to add investigators Gema San Nicolas and Kaylee Deutsch .
Documents Approved:	Mol Viral Protocol v02/20/2014 Study Summary of Progress v01/15/2015
Documents Acknowledged:	Conflict of Interest documents and Human Subject Protection Training for added investigators.
Review Category:	Expedited: Category (5) Research involving materials that have been collected, or will be collected solely for non-research purposes and Category (8)(c) Continuing review of research previously approved by the convened IRB where the remaining research activities are limited to data analysis.
Determinations/Waivers:	Waived the requirement for informed consent. HIPAA Authorization Addendum waived. Study is closed to enrollment - no consents approved. Study is in data analysis phase only.
Submission Approval Date:	2/16/2015
Approval End Date:	3/6/2016
Effective Date:	2/16/2015

The HRRC approved the study from 2/16/2015 to 3/6/2016 inclusive. If modifications were required to secure approval, the effective date will be later than the approval date. The "Effective

Date" 2/16/2015 is the date the HRRC approved your modifications and, in all cases, represents the date study activities may begin.

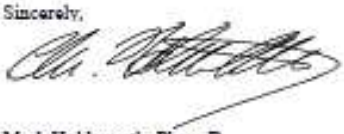
Before 3/6/2016 or within 45 days of study closure, whichever is earlier, you are required to submit a continuing review. You may submit a continuing review by navigating to the active study and clicking the "Create Modification / CR" button.

Please use the consent documents that were approved and stamped by the HRRC. The stamped and approved consents are in a comment within the submission covered by this approval letter.

This determination applies only to the activities described in this submission and does not apply should you make any changes to these documents. If changes are being considered and there are questions about whether HRRC review is needed, please submit a study modification to the HRRC for a determination. A change in the research may disqualify this research from the current review category. You can create a modification by clicking Create Modification / CR within the study.

In conducting this study, you are required to follow the Investigator Manual dated July 31, 2012 (HRP-103), which can be found by navigating to the IRB Library.

Sincerely,



Mark Holdsworth, PharmD
Executive Chair