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Abstract:

Triple-negative breast cancer (TNBC) accounts for 20 percent of all breast cancer cases and is known to be the most invasive form of breast cancer. TNBC's absence of estrogen, progesterone, and human epidermal growth factor-2 receptors makes utilizing hormonal treatments ineffective in suppressing tumor growth. TNBC is associated with poorer prognosis and higher incidences of relapse. Therefore, natural killer cell-mediated immunotherapy shows potential as a treatment option for TNBC. Natural killer cells (NK) are innate lymphoid cells that serves its role in the immune system to eradicate infected and tumor cells. NK cell function is regulated through its receptors interacting with activating and inhibitory ligands on target cells. Lectin-like Transcript-1 (LLT1, CLEC2D) is a ligand that interacts with NKRP1A (CD161) and inhibits NK cell activation. Proliferating Cell Nuclear Antigen (PCNA) is a ligand that interacts with NKp44 and inhibits NK cell activation. We have identified the expression and function of LLT1 and PCNA on TNBC cell lines by flow cytometry, western blot, immunofluorescent microscopy, and chromium-release assay. Our results have demonstrated a higher expression of LLT1 and PCNA on TNBCs than non-tumorigenic breast cell line MCF10A. We have shown that blocking LLT1 interaction with NKRP1A with antibodies and gene knockdown of LLT1, respectively, on TNBCs have increased lysis of TNBCs by primary NK cells. We have also shown that blocking PCNA interaction with NKp44 with antibodies have enhanced killing of TNBCs by NK cells. LLT1 and PCNA expressed on TNBCs sends an inhibitory signal to the NK cell thus serving its role for TNBCs to evade immunosurveillance. Blocking LLT1-NKRP1A or PCNA-NKp44 with antibodies enhances lysis by NK cells and may open a novel immunotherapeutic strategy for patients diagnosed with TNBC.

EXPRESSION AND FUNCTION OF LIGANDS FOR

NATURAL KILLER CELL RECEPTORS ON

TRIPLE-NEGATIVE BREAST

CANCER CELLS

Armando M. Marrufo, B.S.

APPROVED:

_____
Dr. Porunelloor Mathew, Ph.D., Major Professor

_____
Dr. Stephen Mathew, Ph.D., Committee Member

_____
Dr. Harlan Jones, Ph.D., Committee Member

_____
Dr. Nicole Phillips, Ph.D., Committee Member

_____
Dr. Michael Forster, Ph.D., University Member

_____
Dr. Johnny He, Ph.D., Interim Chair, Department of Microbiology, Immunology, and Genetics

_____
Dr. Johnny He, Ph.D., Interim Dean, Graduate School of Biomedical Sciences

EXPRESSION AND FUNCTION OF LIGANDS FOR NATURAL KILLER CELL RECEPTORS ON TRIPLE-NEGATIVE BREAST CANCER CELLS

THESIS

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Armando M. Marrufo, B.S.

Fort Worth, Texas

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INTRODUCTION

Treatment for breast cancer has for a long time been standardized to conventional hormonal and chemotherapeutic treatments. Conventional treatments for breast cancer has been improved over decades in synergy with emerging novelties in technologies for early detection, increasing awareness and education with the public, and novel discoveries and expansion of cancer research. However, despite improvements in conventional treatments, there is still an increasing need to further understand the heterogeneity of breast cancer and partaking methods of treating different types of breast cancers. One patient with breast cancer will differ from another patient with breast cancer in regard to genetics, prognosis, response to conventional treatments, and severity of cancer progression. Hence, the rise of genomics and pathology has emphasized the development of classifications of breast cancer based on the presence or absence of receptors, growth factors, ligands, and other phenotypic characteristics [1]. Of all the subtypes of breast cancer, triple-negative breast cancer (TNBC) is considered the most invasive form of breast cancer and is associated with the worst prognosis [1]. Unfortunately, the challenge to treating TNBC is that endocrine therapy, such as Herceptin and Tamoxifen, are ineffective due to an absence of the estrogen, progesterone, and human epidermal growth factor-2 receptors [2]. In addition, studies have shown that TNBC develop resistance to chemotherapy and relapse occurs after surgery [3,4]. Thus, there is a great need to investigate effective alternative approaches with ultimate goals of eradicating or suppressing growth and proliferation of TNBC while minimizing

side effects of novel therapeutics for patients. Our immune system could supply an undiscovered approach, a “hidden gem”, that may improve treatment for TNBC.

The human immune system consists of a complex network of effector cells, molecules, physical, and chemical barriers that enhances protection against pathogens and tumor cells. The immune system consists of two sectors of immunity: adaptive immunity and innate immunity [5]. Adaptive immunity is known for antigen-specific immune response through recognition of antigens and developing memory to accelerate the immune response to the same or similar antigen in future exposures [5]. Cells of the adaptive immune system consist of B lymphocytes and T lymphocytes [5]. The innate immune system refers to its non-specific immune responses which provides the first line of defense such as mechanical, physical, and chemical barriers [5]. The cells of the innate immune system comprise of basophils, eosinophils, neutrophils, monocytes, and natural killer cells [5].

In particular, natural killer (NK) cells are innate lymphoid cells that secretes cytokines such as interferon-gamma (IFN-γ) and functions in recognizing and differentiating between healthy self-cells and infectious and tumor cells [5,6]. NK cell function is regulated by a ticking balance of inhibitory and activating transduction signals because of NK receptors interacting with activating and inhibitory ligands on target cells [7]. Utilizing NK cells to target tumor cells has shown promise as a strategy in targeting tumor cells [7]. Furthermore, developing immune targeted therapies by activating NK cells to lyse TNBCs with monoclonal antibodies targeting ligands of interest could open a new road to treating TNBC [8]. It is not fully understood how TNBCs evade immunosurveillance from NK cells, but discovering ligands that play a role in TNBC evasion from the immune system could serve as possible targets for monoclonal antibody therapy and recruitment of NK cells to kill these TNBCs.

1.1 Breast Cancer

Breast cancer is a disease in which tumor cells develop within the breast tissues [9]. The anatomy of a female breast consists of lobes and ducts [9]. Lobes are sectioned into lobules which are parts of the breast tissue that produce mammary milk [9]. Ducts are thin tubes that link the lobules and lobes throughout the breast tissue and carry milk from lobules to the nipple [9]. Throughout the breast tissue, there are blood vessels and lymph vessels containing lymph nodes, which contains lymphatic fluid that carries immune cells [9].

Breast cancer is known to be the most common type of cancer among women in the United States [10]. According to the American Cancer Society, there will be an estimated 266,120 new cases of invasive breast cancer diagnosed in women within the United States in 2018 [10]. Furthermore, this updated 2018 figure did not include the additional 63,960 cases of in situ breast cancer, meaning that tumor cells have not left the ducts to invade surrounding breast tissue [10,11]. Breast cancer is the second leading cause of death out of all cancers for women behind lung cancer [10]. American Cancer Society projects that there will be 40,920 deaths in women due to breast cancer for 2018 [10]. There has been an increasing public awareness on breast cancer in recent years and progress has been made since 1989 to decrease the mortality rate of breast cancer [10]. The mortality rate of female breast cancer was at its peak at 33.2 per 100,000 in 1989, but then decreased by 39% to 20.3 per 100,000 in 2015 [10]. The decrease in mortality rate has been due to educating more women about breast cancer, improvements in early detection through mammograms and related technologies, improved treatments, and promoting healthy lifestyles such as exercise, avoiding drugs and smoking, and decreasing use of oral contraceptives [10].

Breast cancer is broadly classified into different types and furthermore into different intrinsic, molecular, and hormone-receptor subtypes. Carcinomas are tumors that originate from epithelial cell layers of an organ such as a breast; furthermore, adenocarcinomas are tumors that come from specialized epithelial cells that secrete substances into ducts such as milk ducts or lobules (milk-producing glands) [12]. Breast cancer can be categorized into either non-invasive, invasive, or metastatic types [13]. The two non-invasive types of breast cancers are ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) [13]. For non-invasive cancers, *in situ* refers to tumor cells that "stays within its original place" [13]. Ductal carcinoma in situ, or DCIS, is a non-invasive breast cancer that begins inside the milk ducts but has not spread to surrounding breast tissue [13,14]. Lobular carcinoma in situ, or LCIS, is defined as having a presence of abnormal cell growth producing within the lobules but has not spread to adjacent breast tissue [13,15]. A patient with LCIS does not necessarily mean that the patient has breast cancer, which is the reason that LCIS is also known as "lobular neoplasia" with neoplasia referring to an abundance of abnormal cell growth and proliferation. Rather, a patient with LCIS has a higher risk for being diagnosed with invasive breast cancer and must be monitored to prevent further progression [15]. LCIS is normally not detected through a mammogram, but instead is found from a biopsy of the breast [15].

The two invasive types of breast cancers (although not limited to these) are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) [13]. Invasive ductal carcinoma, or infiltrating ductal carcinoma, is defined as cancer cells that originate from the ducts and has spread to adjacent breast tissues. IDC is the most common type of breast cancer diagnosed in women with approximately 80 percent of all breast cancers diagnosed as IDCs [16]. Invasive lobular carcinoma, or infiltrating lobular carcinoma, is a type of breast cancer that begins in the

lobules, where mammary milk is produced, and spreads to nearby breast tissue [17]. ILC is ranked as the second most common type of breast cancer behind IDC with an estimated 10% of invasive breast cancers being ILCs [17]. Another type of breast cancer is metastatic breast cancer, also known as stage IV breast cancer [18]. Metastatic breast cancer is known to be the most serious type of breast cancer with tumor cells having spread to other parts or organs of the body [18].

Breast cancer can also be classified into several molecular subtypes. Breast cancer is considered to be a heterogenous disease at the molecular, pathological, and clinical levels [19]. Although classification of breast cancer is complex and debated, most studies have identified five molecular subtypes of breast cancer which include luminal-A, luminal-B, HER2-enriched, triple-negative/basal-like, and claudin-low breast cancers [19-23]. Luminal-like breast cancers are characterized by its expression of estrogen (ER) and progesterone (PR) receptors and genes that encode proteins typically expressed by luminal epithelial cells [21]. Luminal-A is the most common subtype and accounts for approximately 50 to 60 percent of all breast cancers [21]. Luminal-A breast cancers is defined as being positive for either the ER receptor or PR receptor or both, negative for HER2 (human epidermal growth factor-2) receptor, and low levels of Ki-67 protein, which is a nuclear protein involved in cell proliferation [21,23,24]. Luminal-A breast cancer have the best prognosis out of all the subtypes of breast cancers since hormonal treatment can be used to target the ER or PR receptors [21,25,26]. Luminal-B breast cancers is characterized as either ER-positive, HER2-negative, and having high levels of Ki-67 or ER- and HER2-positive and high levels of Ki-67 [21,23]. Luminal-B breast cancers has a higher proliferative index, worse prognosis, and higher occurrence of relapse than luminal-A due to an upregulation of proliferation-related genes [21,27-29].

HER-2 enriched breast cancer is ER-negative and PR-negative, but positive for HER2 receptor and makes up for 15 to 20 percent of all breast cancer subtypes [21,23]. Patients with HER-2 enriched breast cancer have a poorer prognosis with resistance in response to typical hormonal treatment; hence, standard treatment for these patients include chemotherapy drugs such as doxorubicin [21]. Basal-like breast cancers are known to express high levels of basal myoepithelial markers, such as CK5, CK14, CK17, and laminin, while having a triple-negative phenotype which refers to the absence of ER, PR, and HER2 receptors [21,23]. Prognosis for basal-like breast cancers tends to be very poor with studies showing a high occurrence of metastasis to the brain and lungs [21,30]. It is important to note that triple-negative breast cancer and basal-like breast cancer are not the same in terms of how each subtype are defined. Triple-negative breast cancer is defined as breast cancer cells that do not express ER, PR, and HER2 receptor based on immunohistochemical studies [21,31]. On the other hand, basal-like breast cancer is defined based on gene expression microarray analysis [21,31]. Claudin-low breast cancer was identified by gene expression profiling and known to display a triple-negative phenotype [22]. Claudin-low subtype is characterized by the low expression of genes that contribute to the structure of tight junctions and epithelial cell to cell adhesion [22]. Claudin-low refers to the low expression of claudin proteins 3, 4 and 7, occludin, E-cadherin, and luminal epithelial genes, but high expression of genes that correspond to the epithelial to mesenchymal transition (EMT), immune cell infiltration, breast stem cells, and breast tumor initiating cells [20,22,32-35]. Much like basal-like breast cancer and triple-negative breast cancer, claudin-low breast cancer has a poor prognosis with chemotherapy as a standard treatment option [22].

Among all the subtypes of breast cancers, triple-negative breast cancer (TNBC) is recognized as the most invasive subtype of breast cancer due to the lack of expression of the ER,

PR, and HER2 receptors. Due to the absence of these three receptors, TNBCs have limited response to hormonal therapies, chemotherapy, and trastuzumab [2,36,37]. Triple-negative breast cancer accounts for approximately 15 to 20 percent of all breast cancer cases and has the poorest prognosis out of all subtypes of breast cancer [37,38]. TNBC is a heterogenous disease at the molecular, pathologic, and clinical level which increases the difficulty of treating TNBC [39,40].

There are a range of treatments used to treat breast cancer. Depending on the subtype of breast cancer, treatments can include one or a combination of localized surgery, radiation therapy, neoadjuvant or adjuvant chemotherapy, hormonal therapy, targeted therapy, checkpoint inhibitors, and immunotherapy [40]. For hormone-receptor positive breast cancer cells such as luminal-A breast cancer, common treatments can include aromatase inhibitors, fulvestrant, ER receptor blockers such as tamoxifen, Herceptin, and kinase inhibitors [40,41]. Aromatase inhibitors, such as letrozole, anastrozole, and exemestane, degrades aromatase to inhibit production of estrogen [40,41]. Aromatase is an enzyme that converts androstenedione to estrone and testosterone to estradiol [42,43]. Fulvestrant inhibits the dimerization of estrogen and further downregulates estrogen receptors [40,41]. Tamoxifen is one estrogen receptor blocker that prevents binding of estrogen to membrane-bound estrogen receptors [40,41]. Herceptin is a monoclonal antibody that blocks epidermal growth factor from binding to the HER2 receptor [40,41]. Kinase inhibitors, such as lapatinib, prevents phosphorylation of the HER2 receptor domain that would have induced the phosphoinositide 3-kinase (PI3K/AKT/mTOR) pathway which would have accelerated the growth of breast cancer cells [40,41]. In addition to endocrine therapies and drugs that inhibit estrogen production, chemotherapy can be used either before surgery or after surgery [40]. Examples of chemotherapeutic drugs include anthracyclines such

as doxorubicin, taxanes such as docetaxel, and platinum agents such as cisplatin and carboplatin [33].

As conventional treatments are used in clinical practice to treat breast cancer patients, there are new revelations in developing experimental therapeutics. Immunotherapy has contributed to revolutionizing cancer treatments and has now given patients another option to consider. Different types of immunotherapy treatments developed include immune checkpoint inhibitors, monoclonal antibodies, chimeric antigen receptor T cells, and manipulating chemokines and cytokines within tumor microenvironments [3,44]. For difficult-to-treat cancers such as triple-negative breast cancer, immunotherapy can introduce effective alternative options that could regress tumor growth and inhibit metastasis. Immunotherapy can also be used as an alternative for chemotherapy and radiation therapy where side effects are numerous and adverse. There are still ongoing studies on determining the efficacy, bioavailability, and safety of experimental immunotherapeutics, but clinical trials have shown promise for all types of cancers especially breast cancer.

1.2 Triple-Negative Breast Cancer

Triple-negative breast cancer (TNBC) accounts for approximately 12 to 20 percent of all breast cancer cases [37,38]. Triple-negative breast cancer is defined as tumors that have an absence of expression of both endocrine receptors, estrogen (ER) and progesterone (PR), and human epidermal growth factor-2 receptor (HER2) [36]. As these tumors lack these three receptors, treatments targeting any of these three receptors is ineffective and limited such as trastuzumab targeting HER2, tamoxifen, and aromatase inhibitors [2]. Chemotherapy has been the standard treatment for TNBC with taxanes and anthracyclines being the most common treatments [36]. TNBC has been identified and diagnosed based on assessment of ER, PR, and

HER2 protein expression levels by immunohistochemistry and/or fluorescence in situ hybridization [36]. It has been revealed that TNBC has a high level of molecular heterogeneity which contributes to the difficulty of treating this disease [36,45]. Although chemotherapy is commonly used for treatment, patients with TNBC have the poorest prognosis among patients of all subtypes of breast cancer [37,38]. There is an increasing need to better understand TNBC at the molecular, clinical, and pathological level in order to enhance or introduce new treatment options for patients with this aggressive form of breast cancer.

Basal-like breast cancer is a subtype characterized by low or absence of expression of ER-, PR-, and HER2-related genes [2]. Basal-like breast cancer is not synonymous with triple-negative breast cancer even though both types of cancers have a 'triple-negative' phenotype [2]. Basal-like breast cancer had been characterized based on gene expression array profiling that enables categorization of subtypes of breast cancer according to intrinsic gene expression patterns [2,46-50]. Genetic profiling of basal-like breast cancer cells demonstrated that epidermal growth factor receptor HER1, cytokeratin 5, 14, 17, vimentin, p-cadherin, fascin, caveolins 1 and 2, and myoepithelial markers (such as receptors, hormones, and proteins) smooth muscle actin, p63, and CD10 (neprilysin) are expressed [2,46,48,51]. Triple-negative breast cancer was defined and characterized based upon immunohistochemistry where basal-like breast tumors do not show expression of ER, PR, and HER2 receptors [2]. In other words, triple-negative breast cancer is defined based on clinical assays and is encompassed under the 'basal-like' breast cancer intrinsic subtype [2].

Majority of TNBCs diagnosed are classified as invasive ductal carcinomas [36,52]. Histopathological analysis of breast cancer uses two tumor intrinsic characteristics which are histological tumor grade and tumor type [52-54]. Histological tumor grade is defined as the stage

of differentiation based on tubule formation and nuclear pleomorphism and status of proliferation calculated based on mitotic index [52,53]. In other words, histological tumor grade is correlated with tumor aggressiveness [52,53]. TNBC typically has a higher histological tumor grade and higher mitotic count than other subtypes of breast cancer [2]. TNBC is highly heterogenous at the molecular level, but there is not one specific classification system that completely describes the molecular landscape of TNBC [36].

One classification developed by Lehmann *et al*. has further categorized TNBC into six subtypes based on genetic expression profiling [36,55]. Lehmann *et al*. classified TNBC into two basal-like subtypes (BL1 and BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR) subgroups [36,55]. Each of these subtypes are characterized based on which genes are highly enriched and its respective function [56]. BL1 TNBCs have highly enriched expression of genes related to cell division pathway and DNA damage response [55,56]. BL2 TNBCs contain highly expressed genes related to growth factor signaling such as epidermal growth factor, nerve growth factor, hepatocyte growth factor receptor, and insulin-like growth factor 1 receptor pathways [55,56]. In addition, the BL2 subtype is also associated with glycolysis and gluconeogenesis pathways and expression of myoepithelial markers [55,56]. IM TNBCs contain high expression of genes that are related to the immune cell processes [55,56]. These immune processes include signaling pathways such as T_H1 and T_H2 pathways, NK cell pathways, B cell receptor signaling pathways, cytokine signaling, antigen processing and presentation, and immune signal transduction signaling (NFκB, TNF, and JAK/STAT) [55]. M TNBCs have enriched levels of gene expression associated with cell motility, extracellular matrix receptor interaction, and cell differentiation pathways such as Wnt and TGF-β signaling [55]. MSL TNBCs include both elevated expression

of genes related to cell motility and genes associated with stem cells, HOX genes, and mesenchymal stem cell-specific markers [55]. The LAR subtype has gene ontologies that are heavily enriched in hormonal pathways such as steroid synthesis and androgen receptor signaling [55,57]. This study serves as one example of the many different classifications of TNBC. The importance of this specific classification by Lehmann *et al*. is that biomarkers can be identified in TNBC that will allow selection of patients in clinical trials for therapies targeting these biomarkers [55]. In addition, this gene ontology analysis allows development of therapies that can target certain signaling pathways or proteins [55].

Majority of TNBCs display a common set of mutations that contributes to its genomic instability [56]. Studies such as Cancer Genome Atlas have demonstrated that TNBCs has 1.68 somatic mutations per Mb of coding regions which translates to approximately 60 somatic mutations in each tumor [36,56,58]. It has been shown that there is a frequent expression of multiple copy-number aberrations in genes related to signaling pathways ranging from DNA repair pathways as a result of *BRCA1* and *BRCA2* mutations or deletions to cell-cycle checkpoints [36,56,58]. The most frequent mutation in TNBCs occurs in the *TP53* gene which comprises approximately 60 to 70 percent of mutations in TNBCs [36,45,58]. Mutations in the *TP53* gene leads to the loss of function for the tumor suppressor protein 53 and, hence, favors uncontrolled cell division and tumor growth [36,56]. The second most frequent mutation in TNBCs occurs in the *PIK3CA* gene in form of gene amplification [36,59]. *PIK3CA* is responsible for encoding the p110α catalytic subunit of the phosphatidylinositol-3 kinase (PI3K) [59]. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate in order to form phosphatidylinositol-3,4,5-triphosphate (PIP_3) which then activates protein kinase B (AKT) [60]. AKT is known to play a major role in cell survival, the cell cycle, and metabolism [61]. Activation of AKT

promotes cell survival and inhibits apoptosis by phosphorylating BAD, which is a pro-apoptotic protein of the Bcl-2 family [61]. The phosphorylation of BAD leads to a loss of pro-apoptotic function and allows AKT to further activate subsequent pathways that activates genes that help with cell survival [61]. Thus, the mutation in *PIK3CA* allows formation of PIP_3 which inhibit apoptosis and promote cell growth and proliferation enhancing tumorigenesis [59-61].

BRCA1 and *BRCA2* germline mutations occur in an estimated 10 percent of patients with TNBC [45,58,62]. *BRCA1* contributes to the role of DNA repair, maintaining genomic stability, and sending DNA damage signals [36,63]. *BRCA2* plays a role in conjunction with accessory protein RAD51 in repairing double stranded breaks in DNA by homologous recombination [36,63]. Germline mutations in tumor suppressor genes *BRCA1* and *BRCA2* in addition to mutations to *ATM* (encodes a serine/threonine kinase that is recruited to DNA double-strand breaks) and *TP53* contributes to tumorigenesis of TNBCs [36].

Since TNBC lacks the expression of ER, PR, and HER2 receptors, hormonal treatments have been ineffective in treating this subtype of breast cancer. Chemotherapy remains the standard treatment for TNBC showing benefit for patients if given neoadjuvant, adjuvant, or in metastatic situations with taxanes and anthracyclines commonly given [36,64,65]. Interestingly, patients with TNBC have higher success in responding to chemotherapy in their first treatment than other types of breast cancers [36]. Studies conducted by Liedtke *et al*. compared the response and survival of patients diagnosed with TNBC versus those with non-TNBC when given neoadjuvant chemotherapy [66]. Liedtke *et al*. have found that patients with TNBC treated with neoadjuvant chemotherapy have higher pathologic complete response (pCR) than patients with non-TNBC and similar survival probability (24% chance of survival) as those with non-TNBC [66]. However, this finding is only limited to patients with TNBC who have achieved

pCR meaning that there are no residual cancer cells in the tissue samples after treatment [66]. Otherwise, patients with residual disease after neoadjuvant chemotherapy, such as those with metastatic TNBC, have worse overall survival than those with non-TNBC within the first 3 years of treatment [66].

This type of observation has been referred to as the 'TNBC paradox' where patients with TNBC show a higher response in their first treatment to chemotherapy, but in recurrence show poor response to treatment [67]. This paradox can be explained by TNBCs developing resistance to chemotherapy through various mechanisms and predisposition factors such as genes that favor chemoresistance, an accumulation of mutations from the first treatment, or DNA repair pathways and checkpoint pathways that are dysfunctional and lead to tumor growth and proliferation [36]. Regarding concerns with chemoresistance, there has been other therapies in development such as platinum agents, PARP inhibitors, anti-androgen therapy, PI3K inhibitors, MEK inhibitors, and immunotherapies such as monoclonal antibodies targeting ligands and programmed cell death 1 ligand (PD-L1) [36]. With TNBC associated with poor prognosis, higher chance of relapse, and development to chemoresistance, there is a great need to develop innovate therapies and strategies for patients with this serious type of breast cancer.

1.3 Principles of Immunotherapy

Immunotherapy has revolutionized cancer treatment and has expanded options for patients with a number of cancers to utilize. Immunotherapy has been shown to have greater efficacy and improvements over time in treatment of cancers with novel therapies including immune checkpoint inhibitors, monoclonal antibodies, chimeric antigen receptor T cells, and manipulating chemokines and cytokines within the tumor microenvironment or intratumor

environment [3,68]. Chemotherapy and radiation therapy has long been the conventional treatments for cancer and have proven a certain degree of success [3].

Despite the overall positive responses from these two standard treatments, tumor cells do eventually develop resistance to these treatments and patients undergo relapse even after treatment [3,4]. Resistance to chemotherapy can either be intrinsic or acquired [4]. Intrinsic resistance refers to tumor cells that have predisposition factors, such as oncogenes and evasion mechanisms, that was not developed as a result of treatment [4]. Acquired resistance is defined as tumor cells that accumulate mutations or develop mechanisms such as a dysfunctional DNA repair system or checkpoints in the cell cycle that result after initial treatment [4,69]. To add to the complexity of relapse, recurrence usually occurs due to metastasis [4]. Since the effectiveness of chemotherapy is limited after initial treatment and notable adverse side effects is of major concern, there is a need to counter this resistance through novel therapies. Immunotherapy is unique from conventional targeted treatments due to provoking a systemic immune response against cancer cells [3]. The major advantage is that tumor cells throughout a patient's body can be targeted, but the limitations include off-target effects of targeting healthy cells, creating a harmful immune overresponse, or the immune system may get compromised [3]. Nevertheless, immunotherapy does provide advantages, but there is a need to focus greater attention on targeting tumor cells while sparing healthy cells in tissues being treated.

The immune system consists of mechanisms that allows effector cells to: (1) recognize target cells as either its own body's healthy cells, infected cells, dead cells, or tumor cells and (2) develop and trigger mechanisms that eradicate infected, dead, or tumor cells while sparing the healthy cells [5]. Specifically, the process of understanding how the immune system recognizes and eradicates tumor cells has been debated and initially not fully developed. Among the first

findings was that interferon-γ (IFN-γ) plays a role in tumor immunosurveillance [70,71]. Dighe *et al*. had demonstrated in tumor-mice models that neutralizing antibodies binding to IFN-γ resulted in faster progression and growth of tumors compared to mice without antibodies neutralizing IFN-γ [70,71]. Furthermore, mice deficient in the expression of perforin, defined as proteins released by immune effector cells that targets cells by creating pores in the plasma membrane, had a higher number of spontaneous B cell lymphomas compared to mice expressing perforin [72-74]. Takeda *et al*. had also shown that tumor necrosis factor related apoptosis-inducing ligand (TRAIL) plays a role in inhibiting tumor cells from growing [75].

In addition to observing cytokines and chemokines eradicating tumor cells by quantity, there is an additional element to cancer immunosurveillance which involves decreasing the tumor aggressiveness or quality, otherwise known as immunogenicity [70]. The cancer immunoediting concept is defined as the immune system employing its mechanisms that affects tumor immunogenicity [70,76]. The immunoediting concept plays its part after intrinsic mechanisms of tumor suppression have failed [76]. The immune system could suppress tumor growth at this point by intrinsic tumor suppression mechanisms that involves DNA repair of mutations or apoptosis [76]. However, in the event that tumor cells fail to die through these intrinsic mechanisms, the cancer immunoediting process now gets involved in eradicating tumor cells [76]. The cancer immunoediting process does not only describe how the immune system kills the tumor cells by extrinsic mechanisms such as cytokines, but also entail how tumors develop dormancy and escape immunosurveillance [76]. Cancer immunoediting consists of three stages, which are elimination, equilibrium, and escape stages [70,76].

In the elimination phase, the effector cells of the immune system are involved in recognizing tumor cells based on ligands or tumor associated antigen expression [70,76].

Effector cells that are involved in the elimination process include $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, NKT cells, NK cells, macrophages, and γδ T cells [70,76]. Some to all these effector cells are responsible for recognizing the tumor cells and successfully eliminating these tumor cells before clinical effects occurs [76]. The elimination process requires a coherent collaboration between the adaptive and innate immune systems. Besides effector cells directly attacking tumor cells, other methods of extrinsic tumor suppression include using cytokines such as IFN-γ, IFN-α/β, IL-12, and TNF or proteins such as perforin to directly kill the tumor cells [76]. If tumor cells are not eliminated in this first stage by direct killing, then the adaptive immune system will contribute to the equilibrium phase [76]. In the equilibrium phase, tumor growth will be inhibited by cytokines IL-12 and IFN-γ which will allow the tumor immunogenicity to decrease [70,76]. Even after the equilibrium phase, not all the tumor cells will be eradicated. Some tumor cells that were able to survive through both the elimination and equilibrium phases have several characteristics that allow them to evade immunosurveillance [70,77,78]. Some tumors may no longer be recognized by the immune system due to antigen loss, MHC loss, or inhibitory ligand expression [70,77,78]. Tumors in the escape stage also may have developed resistance to effector cells mechanisms designed to kill the tumor cells in the equilibrium stage [70,77,78]. Another way that tumors can escape immunosurveillance is creating a microenvironment that suppresses effector cells [70,77,78]. Tumors that reach the escape stage in general lead to clinical outcomes seen in cancer patients [70].

With a better understanding of the complex ways that cancer cells develop resistance to chemotherapy and evasion mechanisms from the immune system, novel therapies have been developed and are currently undergoing experimentation in hopes of successful outcomes. In order to suppress tumor growth and prevent metastasis, immunotherapy requires (1) activation of

the immune system by secreting cytokines and chemokines or receptor-ligand interactions, (2) expansion of immune effector cells, (3) directing activated effector cells to the site of tumor growth, and (4) effective eradication of tumor cells [3]. For immunotherapy to be successful in accomplishing the four requirements, there has been much emphasis on understanding the tumor microenvironment [3]. Tumor microenvironments present challenges to the immune system because a cluster of tumors can create an additive inhibitory signal that will prevent immune effector cells from acting on the tumors [3,79]. Tumor microenvironments can also create a barrier that prevents immune cells from entering [3,79].

Immune checkpoints have been targeted for immunotherapy due to an advantage that tumor cells can utilize to prevent being killed by T cells [3]. These checkpoints utilize a cascade of pathways that regulate T cell activity and prevent autoimmune response against healthy cells [3,80]. It has been shown that targeting programmed cell death protein 1 (PD-1) and programmed cell death protein 1 ligand (PD-L1) has positive outcome on eradication of tumor cells [3]. PD-L1 can be upregulated in tumor cells which interacts with PD-1 on T cells allowing inhibition of T cell targeting against tumors [3]. Anti-PD-1 antibodies in combination with anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), another checkpoint molecule expressed by regulatory T cells, has shown the greatest success in clinical trials [3,81-83]. Anti-PD-1 antibodies, known as nivolumab, and anti-CTLA-4 antibodies, known as ipilimumab, have been successful in treating advanced stages of melanoma in clinical trials [81-83]. By blocking CTLA-4 on tumor cells, regulatory T cells will be depleted allowing an increase in cytotoxic T cells [3]. In combination with the anti-PD-1 antibody, the interaction between PD-L1 on tumor cells and PD-1 on cytotoxic T cells will be blocked thus allowing the cytotoxic T cells to kill the tumor cells [3].

CAR T cells has also become another concept of immunotherapy that show promise. CAR T cells are engineered chimeric antigen receptors (CARs) that activates T cells when T cells recognize tumor antigens [3,84,85]. Other concepts in immunotherapy that are being studied include targeting chemokines and cytokines within and around tumor microenvironments, pattern recognition receptor agonists that allow immune cells to be recruit and induce cytokines around tumors, and monoclonal antibodies targeting ligands on tumor cells [3]. The tumor microenvironment serves as an important model to study due to the reliance of immunotherapy to target this environment. While many experimental therapeutics are in still in study, there needs to be a further understanding of the interaction between the tumor microenvironment and immune cells for immunotherapy to be successful [3]. Immunotherapy has shown promise in clinical trials, *in vivo*, and *in vitro*, but there still needs to further studies on the efficacy of these novel therapeutics and minimizing side effects for patients being treated.

1.4 Natural Killer Cell-Based Immunotherapy

Expanding the capability of natural killer (NK) cells in targeting tumor cells has become a novel strategy in tumor immunology since the 1980s (6). Utilizing NK cells to target tumor cells has shown potential in treating different types of cancers such as prostate cancer, glioblastoma, and diffuse B cell lymphoma [6,7,88-90]. NK cells are innate lymphoid cells that contribute to the immune system by controlling microbial infection, suppressing tumor progression, secreting cytokines such as IFN-γ and IL-12 that enhances its own function, and uses its ability to recognize self-healthy cells and eradicate infected, tumor, dysfunctional, and damaged cells [6,88].

NK cells employ its capability of recognizing tumor or infected cells through its NK receptors interacting with ligands or antigens on the surface of target cells [6]. NK cell receptors

interact with molecules such as inhibitory and activating ligands, adhesion receptors, major histocompatibility complex class I receptors (MHC), and cytokine receptors to recognize the target cell [91]. The interaction between NK receptors and its ligands sends a transducing signal that either inhibits or activates the NK cell from inducing lysis of the target cell [6,7,91]. A self-healthy cell presents a 'self-peptide' on its MHC I molecule and interacts with NK inhibitory receptors allowing the healthy cell to be spared by NK cells [6]. In contrast, a cell missing a self-antigen on its MHC or missing the MHC complex altogether allows the NK cell to recognize the 'non-self' cell and induce lysis [6]. NK cell function is regulated by a net balance of inhibitory and activating ligands on target cells interacting with NK cell receptors (Figure 1.1) [6,7,91].

When there is a net sum of activating ligands interacting with NK receptors more than inhibitory ligands interacting with NK receptors, then NK cell function will be activated. Likewise, if there is a net sum of inhibitory ligands interacting with NK receptors, then NK cell function will be inhibited, and no lysis of the target cell will occur. Target cells that are abnormal, distressed, or damaged can induce NK cell activation by either losing or missing a 'self-antigen' or MHC I receptors or upregulating proteins that bind to activating NK receptors for apoptosis [6]. Cells that are damaged or dysfunctional can release damage-associated molecular pattern molecules (DAMPs) that will signal effector cells to eradicate the affected cell [5]. Killer immunoglobulin-like receptors (KIRs) and CD94/NKG2A are two inhibitory receptors for human leukocyte antigen class I molecules (HLA I) and both receptors utilize the immunoreceptor tyrosine-based inhibition motifs (ITIMs) for transduction [92]. When a HLA I antigen binds to KIRs or CD94/NKG2A, ITIMs that were tyrosine-phosphorylated will recruit tyrosine phosphatases that will dephosphorylate adaptor molecules attached to the ITIM tail [92]. This dephosphorylation at these tyrosine sites will inhibit transduction for NK activation [92].

In contrast, NK activation occurs when its activating receptors interact with specific ligands on target cells. Natural cytotoxicity receptors (NCRs) are a family of activating receptors expressed by NK cells that recognize ligands on target cells [6]. Within the NCR family, activating receptors include NKp30, NKp44, and NKp46. Other well-known activating NK receptors also include NKG2D (CD314, KLRK1), DNAM1 (CD226), and CD16 (also known as FcγRIIIA, which recognizes the Fc region of IgG antibodies) [6]. The common characteristic of these NK activating receptors is that they have cytoplasmic immunoreceptor tyrosine-based activation motif tails (ITAMs) [6]. Protein tyrosine kinases of the Syk family (Syk and ZAP70) phosphorylate ITAMs at tyrosine residues which then induces adaptor proteins such as SH2 and SH3 to bind to the ITAMs and continue the signaling cascade which activates the NK cell function [6,93]. In summary, activation and inhibition NK signaling depends on the interaction of its NK receptors interacting with ligands on target cells (Figure 1.2).

Another mechanism that natural killer cells utilize is antibody-dependent cellular cytotoxicity (ADCC). CD16, or also known as FcγRIIIA receptor, recognizes the Fc region of IgG antibodies and is responsible for ADCC [6]. When CD16 on natural killer cells binds with the FcRγ chains attached to a ligand on a target cell, activation of the natural killer cell occurs due to a signaling cascade involving phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tail by Src-family kinases [94]. This signaling cascade induces natural killer cell cytotoxicity and allows lysis of the target tumor cell. This signaling pathway is one of few pathways that contribute to the activation of natural killer cells. Natural killer cell cytotoxicity against tumor cells is a tightly regulated process. The interaction between natural killer cell receptors and ligands on the tumor cells that induces cytotoxicity of natural killer cells requires activation of intracellular signaling pathways. The activation of the signaling pathways

within the natural killer cells results in polarization and granule release towards the tumor cell [94].

Developing immune targeted therapies by stimulating NK cells to lyse TNBCs with monoclonal antibodies or upregulating stimulatory cytokines could open a new avenue to treating TNBC [8]. Fang *et al*. has noted that the primary approaches used for NK-based immunotherapy involve antibodies, cytokines, and adoptive transfer of *ex vivo* NK cells that will either increase the number of tumor cells being eradicated by NK cells, improve specific target of tumor cells instead of healthy cells, induce activation of NK cells, and maintain persistency in prolonging NK response in order to effectively decrease tumor cell count [8]. Cytokines can improve the NK cell quantity against a tumor population and increase NK function to decrease tumor immunogenicity [8]. Among the most popular cytokines used is interferon-2 (IL-2). IL-2 was the first cytokine that was approved for use in patients but has its limitations [95]. IL-2 allows expansion and activation of NK cells, which are known as lymphokine-activated killer cells (LAK) [8]. The limitation to using IL-2 is that this interferon also activates T cells especially regulatory T cells, which is known to suppress immune effector cells [8]. It has also been observed that increasing IL-2 treatment causes damage to organs and increased vascular endothelium [95-97]. Another cytokine of use is IL-15. It has been shown that IL-15 contributes to enhancing cytotoxicity of NK cells [95]. The advantage that IL-15 has over IL-2 is that IL-15 does not expand regulatory T cell numbers and favors expansion of NK cells and CD8+ T cells with less side effects such as lower capillary leakage as reported [8,98,99]. In addition to IL-2 and IL-15, IL-12 was also used to stimulate NK cells while also enhancing CD8+ T cells [8,100].

Another set of NK-based immunotherapies in development are antibodies [8]. Antibodies provide a major advantage in ways where ADCC can be utilized, blocking interaction of

inhibitory ligands increases NK response, and blocking proteins that favors tumorigenesis or growth [8]. The use of antibodies has come into play in treating ovarian cancer [101,102]. Several tumor-associated antigens were identified on ovarian cancer including NY-ESO-1, CA 125, MUC1, and epithelial cell adhesion molecule (EpCAM) [101,102]. These particular antigens were targeted with monoclonal antibodies which allowed activation of NK cells through the ADCC mechanism [101]. Monoclonal antibodies were also used in blocking interactions between ligands and NK receptors. Mathew *et al*. had demonstrated that Lectin-like Transcript-1 (LLT1) ligand was overexpressed in prostate cancer [88]. They had shown that blocking LLT1 inhibitory interaction with NK receptor CD161 (NKRP1A) with monoclonal antibodies had enhanced lysis of prostate cancer cells by NK cells [88]. Antibodies can target immune checkpoints, which serve as signaling pathways that prevents immune effector cells from targeting self-tissue [101]. There are antibodies have been developed to target checkpoint pathways such as CTLA-4, PD-1, NKG2A/CD94 complex, and CD96 receptors [96,101,103].

In addition, adoptive transfer of NK cells has been explored. This strategy presents a major challenge as already shown in clinical trials with patients who have undergone chemotherapy [8]. NK cells can be derived from peripheral blood mononuclear cells (PBMCs) and treated with cytokines in medium to amplify the population and increase effector function [8]. Interestingly, Lee *et al*. demonstrated that large-scale expansion of purified NK cells with increased antitumor function was derived from irradiated PBMCs [104]. They have shown that irradiating PBMCs with a radiation dose of 25 Gy inactivates T cells and upregulates NKG2D ligands and CD48 ligands [104]. By using a combination of anti-CD16 monoclonal antibodies with the irradiated PBMCs, there was nearly a 6,000-fold expansion in the NK cell population 21 days after PBMC irradiation [104]. They have also shown that NK cell activating receptors, 2B4,

NKp30, NKp44, NKp48, NKG2D, and DNAM-1, were upregulated when treated with anti-CD16 monoclonal antibodies [104]. This study took it one step further where these purified NK cells were transferred into tumor mice models [104]. Lee *et al*. observed that these expanded NK cells have higher antitumor activity such as increased expression of CD107a (a degranulation marker) and higher secretion of IFN-γ against lung and colon cancer xenografts in NOD/SCID mice than NK cells not derived from irradiated PBMCs [104]. Although adoptive transfer of NK cells has shown some prospect in xenograft mice models, yielding a large number of NK cells is challenging for transfer into humans [8]. In conclusion, NK-based immunotherapy has shown some promise as another option for patients. There still needs to be further studies on the efficacy of this particular novel therapy, but more important is how this type of experimental treatment can effectively translate from xenograft models and cell lines to clinical patient models.

1.5 Lectin-like Transcript-1 (LLT1) and NKRP1A Interaction

One particular ligand of interest for NK cell targeting on tumor cells is Lectin-like Transcript-1 (LLT1, CLEC2D, OCIL). LLT1 is part of the C-type lectin-like receptor superfamily which is encoded by CLEC2D genes within the human natural killer gene complex [105]. LLT1 has five alternative spliced variants (excluding isoform 3 which is a RNA decay product) of the CLEC2D gene with isoform 1 identified to encode for cell surface LLT1 interacting with NKRP1A (CD161) receptor [106,107]. LLT1 is expressed on lymphocytes such as B cells, NK cells, and T cells, and activated dendritic cells [106,107]. Interestingly, Llibre *et al*. reported high expression of LLT1 on human germinal center B cells, early plasmablasts, and germinal center-derived lymphomas [105]. The same study has also reported that LLT1 expression is also found on follicular dendritic cells and that upregulation of LLT1 promotes B

cell activation [108]. Crystallography has revealed that LLT1 forms a homodimer at its cell surface which enables LLT1 to serve as a ligand for the NKRP1A receptor on NK cells [105,108-110].

Natural killer cell receptor NKRP1A is encoded by a single gene KLRB1 and is expressed on NK cells, $CD4^+$ and $CD8^+$ T cells, invariant NKT cells, $\gamma\delta$-TCR^+ T cells, and a subset of $CD3^+$ thymocytes [107,111]. Studies have shown that NKRP1A expression contribute to the role of differentiation of lymphocytes [112]. NKRP1A expression was detected on dendritic cells during monocyte differentiation from both the bone marrow and precursors in the thymus [112]. It has been demonstrated by Poggi *et al*. that antigens binding to NKRP1A leads to an increase in production of interleukins IL-1β and IL-12 by non-activated monocytes and dendritic cells [112]. This induced production of IL-12 leads to an upregulation of NKRP1A expression in human NK cells which can contribute to the role in regulating NK cell activation [112,113].

LLT1 on target cells interacting with natural killer cell receptor NKRP1A leads to inhibition of NK-cell mediated cytolytic targeting (Figure 1.3) [110]. The role of interaction between LLT1 and NKRP1A in modulating immune responses was observed when upregulation of LLT1 was induced by pathogens and expression of NKRP1A was found on NK, T_H1, and T_H17 cells [114]. Furthermore, overexpression of LLT1 was observed on prostate cancer cells and leads to inhibition of NK-cell mediated cytolytic killing against these prostate cancer cells [6].

1.6 Proliferating Cell Nuclear Antigen (PCNA) and NKp44 Interaction

Proliferating cell nuclear antigen (PCNA) is a well-studied accessory protein that contributes to the role of DNA repair mechanism, DNA replication, cell cycle regulation, and

chromatin remodeling [115]. PCNA plays a key role in DNA replication as a nuclear protein associated with the progression of the replication forks [116]. To initiate DNA synthesis, DNA polymerase α-primase serves as a priming enzyme that synthesizes a short RNA primer of 10 nucleotides for further DNA synthesis [116]. The recruitment and function of polymerase α-primase does not need PCNA, but it is in the preceding steps of DNA synthesis where PCNA will begin to play an important role [116].

When priming of the DNA is complete, PCNA as an accessory protein assists in the recruitment and exchange of polymerases δ and ε at the replication forks [116]. Upon exchange, polymerase δ is involved in discontinuous synthesis of the lagging strand while polymerase ε is involved in the continuous synthesis of the leading strand [116,117]. PCNA with assistance of helper proteins at the replication fork enables PCNA to be clamped around DNA by replication factor C protein complex [116]. It is the combination of replication factor C protein complex and PCNA that facilitates the exchange of polymerases α with δ and ε and further allow DNA synthesis to continue [116]. PCNA is also involved in recruiting and facilitating enzymatic reactions that ligate Okazaki fragments [116]. PCNA interacts with DNA ligase I and induces a conformation change that allows the ligase to seal the Okazaki fragments [116].

In addition to DNA replication, PCNA contributes its role in DNA repair through several repair pathways including base excision repair, mismatch repair, and nucleotide excision repair [116]. PCNA is also involved in chromatin assembly and maintenance [116]. It has been studied that one of the chromatin remodeling factors CAF1 (chromatin assembly factor 1) interacts with PCNA which could lead one to suggest that chromatin remodeling may be coupled with DNA replication [116,118,119]. Furthermore, PCNA function is regulated by cell-cycle regulators such as p21 [116]. Cell-cycle regulator p21 interacts with PCNA in order to inhibit DNA

replication process [116]. Part of the cell-cycle regulation involves inducing apoptosis where PCNA plays another role [116]. PCNA interacts with proteins ING1b (inhibitor of growth 1b) and suppresses anti-apoptotic proteins in order to induce apoptosis [116]. PCNA serves as an important protein in DNA replication, synthesis, and cell cycle regulation, but it can also serve as a biomarker for cancer.

PCNA is known to be overexpressed in certain cancers and has been well documented in various studies that PCNA enhances cancer cell survival and proliferation [116,120,121]. Naryzhny *et al*. demonstrated that three breast cancer cell lines (MDA-MB-468, MDA-MB-231, and MCF7) showed higher expression of PCNA than non-tumorigenic breast cell lines HMEC and MCF10A by SDS-PAGE/western blot [120]. It is important to note that Naryzhny *et al*. emphasized and demonstrated that there is no cancer-associated form of PCNA and non-tumorigenic form of PCNA separately [120]. There is one form of PCNA that is expressed on both cancer cells and non-tumorigenic cells; in other words, there is no difference in post-translational modification of PCNA between cancer cells and non-tumorigenic cells [120]. It was demonstrated that the overall PCNA expression levels were higher in cancer cells than non-tumorigenic cells [120].

Rosental *et al*. showed that PCNA expressed on the cell surface of tumor cells ranging from pancreatic, breast, melanoma, lymphoma, and glioblastoma cells interacts with natural killer cell receptor NKp44 [121]. NKp44 is part of the natural cytotoxicity receptors (NCRs) expressed by activated NK cells and induces cytotoxic lysis of tumor and virally infected cells with its DAP12-binding domain in the NKp44 complex [122-124]. NKp44 binds to ligands PCNA, NKp44L, and viral envelope glycoproteins [6,121,122]. PCNA serves as an inhibitory ligand that interacts with NKp44 on NK cells and sends an inhibitory signal through the

immunoreceptor tyrosine-based inhibitory motif (ITIM) on NKp44 [90,121]. It has been shown that nuclear and cytoplasmic PCNA protein in the target cells gets trafficked to the NK immunological synapse when NKp44 is expressed at the surface of NK cells [121]. Furthermore, Horton *et al*. showed that PCNA colocalizes with human leukocyte antigen I (HLA I) showing association of PCNA and HLA I which results in inhibition of NK cell function when PCNA interacts with NKp44 [90]. PCNA has several functions in relation with DNA replication, chromatin remodeling, DNA synthesis, and cell cycle regulation, but PCNA interaction with NKp44 also inhibits NK cell activity allowing tumor cells to evade NK killing.

1.7 Hypothesis and Specific Aims

Hypothesis

LLT1 and PCNA are expressed on triple-negative breast cancer cells (TNBCs) and allow TNBC cells to evade recognition and cytolytic targeting by natural killer cells. Blocking LLT1-NKRP1A or PCNA-NKp44 interactions enhances killing of TNBCs by NK cells.

Specific Aims

This hypothesis will be tested under the following specific aims.

Specific Aim 1:

Investigate the expression of LLT1 and PCNA on TNBCs.

Specific Aim 2:

Investigate the functional outcome of blocking the interactions of LLT1 and PCNA on TNBCs with NKRP1A and NKp44 on NK cells to target TNBC by primary natural killer cells.

Sub Aim 2.1: Blocking LLT1-NKRP1A or PCNA-NKp44 interaction with antibodies enhances killing.

Sub Aim 2.2: Blocking LLT1-NKRP1A interaction with siRNA enhances killing of TNBCs.

1.8 Significance

The absence of the estrogen (ER), progesterone (PR), and human epidermal growth factor-2 (HER2) receptors on triple-negative breast cancer (TNBC) cells makes it difficult to effectively utilize hormonal therapies. TNBC represents an estimated 15 to 20 percent of all breast cancer cases [125]. Patients diagnosed with TNBC have poorer prognosis, higher chance of relapse, and increased chance of metastasis. Among the subtypes of breast cancers, TNBC is recognized as the most invasive subtype of breast cancer and is the most difficult type of breast cancer to treat. For breast cancer cells that are positive for ER and/or PR receptors, the expression and presence of ER and PR receptors allow estrogen or progesterone to bind to these receptors and begin a cascade of cell signaling pathways that contribute to cell division and growth [37]. Patients with breast cancer that are positive for ER and PR receptors have responded more effectively to hormonal therapies such as tamoxifen, fulvestrant, and aromatase inhibitors such as anastrozole, letrozole, and exemestane. These specific treatments are aimed to either suppress estrogen or progesterone production levels, modify ER and PR receptors, or block these receptors [40]. The limitation is that hormonal therapies work effectively for patients with hormone-receptor positive breast cancer cells. In the case of patients with triple-negative breast cancer, these hormonal treatments are not successful at inhibiting the proliferation and growth of these breast tumor cells and metastasis due to the lack of these three receptors to target with hormonal therapies.

Standard treatment for TNBC includes both radiation therapy and chemotherapy. Chemotherapy initially had been successful for patients that are diagnosed with TNBC for the first time; however, studies have shown that TNBCs develop resistance to chemotherapy and relapse occurs after surgery [3,4]. TNBC resistance to chemotherapy is attributed to its

chromosomal instability due to prevalent chromosome 5q deletions [126]. Chromosome 5q deletions are associated with deregulation of signaling molecules, transcription factors, and cell division genes [126]. It has been demonstrated that this chromosomal instability is associated with poor outcomes in patients with lung, colon, and breast cancer driven by intratumor heterogeneity [126,127]. Chromosomal instability in intratumor heterogeneity for TNBCs increases the chance that genes favoring drug-resistance can accumulate in genomes of arising tumors and could lead to acquired drug resistance during initial chemotherapy treatment [127,128]. It has been reported in studies that TNBCs are more sensitive to chemotherapy treatment than breast cancer cells of other subtypes [40,66,67,129]. Even with TNBC's heightened sensitivity to chemotherapy, chemotherapy presents adverse side effects from physical and immune complications to long-term cognitive impairment [37,40,130-134]. Considering the low success of hormonal therapies and many prevalent side effects from chemotherapy, immunotherapy has been explored as an alternative attractive treatment for patients diagnosed with TNBC.

To overcome limitations of chemotherapy and hormonal treatment, targeting ligands such as LLT1 and PCNA on TNBCs that send inhibitory signals to natural killer cells may contribute to improving therapeutic options. This study is the first that looks at LLT1 expression specifically on triple-negative breast cancer cells. Although PCNA has already been shown to be expressed on triple-negative breast cancer cell lines, this specific study introduces targeting cell-surface PCNA on TBNCs with monoclonal antibodies which would favor activation of NK cells. This study is significant in a way that we are exploiting the expression of inhibitory ligands LLT1 and PCNA from an immunotherapeutic perspective. This project has shown that targeting LLT1 and PCNA with monoclonal antibodies introduces novel targets that will further enhance

immunotherapy treatments. We have also performed combinational antibody treatments by simultaneously targeting all combinations of LLT1, PCNA, and HLA with antibodies which showed synergy and enhanced killing of TNBCs by NK cells *in vitro*. Although there has been some promise *in vitro*, it is necessary to take the next step of targeting LLT1 and PCNA on TNBCs *in vivo* models to see if these results can be replicated or further enhanced in these models. Targeting LLT1 and PCNA with monoclonal antibodies will present another promising option for patients diagnosed with triple-negative breast cancer.

Figure 1.1

NK receptors and ligand interactions dictates net inhibition or activation of NK cells.

NK cell function is regulated by a net balance of inhibitory and activating signals being sent to the natural killer cell. This intricate balance depends on the number of and strength of transducing signals from activating and inhibitory ligands interacting with its NK receptors.

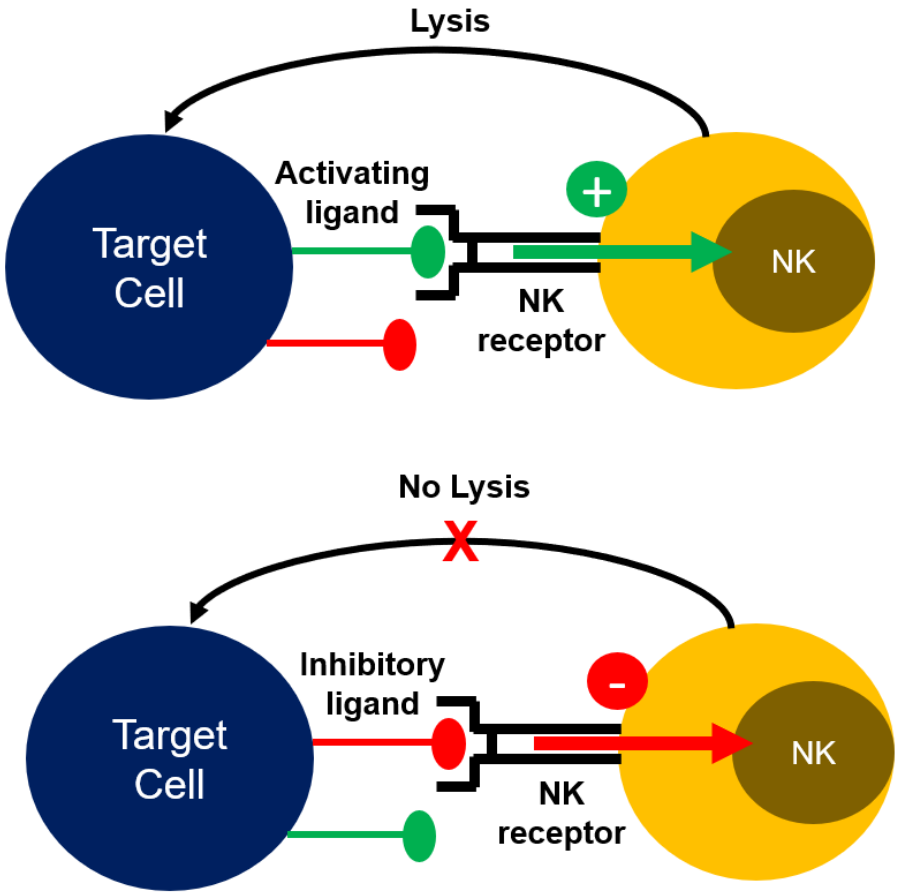


Figure 1.2

NK receptors and ligands that leads to either inhibition or activation of NK function.

Ligands on tumor cells interacts with NK receptors and sends either an activating or inhibitory signal to NK cells.

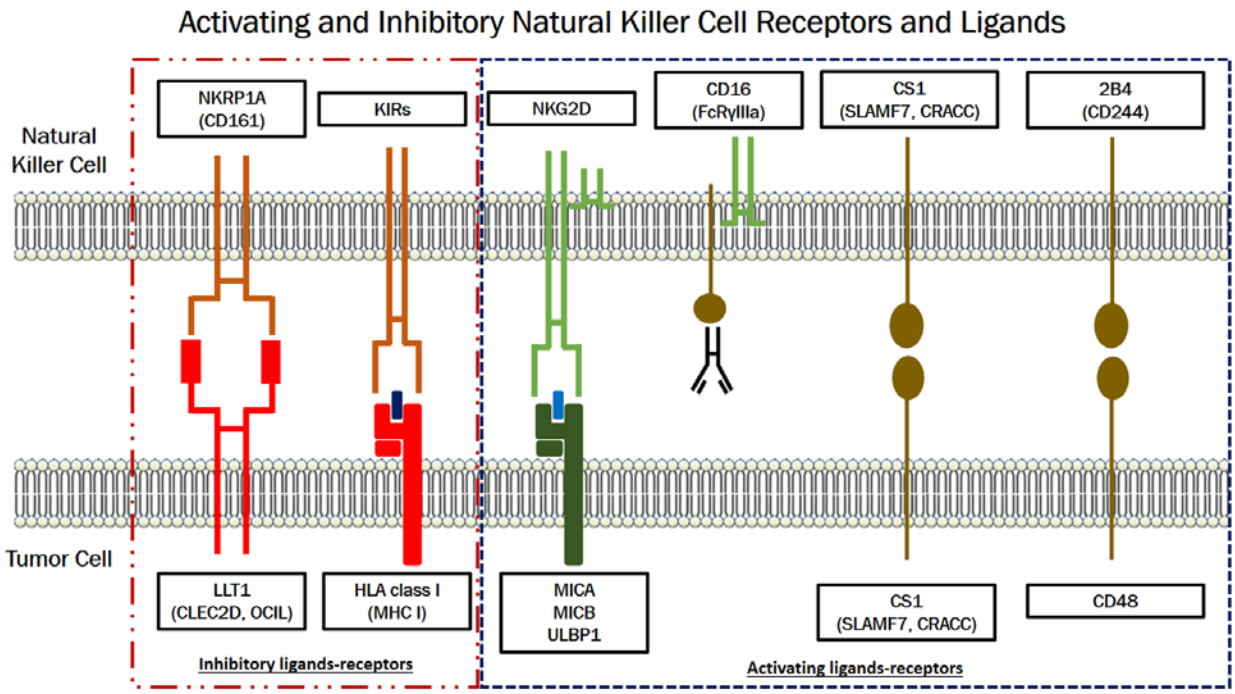


Figure 1.3

Inhibitory ligands LLT1 and PCNA interacts with natural killer cell receptors.

Lectin-like Transcript-1 (LLT1) expressed on tumor cell interacts with natural killer cell receptor NKRP1A (CD161) and Proliferating Cell Nuclear Antigen (PCNA) interacts with natural killer cell receptor NKp44. LLT1-NKRP1A and PCNA-NKp44 interactions send a signal that inhibits natural killer activation thus preventing lysis of the tumor cell.

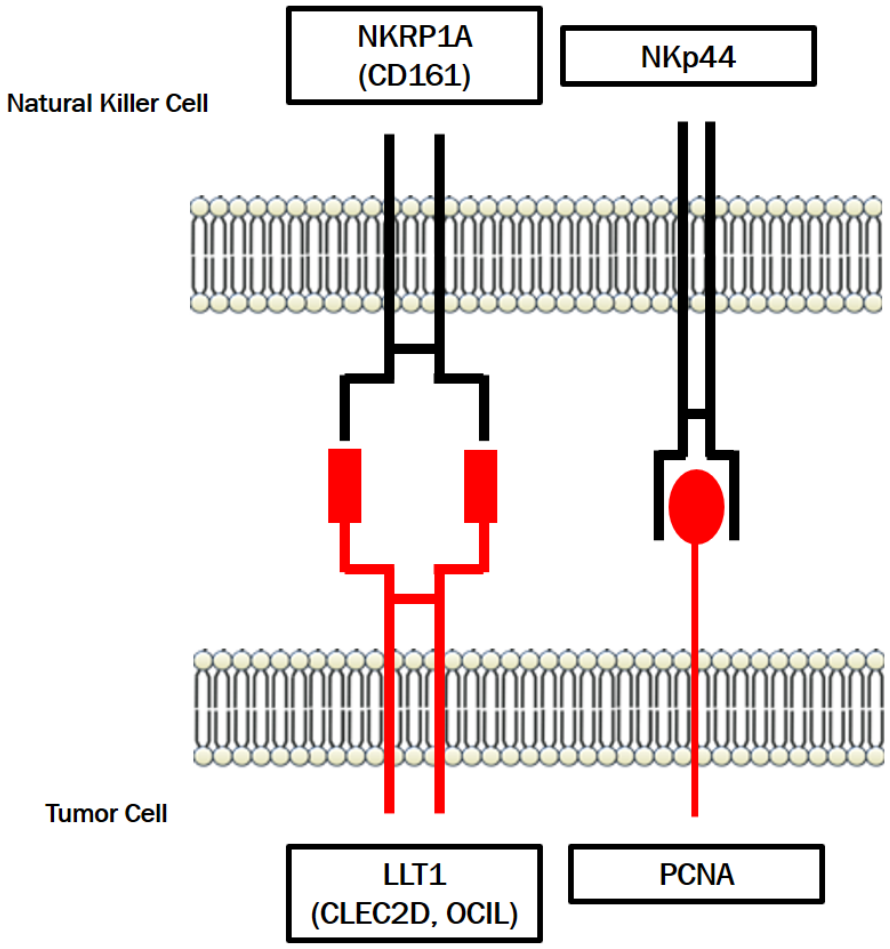


Figure 1.4

Specific Aim 1

Investigate the expression of LLT1 and PCNA on triple-negative breast cancer cells.

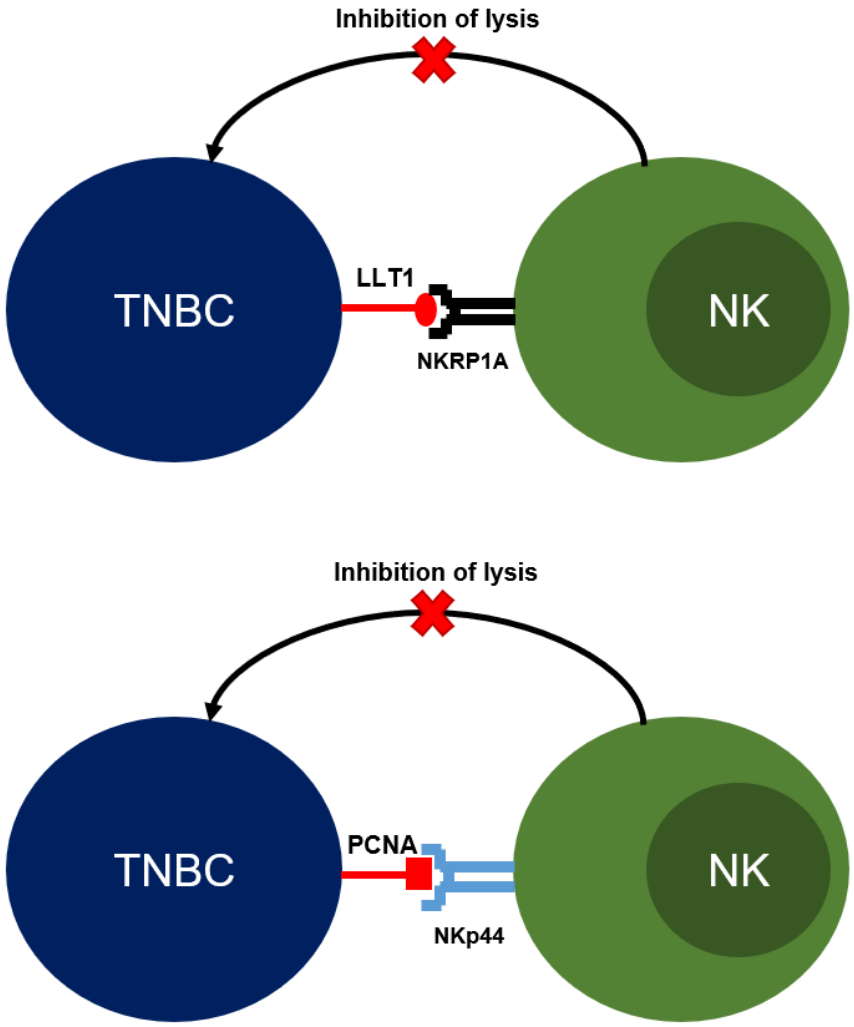
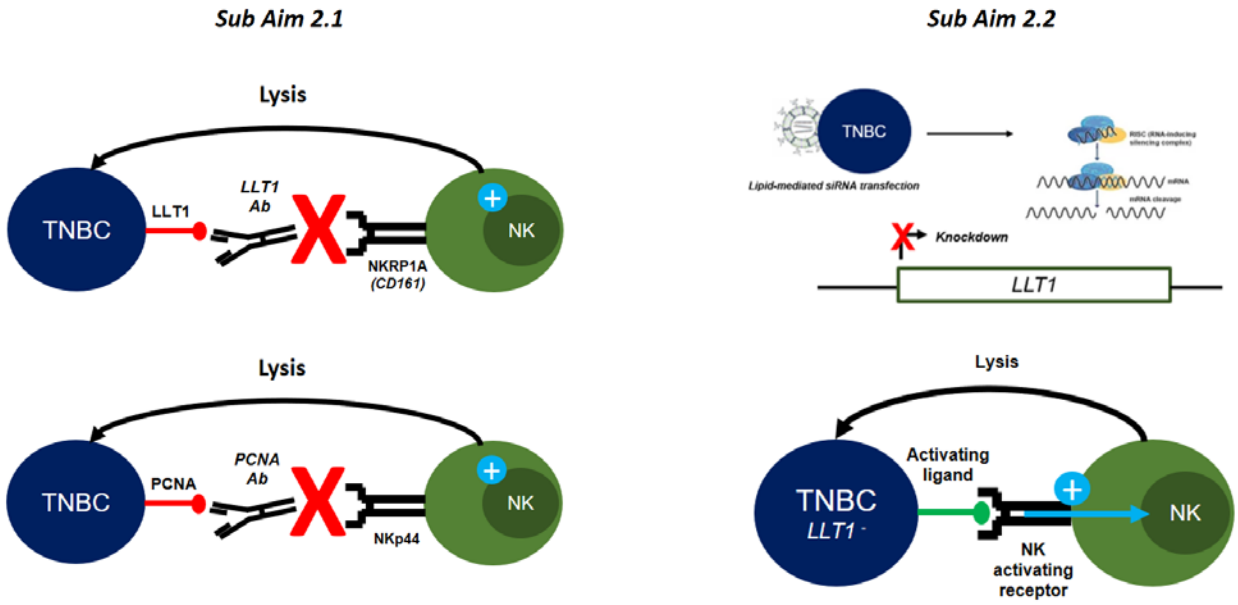


Figure 1.5

Specific Aim 2

Investigate the functional outcome of blocking the interactions of LLT1 and PCNA on triple-negative breast cancer cells with CD161 and NKp44 on NK cells to target TNBC by natural killer cells by anti-LLT1 and anti-PCNA antibodies (Sub Aim 2.1) and gene knockdown of *LLT1* (Sub Aim 2.2).



CHAPTER II

MATERIALS AND METHODS

2.1 Cell Lines

All cells lines were acquired from American Type Culture Collection(ATCC). Human breast cancer cell lines with a triple negative phenotype (ER^- PR^- $HER2^-$) on its cell surface used are MDA-MB-231, MDA-MB-436, and MDA-MB-468. MCF10A, a human non-tumorigenic epithelial breast cell line, and MCF7, a non-triple negative phenotype, were also used. MDA-MB-231 (ATCC HTB-26) is a human breast adenocarcinoma epithelial cell line that was derived from a pleural effusion on October 17, 1973 at M.D. Anderson Hospital (Houston, TX) [135,136]. MDA-MB-436 (ATCC HTB-130) is a human breast adenocarcinoma cell line derived from pleural effusion on January 23, 1976 at M.D. Anderson Hospital [135,136]. MDA-MB-468 (ATCC HTB-132) is a human breast adenocarcinoma cell line derived from pleural effusion on November 4, 1977 at M.D. Anderson Hospital [135,136]. MCF10A (ATCC CRL-10317) is a non-tumorigenic breast epithelial cell line derived from human fibrocystic mammary tissue on August 22, 1984 at Michigan Cancer Foundation [137,138]. MCF7 (ATCC HTB-22) is a non-triple negative breast cancer epithelial cell line derived from pleural effusion and shows positive expression of the estrogen receptor at Michigan Cancer Foundation [139].

2.2 Cell Culturing

Cell lines used were MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, and MCF10A. Primary natural killer cells (NK) isolated from peripheral blood mononuclear cells (PBMCs) of whole blood were also cultured in artificial media. Cells were grown in sterile culture flasks or well plates in a 37ºC 5% CO_2 incubator. Cells were grown to 80 to 90 percent

confluence and then were passaged into new flasks or well plates containing appropriate media for each cell line. For cell lines that are known to adhere to flasks (MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-468), passage occurs upon confluence and was performed by removing media, aspirating the cells with 1X phosphate-buffered saline (PBS) with ethylene-diamine-tetracetic acid (EDTA) or trypsin-EDTA (for MCF10A), and transferring to new sterile flasks or wells with fresh media for further growth or use. For free-floating cells such as primary NK cells, cells floating in the media in the flasks were transferred to a conical tube and were centrifuged for 5 minutes at 250 rpm at 4ºC. Old media was discarded without disrupting the cell pellet and fresh new media was added with the pellet resuspended. NK cells resuspended with fresh new media were transferred to a new flask or well plate where 50 U/ml of recombinant human interleukin-2 (rhIL-2) was added.

MDA-MB-231, MDA-MB-436, and MCF7 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. MDA-MB-468 was cultured in 4+ Roswell Park Memorial Institute 1640 complete medium (RPMI) containing 10% FBS and penicillin-streptomycin. MCF10A was cultured in Medium 171 supplemented with Mammary Epithelial Growth Supplement (MEGS). Primary NK cells were cultured in 4+ RPMI containing 15% FBS and penicillin-streptomycin and 50 U/ml of rhIL-2 added in the flasks or wells containing media. For cell lines that were transfected with small interference RNAs (MDA-MB-231 and MDA-MB-436 transfected with siRNAs), these cell lines were grown in DMEM containing 10% FBS and 2mM L-glutamine, but no penicillin-streptomycin was added.

2.3 Flow Cytometry Analysis

Expression of ligands (LLT1 and PCNA) was detected on the cell surface by flow cytometry analysis. Cells were grown to near confluence on sterile culture flasks or well plates and then were appropriately removed from the flasks or well plates for use. Cells were checked for viability by staining with trypan blue and counted under the hemocytometer. Cells were first treated with human Fc fragment to block CD16 ligands on TNBCs. All samples of cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, and MCF10A) were then stained with either conjugated mouse anti-human LLT1 or PCNA antibodies containing phycoerythrin (PE) or conjugated mouse anti-human isotype antibodies containing PE fluorophore. Cells stained with isotype antibodies were used as controls in flow cytometry analysis to compensate for any non-specific binding of LLT1-PE or PCNA-PE antibodies. Cells stained with either ligand-specific antibodies or isotype antibodies were incubated at 4ºC in the dark for 30 minutes. After incubation, cells were washed with PBS-BSA to remove unbound antibodies before analysis.

Samples underwent detection of bound antibodies using the Beckman Coulter Cytomics FC500 Flow Cytometer in the University of North Texas Health Science Center Core Facility. For all independent experiments, the sampling size of samples from cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 was approximately 20,000 cells in each sample of each experiment. The sampling size of samples from cell line MCF10A was approximately 8,500 cells. All data collections were analyzed using the FlowJo software where the cell population were gated for live cells and then gated for positive detection of ligands LLT1 or PCNA on the cells.

Two quantitative measures, median fluorescence intensity ratio (MFIR) and percentage of cells that are positive for either of the two ligands, were used to determine expression of

ligands for each sample of each cell line. Median fluorescence intensity is a measure of intensity of the fluorophore detected on the conjugated antibody bound to its target protein assigned by the flow cytometer. MFIR, the ratio, is calculated by the number of live cells that are positive for ligands LLT1 or PCNA divided by the number of live cells that are positive for isotype antibodies bound to the cell surface. MFIR above 1.00 indicated positive expression of ligands LLT1 or PCNA for the samples tested. MFIR at or below 1.00 indicated negligible expression of ligands LLT1 or PCNA for the samples tested. To compare the expression of ligands on each of the cell lines, all MFIRs were collected from all independent experiments and the mean and population standard deviation were calculated. One-way ANOVA with Dunnett's multiple comparisons post-hoc test was used for statistical analysis to compare the statistical difference between the means of MFIRs of cell surface ligand expression LLT1 or PCNA on MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 cell lines and control non-tumorigenic breast cell line MCF10A.

2.4 Isolation of Human Peripheral Blood Mononuclear Cells and Primary Natural Killer Cells

Primary natural killer cells were used in co-incubation with TNBCs in the ^{51}Cr release cytotoxicity assay (see section 2.9 for specific details on this assay). Protocols involving isolating natural killer cells from healthy human subjects were approved with voluntary consent and reviewed by the Institutional Review Board. Primary natural killer cells were isolated from peripheral blood mononuclear cells (PBMCs) which were formerly isolated from whole blood [140].

Whole blood was collected from healthy volunteers and then were treated with Histopaque-1077 density gradient. The Histopaque-1077 density gradient was used to separate

whole blood into different layers (serum, PBMCs, platelets, and erythrocytes layers) during the 20-minute centrifugation process. After centrifugation, the white layer consisting of PBMCs was carefully isolated into new sterile conical tubes and processed further according to standard PBMC purification protocol. PBMCs consist of monocytes, dendritic cells, and lymphoid cells such as B lymphocytes, T lymphocytes, and natural killer cells [5]. Upon isolation of PBMCs, PBMCs were counted using a hemocytometer in preparation for the following natural killer cell isolation steps.

Primary natural killer cells were isolated from PBMCs by following standard protocol instructions in the Miltenyi Biotec natural killer cell isolation kit. For every tube containing 10^7 PBMCs, PBMCs were treated with a prepared buffer consisting of phosphate-buffered saline (PBS at pH 7.2), 0.5% bovine serum albumin (BSA), and 2 mM EDTA. Natural killer cell biotin-antibody cocktail was, then, added to the buffer-treated PBMCs and underwent a 5-minute incubation at 4ºC. After incubation, PBMCs were treated with NK cell microbead cocktail before the subsequent magnetic NK cell separation step. The magnetic NK cell separation step utilizes a column in the magnetic field of a MACS Miltenyi Biotec Separator to collect the flow-through fluid containing purified natural killer cells. Upon separation, primary natural killer cells were cultured in 4+ RPMI media supplemented with 15% fetal bovine serum and 50 U/ml of recombinant human interleukin-2 in either sterile well plates or culture flasks. Primary NK cells were kept in culture overnight before use in the ^{51}Cr release cytotoxicity assay the next day.

2.5 Gene Knockdown by Lipid-Mediated siRNA Transfection

Transient transfection of small interference RNAs (siRNA) targeting the *LLT1* gene was performed in order to knockdown or decrease the expression of LLT1 at the cell surface of TNBCs with scramble siRNA used as a control. Cells were initially grown to slightly more than 50 to 60 percent confluency on sterile 96-well plates. SMARTpool: ON-TARGETplus *CLEC2D* (LLT1) 5 nmol siRNAs and SMARTpool: ON-TARGETplus non-targeting 5 nmol siRNA #2 were used in this transfection according to protocol by GE Healthcare Dharmacon, Inc [141]. SiRNAs were diluted from a 20 µM siRNA stock to a 5 µM working stock with 1X siRNA buffer, which was formerly diluted from a 5X siRNA buffer. Transfection optimization conditions were determined by GE Healthcare Dharmacon for cell lines MDA-MB-231 and MDA-MB-436. Manufacturer protocol on transfection was followed according to Dharmacon. Cells were transfected with either *CLEC2D* siRNA or non-targeting siRNA for 63 hours in the 37ºC 5% CO_2 incubator. After transfection for a period of predetermined number of hours, knockdown of cell surface LLT1 on MDA-MB-436 was confirmed by flow cytometry analysis staining transfected cells with either anti-human LLT1-PE antibodies or isotype control mouse IgG_1-PE antibodies.

2.6 Immunofluorescence Confocal Microscopy

Confocal microscopy was performed to visually observe expression of LLT1 at the cll surface and intracellularly. MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 were cultured on coverslips overnight. Cells were fixed with 2% paraformaldehyde and then were incubated with blocking solution containing human Fc fragment to prevent non-specific binding of antibodies. Cells were stained with conjugated mouse anti-human LLT1 antibodies with phycoerythrin (PE) fluorophore attached for detection of ligands at the cell surface. To observe

intracellular and total expression of LLT1, cells were first fixed, permeabilized with 0.2% Triton X-100 (Sigma-Aldrich), and then were stained with mouse anti-human LLT1-PE antibodies. After washing in PBS, the coverslips were mounted on slides using Aqua-Mount solution and imaged on Zeiss LSM 510 Confocal Laser Microscope using the 40x, 1.2 NA, 0.28 WD (water), C-apochromat objective at 488 nm wavelength.

2.7 Cell Extract Preparation and Western Blot Analysis

Cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, and MCF10A were grown to near confluency in sterile flasks. These cells were passaged, aspirated with PBS-EDTA, and collected. Cells were treated with lysis buffer and protease and phosphate inhibitor cocktail at 4ºC for 30 minutes to allow secreted protein to be isolated in preceding steps. After incubating on ice for 30 minutes, cell debris was removed after centrifugation and protein concentrations were analyzed and quantified using the Pierce BCA protein assay (Thermo Scientific). NuPAGE Novex 4-12% Bis-Tris Gels (1.5 mm) readymade gels with MES buffer were used to separate cell protein extracts. Cell extracts were transferred to a nitrocellulose transfer stack for 10 minutes using the Invitrogen iBlot Gel Horizontal Transfer Device. Membrane was then incubated with mouse anti-human unconjugated LLT1 antibody overnight in 5% fat-free milk with 0.05% Tween 20. After washings with Tween 20, the membranes were then incubated with horseradish peroxidase-linked anti-mouse secondary antibody at room temperature for 2 hours. Membranes were next developed, and an image was obtained where bands were analyzed for total LLT1 expression.

2.8 Subcellular Protein Fractionation

Subcellular protein fractionation allows isolation of membrane, cytoplasmic, and nuclear proteins in cell lines being tested according to manufacturer's protocol [142]. Proteins extracted from membrane, cytoplasm, and nucleus are treated with anti-LLT1 and anti-PCNA antibodies and then tested for expression of ligands on the western blot in order to compare the expression of ligands in the three phases.

Cell lines MDA-MB-231, MDA-MB-468, and MCF10A were grown to near confluency in sterile culture flasks. Cells were passaged, aspirated with PBS-EDTA, collected, and counted. Two million cells of each cell line were harvested, centrifuged, and treated with cold cytoplasmic extraction buffer (CEB) containing protease inhibitors. After adding CEB, the samples were incubated at 4ºC for 10 minutes before centrifugation. After centrifugation, supernatant containing cytoplasmic protein was removed and transferred to a pre-chilled Eppendorf tube. The remaining cell pellet was then treated with membrane extraction buffer (MEB). Sample was incubated at 4ºC for 5 minutes before centrifugation. Supernatant containing membrane protein was transferred to a new pre-chilled tube. Nuclear extraction buffer (NEB) was added to the remaining cell pellet, incubated for 30 minutes, and centrifuged. Supernatant containing nuclear protein was transferred to a new tube. Membrane, cytoplasm, and nuclear proteins extracted from the three cell lines are quantified using Pierce BCA protein assay before proceeding with standard western blot protocol and analysis (as described in section 2.7).

2.9 ^{51}Cr Release Assay

The ^{51}Cr release cytotoxicity assay was used to quantify the percentage of cells from the cell lines tested killed by primary natural killer cells (Figure 2.1). Cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF10A were treated with anti-LLT1 and/or anti-PCNA

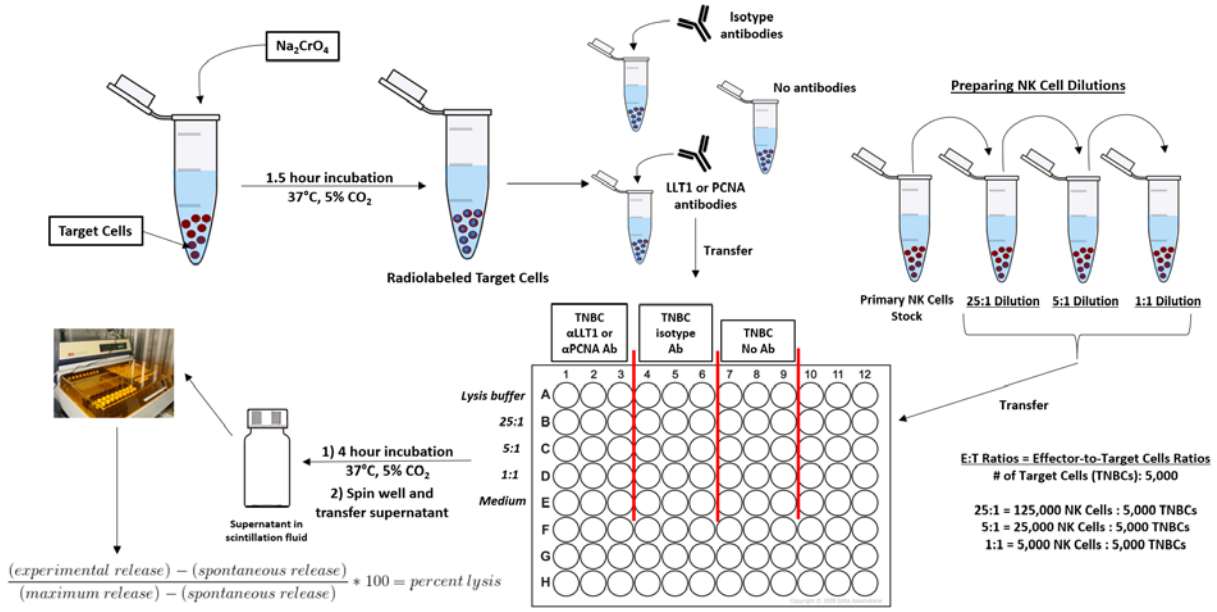
antibodies and were tested in this assay to observe that a decrease in inhibitory signals sent to the natural killer cells would enhance lysis of these cells by natural killer cells.

Cells from the listed cell lines were first collected and then labeled with radioactive isotope ^{51}Cr by incubation in the 37ºC 5% CO_2 incubator for 90 minutes. After incubation, cells were treated with 1 µg (per well) of either goat anti-human LLT1 antibody, mouse anti-human PCNA antibody, goat isotype IgG antibody, or mouse IgG_{2a} antibody. Cells were incubated at 4ºC for 30 minutes to allow antibodies to bind to respective target ligands. Primary natural killer cells were treated with human Fc fragment to block all CD16a receptors on natural killer cells. After antibody incubation, cells from each cell line are then co-incubated with primary natural killer cells for a period of 4 hours at three different effector-to-target ratios (NK-to-5000 Target Cells), 25:1, 5:1, and 1:1 in a 96-well plate.

Co-incubation occurs in a 37ºC 5% CO_2 incubator. After co-incubation and subsequent centrifugation to separate cell debris, 100 µl of supernatant in each well were transferred to each respective scintillation vial containing 2 ml of scintillation fluid. The vials were taken to a scintillation counter where the counts per minute (CPM) was collected. CPM values are converted to percent specific lysis of cells according to the formula (displayed in Figure 2.1). Student t-test with Welch's correction was used to compare the percent specific lysis of cells treated with either anti-LLT1 and/or anti-PCNA antibodies to the isotype control or negative control for each cell line within each of the three effector-to-target ratios.

Figure 2.1

Schematic Representation of ^{51}Cr Release Cytotoxicity Assay Workflow



CHAPTER III

RESULTS

RATIONALE

Breast cancer is known to be one of the most common cancers in women. Triple-negative breast cancer (TNBC) is considered to be the most aggressive form of breast cancer due to the absence of ER, PR, and HER2 receptors and accounts for nearly 20 percent of all breast cancer cases [36]. TNBC is associated with poor prognosis and has poor response to hormonal treatments and increased chance of relapse after initial rounds of chemotherapy. Our long-term goal is to utilize natural killer cells to target ligands with inhibitory function on TNBCs and other types of cancers. The objective of this project is to characterize the expression of ligands on TNBCs that inhibit NK cell function.

Based on previous studies, we have determined the two ligands of interest to target on TNBCs. Two ligands of interest are Lectin-like Transcript-1 (LLT1), which interacts with NK receptor NKRP1A (CD161), and Proliferating Cell Nuclear Antigen (PCNA), which interacts with NK receptor NKp44. The central hypothesis is that LLT1 and PCNA are expressed on TNBCs and allow TNBC cells to evade recognition and cytolytic targeting by natural killer cells. The rationale is that previous studies have shown that LLT1 and PCNA are expressed on the cell surface of different types of cancer cells, as will be discussed throughout this section. Therefore, we rationalized that LLT1 and PCNA may be expressed on TNBCs and that each ligand function as an inhibitory ligand that suppresses NK cells from killing TNBCs.

Prior studies demonstrated that LLT1 expressed on cancer cells interacts with NKRP1A on NK cells. Studies have shown that LLT1 was expressed on glioma cells, non-Hodgkin

lymphoma B cells, and prostate cancer cells which functions to suppress NK cell cytolytic function against these cancer cells [88,89,175]. Mathew *et al.* reported overexpression of LLT1 on prostate cancer cells and showed that blocking interaction between LLT1 and NKP1A have increased killing of prostate cancer cells [88]. Germain *et al.* have also observed LLT1 expression on germinal center B-cell non-Hodgkin lymphomas which also prevents activation of NK cell function [175]. Another study performed by Roth *et al.* have shown that LLT1 expression on malignant glioma cells inhibits antitumor immune activity by interacting with NKRP1A [89]. To the best of our knowledge, there has not been a study that evaluated LLT1 expression on TNBCs. Since there was LLT1 expression on different types of cancers, we would like to determine if there is expression of LLT1 on TNBCs and whether blocking LLT1-NKRP1A interaction will enhance killing of TNBCs by NK cells.

For PCNA, previous studies have shown that PCNA expressed on certain types of cancers has increased cancer cell survival, metastasis, and proliferation [116,120,121]. One study conducted by Naryzhny *et al.* observed higher expression of PCNA on three breast cancer cell lines MDA-MB-231, MDA-MB-468, and MCF7 than normal breast cell lines HMEC and MCF10A by western blot analysis [120]. Although total PCNA expression (combined membrane, cytoplasmic, and nuclear expression) was focused on in the study mentioned, we are interested in looking at cell surface PCNA expression on TNBCs and how blocking PCNA at the cell surface of TNBCs will affect NK cell function. There is also evidence of PCNA expressed on pancreatic, breast, melanoma, lymphoma, and glioblastoma cells shown by Rosental *et al.* [121]. This group has shown that PCNA on these cancer cells interacts with NKp44 and inhibits NK cell function [121]. Furthermore, Horton *et al.* showed that PCNA colocalizes with human leukocyte antigen I (HLA I) and synergistically to inhibits NK cell function when PCNA

interacts with NKp44 [90]. Therefore, because there is evidence of PCNA expression on different types of cancers, we would like to determine if there is expression of PCNA at the cell surface of TNBCs and if blocking PCNA-NKp44 interaction between TNBCs and NK cells will allow NK cells to kill TNBCs. By blocking either LLT1-NKRP1A interaction and PCNA-NKRP1A interaction, a net signal sent to NK cells will then favor activation of NK cell function which then allows NK cells to kill TNBCs.

3.1 EXPRESSION AND FUNCTION OF LLT1 on TNBCs

Introduction

Lectin-like Transcript-1 (LLT1, CLEC2D, OCIL) is a ligand that when expressed on cells interacts with NKRP1A (CD161) on NK cells. The LLT1-NKRP1A interaction sends an inhibitory signal to the natural killer cell through the immunoreceptor tyrosine-based inhibitory tail (ITIM) on the NKRP1A cytoplasmic tail. LLT1 expression and function has been characterized on immune cells such as B cells, T cells, NK cells, and activated dendritic cells [106,107]. Triple-negative breast cancer (TNBC) is the most aggressive form of breast cancer and has the lowest level of success in tumor suppression by conventional chemotherapeutic treatments. With consideration of the cancer immunoediting concept, TNBCs may have established mechanisms that allow tumor cells to escape immunosurveillance by immune cells. There has been a limited number of studies identifying ligands specifically on TNBCs that contribute to the TNBC role of evading targeting by NK cells. Of the available studies that do identify ligands, those studies heavily focused on targeting programmed cell death 1 and its ligand. This study is the first that, we know of, that looks at both the expression and function of LLT1 on triple-negative breast cancer cell lines from an immunotherapeutic perspective. We have found that TNBC cell lines show higher expression of LLT1 at the cell surface compared to

non-tumorigenic breast cell line MCF10A. We have also demonstrated that blocking LLT1-NKRP1A (CD161) interaction enhances killing of TNBCs by primary NK cells. Due to the difference in expression of LLT1 between TNBCs and non-tumorigenic breast cells, LLT1 may serve as a possible target with antibodies, which would suppress inhibitory signals from being sent to the NK cells.

3.1.1 Lectin-like Transcript-1 (LLT1) is Expressed on Triple-Negative Breast Cancer Cells and Inhibits Natural Killer Cell Activation

Cell Surface LLT1 Expression on TNBCs by Flow Cytometry Analysis.

Flow cytometry analysis was performed to detect and quantify expression of LLT1 at the cell surface of TNBCs and non-tumorigenic breast cell lines. All cell lines tested (MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, and MCF10A) are known to adhere to the surface of culture flasks. When cells were harvested from culture, phosphate-buffered saline (PBS) containing EDTA was used instead of trypsin-EDTA to prevent both degradation and cleavage of cell surface LLT1 ligand and block leaking of cytoplasmic LLT1 protein secreted. Cells were treated with a human Fc fragment in order to block potential Fc receptors or Fc-receptor ligands that could be present on cell lines being tested. Any presence of Fc receptors or Fc-receptor ligands on membranes of cell lines being tested may cause potential non-specific binding of anti-LLT1 antibodies to the cell surface. After treating cells with a Fc fragment, cells were stained with either conjugated anti-human LLT1-PE antibodies or isotype IgG_1-PE antibodies.

Expression of LLT1 was quantified and represented by median fluorescence intensity ratios and percentage of cells positive for LLT1. The median florescence intensity (MFI) refers

to the 50th percentile of the antibody-treated cell population whose fluorescence (released by either the fluorophore attached to the antibody or natural fluorescence of the cell) was detected by the flow cytometer. In reference to analyzing results of this experiment, median fluorescence intensity ratio (MFIR) is defined as the MFI of the cell population stained with the anti-LLT1-PE antibody divided by the MFI of the cell population stained with isotype IgG_1-PE antibodies. Cells were gated on a single live cell population since only one antibody was used at a time in staining the cells which does not require compensation.

Three TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were tested for cell surface LLT1 expression and were compared to non-TNBC cytoplasmic estrogen receptor-positive cell line MCF7 and non-tumorigenic mammary epithelial cell line MCF10A. In one representative experiment out of all independent experiments, TNBC cell line MDA-MB-231 displayed the highest cell surface expression of LLT1 at MFIR of 1.84 with 13.3% of 19859 cells positive for cell surface LLT1 expression compared to the other two TNBC cell lines (Figure 3.1). Interestingly, TNBC MDA-MB-468 displayed the lowest expression of cell surface LLT1 out of all the TNBC cell lines tested at MFIR of 1.29 and 2.79% of 19950 cells positive for LLT1 expression. MCF7, a non-TNBC breast cancer cell line, showed the highest expression of LLT1 out of all the cell lines at MFIR of 1.70 and 15.5% of 19835 cells positive for LLT1. All the TNBC cell lines and MCF7 have higher expression of LLT1 than MCF10A. Non-tumorigenic breast cell line MCF10A has the lowest expression of LLT1 with MFIR of 0.80 and 0.17% of 8117 cells having such expression. Of these representatives, since there is a difference between the expression of LLT1 between TNBCs and MCF10A, LLT1 may be a possible target for antibody-blocking in future experiments.

To confirm consistency in cell surface LLT1 expression (Figure 3.2, Table 3.1), 3 independent flow cytometry experiments were performed for each cell line. MFIRs and percentage of cells that have positive expression of LLT1 from all independent experiments were averaged. All histograms and dot plots showing MFIR and percent of cell population that are $LLT1^+$ in each independent experiment of each cell line that comprise the mean of MFIR and percent of cell population $LLT1^+$ values in figure 3.2 and table 3.1 are shown in figure 3.3. Of the three TNBC cell lines, MDA-MB-231 displayed the highest cell surface LLT1 expression based on its mean of MFIRs and mean percent of its population expressing LLT1 (Figure 3.2). MDA-MB-231 mean of MFIRs of 1.80 and its mean percent $LLT1^+$ cells of 14.67% is statistically significantly higher (Mean of MFIRs and % $LLT1^+$ ** $p < 0.01$, Figure 3.2) than non-tumorigenic breast cell line MCF10A respective values of 0.95 and 0.56% $LLT1^+$ cells. MDA-MB-436 does show a higher expression of LLT1 based on mean of MFIRs of 1.34 and mean percent $LLT1^+$ cells of 6.54% than MCF10A, but still much lower than MDA-MB-231 respective values. Estrogen receptor-positive breast cancer cell line MCF7 displays similar expression of LLT1 with MDA-MB-231 and has significantly higher LLT1 than MCF10A (Mean of MFIRs * $p < 0.05$; % $LLT1^+$ ** $p < 0.01$, Figure 3.2). MDA-MB-468 shows the lowest expression of LLT1 for both average MFIRs and % of cells $LLT1^+$. Hence, these independent flow cytometry experiments under the same culture conditions and stained with the same anti-LLT1-PE antibodies have consistently shown that TNBCs, especially MDA-MB-231, show higher cell surface expression of LLT1 than non-tumorigenic breast cell line MCF10A. These results have further demonstrated that LLT1 may serve as a possible target of interest for antibody-blocking treatment on TNBCs.

LLT1 is Expressed at Both the Cell Membrane and Intracellular.

Flow cytometry analysis had revealed that LLT1 is expressed on all the TNBC cell lines tested and has shown higher expression of LLT1 compared to non-tumorigenic breast cell line MCF10A (Figure 3.2). Immunofluorescence confocal studies were performed on TNBC cell lines and MCF7 to observe for expression of LLT1 at both the cell surface and intracellular. Confocal studies could not be performed on MCF10A due to repetitive technical issues; however, future studies should troubleshoot this technical issue and allow one to conduct immunofluorescent studies of LLT1 on non-tumorigenic breast cell line MCF10A. Since a confocal image of MCF10A is not available at this time, we have performed a western blot analysis which confirms total LLT1 expression on MCF10A.

For confocal microscopy studies, TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 as well as non-TNBC breast cancer cell line MCF7 were harvested from culture flasks with PBS containing EDTA. The use of PBS-EDTA instead of trypsin-EDTA allows these adherent cells to be removed without degrading LLT1 at the cell surface. Cells were fixed with 2% paraformaldehyde and then were treated with human Fc fragment to prevent non-specific binding of anti-LLT1-PE antibodies. To observe for cell surface expression, cells were stained with anti-human LLT1-PE antibodies and were then examined with a confocal laser microscope. To observe for intracellular LLT1 expression, cells were treated with 1X BD Perm/Wash buffer after cells were fixed in order to obtain all protein secreted by the cell. These cells were then stained with antibodies for LLT1 detection.

All the TNBC cell lines and MCF7 express LLT1 at the cell surface as shown by shades of red staining on the cell (Figure 3.4). Of all the cell lines, MDA-MB-231 display the greatest cell surface LLT1 expression while both MDA-MB-436 and MDA-MB-468 has the least cell

surface LLT1 expression. MCF7 also shows slightly higher expression of LLT1 than MDA-MB-436 and MDA-MB-468. These immunofluorescent studies were consistent with flow cytometry results showing expression of LLT1 at the cell surface. Both flow cytometry analysis and immunofluorescent cell surface studies show that MDA-MB-231 has the greatest expression of LLT1 at the cell surface while MDA-MB-468 has the lowest cell surface expression of LLT1.

In addition, all the TNBC cell lines and MCF7 express intracellular LLT1 as shown by shades of red staining within the cells (Figure 3.5, A). It is important to note that in these confocal microscopy images, expression of LLT1 is not only limited to intracellular expression, but this LLT1 expression includes both intracellular LLT1 expression and cell surface LLT1 expression since the cells were treated with permeabilizing agent. Interestingly, MDA-MB-231 showed the lowest total LLT1 expression while MDA-MB-468 showed the highest total LLT1 expression. Intracellular staining of LLT1 by flow cytometry analysis using the same reagents for fixation and permeabilization of cells for confocal microscopy analysis showed an increase in LLT1 expression for MDA-MB-231 and MDA-MB-468 cells permeabilized compared to MDA-MB-231 and MDA-MB-468 cells not permeabilized (Figure 3.6).

Western blot analysis testing for total LLT1 expression has also shown that MDA-MB-468 has the highest total LLT1 expression comparable to MCF10A total LLT1 expression (Figure 3.5, B). Total LLT1 expression refers to the combined intracellular LLT1 expression and cell surface LLT1 expression. Western blot analysis has also shown secretion of LLT1 protein for MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 cell lines. This western blot analysis does not distinguish expression of LLT1 at the membrane versus cytoplasmic versus nuclear locations. Flow cytometry analysis from figure 3.1, figure 3.2, and figure 3.3 has already confirmed LLT1 expression at the cell surface of all the TNBC cell lines, MCF7, and MCF10A.

Hence, immunofluorescent studies and western blot showed LLT1 expression at both the cell surface and intracellularly in all the TNBC cell lines, MCF7, and MCF10A. This indicates that LLT1 is secreted and LLT1 may be a target of interest for antibody-blocking treatment especially on the TNBC MDA-MB-231 cell line. Since western blot, flow cytometry, and confocal microscopy confirmed secretion of LLT1 protein, we can target the actively transcribed LLT1 gene for knockdown with a lipid-mediated small-interference RNA in order to decrease expression of LLT1 at the cell surface as a future study.

Figure 3.1

TNBC and non-TNBC cell lines display higher expression of LLT1 than non-tumorigenic breast cell line.

Expression of LLT1 represented by median fluorescence intensity ratios (A) and percentage of cells positive for LLT1 (B) was identified by flow cytometry analysis by staining TNBC cell lines and non-tumorigenic breast cell line with either anti-human LLT1-PE (gray shade in histograms in A) or isotype IgG_1-PE antibodies (white shade in histograms in A).

One representative of all independent experiments shown next page.

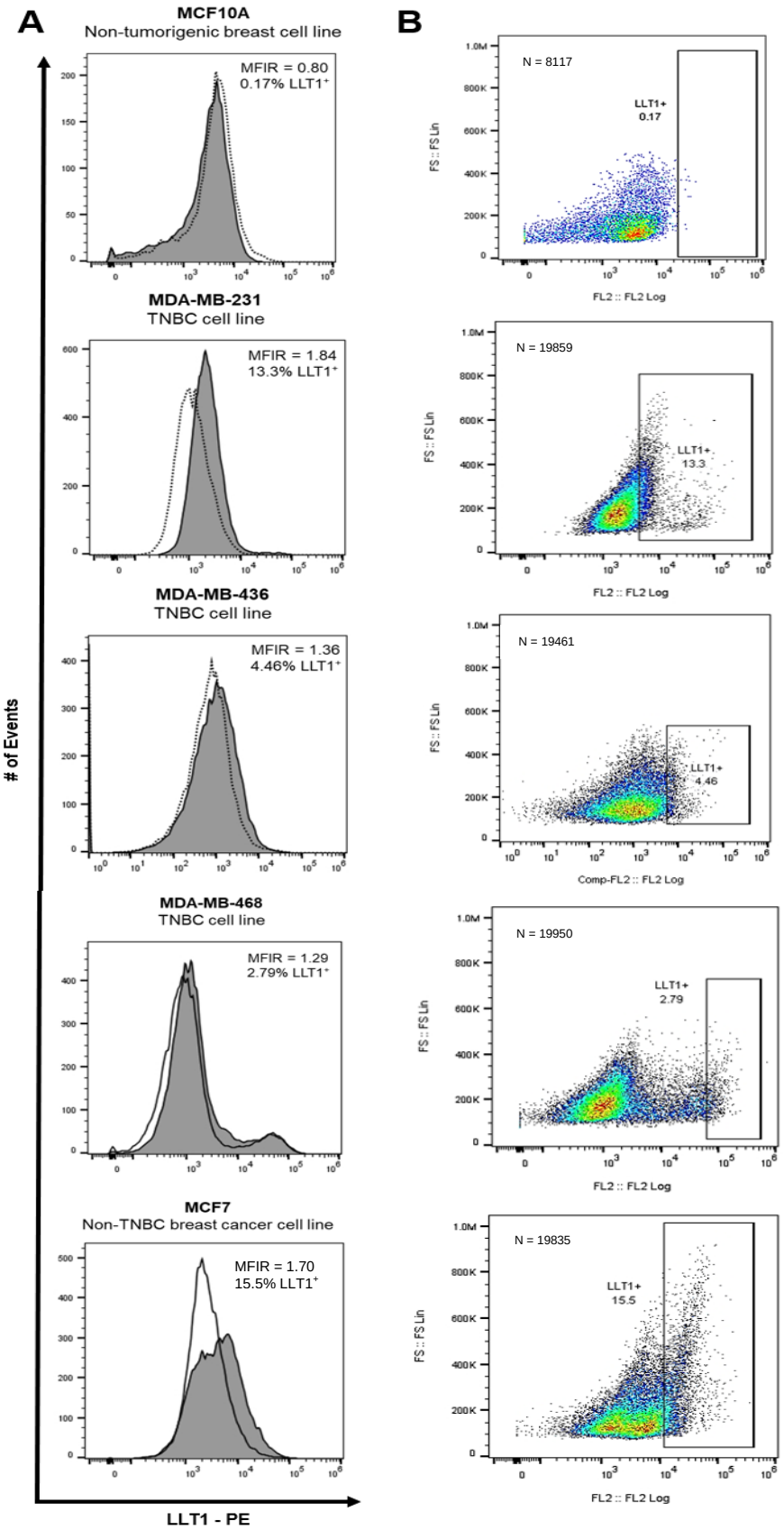

A
MCF10A
Non-tumorigenic breast cell line
MFIR = 0.80
0.17% LLT1+
MDA-MB-231
TNBC cell line
MFIR = 1.84
13.3% LLT1+
MDA-MB-436
TNBC cell line
MFIR = 1.36
4.46% LLT1+
MDA-MB-468
TNBC cell line
MFIR = 1.29
2.79% LLT1+
MCF7
Non-TNBC breast cancer cell line
MFIR = 1.70
15.5% LLT1+
# of Events
LLT1 - PE
B
N = 8117
LLT1+
0.17
N = 19859
LLT1+
13.3
N = 19461
LLT1+
4.46
N = 19950
LLT1+
2.79
N = 19835
LLT1+
15.5
FS :: FS Lin
FL2 :: FL2 Log
Comp-FL2 :: FL2 Log

Figure 3.2

TNBC and non-TNBC cell lines show higher expression of LLT1 than non-tumorigenic breast cell line by average MFIR and percent of cell population LLT1+.

Flow cytometry analysis was used to detect expression of LLT1 at the cell surface of all cell lines tested. Median fluorescence intensity ratios (MFIRs) and percentage of cells that have positive expression of LLT1 from all independent experiments (n=3 for all cell lines) were averaged. TNBC cell lines MDA-MB-231 and non-TNBC breast cancer cell line MCF7 have higher average MFIRs than non-tumorigenic breast cell line MCF10A. MDA-MB-231, MDA-MB-436, and MCF7 cell lines have a significantly higher percent of cell population that display expression of LLT1 than MCF10A. *One-way ANOVA confirmed differences in the means of MFIRs and percent of cell populations LLT1$^+$ at $p < 0.05$ for all cell lines. Dunnett's multiple comparison post-hoc was used to test for statistical significance of MFIRs and percent of cell populations LLT1$^+$ of each cell line compared to the control MCF10A (* $p < 0.05$, ** $p < 0.01$).*

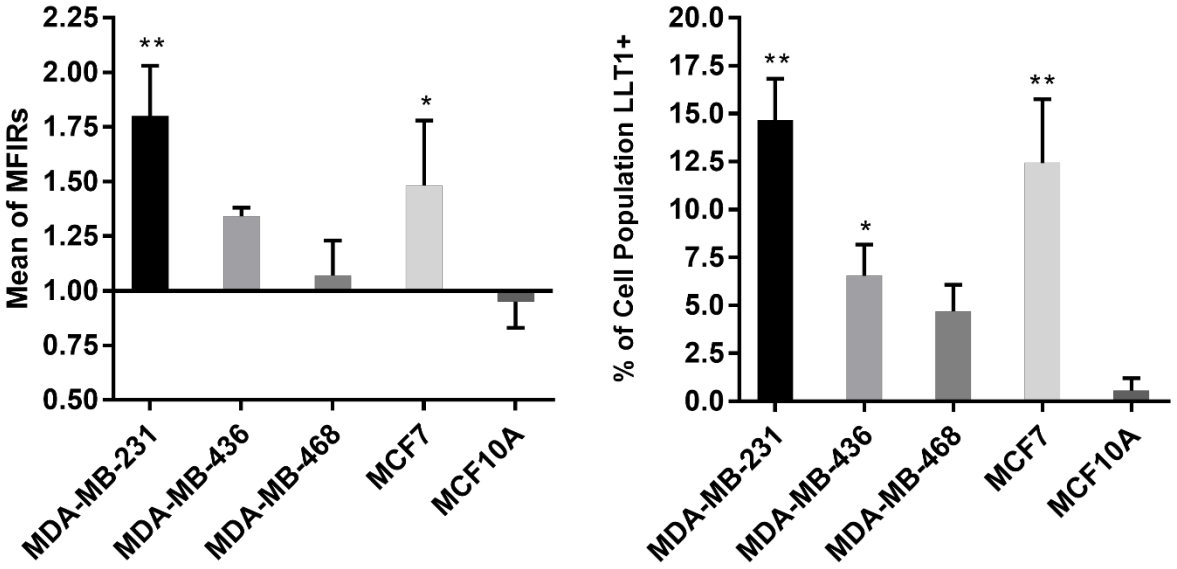
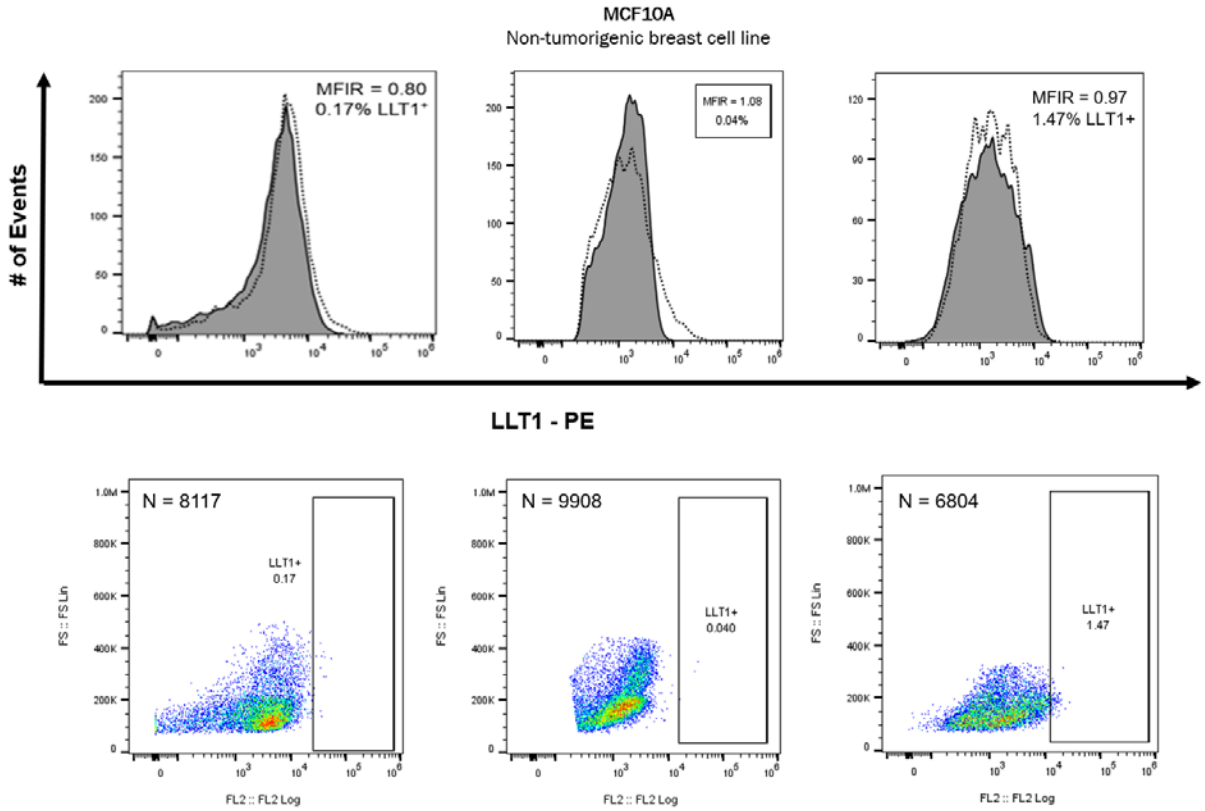


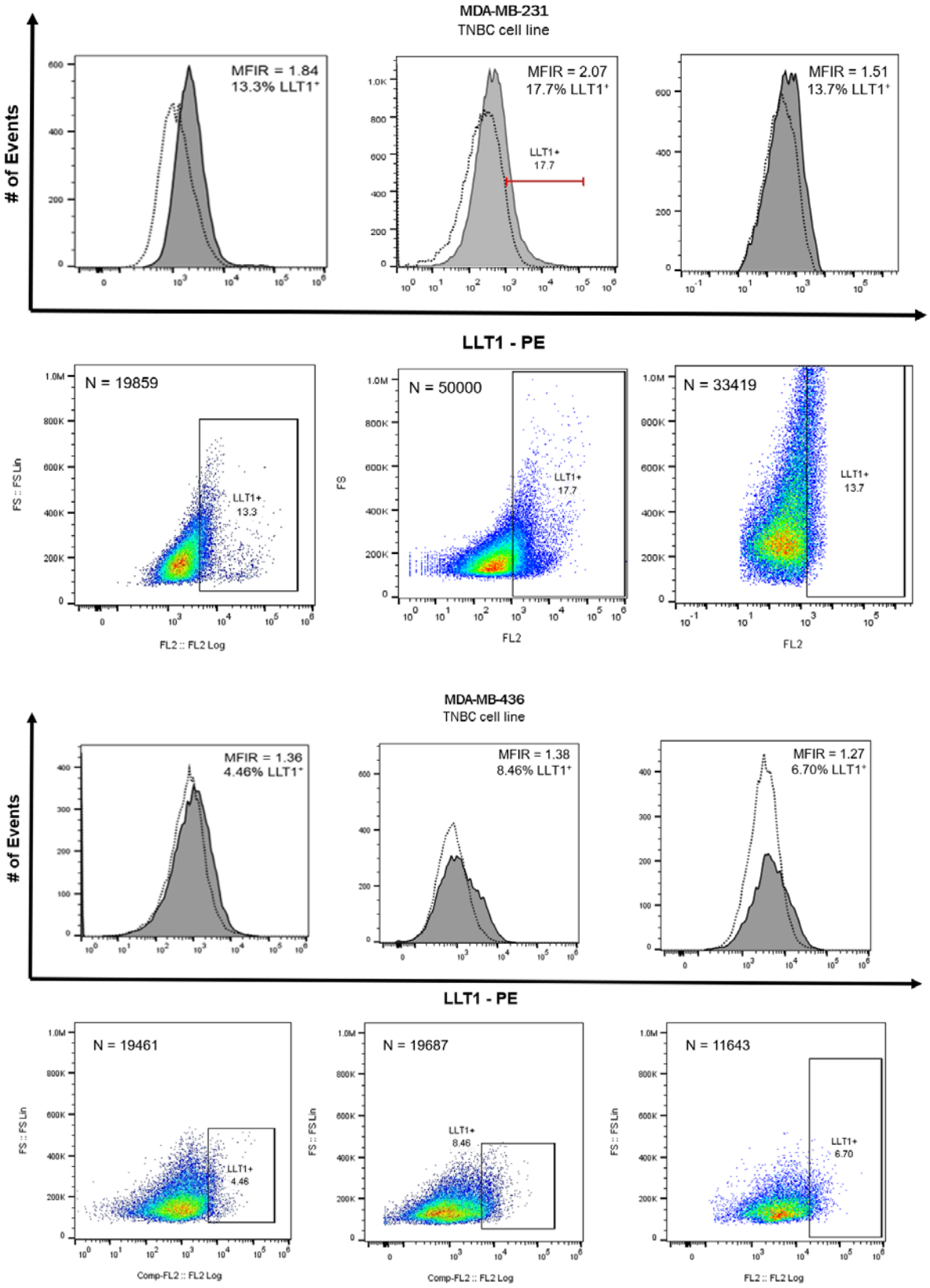
Figure 3.3

Three independent flow cytometry experiments of cell lines tested for cell surface LLT1 expression.

To obtain the mean of MFIRs (median fluorescence intensity ratios) and mean percent of cell population $LLT1^+$, three independent experiments of each cell line MDA-MB-231, MDA-MB-436, MDA-MB-468, & MCF7 as well as two independent experiments of MCF10A were performed. These MFIRs and % $LLT1^+$ values obtained make up the mean of MFIRs and mean % of cell population $LLT1^+$ presented in figure 3.2 and table 3.1.

(Continuing parts of the figure from below are in the next page and forward.)




MDA-MB-231
TNBC cell line
MFIR = 1.84
13.3% LLT1+
MFIR = 2.07
17.7% LLT1+
LLT1+
17.7
MFIR = 1.51
13.7% LLT1+
# of Events
LLT1 - PE
N = 19859
LLT1+
13.3
FS :: FS Lin
FL2 :: FL2 Log
N = 50000
LLT1+
17.7
FS
FL2
N = 33419
LLT1+
13.7
FL2
MDA-MB-436
TNBC cell line
MFIR = 1.36
4.46% LLT1+
MFIR = 1.38
8.46% LLT1+
MFIR = 1.27
6.70% LLT1+
# of Events
LLT1 - PE
N = 19461
LLT1+
4.46
FS :: FS Lin
Comp-FL2 :: FL2 Log
N = 19687
LLT1+
8.46
FS :: FS Lin
Comp-FL2 :: FL2 Log
N = 11643
LLT1+
6.70
FS :: FS Lin
FL2 :: FL2 Log

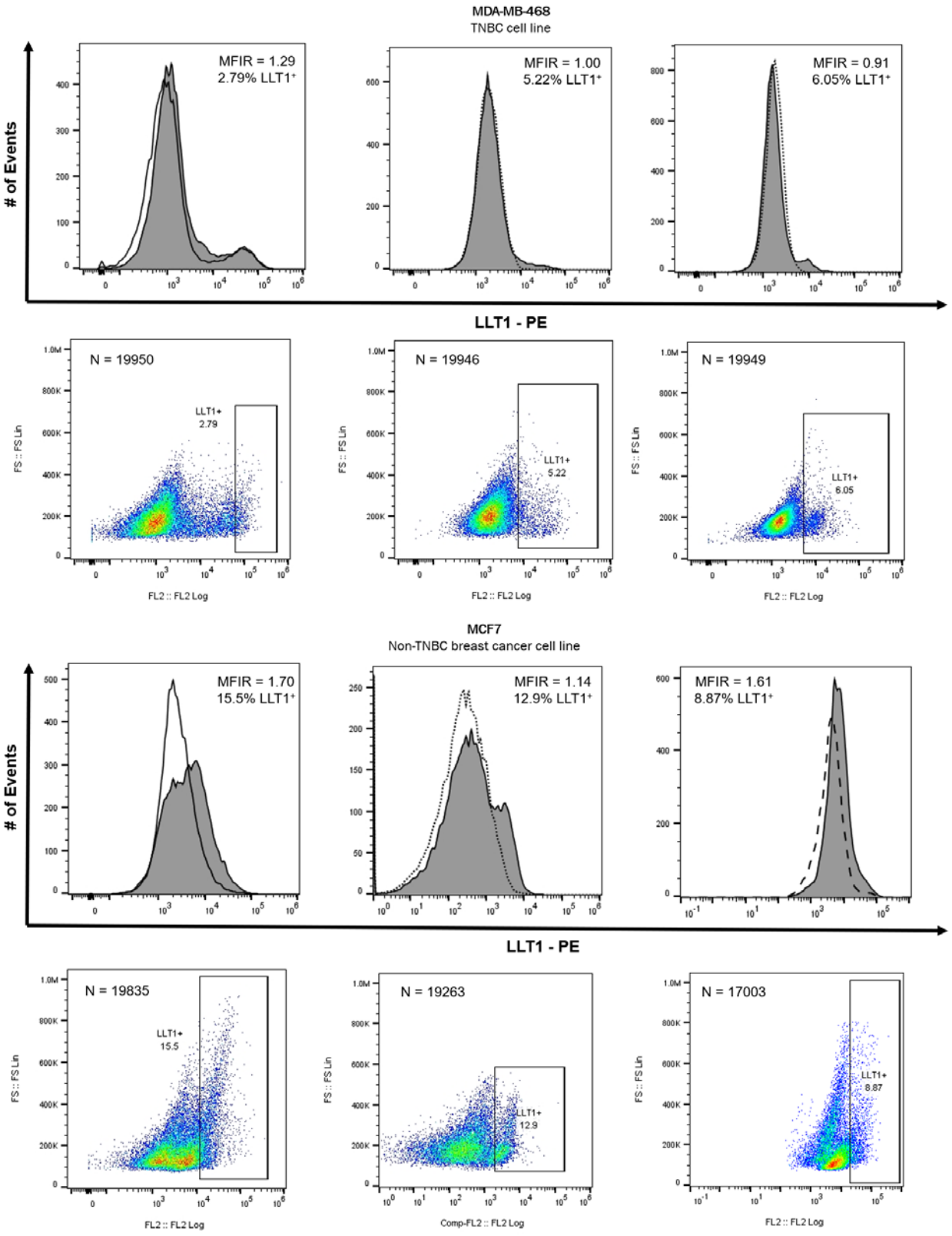

MDA-MB-468
TNBC cell line
MFIR = 1.29
2.79% LLT1+
MFIR = 1.00
5.22% LLT1+
MFIR = 0.91
6.05% LLT1+
# of Events
LLT1 - PE
N = 19950
LLT1+
2.79
N = 19946
LLT1+
5.22
N = 19949
LLT1+
6.05
FS :: FS Lin
FL2 :: FL2 Log
MCF7
Non-TNBC breast cancer cell line
MFIR = 1.70
15.5% LLT1+
MFIR = 1.14
12.9% LLT1+
MFIR = 1.61
8.87% LLT1+
# of Events
LLT1 - PE
N = 19835
LLT1+
15.5
N = 19263
LLT1+
12.9
N = 17003
LLT1+
8.87
FS :: FS Lin
FL2 :: FL2 Log
Comp-FL2 :: FL2 Log

Table 3.1

Means of MFIRs, Percent of Cell Population LLT1$^+$, and Cell Population Size Tested.

Of the three TNBC cell lines, MDA-MB-231 displayed the highest average median fluorescence intensity ratio (MFIRs) and percent of cell population with cell surface expression of LLT1. Non-TNBC cell line MCF7 showed the highest expression of LLT1 out of all cell lines tested. All breast cancer cell lines average MFIRs and percent of cell population have higher expression of LLT1 than non-tumorigenic breast cell line MCF10A.

	Means of MFIRs ± SD	Mean % LLT1$^+$ ± SD	Mean Number of Cells in Population Tested	Number of Independent Experiments
MDA-MB-231 LLT1$^+$	1.80 ± 0.23	14.67 ± 2.15	34414	3
MDA-MB-436 LLT1$^+$	1.34 ± 0.04	6.54 ± 1.64	16930	3
MDA-MB-468 LLT1$^+$	1.07 ± 0.16	4.69 ± 1.38	19948	3
MCF7 LLT1$^+$	1.48 ± 0.30	12.42 ± 3.34	18719	3
MCF10A LLT1$^+$	0.95 ± 0.12	0.56 ± 0.65	8276	3

Figure 3.4

Cell surface expression of LLT1 detected by confocal microscopy on TNBCs.

TNBC cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and non-TNBC cell line MCF7 were fixed, blocked with human Fc fragment, and stained with anti-human LLT1-PE antibody. Cell surface expression of LLT1 was detected on all cell lines tested. Cells were examined with a Zeiss LSM 510 Confocal Laser Microscope at 40x objective. Scale bar is 10 µm.

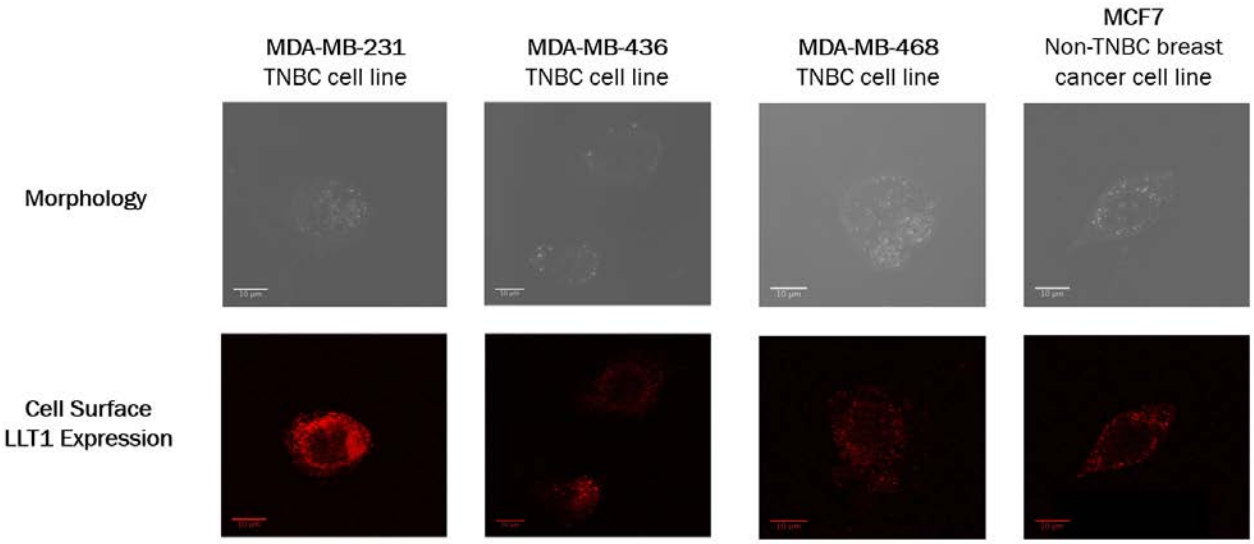


Figure 3.5

Combined cell surface and intracellular expression of LLT1 on TNBCs.

(A) TNBC cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and non-TNBC cell line MCF7 were fixed, blocked with human Fc fragment, permeabilized with 0.2% X-Triton 100, and stained with anti-human LLT1-PE antibody. Total LLT1 was observed by confocal microscopy on all cell lines tested. Cells were examined at 40x objective. Scale bar is 10 µm.

(B) Cell lines were examined for total LLT1 expression on a western blot. MDA-MB-468 and MCF10A displayed highest total expression of LLT1 compared to other cell lines. β-actin served as a loading control.

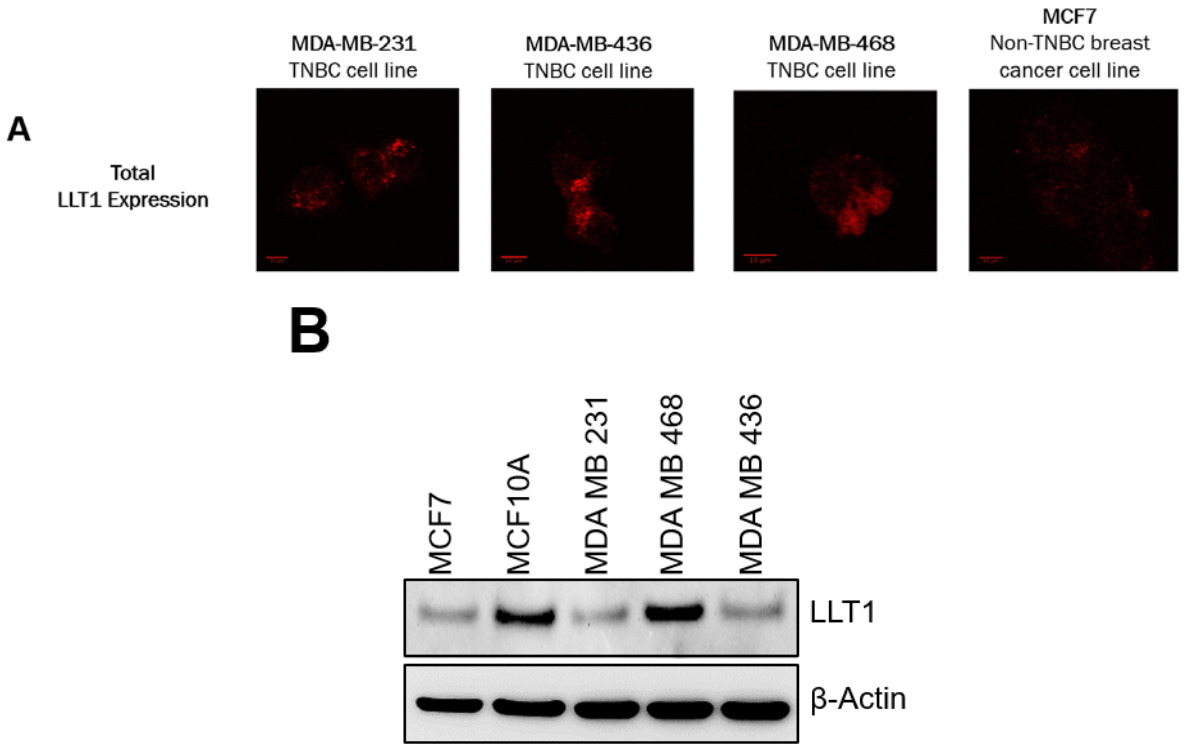
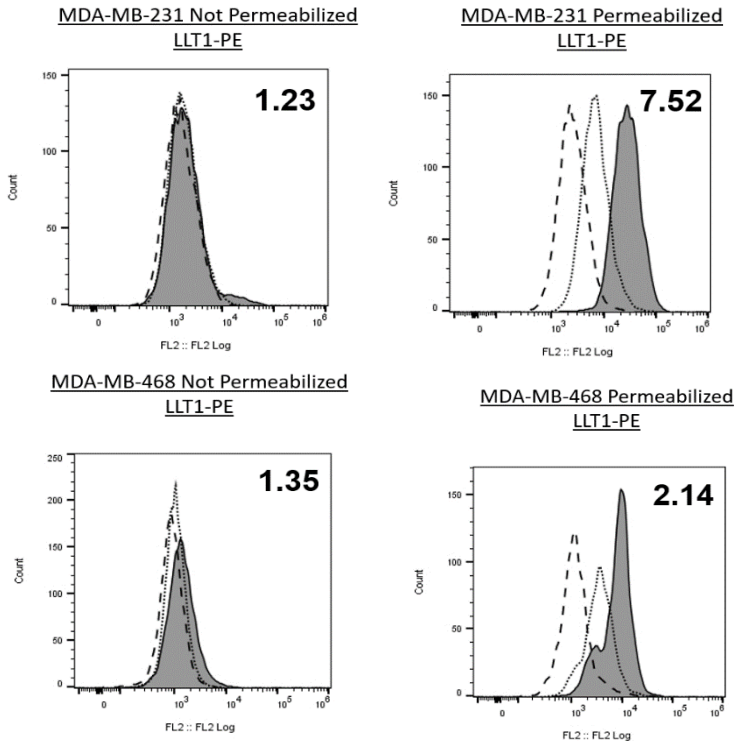


Figure 3.6

Flow cytometry confirms intracellular expression of LLT1 on TNBCs.

TNBC cell lines MDA-MB-231 and MDA-MB-468 were fixed with 2% paraformaldehyde and then treated with 1X BD Perm/Wash buffer for permeabilization. Cells were then stained with LLT1-PE antibodies (gray shade), isotype IgG_1-PE antibodies (dotted line white area), or not stained with any antibodies (dashed line white area) and then was quantified by flow cytometry analysis. Cells not permeabilized were not fixed and not treated with perm/wash buffer. Both cell lines treated with permeabilization agent displayed a higher expression of LLT1 compared to cell lines not treated with permeabilization. This indicates that there is LLT1 expression intracellular and at the cell membrane on these two TNBC cell lines.



3.1.2 Blocking Lectin-like Transcript-1 (LLT1) on Triple-Negative Breast Cancer Cells with Anti-LLT1 Antibodies Induces Natural Killer Cell Activation

This section fulfills specific aim 2 - sub aim 2.1: Blocking interaction of LLT1 and PCNA on triple-negative breast cancer cells and its natural killer cell receptors with antibodies targeting each ligand enhances cytolytic targeting against these TNBCs (Figure 1.7).

Previous flow cytometry analysis and immunofluorescent studies (section 3.1.2) demonstrated that there is higher cell surface expression of LLT1 on TNBC cell lines than non-tumorigenic breast cell line MCF10A with MDA-MB-231 showing a statistically significant difference in LLT1 expression compared to MCF10A (Figure 3.2). Furthermore, confocal microscopy studies confirmed expression of LLT1 for cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 at the cell surface and intracellular locations (Figure 3.4, 3.5). Western blot analysis confirmed total protein expression of LLT1, but interestingly MCF10A and MDA-MB-468 showed the greatest total expression of LLT1 (Figure 3.5B) but showed the least expression of LLT1 at the cell surface based on flow cytometry analysis (Figure 3.3). In summary, LLT1 expression at the cell surface was confirmed through these three methods on TNBCs. Furthermore, there was greater LLT1 expression on TNBCs than MCF10A. The next step was to utilize antibodies targeting the LLT1 ligand on TNBCs and MCF10A and determine if blocking this interaction will enhance killing by primary NK cells.

We utilized the chromium-release cytotoxicity assay which is known as a gold standard assay that quantifies the percent of target cells being killed by immune effector cells of one's choice. In this case, we labeled two TNBC cell lines MDA-MB-231 and MDA-MB-436 and MCF10A with radioactive chromium-51, then treated these cells with human anti-LLT1 antibodies. We used primary NK cells isolated from peripheral blood mononuclear cells derived

from whole blood. Fc receptors on primary NK cells were blocked with human Fc fragment in order to prevent antibody-dependent cell-mediated cytotoxicity (ADCC) from occurring. TNBCs treated with the anti-LLT1 antibodies or isotype control were then co-incubated with NK cells at effector-to-target ratios (E:T) of 25:1, 5:1, and 1:1 for 3.5 hours. Specific lysis was quantified and determined.

We first targeted LLT1 on MDA-MB-231 and MCF10A cells through treatment with 1 µg of anti-LLT1 antibody (Figure 3.7). At 25:1 E:T ratio, there is a statistically significant difference in percent specific lysis of MDA-MB-231 cells between cells treated with anti-LLT1 antibodies compared to cells treated with IgG isotype antibody and cells not treated with antibodies (Figure 3.7; ** $p < 0.01$ compared to isotype control, # $p < 0.05$ compared to no antibodies). At 25:1, 19.708% of MDA-MB-231 treated with anti-LLT1 antibodies were killed by primary NK cells compared to 2.02% of MDA-MB-231 cells treated with IgG isotype antibody and 4.17% of MDA-MB-231 cells not treated with any antibodies. There is a distinct difference between the percent specific lysis of MDA-MB-231 cells treated with anti-LLT1 antibodies compared to non-tumorigenic breast MCF10A cells treated with the same anti-LLT1 antibody at the 25:1 E:T ratio. At 25:1, 7.54% of MCF10A cells treated with anti-LLT1 antibody were killed by primary NK cells compared to 2.956% of MCF10A cells treated with IgG isotype antibody (Figure 3.7; * $p < 0.05$ compared to isotype control). At 5:1 E:T ratio, there is a statistically significant difference in percent specific lysis of MDA-MB-231 cells treated with anti-LLT1 antibodies compared to both cells treated with isotype antibodies and not treated with antibodies (Figure 3.7; ** $p < 0.01$ compared to isotype control, # $p < 0.05$ compared to no antibodies). There is not a significant difference in MDA-MB-231 cells and MCF10A cells

treated with anti-LLT1 antibodies killed compared to the two cell lines treated with IgG isotype antibodies at 1:1 E:T ratio.

Next, we treated MDA-MB-231 cells with anti-LLT1 antibodies at four different concentrations of antibodies (Figure 3.8). We treated with MDA-MB-231 cells with anti-LLT1 antibodies at concentrations of 1 µg, 0.1 µg, 0.01 µg, and 0.001 µg. There is a noticeable decrease in percent of MDA-MB-231 cells killed by primary NK cells as the concentration of anti-LLT1 antibodies decreases at 25:1 E:T ratio. At 25:1, MDA-MB-231 cells treated with 1 µg of anti-LLT1 antibodies exhibited the highest percent of its cells being killed at 71.51% compared to 49% of MDA-MB-231 cells treated with IgG isotype antibodies (Figure 3.8). There is not a statistical significant difference between these two specific groups mentioned based on p-value of 0.05; however, the difference is well distinguished between the two groups with its p-value calculated to be 0.08. Within the 25:1 E:T ratio, there is a difference between MDA-MB-231 cells treated with 0.1 µg, 0.01 µg, and 0.001 µg anti-LLT1 antibodies and cells treated with IgG isotype antibodies. As the concentration of the anti-LLT1 antibodies decreases, the percent specific lysis of MDA-MB-231 cells also decreases at 25:1 E:T ratio from 71.51% [1 µg], 69.28% [0.1 µg], 68.24% [0.01 µg], and 63.11% [0.001 µg]. At 5:1 E:T ratio, there is a difference between MDA-MB-231 cells treated with 1 µg of anti-LLT1 antibodies killed by NK cells compared to cells treated with isotype antibodies. At 5:1 E:T ratio, 42.15% of MDA-MB-231 cells treated with 1 µg of anti-LLT1 antibodies were killed versus 33.15% of cells treated with isotype control. At 5:1, there is not a significant difference in percent lysis of MDA-MB-231 cells at 0.1 µg, 0.01 µg, and 0.01 µg. At 1:1, there is also not a difference observed of MDA-MB-231 cells killed at all antibody concentrations and its isotype control.

We also have tested the effects of blocking LLT1-NKRP1A interaction by treating another TNBC cell line MDA-MB-436 with anti-LLT1 antibodies. There was a lower percentage of MDA-MB-436 cells killed by NK cells than MDA-MB-231 cells at all the E:T ratios. The lower percentage of MDA-MB-436 cells killed can be attributed to the lower expression of cell surface LLT1 on this cell line in contrast to MDA-MB-231 LLT1 expression as shown in flow cytometry analysis (Section 3.1.1). At 25:1 E:T ratio, 8.39% of MDA-MB-436 cells treated with anti-LLT1 antibodies were killed while 4.75% of cells treated with isotype antibodies were killed (Figure 3.7). There was a statistical significant difference between the percent lysis of MDA-MB-436 cells treated with anti-LLT1 antibodies and cells treated with isotype antibodies (Figure 3.7, * $p < 0.05$ compared to isotype control). At 5:1 E:T ratio, although no statistical significant difference was observed, 3.26% of anti-LLT1 treated MDA-MB-436 cells were killed while there was negligible percent lysis of MDA-MB-436 cells treated with isotype antibodies (Figure 3.7). There was also negligible killing of MDA-MB-468 cells when treated with either anti-LTL1 antibodies or isotype antibodies due to the low expression of cell surface LLT1 (Figure 3.7).

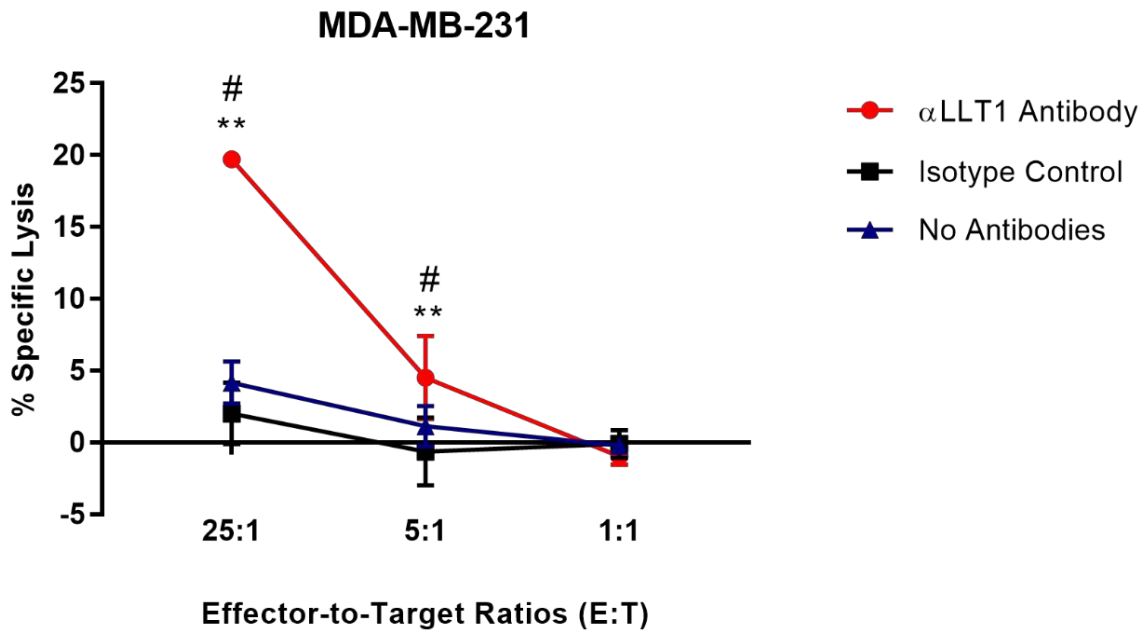
In summary, treating MDA-MB-231 and MDA-MB-436 cells with anti-LLT1 antibodies allowed an increase of killing by primary NK cells. Furthermore, MDA-MB-231 cells treated with anti-LLT1 antibodies had a greater percent of cells killed compared to non-tumorigenic breast cell line MCF10A treated with anti-LLT1 antibodies. The greater percentage of MDA-MB-231 cells killed when targeting LLT1 with antibodies than MCF10A cells is consistent with flow cytometry analysis demonstrating that MDA-MB-231 cells had a statistically significant higher expression of cell surface of LLT1 in contrast to LLT1 cell surface expression on MCF10A. These results show that there is a difference in percent of TNBCs killed versus non-

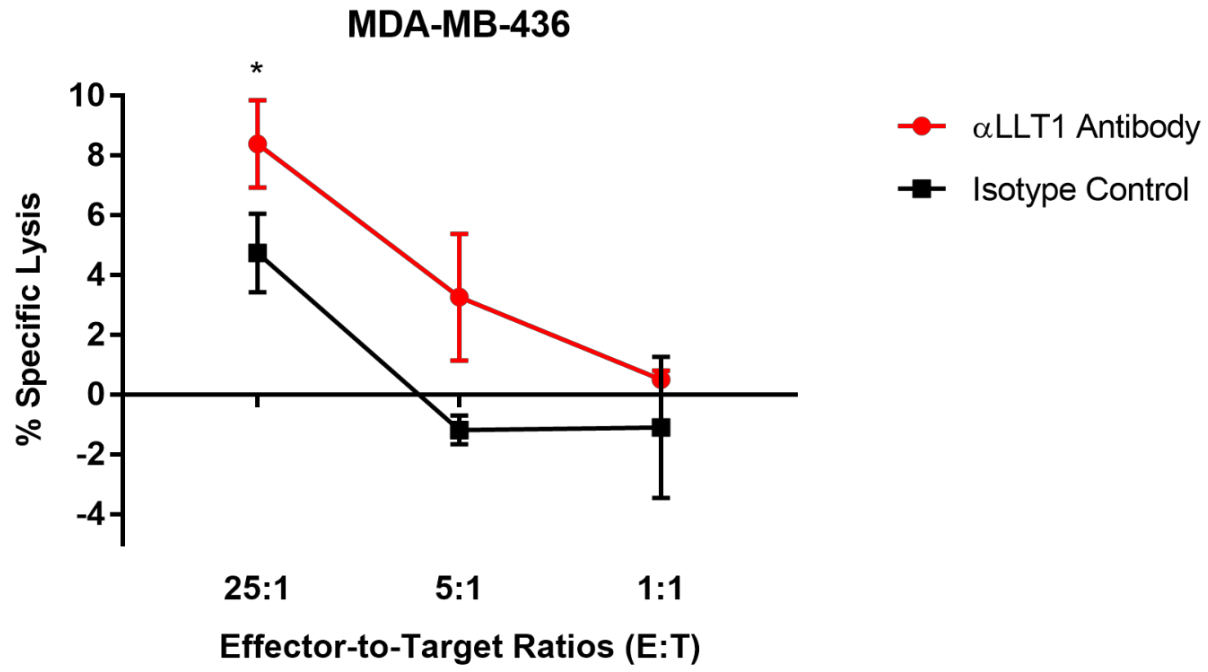
tumorigenic breast cells killed when targeting LLT1 with antibodies. The lower percent of MCF10A being killed when targeting LLT1 with anti-LLT1 antibodies supports that LLT1 may serve as a possible target that would favor killing TNBCs while minimizing killing healthy breast cells. Treating cells with anti-LLT1 antibodies blocks the interaction of LLT1 on TNBCs with NKRP1A (CD161) receptor on NK cells. Blocking this interaction directly with anti-LLT1 antibodies prevents inhibitory signals from LLT1-NKRP1A interaction from being sent to the NK cells. This would allow lower overall inhibitory signals from being sent and favors a net activation signal to be sent to NK cells which then targets these treated TNBCs.

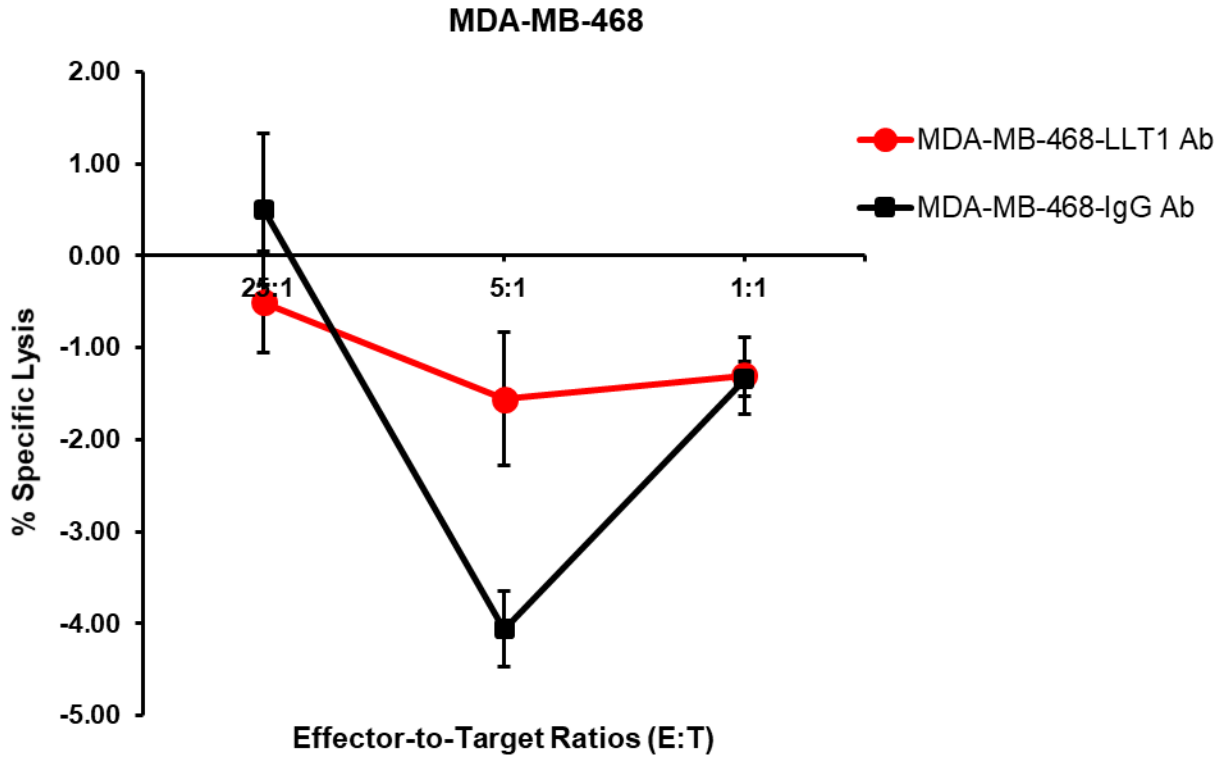
Figure 3.7

Blocking LLT1 with antibodies on TNBCs enhanced killing by primary NK cells.

TNBC cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and non-tumorigenic breast cell line MCF10A were treated with either anti-human LLT1 antibodies (αLLT1) or isotype control antibodies. Cells were labeled with ^{51}Cr and then were co-incubated with primary NK cells isolated from PBMCs derived from whole blood of healthy volunteers at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 3.5 hours. Specific lysis of labeled cells was subsequently quantified and calculated. *Student paired t-test was utilized to test for statistical significance between cells treated with anti-LLT1 antibody versus isotype control and 'no antibodies' control within each E:T ratio. MDA-MB-231: ** $p < 0.01$ compared to isotype control, # $p < 0.05$ compared to 'no antibodies' control; MCF10A and MDA-MB-436: * $p < 0.05$ compared to isotype control.*




MDA-MB-436
*
10
8
6
4
2
0
-2
-4
% Specific Lysis
25:1
5:1
1:1
Effector-to-Target Ratios (E:T)
αLLT1 Antibody
Isotype Control


MDA-MB-468
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
% Specific Lysis
25:1
5:1
1:1
Effector-to-Target Ratios (E:T)
MDA-MB-468-LLT1 Ab
MDA-MB-468-IgG Ab

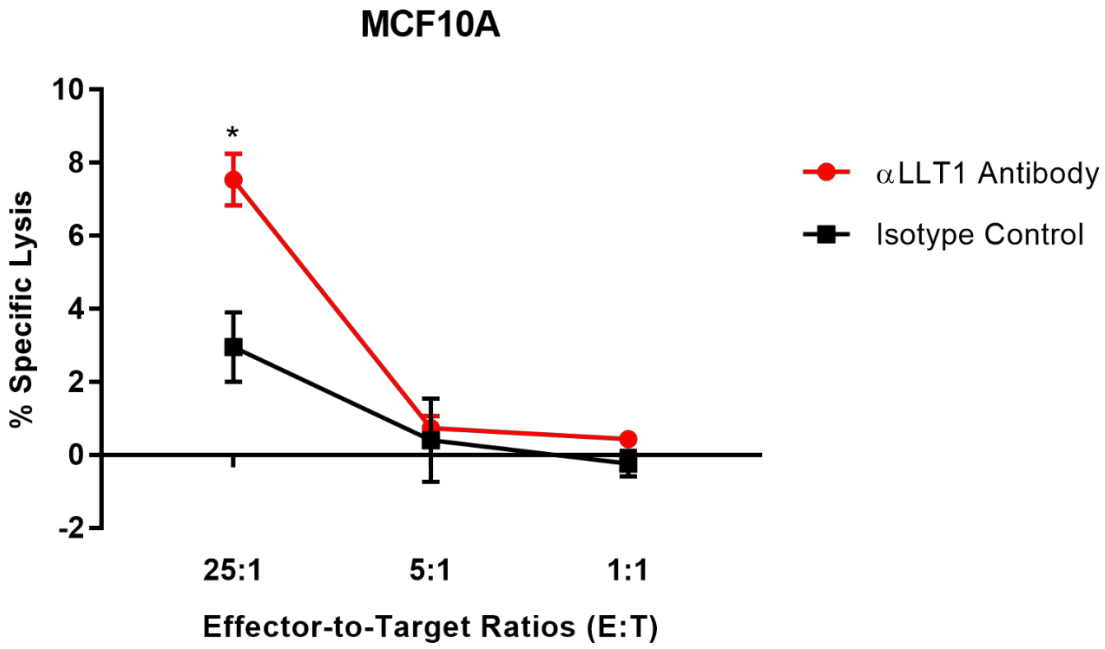
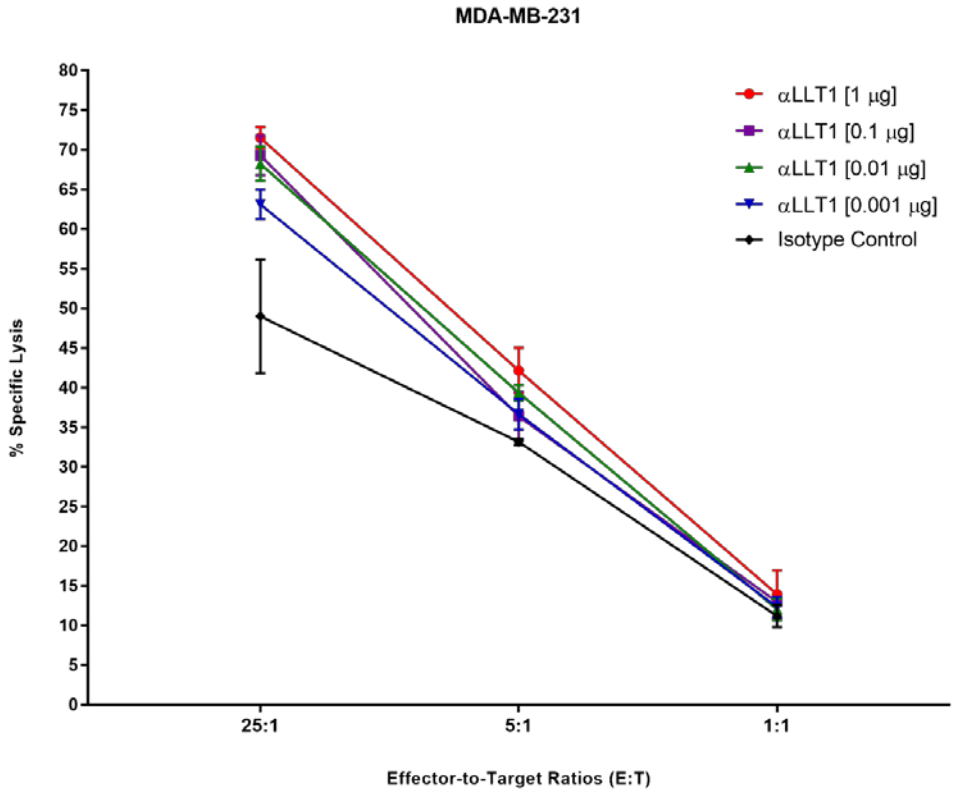

MCF10A
10
8
6
4
2
0
-2
% Specific Lysis
*
25:1
5:1
1:1
Effector-to-Target Ratios (E:T)
αLLT1 Antibody
Isotype Control

Figure 3.8

Anti-LLT1 antibody dose-dependent treatment on TNBC MDA-MB-231 demonstrates enhanced killing by primary NK cells.

TNBC cell line MDA-MB-231 was treated with either anti-human LLT1 antibodies (αLLT1) or isotype control antibodies. MDA-MB-231 cells were treated with anti-LLT1 antibodies at four different concentrations. There is greatest lysis of TNBCs when treated with anti-LLT1 antibodies at 1 µg per well compared to other concentrations and isotype IgG control (P-value = 0.08). Cells were labeled with ^{51}Cr and then were co-incubated with primary NK cells isolated from PBMCs derived from whole blood of healthy volunteers at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 3.5 hours. Specific lysis of labeled cells was subsequently quantified and calculated.



3.1.3 Gene Knockdown of LLT1 Increases Susceptibility of Triple-Negative Breast Cancer Cells to Killing by Natural Killer Cells

This section fulfills specific aim 2 - sub aim 2.2: Blocking interaction of LLT1 on triple-negative breast cancer cells and its natural killer cell receptors by downregulation of LLT1 through knockdown of ligand enhances cytolytic targeting against these TNBCs (Figure 1.7).

Confirmation of LLT1 Knockdown on TNBCs.

We utilized a lipid-mediated small interference RNA (siRNA) complex to target the *LLT1* gene on TNBC cell line MDA-MB-436. The complex consists of either scramble siRNA or LLT1 siRNA within a lipid-membrane vesicle which is designed to attach to the lipid bilayer membrane of MDA-MB-436 cells to prepare for the next step, which is endocytosis. After the vesicle attaches to the membrane of TNBC cells, the vesicle gets encapsulated by the TNBC cell membrane lipid bilayer and buds off within the cytoplasm to form an endosome. At this point, the endosome contains the lipid-vesicle complex containing the siRNA. Within the endosome environment, the initial pH immediately after endosome formation is approximately 7.0. As the endosome further remains in the cytoplasm of the TNBC cell, the endosome environment becomes acidic which then allows degradation of the endosome and lipid-vesicle membranes thus releasing the siRNA into the cytoplasm. The released siRNA in the cytoplasm binds to the RNA-induced silencing complex (RISC) in which one strand of the double-stranded siRNA gets degraded while the other strand of the siRNA serves as the guide RNA strand that binds to complementary regions of the mRNA that comprises the *LLT1* gene. Upon binding to those regions of the *LLT1* gene, the *LLT1* gene transcription is now suppressed which would then downregulate expression of LLT1 at the cell surface of TNBCs.

MDA-MB-436 cells were transfected with either 5 μM scramble siRNA control or LLT1 siRNA for a period of 63 hours in a 96-well plate. To confirm downregulation of cell surface expression of LLT1, flow cytometry analysis was used to detect the difference in expression of LLT1 between MDA-MB-436 cells treated with scramble siRNA control and cells treated with LLT1 siRNA. MDA-MB-436 cells transfected with LLT1 siRNA displayed a lower median fluorescence intensity ratio (MFIR) at 0.31 than MDA-MB-436 cells transfected with scramble siRNA which showed a MFIR of 1.09 (Figure 3.9A). These results demonstrated that cell surface expression of LLT1 was downregulated thus serving as another method of blocking LLT1 interaction with NKRP1A receptor on NK cells. Downregulation of LLT1 at the cell surface prevents LLT1-NKRP1A interaction which suppresses inhibitory signals from being sent to the NK cells. This particular method of blocking LLT1-NKRP1A was next tested in the chromium-release cytotoxicity assay.

Knockdown of Cell Surface LLT1 on TNBCs Enhances Killing by NK cells.

Transfected MDA-MB-436 cells with either scramble siRNA or LLT1 siRNA were co-incubated with IL-2 stimulated natural killer cells (lymphokine activated killer cells) for 3.5 hours. MDA-MB-436 transfected with LLT1 siRNA had a higher percent specific lysis at both 25:1 and 5:1 E:T ratios in contrast to its scramble siRNA transfected cells (Figure 3.9B). At 25:1 E:T ratio, 63.38% of MDA-MB-436 LLT1 siRNA-transfected cells were killed by NK cells compared to 42.18% of MDA-MB-436 scramble siRNA-transfected cells killed (Figure 3.9B, $p = 0.07$ compared to scramble siRNA control). At 5:1 E:T ratio, 21.83% of MDA-MB-436 LLT1 siRNA-transfected were killed compared to 18.87% of scramble siRNA-transfected cells.

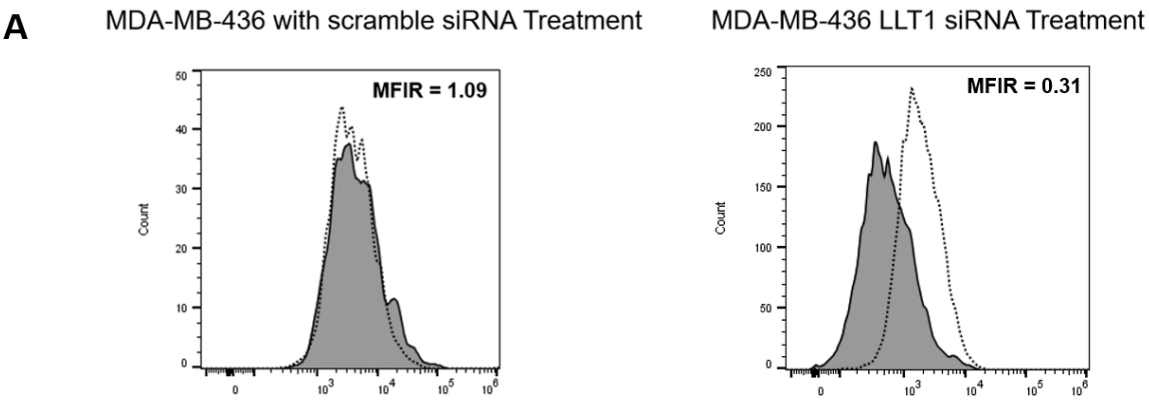
In summary, transfecting MDA-MB-436 cells with siRNA targeting the *LLT1* gene decreases expression of LLT1 at the cell surface. Decreasing the expression of cell surface LLT1 prevents interaction of LLT1 with NKRP1A on primary NK cells. Results show that knockdown of LLT1 enhances killing of TNBCs by NK cells which provides another method of blocking LLT1-NKRP1A interaction.

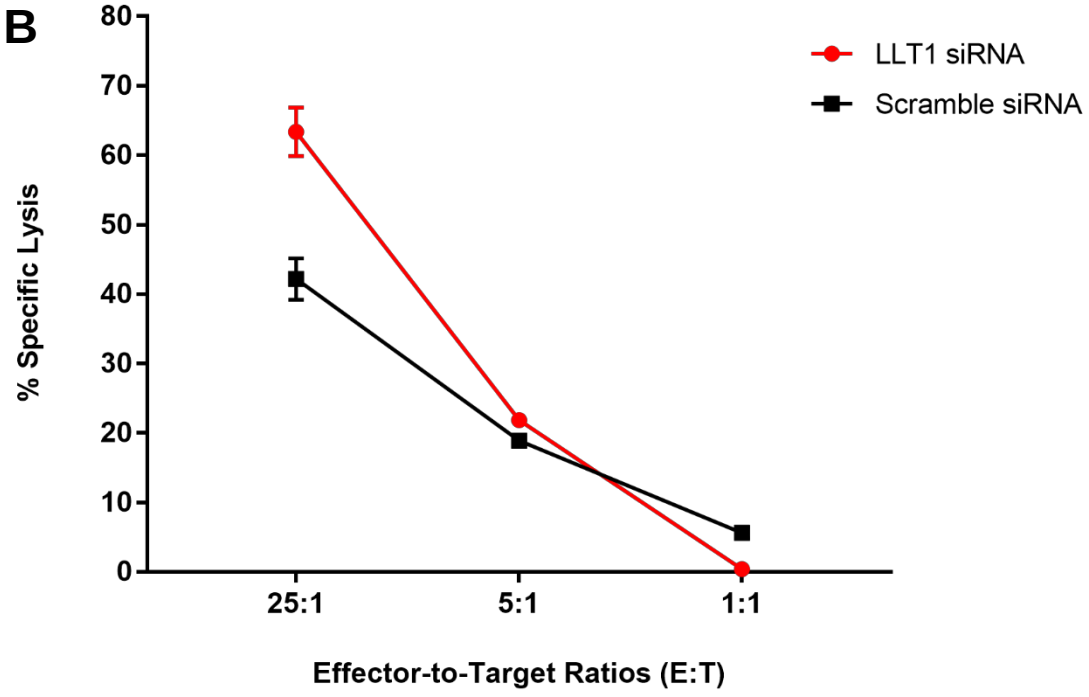
Figure 3.9

Knockdown of cell surface LLT1 on TNBCs increases killing by NK cells.

TNBC cell line MDA-MB-436 was transfected with either 5 μM scramble siRNA control or LLT1 siRNA for 63 hours in 96-well plates. Final siRNA concentration in culture with media is 25 nM siRNA. Knockdown of cell surface LLT1 was confirmed by flow cytometry after transfection (A) in which the median fluorescence intensity ratio (MFIR) for cells treated with LLT1 siRNA was lower (0.31) compared to the MFIR of cells treated with scramble siRNA control (1.09). After confirming knockdown of LLT1 at the cell surface of MDA-MB-436 cells, these transfected cells were radiolabeled with ^{51}Cr and co-incubated with primary NK cells for 3.5 hours (B). MDA-MB-436 cells with LLT1 knockdown had a higher percentage of lysis at both 25:1 and 5:1 ratios in contrast to its scramble siRNA treated cells *($p = 0.07$ at 25:1 by student paired t-test)*

(Figure next page)


A
MDA-MB-436 with scramble siRNA Treatment
MDA-MB-436 LLT1 siRNA Treatment
MFIR = 1.09
MFIR = 0.31
Count
Count


B
% Specific Lysis
Effector-to-Target Ratios (E:T)
25:1
5:1
1:1
LLT1 siRNA
Scramble siRNA

3.2 EXPRESSION AND FUNCTION OF PCNA ON TNBCs

Introduction

Proliferating Cell Nuclear Antigen (PCNA) is an inhibitory ligand that when expressed on cells interacts with NKp44, an activating receptor, on NK cells. The PCNA-NKp44 interaction transduces an inhibitory signal to the natural killer cell [121]. It is known that PCNA contributes to the role of DNA replication as a nuclear accessory protein by stabilizing the replication fork, recruiting and exchanging polymerases at the replication fork, and cell cycle regulation [116]. It has been studied that PCNA was expressed on cell surface of pancreatic, breast, melanoma, lymphoma, and glioblastoma cells [121]. Interestingly, PCNA colocalizes with human leukocyte antigen I (HLA I) which shows that the association of PCNA and HLA I contributes to the inhibition of NK cell function when PCNA interacts with NKp44 [90].

Thus, we introduce another cancer model to study using triple-negative breast cancer cells, because PCNA expression at the cell surface and its function has not been looked at from a cancer immunotherapeutic perspective. We have found that there was a higher percent of TNBCs from cell lines MDA-MB-231 and MDA-MB-436 that show expression of cell surface PCNA than both TNBC cell line MDA-MB-468 and non-tumorigenic breast cell line MCF10A. We have demonstrated that blocking PCNA-NKp44 interaction enhances killing of TNBCs by primary NK cells. MCF10A showed a higher expression of cell surface PCNA than its cell surface LLT1 based on flow cytometry analysis, which indicates that more studies will need to be done to confirm cell surface expression of PCNA on non-tumorigenic breast cells and if PCNA can be used as a possible target.

Results

3.2.1 Proliferating Cell Nuclear Antigen (PCNA) is Expressed on Triple-Negative Breast Cancer Cells and Inhibits Natural Killer Cell Activation

This section fulfills specific aim 1: Investigate the expression of LLT1 and PCNA on triple-negative breast cancer cells. (Figure 1.6)

Cell Surface Expression of PCNA on TNBCs by Flow Cytometry Analysis.

Flow cytometry analysis was performed to detect and quantify expression of PCNA at the cell surface of TNBCs and non-tumorigenic breast cell lines. All cell lines tested (MDA-MB-231, MDA-MB-436, MDA-MB-468, MCF7, and MCF10A) were treated with a human Fc fragment in order to block potential Fc receptors or Fc-receptor ligands that could be present on cell lines being tested. Blocking Fc receptors or Fc ligands will prevent or minimize non-specific binding of anti-PCNA antibodies. After treating cells with a Fc fragment, cells were stained with either conjugated anti-human PCNA-PE antibodies or isotype IgG_{2a}-PE antibodies. Cell surface expression of PCNA was quantified and represented by median fluorescence intensity ratios (MFIR) and percentage of cells positive for PCNA. For this case, MFIR is defined as the MFI of the cell population stained with the anti-PCNA-PE antibody divided by the MFI of the cell population stained with isotype IgG_{2a}-PE antibodies. Cells were gated on a single live cell population because only one antibody was used in staining the cells which does not require compensation.

Three TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were tested for cell surface PCNA expression and were then compared to non-TNBC breast cancer cell line MCF7 and non-tumorigenic breast cell line MCF10A. Three independent experiments testing for

expression of cell surface PCNA were tested for each cell line used. MFIRs and percent of cells that are $PCNA^+$ expression at the cell surface were averaged. Histograms and dot plots displaying the MFIRs and percent of cell population that are $PCNA^+$ in each independent experiment of each cell line that form the mean of MFIR and percent of cell population $PCNA^+$ values in figure 3.11 and table 3.2 are shown in figure 3.10.

MDA-MB-436 showed the highest cell surface PCNA expression since its mean of MFIRs and mean percent of its cell population $PCNA^+$ are higher than all other cell lines (Table 3.2, Figure 3.11). MDA-MB-436 mean of MFIRs and percent $PCNA^+$ are 1.89 and 23.2% respectively. Although the MFIRs for MDA-MB-436 are higher than the other cell lines, MCF7 displayed relatively similar expression of PCNA at mean MFIR of 1.85 and percent $PCNA^+$ of 14.53% compared to MDA-MB-436 and MCF10A. It is interesting to note that MCF10A displayed a high mean of MFIR at 1.75, but a low percentage of the cell population that show positive expression for PCNA at 3.65%. Based on the percent of the cell population that was $PCNA^+$ for the MCF10A, it still shows the lowest expression of cell surface PCNA in comparison with the TNBCs. The concern is that MCF10A cells show expression of PCNA at 3.65% which may indicate that further studies would need to be performed to check if targeting cell surface PCNA would serve as a possible target. However, only one independent experiment checking for PCNA expression on MCF10A was performed. A repeat of testing for PCNA expression of MCF10A will need to be conducted.

Hence, these independent flow cytometry experiments under the same culture conditions and stained with the same anti-PCNA-PE antibodies have consistently shown that TNBCs, especially MDA-MB-436, show higher cell surface expression of PCNA than non-tumorigenic breast cell line MCF10A based on percent of cell population that are $PCNA^+$. These results have

further demonstrated that PCNA may serve as a possible target of interest for antibody-blocking treatment on TNBCs, but additional studies need to further address and clarify if there is PCNA cell surface expression on normal breast tissue through immunohistochemical staining and analyzing for cell surface PCNA expression on additional non-tumorigenic breast cell lines besides MCF10A.

Cell Surface, Cytoplasmic, and Nuclear PCNA Expression by Subcellular Protein Fractionation.

Flow cytometry analysis testing for PCNA expression was tested on TNBCs and MCF10A. However; the means of MFIRs and percent of the cell population that showed positive expression of PCNA for the non-tumorigenic breast cell line MCF10A contradicted each other, because the mean MFIR was 1.75 and percent $PCNA^+$ was 3.65%. At that point, it was unclear if there is PCNA expression at the cell surface of MCF10A cells. Thus, we have proceeded with performing a subcellular protein fractionation method that allowed us to observe for expression of PCNA at the membrane, cytoplasmic, and nuclear locations. We have tested for expression of PCNA at these locations on MCF10A, MDA-MB-231, and MDA-MB-468.

Proteins extracted from membrane, cytoplasm, and nucleus were treated with anti-PCNA antibodies and then tested for expression of PCNA on the western blot in order to compare the expression of ligands at the three locations (Figure 3.12). For membrane expression on the western blot, expression of Na^+/K^+ ATPase pump, which is constitutively expressed at the membrane of cells, served as a loading control. GAPDH served as the loading control for cytoplasmic expression and lamin A/C, which is a protein that makes up the nuclear lamina, served as the loading control for nuclear expression. PCNA expression in the nucleus served as our positive control for quality since PCNA is constitutively expressed in the nucleus due to its

role in DNA replication, cell cycle regulation, and DNA repair mechanisms. For all cell lines tested, expression of all loading controls at appropriate locations was observed and PCNA expression at the nucleus was observed on the western blot (Figure 3.12).

For MDA-MB-231, PCNA expression was observed in the cytoplasm, but there was minimum to no expression observed at the membrane. This is contradictory to PCNA expression observed by flow cytometry analysis (Figure 3.10). However, the limitation to flow cytometry analysis testing for PCNA expression is that there is not a distinction between PCNA expression on the cell membrane versus PCNA expression on the exosomes. PCNA is known to either be expressed on the cell surface of cells or be released in soluble form as exosomes to interact with NKp44 on natural killer cells. Horton *et al.* had observed through confocal microscopy using CD63 as an exosomal marker colocalization of PCNA with CD63 on the MDA-MB-231 cell line. Subcellular protein fractionation cannot distinguish between the expression of PCNA on the cell membrane versus on the exosome. What appears to be a discrepancy between flow cytometry analysis of PCNA expression on MDA-MB-231 and no expression at the membrane component in the subcellular protein fractionation may indicate that there could be higher expression of PCNA on exosomes released by MDA-MB-231 cells than cell membrane PCNA ligand expression. In order to distinguish and quantify the differences in these expressions of PCNA, exosomes will need to be isolated from MDA-MB-231 cells and be tested for PCNA expression by flow cytometry, western blot, and microscopy studies. To show that one is working with exosomes, one would need to show expression of typical exosome markers such as CD63, CD9, and CD81, and secretion of intracellular vesicle proteins that forms early endosomes, such as ESCRTs (Hrs, TSG101), and late endosomes (SNAREs like VAMP7) [143].

It may be possible that there could be a difference in PCNA expression as a ligand versus exosomes which needs to be looked at for all cell lines tested.

For MDA-MB-468, PCNA expression was observed in both the cytoplasm and the membrane. MDA-MB-468 had a higher expression of PCNA at the membrane than MDA-MB-231 and MCF10A which does indicate some potential of targeting PCNA on TNBCs. Non-tumorigenic breast cell line MCF10A displayed the lowest PCNA expression at both the membrane and cytoplasm in comparison with MDA-MB-231 and MDA-MB-468 cell lines at the same locations. From flow cytometry analysis, we have observed the lowest expression of PCNA for the MCF10A based on the percent of its cell population $PCNA^+$ of 3.65% (Figure 3.10). From subcellular protein fractionation in combination with flow cytometry analysis, results indicate that PCNA is minimally expressed on non-tumorigenic breast cell line MCF10A and there is greater PCNA expression on TNBC cell lines tested. Hence, we proceeded to target PCNA with antibodies in next experiments to observe for effects of blocking PCNA-NKp44 interaction.

Figure 3.10

TNBC and non-TNBC cell lines display higher expression of PCNA than non-tumorigenic breast cell line.

Expression of PCNA represented by median fluorescence intensity ratios (MFIRs) and percentage of cells positive for PCNA was identified by flow cytometry analysis by staining TNBC cell lines and non-tumorigenic breast cell line with either anti-human PCNA-PE (gray shade in histograms) or isotype IgG_{2a}-PE antibodies (white shade in histograms). To obtain the mean of MFIRs and mean percent of cell population $PCNA^+$, three independent experiments of each cell line MDA-MB-231, MDA-MB-436, MDA-MB-468, & MCF7 as well as two independent experiments of MCF10A were performed. These MFIRs and % $PCNA^+$ values obtained make up the mean of MFIRs and mean % of cell population $PCNA^+$ presented in figure 3.13 and table 3.2.

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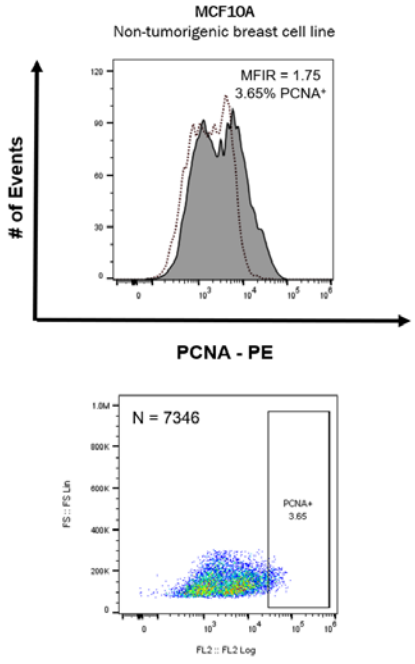
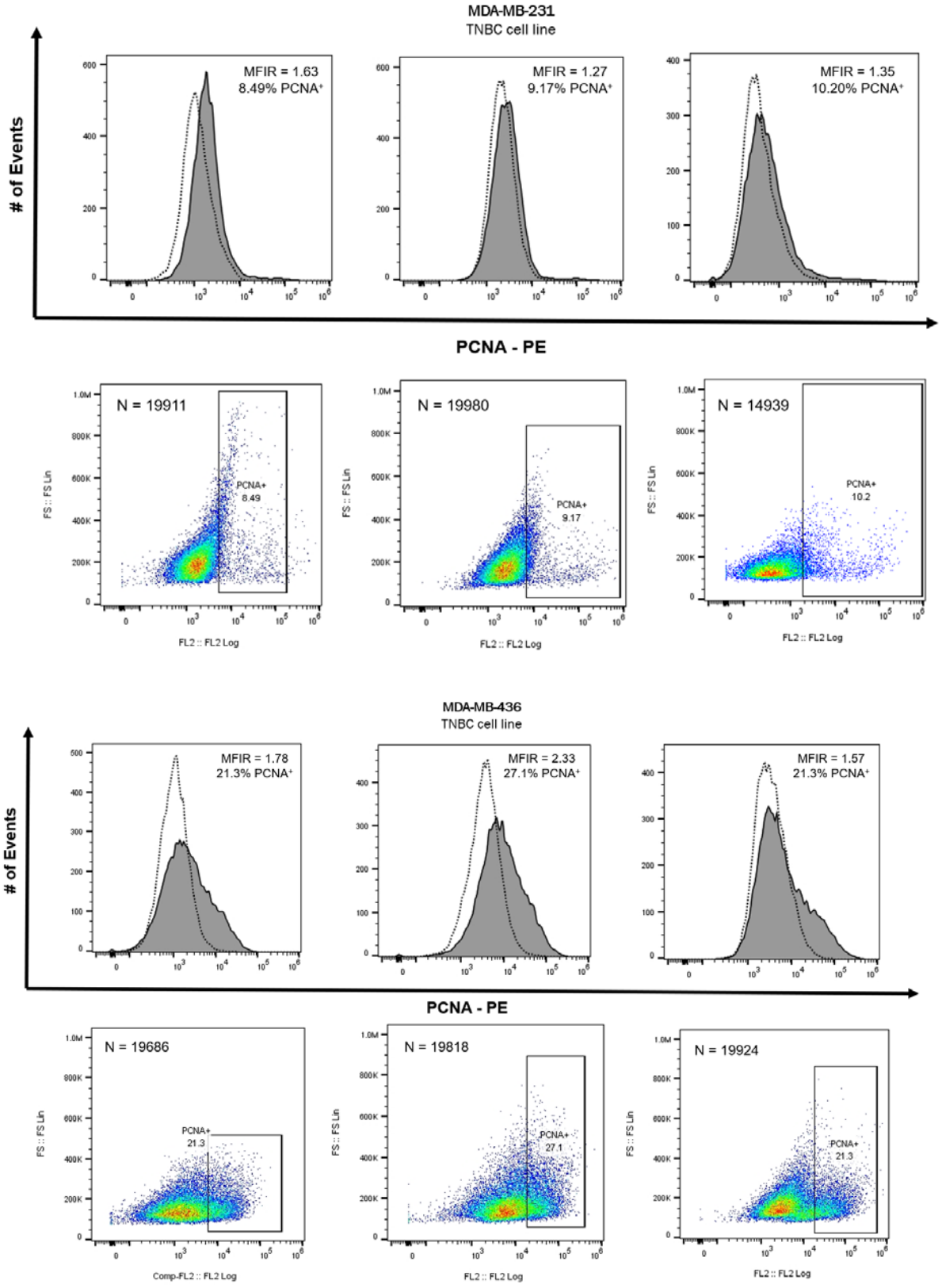



MDA-MB-231
TNBC cell line
# of Events
MFIR = 1.63
8.49% PCNA+
MFIR = 1.27
9.17% PCNA+
MFIR = 1.35
10.20% PCNA+
PCNA - PE
N = 19911
PCNA+
8.49
N = 19980
PCNA+
9.17
N = 14939
PCNA+
10.2
FS :: FS Lin
FL2 :: FL2 Log
MDA-MB-436
TNBC cell line
# of Events
MFIR = 1.78
21.3% PCNA+
MFIR = 2.33
27.1% PCNA+
MFIR = 1.57
21.3% PCNA+
PCNA - PE
N = 19686
PCNA+
21.3
N = 19818
PCNA+
27.1
N = 19924
PCNA+
21.3
FS :: FS Lin
Comp-FL2 :: FL2 Log
FL2 :: FL2 Log

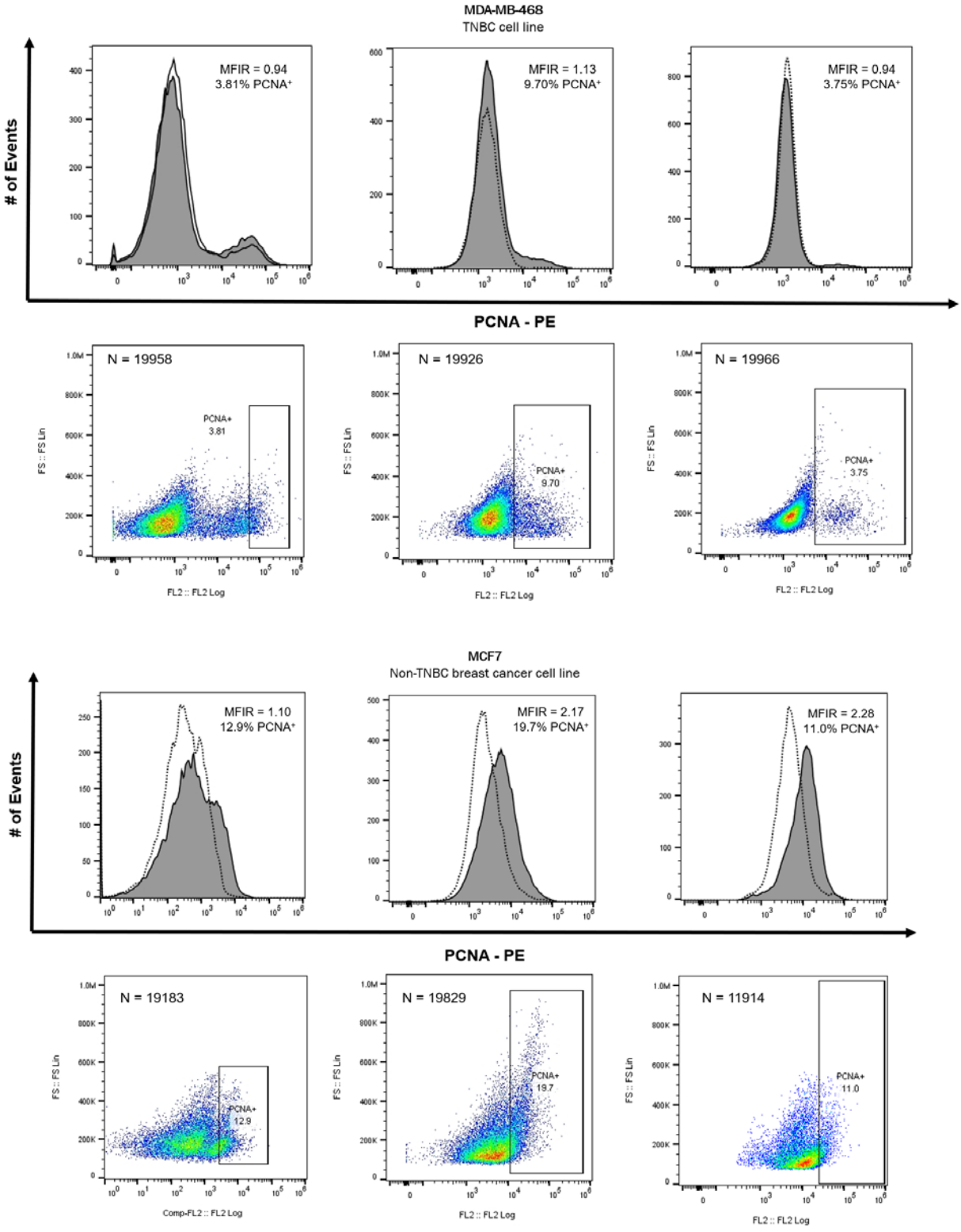

MDA-MB-468
TNBC cell line
# of Events
MFIR = 0.94
3.81% PCNA+
MFIR = 1.13
9.70% PCNA+
MFIR = 0.94
3.75% PCNA+
PCNA - PE
N = 19958
PCNA+
3.81
N = 19926
PCNA+
9.70
N = 19966
PCNA+
3.75
FS :: FS Lin
FL2 :: FL2 Log
MCF7
Non-TNBC breast cancer cell line
# of Events
MFIR = 1.10
12.9% PCNA+
MFIR = 2.17
19.7% PCNA+
MFIR = 2.28
11.0% PCNA+
PCNA - PE
N = 19183
PCNA+
12.9
N = 19829
PCNA+
19.7
N = 11914
PCNA+
11.0
Comp-FL2 :: FL2 Log

Figure 3.11

TNBC and non-TNBC cell lines show higher expression of PCNA than non-tumorigenic breast cell line by percent of cell population PCNA$^+$.

Flow cytometry analysis was used to detect cell surface expression of PCNA of all cell lines tested. Median fluorescence intensity ratios (MFIRs) and percentage of cells that have positive expression of PCNA from all independent experiments (n=3 for all cell lines; MCF10A n=1) were averaged. TNBC cell lines MDA-MB-436 and non-TNBC breast cancer cell line MCF7 have higher average MFIRs than non-tumorigenic breast cell line MCF10A. MDA-MB-231, MDA-MB-436, and MCF7 cell lines have a higher percent of cell population that display expression of PCNA than MCF10A. *One-way ANOVA confirmed differences in the percent of cell populations LLT1$^+$ at $p < 0.05$ for all cell lines. Dunnett's multiple comparison post-hoc was used to test for statistical significance of percent of cell populations LLT1$^+$ of each cell line compared to the control MCF10A (** $p < 0.01$).*

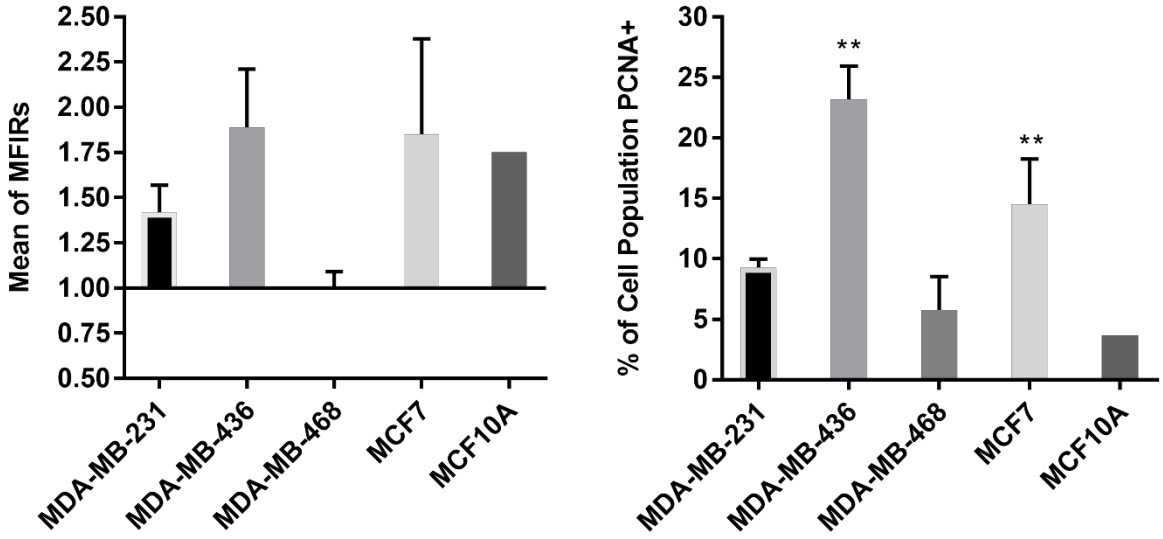


Table 3.2

Means of MFIRs, Percent of Cell Population PCNA$^+$, and Cell Population Size Tested.

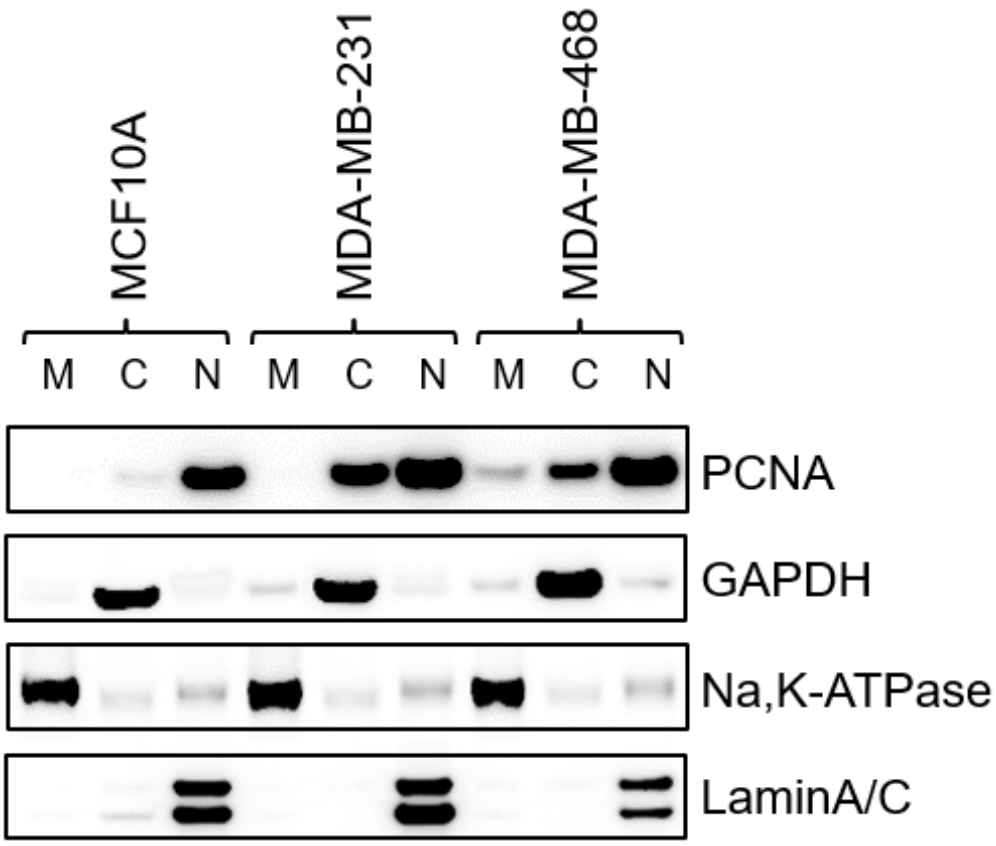
Of the three TNBC cell lines, MDA-MB-436 displayed the highest average median fluorescence intensity ratio (MFIRs) and percent of cell population with cell surface expression of PCNA. All breast cancer cell lines tested have a higher percent of cell population with cell surface expression of PCNA than non-tumorigenic breast cell line MCF10A.

	Means of MFIRs ± SD	Mean % PCNA$^+$ ± SD	Mean Number of Cells in Population Tested	Number of Independent Experiments
MDA-MB-231 PCNA$^+$	1.42 ± 0.15	9.29 ± 0.70	18276	3
MDA-MB-436 PCNA$^+$	1.89 ± 0.32	23.2 ± 2.73	19809	3
MDA-MB-468 PCNA$^+$	1.00 ± 0.09	5.75 ± 2.79	19950	3
MCF7 PCNA$^+$	1.85 ± 0.53	14.53 ± 3.73	16975	3
MCF10A PCNA$^+$	1.75 ± 0.00	3.65 ± 0.00	7346	1

Figure 3.12

Membrane, cytoplasmic, and nuclear expression of PCNA for TNBCs.

Proteins extracted from membrane, cytoplasm, and nucleus were treated with anti-PCNA antibodies and then tested for expression of PCNA on the western blot in order to compare the expression of ligands at the three locations on MCF10A, MDA-MB-231, and MDA-MB-468 cell lines. GAPDH, Na^+/K^+ ATPase pump, and lamin A/C served as loading controls for cytoplasmic, membrane, and nuclear expressions, respectively. MDA-MB-468 and MDA-MB-231 showed higher cytoplasmic expression of PCNA than MCF10A. MDA-MB-468 displayed membrane expression of PCNA while MDA-MB-231 and MCF10A did not show PCNA expression at the same locations.



3.2.2 Blocking Proliferating Cell Nuclear Antigen (PCNA) on Triple-Negative Breast Cancer Cells with Anti-PCNA Antibodies Induces Natural Killer Cell Activation

This section fulfills specific aim 2 - sub aim 2.1: Blocking interaction of LLT1 and PCNA on triple-negative breast cancer cells and its natural killer cell receptors with antibodies targeting each ligand enhances cytolytic targeting against these TNBCs (Figure 1.7).

Previous flow cytometry analysis has shown that there was higher cell surface expression of PCNA on TNBC cell lines than non-tumorigenic breast cell line MCF10A based on percent of the cell population $PCNA^+$ with MDA-MB-436 showing a statistically significant difference in PCNA expression compared to MCF10A (Figure 3.11). Since our flow cytometry analysis alone cannot effectively conclude that there was low expression of cell surface PCNA on MCF10A than other cell lines due to conflicting results between MFIR and percent $PCNA^+$, we have proceeded with subcellular protein fractionation. To verify if there is low expression of PCNA at the cell surface of MCF10A cells, subcellular protein fractionation allowed us to distinguish between PCNA expression at the cell membrane, cytoplasmic, and nuclear locations. Subcellular protein fractionation results revealed that MCF10A cells do have lower expression of PCNA at the cell surface than MDA-MB-468 and MDA-MB-231 (Figure 3.12). Hence, greater PCNA cell surface expression on TNBCs than MCF10A was confirmed. We, then, proceeded with targeting PCNA on TNBCs with anti-PCNA antibodies with intent to block PCNA-NKp44 interaction.

Similar to testing the effects of blocking LLT1-NKRP1A interaction, we used a conventional chromium-release cytotoxicity assay with primary NK cells isolated from blood-derived PBMCs. To prevent antibody-dependent cell-mediated cytotoxicity from occurring, Fc receptors on primary NK cells were blocked with human Fc fragment. TNBCs treated with the

anti-PCNA antibodies or isotype control were then co-incubated with NK cells at effector-to-target ratios (E:T) of 25:1, 5:1, and 1:1 for 4 hours. Specific lysis was quantified and determined.

We targeted PCNA on MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells through treatment with 1 µg of anti-PCNA antibody (Figure 3.13). At 25:1 E:T ratio, there is a statistically significant difference in percent specific lysis of MDA-MB-231 cells between cells treated with anti-PCNA antibodies compared to cells treated with IgG2a isotype antibody and cells not treated with antibodies (Figure 3.13; ** $p < 0.01$ compared to isotype control). At 25:1, 24.721% of MDA-MB-231 treated with anti-PCNA antibodies were killed by primary NK cells compared to 2.08% of MDA-MB-231 cells treated with isotype antibody. There was not a significant difference in MDA-MB-231 cells treated with anti-PCNA antibodies at both 5:1 and 1:1 E:T ratios.

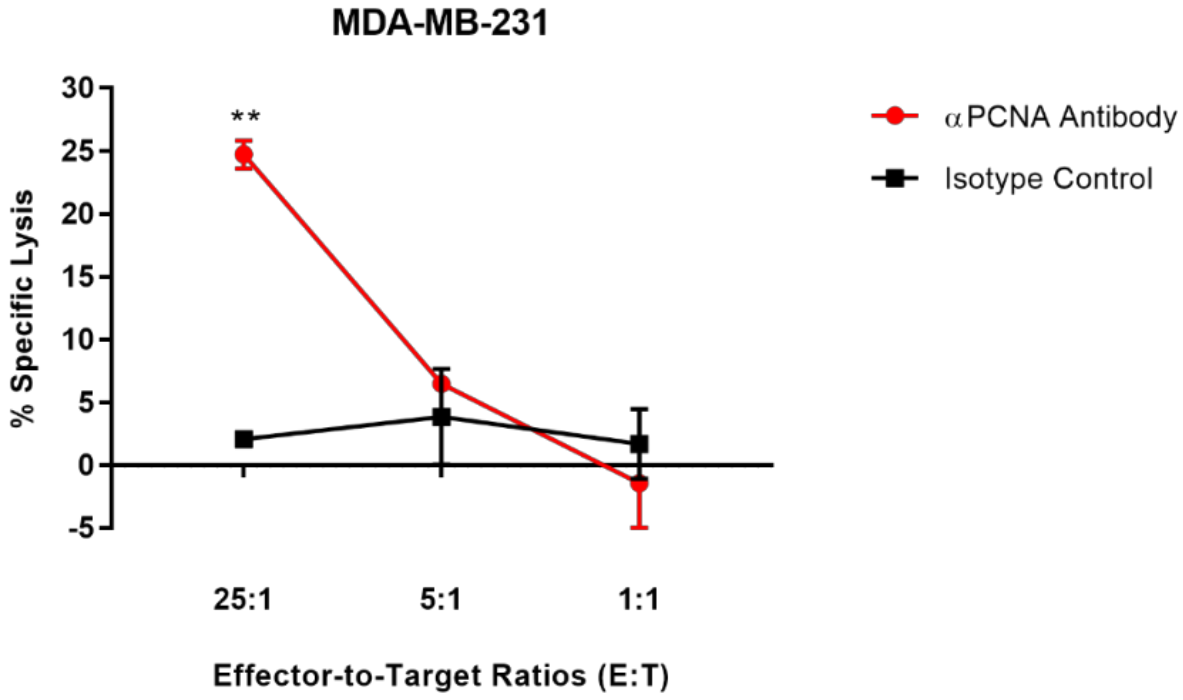
We also have tested the effects of blocking PCNA-NKp44 interaction by treating another TNBC cell line MDA-MB-436 with anti-PCNA antibodies. At 5:1 E:T ratio, 24.70% of MDA-MB-436 cells treated with anti-PCNA antibodies were killed while 19.260% of cells treated with isotype antibodies were killed (Figure 3.13). At 1:1 E:T ratio, 13.49% of MDA-MB-436 cells treated with anti-PCNA antibodies were killed compared to 7.02% of cells treated with isotype control. There was statistical significant difference observed between the percent lysis of MDA-MB-436 cells treated with anti-PCNA antibodies and cells treated with isotype antibodies at 5:1 and 1:1 ratios (Figure 3.13, * $p < 0.05$ & ** $p < 0.01$ compared to isotype control). We further tested the same concept on MDA-MB-468. Although no statistical difference was observed at any E:T ratios, MDA-MB-468 cells treated with anti-PCNA antibodies has a higher percentage of cells killed than cells treated with isotype control at 25:1 and 5:1 ratios.

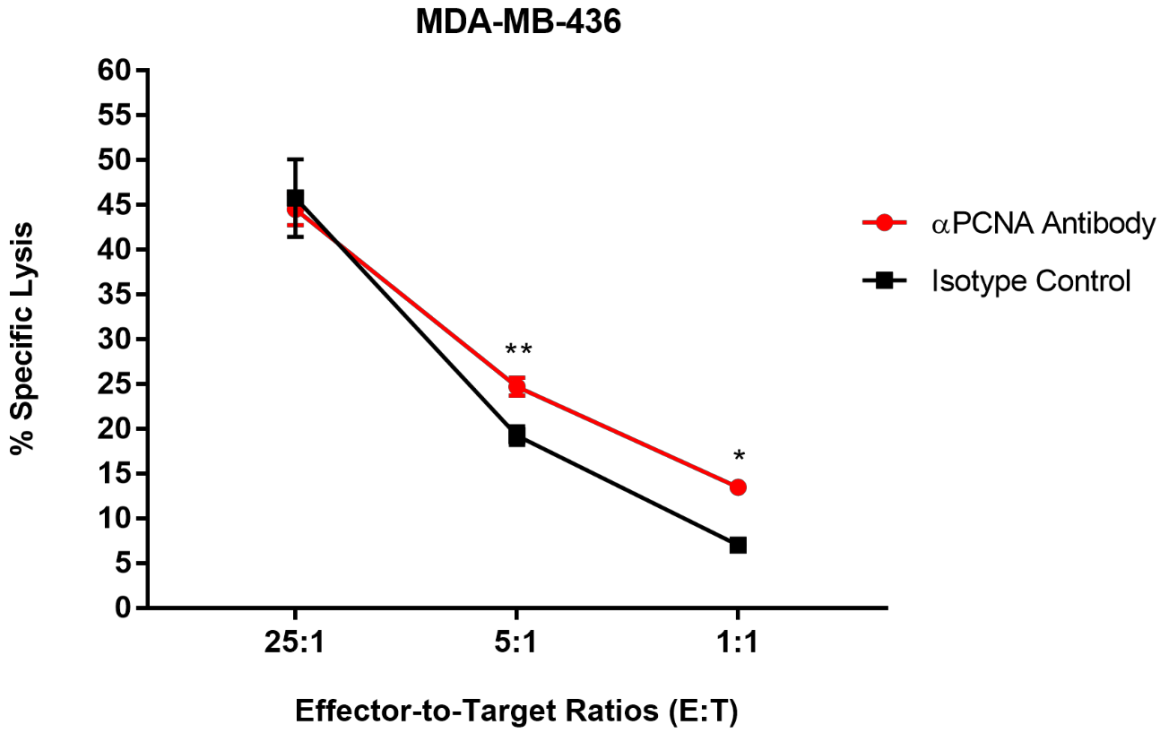
In summary, treating TNBC cell lines with anti-PCNA antibodies allowed an increase of killing by primary NK cells. Furthermore, MDA-MB-231 cells treated with anti-PCNA antibodies had a greater percent difference of cells killed compared to other TNBC cell lines. MDA-MB-436 and MDA-MB-468 with cell surface PCNA blocked by antibodies were killed at higher percentages than the same cells without blocking PCNA-NKp44 interaction. Therefore, treating TNBCs with anti-PCNA antibodies blocks the interaction of PCNA on TNBCs with NKp44 receptor on NK cells which prevents inhibitory signals from this interaction from being transduced to the NK cells. Inhibiting this interaction then enhanced killing of TNBCs by NK cells.

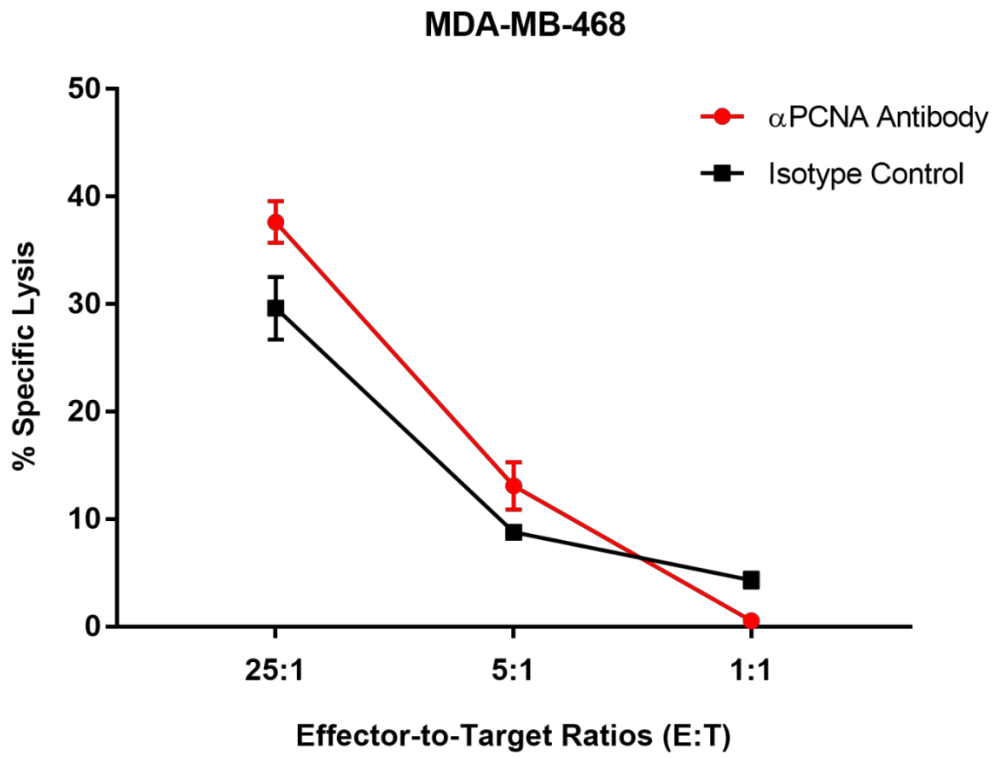
Figure 3.13

Blocking PCNA with antibodies on TNBCs increased killing by primary NK cells.

TNBC cell line MDA-MB-231, MDA-MB-436, and MDA-MB-468 were treated with either anti-human PCNA antibodies (αPCNA) or isotype control antibodies. Cells were labeled with ^{51}Cr and then were co-incubated with primary NK cells isolated from PBMCs derived from whole blood of healthy volunteers at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 4 hours. Specific lysis of labeled cells was subsequently quantified and calculated. *Student paired t-test was utilized to test for statistical significance between cells treated with anti-PCNA antibody versus isotype control within each E:T ratio. * $p < 0.05$ & ** $p < 0.01$ compared to isotype control.*




MDA-MB-436
% Specific Lysis
60
55
50
45
40
35
30
25
20
15
10
5
0
**
*
25:1
5:1
1:1
Effector-to-Target Ratios (E:T)
αPCNA Antibody
Isotype Control


MDA-MB-468
% Specific Lysis
50
40
30
20
10
0
25:1
5:1
1:1
Effector-to-Target Ratios (E:T)
αPCNA Antibody
Isotype Control

3.2.3 Blocking Inhibitory Ligands Interaction with NK Receptors Using Combinational Antibodies Further Enhances Natural Killer Cell-Mediated Killing against TNBCs

This section fulfills specific aim 2 - sub aim 2.1: Blocking interaction of LLT1 and PCNA on triple-negative breast cancer cells and its natural killer cell receptors with antibodies targeting each ligand enhances cytolytic targeting against these TNBCs (Figure 1.7).

Since TNBCs show expression of LLT1 and PCNA at the cell surface, as a preliminary experiment, we have decided to test the combination treatment of antibodies. It has been shown that PCNA colocalizes with human leukocyte antigen and inhibits NK cell function based on studies conducted by Horton and colleagues [90]. Hence, we have decided to combine three antibodies, anti-LLT1, anti-PCNA, and anti-HLA-A,B,C in one treatment to see the effects of increased killing by NK cells.

We used a chromium-release cytotoxicity assay with co-incubation of TNBC cell line MDA-MB-231 and primary NK cells derived from whole blood-derived PBMCs. Fc receptors on NK cells were blocked with human Fc fragment in order to prevent ADCC from occurring. We have observed a higher percentage of MDA-MB-231 cells treated with the combination of the three antibodies killed than MDA-MB-231 cells without treatment of any antibodies (Figure 3.14). At 25:1 E:T ratio, 42.10% of MDA-MB-231 cells treated with the combination of three antibodies were killed compared to 29.31% of MDA-MB-231 cells without antibody treatment killed by NK cells. There was a statistical significant difference observed at the 25:1 E:T ratio (Figure 3.12, * $p < 0.05$ compared to no antibodies).

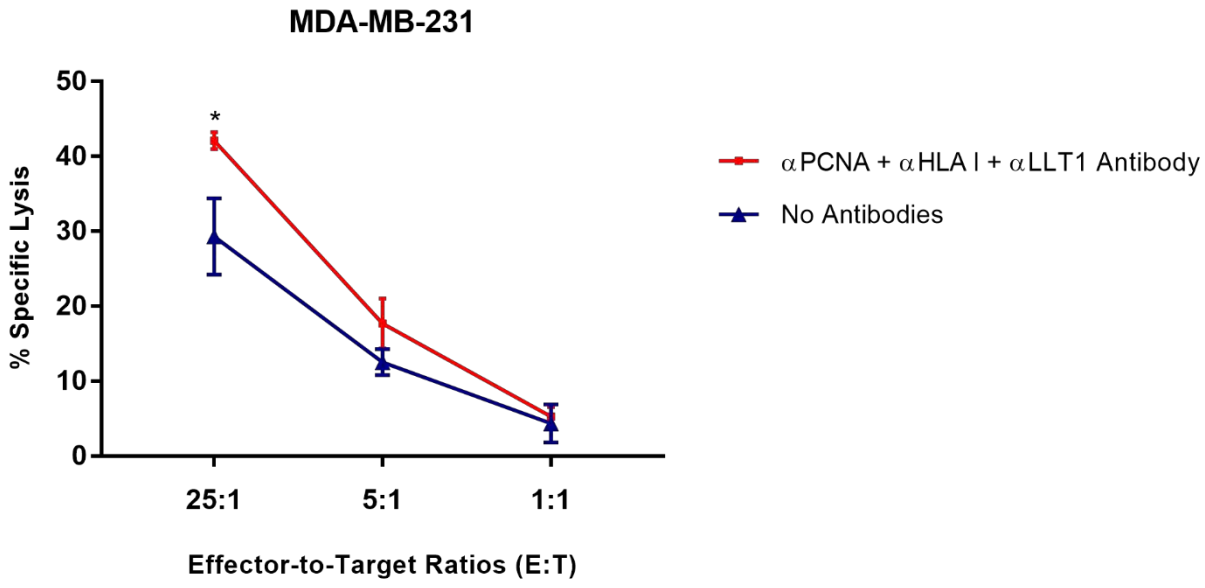
Even though there was no significant difference seen between the two groups at the 5:1 and 1:1 E:T ratios, we still observed a higher percentage of cells killed for TNBCs treated with the combination of antibodies. At 5:1 E:T ratio, 17.69% of MDA-MB-231 cells with

combinational treatment were killed compared to 12.53% of MDA-MB-231 cells without antibody-treatment. At 1:1 E:T ratio, 5.29% of MDA-MB-231 cells with combination treatment were killed compared to 4.37% of MDA-MB-231 cells without antibody-treatment. Since this was a preliminary study utilizing a combination of anti-LLT1, anti-PCNA, and anti-HLA antibodies, further testing on other TNBC cell lines and a non-tumorigenic breast cell line would need to be done in order to determine if a combinational treatment of antibodies approach could introduce another strategy of treating TNBCs by blocking both LLT1-NKRP1A and PCNA-NKp44 interactions.

Figure 3.14

NK-mediated killing of TNBCs was further expanded by using combinational blocking of PCNA, HLA, and LLT1 with antibodies.

TNBC cell line MDA-MB-231 was treated with a combination of 1 µg anti-human PCNA antibodies (αPCNA), 1 µg anti-human leukocyte antigen I (αHLA I), and 1 µg anti-LLT1 antibodies (αLLT1) together and compared to cells not treated with any antibodies. Cells were labeled with ^{51}Cr and then were co-incubated with primary NK cells isolated from PBMCs derived from whole blood of healthy volunteers at effector-to-target ratios (NK-to-5000 TNBCs) of 25:1, 5:1, and 1:1 for 4 hours. Specific lysis of labeled cells was subsequently quantified and calculated. MDA-MB-231 cells treated with combination of the three antibodies had a higher percent of cells killed by NK cells than cells without antibody-treatment *Student paired t-test was utilized to test for statistical significance between cells treated with anti-LLT1 antibody versus 'no antibodies' control within each E:T ratio. MDA-MB-231: * p < 0.05 compared to no antibodies.*



CHAPTER IV

DISCUSSION

4.1 Challenges of Utilizing Endocrine Therapy on TNBC.

Triple-negative breast cancer (TNBC) is considered the most invasive type of breast cancer out of all subtypes [37,38]. TNBC's absence of three receptors, that is the estrogen (ER), progesterone (PR), and human epidermal growth factor-2 receptor (HER2), typically used for hormone-targeted treatments renders it difficult to treat TNBC [2]. For patients with estrogen-positive breast cancer, tamoxifen is one of the standard drugs administered with intent to slow down tumorigenesis in these patients [2]. Estrogen plays a role in eliciting proliferation of breast cancer tumors though a mechanism outlined by Dutertre & Smith and their colleagues [144,145].

Estrogen receptors in either ER-α or ER-β forms both have similar structures that consist of two binding domains, which are known as the DNA-binding domain and the ligand-binding domain [144,145]. When estrogen binds to the ligand-binding domain, the estrogen receptor interacts with the specific DNA sequences called estrogen response elements (EREs) at the DNA-binding domain of the ER receptors [144,145]. Activation functions AF-1 and AF-2 within the estrogen receptor then recruits co-activators which further allows transcription of genes favoring cell growth [144,145]. In the case of ER-positive breast cancer, this specific mechanism causes transcription of oncogenes that favors proliferation of the tumor cells [144,145]. The understanding of this molecular mechanism allowing proliferation of breast cancer cells had propelled the development of anti-estrogen drugs in particular tamoxifen.

Interestingly, tamoxifen was not initially designed for treatment of breast cancer, but instead was designed as a contraceptive when it was discovered that treating rats with this new drug in the 1960s resulted in a decrease in fertility [145,146]. Although when tamoxifen was tested in humans, tamoxifen failed to show anti-fertility effects and was found to induce ovulation in women instead [145]. Although the drug was considered a failure as a contraceptive, it was the chemical structure of this newly designed drug that revealed a "hidden jewel" that will soon revolutionize cancer treatment [145]. Dr. Michael Harper and Dr. Arthur Walpole had both found that the cis isomer of the chemical structure is an estrogen while the trans isomer is an anti-estrogen [145]. This discovery laid the foundation on utilizing drugs that are considered anti-estrogen to successfully compete with estrogen for estrogen receptors on breast cancer cells [145,147].

Tamoxifen known as a Selective Estrogen Receptor Modulator (SERM) competes with estrogen for the estrogen receptor binding site [145]. Upon tamoxifen binding to the estrogen receptor, tamoxifen blocks the estrogen from binding to the ligand-binding domain. Since estrogen could not bind to the ligand-binding domain of the estrogen receptor complex, oncogenes such as *c-ergB-2* could not be initiated thus suppressing tumorigenesis of these ER-positive breast cancer cells [148]. The suppression of oncogene *c-ergB-2* is caused by tamoxifen effects on cell cycle arrest and downregulation of TGFα (transforming growth factor α), whose initial function before downregulation was to start the phosphatidylinositide 3-kinase (PI3K) signaling cascade to favor cell cycle progression and cell growth [148,149]. The intricate mechanism that makes tamoxifen effective for ER-positive breast cancer cells lays the forefront in terms of treating other types of breast cancers. Unfortunately, this does not apply to triple-

negative breast cancer where tumor cells do not depend on estrogen for proliferation of its tumor cells.

The absence of ER was first identified and assessed based on its corresponding expression levels by immunohistochemistry and/or fluorescence in situ hybridization [36]. Although TNBCs do not display estrogen receptors on either the membrane or cytosolic, there has been studies that uses tamoxifen in combination with other treatments in its experimental phases. It has been well established that tamoxifen is involved in cell cycle arrest and suppression of tumor growth by blocking estrogen from binding to the estrogen receptor. This mechanism has been known and applied to cancer cells that express estrogen receptors. However, there has been recent evidence that anti-tumor activity from tamoxifen may also be estrogen receptor-independent meaning that cancer cells without estrogen receptors could theoretically be treated with tamoxifen [150]. It has been shown that tamoxifen had anti-tumor results against ER-negative gastric cancer cells, cholangiocarcinomas (bile duct cancer), and colon cancer cells [151-153]. One rationale behind this first began with studying the development of multidrug resistant tumors [151]. Mechanisms contributing to multidrug resistant tumors has pointed to the upregulation of the PI3K signaling that also upregulates P-glycoprotein (P-gp) [151]. P-gp is an integral membrane protein that is part of the multidrug resistant tumor phenotype and is involved in drug efflux of tumor drugs [151,154,155]. It has been found that tamoxifen was able to effectively reverse multidrug resistant tumors by binding to and inhibiting P-gp and suppressing PI3K signaling [151]. The question is: can the same principle of using tamoxifen effectively reverse mechanisms of multidrug resistance in triple-negative breast cancer?

A study conducted by Wang and colleagues investigated that treating mesenchymal triple-negative breast cancer with tamoxifen will have antitumor effects [150]. The epithelial-to-mesenchymal transition (EMT) is one feature that has been focused in studying cancer cells phenotype and metastasis [156]. One mechanism that tumor cells utilize within the EMT is the decrease in the expression of epithelial marker E-cadherin and increased expression of the mesenchymal marker vimentin [156]. The increase in vimentin has been associated with cancer cells developing mesenchymal characteristics that enables them to migrate to other tissues and play a role in increasing drug resistance in tumor cells [156]. The major basis of the study by Wang *et al*. is that majority of TNBCs have mesenchymal characteristics and that the EMT contribute to its resistance to drugs [150]. Their results have revealed that treating TNBC cell line MDA-MB-231 with tamoxifen (5 μmol/L) has increased expression of E-cadherin and decreased expression of vimentin which further results in a decrease in migration rate of these cells in contrast to MDA-MB-231 cells without tamoxifen treatment showing the opposite result [150, Figure 2B-D in Wang *et al*.]. This is due to the reversal of the EMT process in the MDA-MB-231 cells. This group went further to show how reversing the EMT impacts the TNBCs response to chemotherapy drugs epirubicin and 5-fluorouracil [150, Figure 3 in Wang *et al*.]. They have found that TNBCs treated with tamoxifen have an increased response to these two chemotherapy drugs and have shown in additional experiments that tamoxifen may have reversed EMT by upregulating miRNA miR-200c [150]. Although this study assessed a mechanism of how tamoxifen reverses the EMT and allow TNBCs to lose its tumorigenicity, some additional questions that needs to be addressed include: (i) will tamoxifen have the same effect in reversing EMT once TNBCs develop resistance to tamoxifen? (ii) since TNBCs lack the estrogen receptor, where does tamoxifen bind to initiate EMT reversal? It has been established

that in ER-positive breast cancer, tamoxifen binds to the ligand-binding region of ER complex, so where does tamoxifen bind in the case of ER-negative breast cancer?

The complex issue is that showing that tamoxifen treatment on ER-negative breast cancer cells having antitumor effects now competes with the established principle that tamoxifen only works on ER-positive breast cancer cells. There are overwhelming studies that support the tamoxifen treatment on women with ER-negative breast cancers including TNBC has not been effective in suppressing tumorigenesis, metastasis, and recurrence [157]. TNBC phenotype of lacking an estrogen receptor makes utilizing tamoxifen for treatment largely ineffective in women with this type of breast cancer. Likewise, the absence in expression of progesterone receptor makes utilizing anti-progesterone therapy ineffective. Progesterone is known as an ovarian hormone needed for breast development and functions such as lactation [158]. Unlike the role of estrogen in breast cancer being well established, the role of progesterone in breast cancer is debated and controversial [158].

There is still a consensus that the TNBC lack of expression of progesterone receptor makes it difficult to use antiprogestins for safe and effective treatment against TNBC. Patients with breast cancer that are positive for ER and/or PR receptors have responded more effectively to hormonal therapies such as tamoxifen, fulvestrant, and aromatase inhibitors such as anastrozole, letrozole, and exemestane. These specific treatments are aimed to either suppress estrogen or progesterone production levels, modify ER and PR receptors, or block these receptors from interacting with estrogen [40]. Hence, the TNBC phenotype that consist of an absence of both ER and PR have summoned the use of chemotherapy as a standard treatment. However, chemotherapy does have adverse side effects, compromise the immune system, and TNBCs do develop resistance to chemotherapy (as explained in the next section). Hormonal

therapies involving anti-estrogen receptor drugs and anti-progesterone receptors drugs have not shown much success in clinical treatment of patients with TNBC and chemotherapy also display disadvantages, thus it will be important to develop alternative approaches such as immunotherapy in treating TNBC.

4.2 TNBCs Develop Chemoresistance

Current conventional treatments for TNBC include both radiation therapy and chemotherapy. There are benefits for patients if given chemotherapy neoadjuvant, adjuvant, or in metastatic settings where taxanes and anthracyclines were commonly given [36,64,65]. Deciding which form of chemotherapy to give to a patient is generalized and not personalized to one's genetic profile in current clinical practice [159]. Various chemotherapeutic strategies include targeting DNA repair systems with platinum compounds, suppressing dysfunctional tumor suppressor genes with taxanes, and arresting the cell cycle of tumor cells with anthracyclines as a few examples [159,160].

It is interesting to note that patients with TNBC have a higher success rate in terms of tumor cells getting eradicated from chemotherapy in initial treatment than other subtypes of breast cancer [36]. One study conducted by Liedtke and colleagues had observed the response to neoadjuvant chemotherapy and survival rates between 1,118 total patients who received this treatment for breast cancer at M.D. Anderson Cancer Center [66]. This detailed study compared the responses of patients with TNBC versus patients with other subtypes of breast cancer by a thorough analysis of each of its pathologic complete response rates (pCR), survival rates, and rates of relapse [66]. Of the total number of patients, 255 patients (23% of total) had TNBC. Results of this study observed that patients with TNBC treated with neoadjuvant chemotherapy had a higher pCR rate at 22% versus non-TNBC patients at 11% [66]. Patients with TNBC that

were able to reach pCR had a 24 percent chance of survival which is similar to patients with non-TNBC [66]. However, TNBC patients with residual disease had a lower overall 3-year survival rate at 68% probability of survival (3 years after surgery) than non-TNBC patients with residual disease at 88% probability of survival [66]. Although TNBC patients exhibited a higher response to chemotherapy in initial treatments, it has been shown that TNBC patients with any residual disease after the first round of neoadjuvant chemotherapy have a higher recurrence than non-TNBC patients [66]. One reason for this is due to TNBC's development of resistance to chemotherapeutic drugs [67].

For decades, chemotherapy has been one of the most predominant treatments that has been used for patients with all types of cancers. Although results after initial round of chemotherapy appears to be optimistic for the patient, it is the higher number of recurrence cases after chemotherapy that raises concerns over multidrug resistant tumors [4]. In order to understand how this concern applies to TNBCs, we must first understand the basic principles of pharmacokinetics that contribute to tumors developing resistance to drugs. Several pharmacokinetic factors such as drug absorption, distribution, metabolism, and elimination determine if a drug is able to target a tumor [4].

Pharmacokinetics refers to factors that affect the influx and efflux of an administered drug into and out of the tumor cell [4]. With regards to how tumors develop drug resistance, cell membrane transporters play a key role in regulating the flux of drugs in and out of tumor cells [4]. A family of transporters called the ATP-binding cassette (ABC) had been the main focus on increasing efflux of chemotherapeutic drugs [4]. Among the ABC transporter family members, P-glycoprotein (P-gp from our discussion in section 4.1; also known as MDR1), MDR-associated protein 1 (MRP1), and breast cancer resistance protein (BCRP) have been studied to see how

these transmembrane proteins allow tumors to pump out chemotherapeutic drugs [4,161]. Studies have found that P-gp overexpression in different types of cancer cells such as kidney, colon, liver, and leukemias, either caused by chemotherapy or intrinsic gene expression before chemotherapy was given [4].

ABC transporters had been extensively studied in TNBCs. Three ABC transporters have been shown to be expressed on TNBCs which are MRP1, BCRP, and P-gp (MDR1) [4,157]. P-glycoprotein had been a major focus in targeting TNBCs. A clinical study conducted by Thomas *et al*. looked at the impact of P-gp inhibitors on cancer patients [162]. Results have shown that first-generation inhibitors such as cyclosporin and verapamil were not successful due to high toxicity effects while second-generation inhibitors such as valspodar and biricodar showed slightly better results [162]. There was still a great need to improve upon the second-generation drugs for better targeting against P-gp instead of other transporters [162]. According to the study, third-generation P-gp inhibitors such as tariquidar have high specific targeting towards P-gp [162]. Beyond the third-generation P-gp inhibitors that seemed to show promise for both TNBCs and other types of cancers, fourth-generation P-gp inhibitors are in development in hopes of lower toxicity, higher potency, and higher specificity against P-gp [163].

Another study conducted by Zhang *et al*. looked at how the overexpression of P-gp on epirubicin-resistant TNBCs increase its autophagy by autophagosome-like vacuoles which contributes to its resistance to epirubicin [164]. Results from this study have demonstrated that epirubicin-resistant modified TNBC cell line MDA-MB-231 showed overexpression of P-gp and contained a large number of autophagosome-like vacuoles [164]. They have found that downregulating the expression of P-gp on this TNBC cell line by using small hairpin RNA targeting P-gp (*sh-MDR1*) increased its sensitivity to epirubicin [164]. Thus, this group observed

that suppressing both P-gp expression and autophagy enhanced sensitivity of TNBCs response to epirubicin [164].

While there are many studies that looks at the ABC transporters and how they contribute to the development of drug resistance for TNBCs, we also need to look at the molecular level to understand how genetic changes in TNBCs could enhance drug resistance. Chemoresistance can be divided into two categories which are acquired resistance and intrinsic resistance [4]. Acquired resistance refers to tumors developing resistance to chemotherapy as a result of initial treatment [4]. Examples of acquired resistance in TNBCs include an accumulation of mutations, dysfunctional DNA repair system where mutation errors could not be fixed, transcription of oncogenes or suppression of tumor suppressor genes, and tumor's pharmacodynamic adjustments as a result of the effects of drug treatment [4]. Intrinsic resistance is defined as predisposition factors that favors the tumor to resist the effects of chemotherapy. Examples of intrinsic resistance include already-developed mutations such as the expression of *BRCA1/2* or overexpression of ABC transporters which are known to be expressed before any chemotherapy treatment [4].

The molecular heterogeneity of TNBC contributes to the difficulty of treating TNBC with conventional treatments. Genomic analyses have revealed that there is molecular heterogeneity for TNBCs both within the tumor and its surrounding outside environment [39]. One factor that contributes to TNBC's chemoresistance is its chromosomal instability [126]. When one compares the genomic instability between TNBCs and non-TNBCs, there is strong evidence that TNBCs have higher levels of genomic instability based on its short chromosome regions, a high number of copy number aberrations, and a higher number of gains and losses of function than non-TNBCs [4,165]. TNBC chemoresistance has been associated with the high number of

chromosome 5q deletions as determined by the Molecular Taxonomy of Breast Cancer International Consortium [4,166]. Another factor that contributes to TNBCs developing resistance are the number of mutations in certain genes especially *TP53*, *PIK3CA*, and *PTEN* [4, 58]. Majority of TNBCs display a common mutation in the *TP53* gene, which leads to the loss of function for the tumor suppressor protein 53 [39]. The result of this significant mutation is that tumor suppressor protein 53 will not be able to control cell division and tumor growth [39]. There is also a high frequency of deleterious mutations in genes that are involved in homologous recombination, including *BARD1*, *PALB2*, *RAD51D*, *RAD51C*, *BRIP1*, and *BRCA1/2* genes [39,167].

Both deleterious mutations in genes involved in homologous mutations and chromosomal instability are among several mechanisms that lead TNBCs to develop resistance to chemotherapeutic drugs. Interestingly, this type of genomic instability favors patients with TNBC being treated with chemotherapy the first time, but significantly lowers the chance of survival in cases of recurrence [67]. Carey *et al*. observed that patients with TNBC that are given anthracycline before surgery responded well in the long term only if these patients achieved near 100% pathologic complete response [67]. For patients that weren't able to achieve 100% pathologic complete response, these patients' tumor still showed increased sensitivity to treatment initially; however, with residual disease remaining, these same patients had a much poorer prognosis with higher incidences of relapse and lower survival rates [67]. This is due to the TNBC's developing resistance to chemotherapeutics since they were positively selected to survive past the initial eradication phase by chemotherapy. Carey *et al*. had coined this type of observation as the 'TNBC paradox' where patients with TNBC show a higher response in their first treatment to chemotherapy, but in recurrence show poor response to treatment [67].

With these observations in TNBC patients becoming more frequent, concerns surrounding TNBC chemoresistance highlights the need to understand mechanisms of resistance and utilize alternative options besides chemotherapy to kill these tumor cells. In addition, the use of chemotherapy presents adverse side effects from physical and immune complications to even long-term cognitive impairment [37,40,130-134]. Considering the low success of hormonal therapies and many prevalent side effects from chemotherapy and concerns surrounding chemoresistance, immunotherapy has been strongly considered and explored as an attractive treatment option for patients diagnosed with TNBC. Immunotherapeutic options now include utilizing monoclonal antibodies targeting ligands of interest such as proteins that suppress immune response or inhibit the tumor cell from self-apoptosis. It is the concerns of TNBCs developing chemoresistance that should drive the scientific community into developing therapeutics that will bypass this challenge and possibly decrease heavy reliance on chemotherapy and increase one's chance of survival while minimizing the number of serious side effects.

4.3 LLT1-NKRP1A Interaction on TNBCs and Its Function.

Role of LLT1 and CD161 in Modulating Immune Functions

Lectin-like Transcript-1 (LLT1, CLEC2D, OCIL) is a ligand that when expressed on cells interacts with its corresponding NK cell receptor NKRP1A (CD161) [105]. LLT1 is part of the C-type lectin-like receptor family encoded by *CLEC2D* genes within the human NK gene complex [105]. It has been studied that LLT1 has a key role in the human immune system [105]. It is interesting that there are two C-type lectins found in the NK gene complex which are LLT1

and NKRP1A, because it has been well established through crystallography and cloning that LLT1 and NKRP1A interacts with each other in many different situations [105,168].

Boles and colleagues have demonstrated that LLT1 is expressed on NK cells, T, and B cells [168]. Furthermore, Mathew and colleagues have determined the function of LLT1 on human NK cells by generating a monoclonal antibody (L9.7) that binds to the LLT1 receptor on NK cells [169]. They have found that when the antibody binds to the LLT1 receptor on both resting NK cells and IL-2 activated NK cells, IFN-γ production was induced, but it did not induce cytotoxicity in NK cells [169]. This suggests that there are two separate signaling mechanisms that regulate IFN-γ production and cytotoxicity function when anti-LLT1 antibody crosslinks with the LLT1 receptor [169]. The next question begs: what is the role of IFN-γ production after LLT1 crosslinking with its monoclonal antibody?

It may be that IFN-γ production, because of LLT1 interacting with a protein or antibody, may play a role in early innate immune response to pathogens [169]. The function of LLT1 in modulating immune responses has been further studied by determining the expression of LLT1 on immune effector cells, if the expression of LLT1 was upregulated after stimulation, and what is its role on specific immune cells [114]. LLT1-NKRP1A (CD161) interaction has two functions where NK cell activation is suppressed while costimulating T cells in response to pathogens [114]. In addition to LLT1 being expressed on T cells, LLT1 is also expressed on antigen presenting cells specifically dendritic cells and B cells [114]. Plasmacytoid dendritic cells (pDCs) and monocyte-derived dendritic cells express LLT1 when toll-like receptors (TLRs) 9 was stimulated by CpG DNA [114]. Interestingly, when pDCs TLRs 7 and 8 gets stimulated by inactivated influenza virus and herpes simplex virus, LLT1 is induced on activated pDCs which

then in response to those viruses produce increasing amounts of type I interferons such as IFN-α and IFN-β [114,170].

LLT1 and CD161 interaction constitutes a critical role in human germinal centers in promoting B cell activation and chemokine receptor CXCR4 downregulation [171]. Germinal centers are located within secondary lymphoid organs where B cells expand and mature as they interact with antigens being presented [171]. B cells have its own B-cell receptors that can change its affinity for antigens [171]. Germinal centers consist of a light zone, where B cells compete for antigens and interactions with T follicular helper cells, and the dark zone, where B cells hypermutate and proliferate [171, 172]. It has been determined that LLT1 expression was observed on all germinal center B cells and that expression of LLT1 might be associated with germinal center B cells with high-affinity B-cell receptors [171]. It is these B cells that can more effectively serve as antigen-presenting cells and present antigens to T follicular helper cells [171].

This study was extended further to see how CD161 expression on follicular dendritic cells contributes to B cells migrating between the light zones and dark zones of germinal centers [171]. Downregulation of CXCR4 expression on dark zone B cells has allowed these B cells to migrate back to the light zone, but the question is: does LLT1 expression have to do with this migration? Crosslinking of LLT1 on dark zone B cells has allowed expression of CXCR4 to decrease which would favor migration of those B cells back into the light zone [171]. Upon the B cells returning to the light zone, LLT1 on these B cells interact with CD161 on T follicular helper cells and follicular dendritic cells which causes B cells to better receive costimulatory signals [171]. Expression of LLT1 has been linked to proliferation of B cells which serves as a possible marker for non-Hodgkin B cell lymphoma [171,173].

The role of LLT1 expression on immune cells in general is important in regulating immune functions in NK cells, germinal B cells, T cells, and plasmacytoid dendritic cells as discussed. If the immune cells display LLT1 expression and upregulate LLT1 expression under given circumstances that would enhance immune cell function against pathogens, the next question becomes: can tumor cells express LLT1 as method to evade targeting by immune effector cells? Through the cancer immunoediting concept, once tumors are able to survive through the eradication and equilibrium phases, tumors are now in the escape phase where it can display ligands or secrete cytokines that can inhibit immune cell function. If immune cells can utilize its own mechanisms to kill tumor cells and pathogens and protect itself from other immune cells, then tumor cells can adapt and enhance its survival by displaying those same ligands and receptors. For the case of LLT1-NKRP1A interaction between target cells and NK cells, it is known that LLT1 expressed on target cells inhibits NK cell function when LLT1 interacts with NKRP1A as discussed. NK cells also display LLT1 which when crosslinked with its counter-receptor or antibody induces IFN-γ production that enhances NK cell function but does not increase NK cytotoxicity function [169]. One may ask if different types of cancer cells also express LLT1 with the same role of inducing IFN-γ production while inhibiting NK cytotoxicity function. In particular, there are no prior studies to our knowledge that looks at LLT1 expression on TNBCs. We have then decided to study the possibility of this immunosurvillence evasion by determining if there is LLT1 expression on TNBCs and observing for the effects of blocking this inhibitory interaction of LLT1-NKRP1A as will be discussed in our analysis in the next section.

Expression and Function of LLT1 on TNBCs

There have been numerous studies that show the expression and function of LLT1 on immune cells such as germinal center B cells, plasmacytoid dendritic cells, NK cells, and T cells [114,168,170,171]. Immune cells express and upregulate LLT1 in order to enhance its own function of targeting pathogens, presenting antigens to other cells, secrete cytokines, or enhance its receptor interactions with ligands on immune cells with intent for co-stimulation [114,169-171]. In addition, certain immune cells have anti-tumor effects such as $CD8^+$ T cells and NK cells by recognizing tumor-associated antigens presented on the major histocompatibility complex I of tumor cells [174]. It may sound convenient for immune systems to recognize every tumor cell by its tumor-associated antigens being presented, but this is not always the case. The cancer immunoediting process describes how tumor cells can develop resistance to the immune system by acquiring mutations, ligands, receptors, and producing cytokines that will prevent immune systems from recognizing them [70,76]. For the case of the interaction between NK cells and tumor cells, the expression of inhibitory ligands such as LLT1 on cancer cells allows cancer cells to escape immune surveillance.

LLT1 expression on different types of cancers have been reported. Mathew and colleagues have reported the overexpression of LLT1 on prostate cancer cells and have determined that disrupting the LLT1-NKRP1A interaction have increased killing of prostate cancer cells [88]. Germain and colleagues have also observed the expression of LLT1 on germinal center B cell non-Hodgkin lymphomas which contributes to inhibiting NK cell function [175]. In addition, Roth and colleagues have shown that LLT1 expression on malignant glioma cells which plays a role in inhibiting antitumor immune activity [89]. To our knowledge, there has not been a study that looks at LLT1 expression on TNBC, but since LLT1 expression has

been tested in various types of cancers, we would like to determine if there is LLT1 expression on TNBC and observe for its function from an immunotherapeutic perspective.

We have observed expression of LLT1 at the cell surface of TNBC, non-TNBC breast cancer, and non-tumorigenic breast cell lines by flow cytometry analysis and immunofluorescent confocal studies. We have tested for LLT1 expression on three TNBC cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 as well as a non-TNBC cell line MCF7 and compared its expression to non-tumorigenic breast cell line MCF10A. In order to determine if LLT1 could serve a possible candidate for targeting with antibodies, comparing the expression of LLT1 on TNBCs to MCF10A was needed in hopes of minimizing off-target effects against non-tumorigenic breast cells. Based on flow cytometry analysis of three independent experiments under the same culturing conditions and using the same anti-LLT1 antibody, the non-tumorigenic breast cells MCF10A expressed significantly low levels of LLT1 than all the breast cancer cell lines tested (except for MDA-MB-468). Since LLT1 is expressed at low levels on non-tumorigenic normal breast cells at the cell surface and LLT1 is not expressed on 99.44% of MCF10A cells (N=8276 MCF10A cells, mean population size of 3 independent experiments, Table 3.1), LLT1 may serve as a possible ligand to target to examine its function on TNBCs.

By flow cytometry, normal breast cell line MCF10A has consistently shown very low expression of LLT1, there has been a study that performed immunohistochemical staining of LLT1 on healthy breast tissues [176]. The staining performed by Llibre and colleagues has shown that healthy breast tissues do express some levels of LLT1, but it is not known how many different types of healthy breast tissues was observed and there was no quantification of expression of LLT1 on the healthy breast tissue examined [176]. Our study has quantified the expression of LLT1 at the cell surface of one normal breast cell line, but there needs to be further

comprehensive study on the expression of LLT1 on multiple normal breast cell lines as well as healthy breast tissue samples. Furthermore, it has been established that normal breast cells display a self-antigen peptide on its human leukocyte antigen class A, B, or C receptor to prevent cytotoxic T cells from killing normal cells [5]. Because healthy breast cells utilize HLA-A, HLA-B, or HLA-C to inhibit both cytotoxic T cells and NK cells from killing, healthy breast cells rely less on expressing LLT1 at the cell surface to inhibit killing by immune cells [5].

Based on our findings, we are interested in determining the function of LLT1 expressed on TNBCs. Therefore, we have used an anti-LLT1 antibody specific for binding to LLT1 on all three TNBC cell lines in a chromium-release cytotoxicity with primary NK cells to see the effects of blocking LLT1-NKRP1A interaction. Based on the previous studies detailing the function of the LLT1-NKRP1A interaction, we have hypothesized that LLT1 functions as an inhibitory ligand on TNBCs and that blocking LLT1-NKRP1A interaction will enhance killing of TNBCs. We have observed that anti-LLT1 antibodies binding to LLT1 on most cell lines (except MDA-MB-468 due to low LLT1 cell surface expression) have prevented LLT1 from interacting with NKRP1A on primary NK cells. Utilizing antibodies to block interaction between LLT1 and NKRP1A has increased lysis of TNBC cell lines MDA-MB-231 and MDA-MB-436 more than the same cell lines treated with corresponding isotype controls. Furthermore, targeting LLT1 on TNBCs has shown greater killing than normal breast cells MCF10A being treated with anti-LLT1 antibodies. Our results have demonstrated that the lower killing of normal breast cells and greater killing of TNBCs indicate that using LLT1 as a target can be distinguished between normal breast cells versus TNBCs due to this difference in expression. However, further testing of LLT1 expression on more normal breast cell lines and healthy breast tissues needs to be done in order to confirm that there is very low expression of LLT1 on a minority of normal breast

cells. The goal is to minimize off-target effects as much as possible when targeting ligands of interest that would increase cancer cells' susceptibility to lysis by NK cells.

We have also utilized another method of blocking LLT1-NKRP1A interaction by using small interference RNA targeting the *LLT1* gene. We have determined that downregulation of LLT1 cell surface expression have prevented LLT1 from interacting with NKRP1A on primary NK cells. By preventing this interaction, we have observed an increase in killing of TNBCs. Using siRNA-mediated downregulation of LLT1 has been shown to be successful in NK-mediated killing of glioma cells [89]. Likewise, when used siRNA-mediated downregulation (*CLEC2D* siRNA purchased from Dharmacon), we have seen an increase in killing of TNBC MDA-MB-436 LLT1-siRNA-transfected cells by 21% compared to the same cell line treated with scramble siRNA with LLT1 expressed (Figure 3.9).

Hence, our results have tested for the expression and function of LLT1 on TNBCs. We have determined that there is greater LLT1 expression at the cell surface on all three TNBC cell lines and non-TNBC cell line MCF7 than normal breast cells. The significant difference in cell surface expression of LLT1 between TNBCs and normal breast cells indicates that LLT1 can be used to target TNBCs while sparing majority of normal breast cells based on our findings. This difference in cell surface LLT1 expression between TNBCs and normal breast cells has been tested when TNBCs and normal breast cells were treated with anti-LLT1 antibodies. Based on our findings, the significant difference in a higher percentage of TNBCs killed versus the lower percentage of MCF10A cells killed has supported using LLT1 as a reasonable target against TNBCs. Our findings have demonstrated that cell surface LLT1 expression on TNBCs serves as an inhibitory ligand that suppresses NK cell activation when it interacts with NKRP1A. We have also shown that blocking LLT1-NKRP1A interaction by two methods, with anti-LLT1 antibodies

and siRNA, have enhanced killing of TNBCs by primary NK cells. Furthermore, much like what other studies have shown when LLT1 was expressed on other types of cancers, TNBCs utilize LLT1 as one of its mechanisms in evading immunosurveillance from NK cells. The function of LLT1 on TNBCs needs to be further characterized in terms of cytokine secretion by TNBCs, upregulation of ligand, and effects of LLT1 expression on other receptors or ligands on TNBCs in future studies. Therefore, targeting LLT1 on TNBCs with monoclonal antibodies may introduce another strategy for patients diagnosed with TNBC.

Future Studies

Based on this study, LLT1 is expressed on TNBCs and blocking LLT1-NKRP1A interaction has enhanced killing of TNBCs by primary NK cells. To our knowledge, this is the first study that targeted LLT1 on TNBCs from an immunotherapeutic perspective. We have determined that cell surface expression of LLT1 on TNBCs inhibit NK cell function. However, it is not known what mechanisms TNBCs use as a result of LLT1-NKRP1A interaction. Our study demonstrates that disrupting the LLT1-NKRP1A interaction, with two methods by siRNA-mediated downregulation of LLT1 and antibody binding to LLT1, has increased susceptibility of TNBCs to be killed by NK cells and that LLT1 on TNBCs allow tumor cells to evade antitumor immune responses by NK cells. Future studies should address the following: (i) What cytokines are secreted by TNBCs when LLT1 interacts with NKRP1A? (ii) Is there upregulation of LLT1 on TNBCs and under what conditions does upregulation occur? (iii) Do we see LLT1 expression on TNBC patient tissue samples? (iv) Can we better enhance the quality of the anti-LLT1 antibody? (v) Does LLT1-NKRP1A interaction inhibit NK cell function in vivo?

Previous studies have confirmed that LLT1 is an inhibitory ligand that interacts with NKRP1A on NK cells on other types of cancer. The general function of LLT1 expressed on

TNBCs remains the same as LLT1 expressed on glioma cells, non-Hodgkin lymphoma B cells, and prostate cancer cells which serves to inhibit NK cell function [88,89,175]. There is a need to further characterize the function of LLT1 on TNBCs because every type of cancer cell may have different mechanisms of inhibiting NK function as a result of the expression of LLT1. For example, LLT1 expression on glioma cells gets upregulated by transforming growth factor-β (TGF-β) which is known as an immunosuppressor produced by glioma cells to evade T cells and NK cells [89,177]. Among the future studies, we should look at what cytokines are produced by TNBCs when LLT1 interacts with NKRP1A on NK cells and separately without LLT1-NKRP1A interaction *in vitro*. As observed in glioma cells, secretion of TGF-β by TNBCs is of interest since it is well-established that production of TGF-β allows glioma cells to evade immunosurveillance [177]. Another cytokine of interest is secretion of IL-12 when LLT1 interacts with NKRP1A, because IL-12 upregulates the expression of NKRP1A on NK cells which leads to inhibition of NK function [113]. Other cytokines that should be tested with and without LLT1-NKRP1A interaction include IL-2, tumor necrosis factor-α (TNF-α), IL-15, and other cytokines that would regulate function of NK cells [5]. Looking at the cytokine profile upon LLT1-NKRP1A interaction would allow us to understand mechanisms that suppress NK cell function and may provide additional targets that would downregulate expression of LLT1 on TNBCs. This type of study can be performed by ELISA method. A study of cytokine secretion by TNBCs or NK cells due to LLT1-NKRP1A interaction will allow us to determine which cytokines produced is involved in the upregulation of LLT1 on TNBCs.

Another future study should look at the expression of LLT1 on tissues of TNBC patients and compare with the expression of LLT1 on healthy breast tissues. Llibre and colleagues has observed that healthy breast tissues do express LLT1, but it is not known quantitatively how

much expression of LLT1 is on these healthy breast tissue since immunohistochemical staining was performed [176]. Our results by flow cytometry of non-tumorigenic normal breast cell line MCF10A has consistently showed in three independent experiments very low expression of LLT1. Furthermore, there was also a much lower percentage of killing of MCF10A cells when treated with anti-LLT1 antibody than TNBC cell line MDA-MB-231 treated with anti-LLT1 antibodies. We can further confirm negligible expression of LLT1 in additional non-tumorigenic breast cell lines such as HMEC (human mammary epithelial cells). For clinical relevance, immunohistochemical staining of LLT1 and obtaining quantitative measures of LLT1 expression such as flow cytometry, mRNA expression, and western blot on tissue samples would allow us to go beyond *in vitro* models and study clinical samples. If we see a difference in LLT1 expression between healthy breast cells and TNBCs in these clinical samples, then this would further strengthen using LLT1 as a target for future monoclonal antibody treatment in the future.

The use of antibodies purchased commercially is convenient, but sometimes does present issues of quality control from the company's end. The anti-LLT1 antibodies we used throughout the project were purchased from a company. In future studies, we would like to also produce antibodies specific for binding to LLT1 from hybridomas. Hybridoma technology has allowed the scientific community to generate high-quality monoclonal antibodies that is specific to binding an antigen of interest [5,178]. Generating anti-LLT1 antibodies from hybridomas specific for secreting antibodies binding to LLT1 will allow us to perform the same experiments using our own enhanced-quality antibodies.

Lastly, transitioning from *in vitro* model to *in vivo* models in targeting LLT1 with generated monoclonal antibodies from hybridomas is important. We are interested in testing our hypothesis of blocking LLT1-NKRP1A interaction with monoclonal antibodies in appropriate

mice models. Future studies should include measuring TNBC tumor size and volume before and after treatment and appropriately quantifying the percent of TNBCs in a tumor microenvironment that are killed by NK cells when TNBCs are treated with anti-LLT1 antibodies. These are additional studies that should be taken into consideration in order to further determine if LLT1 is an ideal target with monoclonal antibodies in future clinical immunotherapeutic treatments.

4.4 PCNA-NKp44 Interaction on TNBCs and its Function

Expression and Function of Cell Surface PCNA on TNBCs

Proliferating Cell Nuclear Antigen (PCNA) has been known as a nuclear protein that serves various roles in replication, cell cycle regulation, and DNA repair mechanism [115]. In addition to PCNA's role in the viability of cells, PCNA can also be overexpressed in different types of cancers which allows the cancer cells to survive and proliferate [116, 120, 121]. Rosental and colleagues have observed that PCNA was expressed on the cell surface of pancreatic, breast, melanoma, lymphoma, and glioblastoma cells [121]. Furthermore, it has also been shown that PCNA serves as an inhibitory ligand that interacts with NKp44 on NK cells and inhibits NK cell function [121]. In this project, we have looked at cell surface PCNA expression on three TNBC cell lines and non-tumorigenic normal breast cells.

We have determined that there is greater PCNA cell surface expression on two TNBC cell lines than on normal breast cell line MCF10A based on subcellular protein fractionation and flow cytometry analysis. In particular, TNBC cell lines MDA-MB-436 and MDA-MB-231 has shown greater PCNA cell surface expression based on flow cytometry analysis looking at percent of cell population $PCNA^+$ than PCNA cell surface expression on normal breast cell line MCF10A. However, based on subcellular protein fractionation, we did not detect PCNA cell

surface expression on MDA-MB-231, but did detect PCNA cell surface expression on TNBC MDA-MB-468. Due to low cell count at the time, MDA-MB-436 could not be tested by subcellular protein fractionation. Based on subcellular protein fractionation, we did not detect PCNA cell surface expression on normal breast cells MCF10A. This subcellular protein fractionation was consistent with flow cytometry analysis of % $PCNA^+$ for MCF10A.

Based on this evidence, we have decided that PCNA may be a possible target for antibody treatment against TNBCs. Even though our flow cytometry analysis showed a higher MFIR value of PCNA cell surface expression on MCF10A than TNBCs, flow cytometry analysis was only performed once and by our gating strategy have determined that there was a significant difference in the lower percentage of MCF10A cells that are $PCNA^+$ than TNBC cell lines that are $PCNA^+$. Naryzhy and colleagues have confirmed that there was higher expression of PCNA on MDA-MB-231 and MDA-MB-468 cell lines than normal breast cell lines MCF10A and HMEC on western blot [120, Figure 1 in Naryzhy *et al*.]. However, the figure focused on total expression of PCNA which encompasses PCNA expression at the nuclear, cytoplasmic, and membrane locations. We have determined that there was low expression of PCNA at the cell membrane of MCF10A than TNBCs from subcellular protein fractionation. This allowed us to target PCNA on the cell membrane of TNBCs in the chromium-release cytotoxicity assays.

The function of PCNA cell surface expression on TNBCs was tested by blocking PCNA-NKp44 interaction with anti-PCNA antibodies binding to PCNA on TNBCs. We have observed that blocking PCNA-NKp44 interaction on TNBC cell lines has enhanced killing of TNBCs by primary NK cells. Treating TNBCs with anti-PCNA antibodies have prevented PCNA on the cell surface of TNBCs from interacting with NKp44 receptor on primary NK cells. By disrupting the PCNA-NKp44 interaction on TNBCs, inhibitory signals are being blocked to the NK cells

allowing the net overall signals to favor activation of NK cell function. Future studies should test targeting PCNA on normal breast cells MCF10A or another similar cell line to see if there is a much lower killing of normal cells than TNBCs. We need to address that targeting PCNA with antibodies will kill more TNBCs than normal breast cells. The goal is to minimize the off-target effects of using antibodies targeting certain ligands. At this time, we have observed a higher percentage of TNBCs killed when PCNA-NKp44 interaction was disrupted than TNBCs with this interaction intact, but it is unknown if normal breast cells are spared from killing by NK cells when treated with anti-PCNA antibodies.

We have already shown that blocking LLT1-NKRP1A interaction on TNBCs has enhanced killing of TNBCs by reducing the inhibitory signal from this interaction from being sent to the NK cells. Likewise, we have shown that blocking PCNA-NKp44 interaction on TNBCs has also increased killing of TNBCs through the same mechanisms. Horton *et al*. has shown that PCNA does synergize with human leukocyte antigen I (HLA I) to inhibit NK cell function [90]. Based on previous studies and results from this project, we have performed a preliminary study on the use of combinational antibodies targeting LLT1, PCNA, and HLA to observe for enhanced killing of TNBCs. We have found that there is increased killing of TNBCs with the combination treatment than TNBCs not treated with any antibodies. Future studies should address specific combinational treatments such as targeting LLT1 and PCNA, targeting PCNA and HLA, and targeting LLT1 and HLA to see which of these interactions are most responsible for increased killing of TNBCs when all three are used.

In summary, this part of our major study has looked at the expression and function of PCNA on TNBCs. We have demonstrated that there is greater expression of cell surface PCNA on TNBCs than normal breast cells based on *in vitro* models. We have also shown that blocking

PCNA-NKp44 interaction with antibodies targeting cell surface PCNA have enhanced killing of TNBCs by primary NK cells. This is due to the lower number of inhibitory signals being sent to NK cells. A combinational treatment targeting LLT1, PCNA, and HLA has also increased killing of TNBCs by primary NK cells, but has similar lysis percentages to TNBCs treated with either anti-LLT1 or anti-PCNA antibodies separately. We have observed that the function of cell surface PCNA on TNBCs is to allow TNBCs to evade immunosurveillance by NK cells through PCNA interaction with NKp44. Future studies should further characterize the function of cell surface PCNA on TNBCs by looking at cytokine secretion and which of the two scenarios, cell surface PCNA interacting with NKp44 or soluble PCNA interacting with NKp44, contributes more to inhibiting NK cell function. Hence, targeting PCNA on TNBCs with monoclonal antibodies may introduce another avenue for patients with TNBCs, but further studies need to be performed to further confirm if PCNA is an ideal target.

Future Studies

From this study, results have shown that PCNA is expressed on TNBCs at the cell surface and blocking PCNA-NKp44 interaction has enhanced killing of TNBCs by primary NK cells. We have demonstrated that cell surface expression of PCNA on TNBCs function to inhibit NK cell function. Like our study on LLT1-NKRP1A interaction on TNBCs, we do not know the exact mechanisms that TNBCs use as a result of PCNA-NKp44 interaction. Based on our observations, our study can conclude that disrupting the PCNA-NKp44 interaction by antibody binding to PCNA has increased susceptibility of TNBCs to be killed by NK cells and that PCNA expressed at the cell surface on TNBCs allow tumor cells to evade antitumor immune responses by NK cells. Future studies should address the following: (i) What cytokines are secreted by TNBCs when PCNA interacts with NKp44? (ii) Is there upregulation of PCNA on TNBCs and

under what conditions does upregulation occur? (iii) Do we see PCNA expression on TNBC patient tissue samples? (iv) Is cell surface PCNA or soluble PCNA responsible for inhibiting NK cell function?

Like the LLT1-NKRP1A future studies, we should look at the cytokine profile as a result of PCNA-NKp44 interaction. Cytokines such as TGF-β, TNF-α, IL-2, IL-15, IL-18, and others could be released by either TNBCs or NK cells from PCNA-NKp44 interaction [5]. A study of cytokine secretion by TNBCs or NK cells from this ligand-receptor interaction will help us to determine which cytokines produced is involved in the upregulation of PCNA on TNBCs. In addition, data collected by previous colleagues in our lab looked at the expression of PCNA on exosomes and at the cell surface on several cell lines in different types of cancers including TNBC MDA-MB-231 and MCF7. It could be possible that cytokine secretion by either TNBC or NK cells may favor either exosomal PCNA expression released by TNBCs or cell surface expression of PCNA on TNBCs. By determining which cytokines are secreted, a mechanism for understanding how PCNA is expressed and how it is inhibiting NK cell function from this interaction with NKp44 can be determined.

Another future study should also look at the expression of PCNA on patient tissue samples. A study that focuses on immunohistochemical staining of cell surface PCNA on tissue samples from patients with TNBC and comparing it to the expression of PCNA on healthy breast tissue is important in determining if targeting PCNA with monoclonal antibodies will effectively kill TNBCs while minimizing off-target effects of killing healthy breast cells. In addition to immunohistochemical staining, flow cytometry analysis and protein expression analysis on patient tissue samples can also give a quantification of cell surface PCNA expression on both TNBC and healthy breast tissue samples.

As discussed, PCNA can be expressed on exosomes released by tumor cells or at the cell surface. Previous data from our lab has looked at the presence of CD63 markers colocalizing with PCNA expression on various cell lines including MDA-MB-231 and MCF7. CD63 is one of the markers used on exosomes. There is much debate on how to prove that one is working with exosomes. It has been studied that exosomes are detected within tumor microenvironments and that exosomes may play a role in enhancing a tumor survival, immunity, and proliferation [179]. Exosomes are known to be formed from inside the cell as endosomes and contains some RNA, DNA, and proteins that are shuttered to outside the cell as vesicles [179,180]. The function of exosomes in cancer has been highly debated, but there seems to be a consensus that exosomes are somehow involved in tumor progression [179].

Several studies have reported increased concentration of exosomes in patients with ovarian, pancreatic, and breast cancers [179,181,182]. An example that proteins in exosomes may play a role in cancer development is proteoglycan glypican-1 (GPC-1) detected in exosomes of serum of patients with pancreatic cancer and breast cancer [181]. This study has detected a difference in expression of GPC-1 between early and late stages of pancreatic cancer versus low levels of GPC-1 in exosomes from healthy patients [181]. Likewise, it could be possible that PCNA detected in exosomes on TNBCs may differ in its expression depending on the stage of tumor progression. A future study should look at the difference in expression of exosome PCNA in different stages of tumor progression on TNBCs and compare it to the expression of exosome PCNA from healthy donors.

Exosomes could play a role in inhibiting NK cell function. Exosomes derived from cancer cells activate NK cell function by presenting a stress protein, heat shock protein 70 (HSP70), to NK cells [179,183]. It has also been studied that exosomes can inhibit NK cell

function by downregulating NKG2D which is known as a receptor that detects infected and tumor cells from its interaction with stress-induced ligands expressed on target cells [184]. Future studies should look at if PCNA expressed in exosomes released from TNBCs also inhibit NK cell function by downregulating NKG2D upon PCNA-NKp44 interaction. Another future study should also address whether which PCNA, cell surface or exosome PCNA, is more responsible for interacting with NKp44 and inhibiting NK cell function. By determining this, we can then focus on either targeting cell surface expression of PCNA or exosome PCNA or both on TNBCs.

In summary, we have determined that there is expression of cell surface PCNA on TNBCs. Furthermore, we have also demonstrated that disrupting PCNA-NKp44 interaction by using antibodies targeting cell surface PCNA on TNBCs has increased killing of TNBCs by primary NK cells. Further studies should look at the mechanism of how PCNA expressed by TNBCs inhibit NK cell function and if PCNA serves as an ideal target in future clinical treatments.

4.5 Conclusion

My project focused on the expression and function of ligands for natural killer cell receptors on TNBCs. Two ligands of focus in my project were Lectin-like Transcript-1 (LLT1) and Proliferating Cell Nuclear Antigen (PCNA). Prior studies have shown that expression of LLT1 and PCNA, respectively, in different types of cancers contributed its role of inhibiting NK cell function. LLT1 as a ligand interacts with NKRP1A (CD161) receptor on NK cells and PCNA as a ligand interacts with NKp44 on NK cells. This study has focused on targeting LLT1 and PCNA on TNBCs with a goal to enhance killing of TNBCs. We have found LLT1 expression on TNBCs and have found that there is significantly greater LLT1 expression on

TNBCs than normal breast cells. Our results have demonstrated that disrupting LLT1-NKRP1A interaction by using antibodies specific for binding to LLT1 has enhanced killing of TNBCs. In addition, we have also observed that downregulating expression of LLT1 by siRNA-mediated knockdown of the *LLT1* gene has disrupted LLT1-NKRP1A interaction and increased killing of TNBCs by primary NK cells. Therefore, we have concluded that LLT1 is expressed on TNBCs allow TNBCs to evade immunosurveillance by NK cells. Hence, we also conclude that blocking LLT1 by both antibody treatment and siRNA-mediated knockdown has prevented LLT1 from interacting with NKRP1A and enhanced killing of TNBCs by NK cells. Blocking LLT1-NKRP1A interaction between TNBCs and NK cells has lowered inhibitory signals from being sent to NK cells thus favoring the net overall balance towards NK cell activation.

Our results demonstrated PCNA expressed at the cell surface of TNBCs. PCNA cell surface expression on TNBCs is greater than PCNA cell surface expression on normal breast cells. Our results demonstrated that disrupting PCNA-NKp44 interaction by using antibodies specific for binding PCNA has enhanced killing of TNBCs by primary NK cells. Therefore, we conclude that blocking PCNA by antibody treatment has prevented PCNA from interacting with NKp44 and increased killing of TNBCs. Blocking PCNA-NKp44 interaction between TNBCs and NK cells has decreased inhibitory signals from being sent to NK cells favoring NK cell activation. PCNA expressed on the cell surface of TNBCs allowed TNBCs to evade immunosurveillance from NK cells.

In conclusion, we have determined that both ligands LLT1 and PCNA are expressed on TNBCs and contribute to TNBCs evasion from NK cells as one of its evasion mechanisms. Future studies should further characterize the function of LLT1 and PCNA by looking at cytokine profiles and understanding how each ligand gets upregulated and affects TNBC

immunogenicity. Targeting LLT1 and PCNA on TNBCs with antibodies will activate NK-mediated lysis and could potentially lead to a new immunotherapeutic treatment for patients diagnosed with TNBC.

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