Toll-like Receptor 3 Signaling in Breast Cancer Cells and the Recruitment of Leukocytes
to the Tumor Microenvironment

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Abstract

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Toll-like Receptor 3 Signaling in Breast Cancer Cells and the Recruitment of Leukocytes to the Tumor Microenvironment

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Advanced stages of breast cancer are often associated with chronic inflammation in the tumor microenvironment that in turn attracts proangiogenic factors, resulting in neoangiogenesis. The upstream signaling that triggers this chronic inflammation is poorly understood. Toll-like receptors are a group of proteins involved in innate immunity and it was hypothesized that the over-stimulation of downstream TLR3 signaling could lead to the production of chemokines that cause leukocyte infiltration into the tumor microenvironment. In vitro stimulation of TLR3 using poly(I:C) and poly(A:U) yielded supportive results, showing an increase in the expression of RANTES, IL-6 and MIP-2 in both mouse mammary tumor cell line 4T1 and human breast cancer cell line MCF7. Further, treated 4T1 cell samples caused a greater percentage of migration of mouse dendritic cells and macrophages as compared to untreated samples in vitro. Lastly, TLR3 expression was knocked down by using siRNA. In the future, TLR3 knock down studies in vivo will help understand the effectiveness of targeting TLR3 in drug development.

Approved: _____________________________________________________________

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1. Introduction

1.1. Cancer and the Immune System

Cancer is a subversive disease which disrupts the homeostasis of a biological system [1]. While the immune system has the ability to defend against and cope with several such attacks on the delicate balance maintained in an organism, it often fails to eliminate these insubordinate cancer cells [1]. The relationship between the immune system and cancer has been investigated for decades. Although the involvement of the immune system in cancer is universally acknowledged, several schools of thought exist regarding whether active immune cells help in combating or promoting tumor development [2].

1.1.1. Contradicting Roles of the Immune System in Cancer

The primary initial response of the organism upon infection is the activation of innate immunity, executed by cells such as macrophages, dendritic cells (DC) and neutrophils [3]. Innate immunity involves the recruitment of these cells to the site of disease or infection and their non-specific activation and response [3]. An adaptive immune response, on the other hand, is a more specific response to a particular disease or infection, involving T cells, which carry out cell-mediated immunity as well as B cells, which carry out humoral immunity [4]. It has been shown that at every stage in cancer, in theory, the immune system has the ability to alleviate, if not eliminate tumorigenesis by a combination of adaptive and innate immune responses [5]. Why then are we not successfully able to eradicate cancer? Studies have shown that the appropriate activation of the adaptive immune system can potentially eradicate tumorigenesis and even protect
against future cancer development [6-13]. However, this is in an ideal scenario, in which tissue homeostasis is maintained and remains unperturbed by the tumorigenic condition. In a more realistic situation, tumor cells possess the ability to hijack the immune system and trigger a plethora of signals that can result in the chronic activation of the innate immune system [14]. It has been observed that this can contribute to angiogenesis by an upregulation of several chemokines, cytokines and growth factors that support neovascularization. Moreover, it also helps in the recruitment of more macrophages and dendritic cells to the diseased site, which in turn assist in angiogenesis by overexpressing angiogenic factors [14]. Therefore, on the one hand, there have been several attempts to activate the B cells and T cells in order to increase the ability of the immune system to eliminate tumor growth [15], while on the other hand, researchers have been trying to determine and eliminate the causes for the chronic activation of the innate immune cells, also known as antigen presenting cells [16-20].

1.1.2. Chronic Activation of Innate Immunity in Different Types of Cancer

As previously mentioned, the chronic activation of innate immune cells such as dendritic cells and macrophages has been shown to contribute to tumor growth. There are many examples in the literature, demonstrating the cause-effect relationship of these leukocytes and different cancer types. For instance, Hepatitis B or Hepatitis C infections can lead to hepatocellular carcinoma [21], while an infection with the human papillomavirus (HPV) could result in cervical cancer [22]. Being inflammatory in nature, these conditions are characterized by an active involvement of the innate immune system. Hence, a typical innate immune response is generated, consisting of the recruitment of
macrophages, dendritic cells and neutrophils to the diseased site by a process called leukocyte infiltration [20, 23]. This phenomenon of the migration of immune cells to the site of infection is facilitated by several factors that fall into the categories of cytokines, chemokines and cell-adhesion molecules[24, 25]. Chemokines are a family of chemo-attractant proteins, whose function is to facilitate the migration of specific cells in a desired direction, determined by the signals received from cytokines [26]. Cell-adhesion molecules in turn help in the tethering and rolling of the cells over the endothelial cells [25]. A pre-existing chronic inflammatory condition usually involves immune cells such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), B cells and regulatory T cells, which have been shown to possess tumor-promoting abilities [17]. Such an influx of leukocytes is also prevalent in non-infective chronic inflammatory conditions such as that associated with smoking, which increases the risk of carcinogenesis [27].

1.1.3. The Tumor Microenvironment and its Tendency to Host Inflammatory Cells

Just as the correlation of a chronic inflammatory condition with cancer is evident and well documented, the converse is also true. In other words, it has been found that an existing tumor growth is often associated with a large proportion of leukocyte infiltration [28]. This area surrounding a tumor, which consists of both cancerous and non-tumorigenic cells such as fibroblasts, endothelial cells, etc. along with connective tissues, the extra-cellular matrix (ECM) as well as infiltrating leukocytes, cytokines and growth factors is termed as the tumor microenvironment [29]. There is a general consensus among researchers that the tumor microenvironment, harbored and nourished by the
tumor cells themselves, significantly contributes to tumor development [30]. For this reason, several groups have dedicated their studies to understanding the tumor microenvironment so as to propose manipulative techniques that might assist in the alteration of the microenvironment to eliminate tumor growth [31].

The immune system directs the leukocytes of the first line of defense to the site of tumor growth in an effort to neutralize, endocytose or kill the rapidly proliferating tumor cells [32]. Although in theory, this action should be sufficient to render protection against cancer growth and is even true to a certain extent, further examination of these tumor-associated leukocytes in the tumor microenvironment has revealed that they gradually develop a different phenotype that, over time, collaborates with, rather than fight the tumor growth [20]. As mentioned earlier, the key to fighting tumor growth is to stimulate a specialized adaptive immune response rather than an array of generic inflammatory responses mediated by the innate immune cells. In an attempt to determine the cause for this leukocyte influx, there have been several studies to examine the upstream signals generated in the tumor microenvironment, insomuch that cancer signaling has branched out as a separate specialization by itself[33-38].

1.2. Breast Cancer, Leukocytes and Cancer Cell Signaling: How they are Related

The deregulation, disruption or over-activation of any of the intrinsic cell signaling pathways can be a key factor in a complex disease such as cancer. This has sparked interest among researchers to study the upstream as well as downstream signaling associated with different types of cancer and put together the big picture. For instance, it has been shown that the PI3K/AKT pathway and the Wnt/β catenin pathway
are frequently altered in many cancers [35, 39]. Similarly, Stat3 has been extensively studied to define its function in cancer stem cells [38], while components of the NF-κB pathway have been shown to be interlinked with the immune response to cancer [16]. While the exact mechanism involved in the attraction of leukocytes to the tumor microenvironment is not well understood, it has been identified as being closely related to the deregulation of cellular signals. The below paragraphs examine cell signaling in breast cancer and how it affects and manipulates the immune system.

### 1.2.1. The Involvement of Leukocytes in Breast Cancer

The treatment modalities for breast cancer, the second leading cause of cancer death among women, depend largely on the stage at which it is detected. The five-year survival rate associated with an early detection is nearly a hundred percent. However, this encouraging statistic drops exponentially, as the disease becomes more advanced [40]. Hence, it is important to understand the numerous cellular and molecular events that occur with the progress of the disease.

Studies have indicated that the invasive nature of breast cancer is typically associated with extensive leukocyte infiltration, predominated by TAMs and dendritic cells [41, 42]. Generally correlated with a poor survival rate [43-45], this predictably enhances the proliferative ability of tumor cells and creates an environment that supports metastasis. Furthermore, antigen presenting cells (APCs), abundantly found in the tumor microenvironment, are known to be related to neoangiogenesis in both human cancers and mouse tumor models, as mentioned previously [21, 46-50]. Neoangiogenesis is the process of formation of new blood vessels that nourish and harbor the proliferation of
tumor cells in the tumor microenvironment. However, the mechanism by which inflammatory cells are attracted to the tumor microenvironment is unclear and remains widely debated. As observed in many studies, the cellular signaling in a tumor microenvironment is aberrant. It is proposed that both the tumor cells and non-cancerous cells in the tumor microenvironment spew out signaling molecules – chemotactic factors and other cytokines – that promote immunological reactions and activity by attracting leukocytes to the tumor microenvironment. It has further been postulated that these inflammatory cells in turn produce more cytokines, growth factors and pro-angiogenic factors that contribute to cancer progression. Since the progression of breast cancer is incumbent upon the initiation and sustenance of neovascularization, a better understanding of these initial interactions between the tumor cells and the immune system is important in designing effective anti-tumor therapies.

1.2.2. Cytokines and Cellular Signaling Associated with Leukocyte Infiltration in Breast Cancer

It has been observed that different stages in the advancement of breast cancer are associated with different expression levels of some notorious cytokines and other proteins. In other words, the progression of the disease correlates with the abnormal expression of specific proteins. For instance, the C-C chemokine family molecule RANTES (Regulated upon Activation Normal T-cells Expressed and Secreted), also called CCL5 has been found to be overexpressed in advanced breast carcinoma cases [51]. It has furthermore been observed that RANTES, along with another chemokine, CCL2, exhibits promalignant properties that help in the progression of the disease by
attracting a large number of monocytes by its chemotactic properties [18, 52]. Another proinflammatory cytokine, IL-1α has also been reportedly involved in promoting tumor malignancy and inducing atrophy in tissues. While normally expressed constitutively in epithelial cells, its overexpression in breast cancer has been suggestive of tumor growth [53]. Other studies have cited the correlation of the levels of specific breast cancer biomarkers with the disease stage. For instance, one particular study shows high expression levels of IL-6, IL-8 and IL-10 in breast cancer patients and a direct relationship between the concentration of these cytokines and the stage of the disease [54].

It is noteworthy to mention here that cytokines can have either a supporting or an inhibitory role in tumor development, depending on their expression level, their general functions which are usually numerous and the presence of certain other factors in the surrounding microenvironment. Therefore, several studies have focused on evaluating the utility of cytokines as prognostic tools in predicting the progression of the disease in a patient. For instance, in one study, it has been shown that patients with recurring breast cancer have an observable change in the level of IL-6 and IL-8, that is prognostic as well as indicative of the response to treatment [55]. Also, in another clinical study using immunohistochemistry on tissue samples from patients affected with breast cancer at different stages, it was found that RANTES/CCL5 can be used as a prognostic marker in conjunction with other factors for stage II breast cancer patients [56]. IFN-β and IL-2 are some other cytokines that have not only been identified as markers, but also been
administered to patients in some clinical trials in order to assess their therapeutic potential [57].

Not only have cytokines simply been observed in elevated or depleted levels in a tumor condition, they have also been shown to actively induce angiogenesis and inflammation. The tumor associated fibroblasts in breast cancer have been indicated in the excessive production of SDF-1, which in turn recruits endothelial progenitor cells that contribute to angiogenesis and invasiveness of the tumor [58]. In clinical samples from breast cancer affected patients, immunohistochemical analyses revealed high expression levels of macrophage chemoattractant protein-1 (MCP-1) in the tumor cells. Further examination also showed the massive macrophage-infiltrations caused by MCP-1, most of the macrophages also showing high expression of MCP-1 [59].

From all these studies, it is evident that just as normal cells produce certain cytokines and growth factors for their growth, nourishment, sustenance and survival, so do tumor cells for the very same reasons. As previously described, abnormal or aberrant levels of cytokine production are a result of aberrant cellular signals. Therefore, many attempts have been made to understand the various cellular signals that could have been derailed and also could possibly induce cells in the tumor microenvironment to produce the cytokines in question.

1.3. Toll-Like Receptors

Toll-like receptors (TLRs) are an important class of protein molecules involved in the process of inflammation [60]. These are a family of pattern recognition receptors (PRR) which can activate an innate immune response and confer protection against
various microbes [60]. The TLRs carry out this protective function by the recognition of exogenous or cellular products of microbes, called pathogen-associated molecular patterns (PAMPs) [61, 62]. For instance, TLR3 plays a role against viral infection by activating immune cell interactions and antiviral responses upon recognizing dsRNA from virus infected cells [62]. Recently, some endogenous ligands such as nucleic acids and heat shock proteins have also been identified as potential activators of TLRs [63, 64]. Mostly found in association with inflammatory cells, these receptors may be expressed on either the cell surface or in endosomal compartments.

1.3.1. Types, Localization, Structure and Downstream Signaling of TLRs

So far, at least 13 TLR subtypes have been recognized in mice and 11 in humans [65]. Each TLR subtype recognizes and is activated by a particular ligand, in turn triggering the release of a cascade of cytokines that affect cellular functions. Table 1 shows a list of TLRs, their location and some of the ligands they recognize.

As depicted in Table 1, TLR1, TLR2, TLR4, TLR5 and TLR6 are all located on the cell surface, the majority of which recognize lipid-related ligands. These are type 1 transmembrane receptors, whose cytoplasmic domain resembles that of IL-1R. Therefore, this domain is also called the TIR domain, which stands for Toll IL-1R domain [61]. They are also usually characterized by leucine-rich repeats in the extracellular domain. TLRs are structurally and evolutionarily conserved across several species, even though some subtypes are exclusive to some species [66]. For instance TLR11, TLR12 and TLR13 are expressed in mouse but not in humans, even though a gene for TLR13 exists in humans, but fails to be transcribed due to the presence of stop codons [67]. On the
other hand, TLR3, TLR7, TLR8 and TLR9 are located in endosomal compartments. This is apt for their function, as they are mostly involved in recognizing viral and bacterial DNA. Their localization helps them distinguish between host and foreign nucleic acid due to the fact that the elimination of viral particles and bacterial cells requires them to be endocytosed into late endosomal compartments [65]. Moreover, some studies have also shown that the expression of TLR9 on the cell surface as a transmembrane protein will result in the indiscriminate recognition of self DNA, which could be implicated in certain types of autoimmune diseases [68].
Table 1: List of Toll-Like Receptors, their Location and Ligands [61, 69]

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Location</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Cell surface</td>
<td>Triacyl Lipopeptides</td>
</tr>
<tr>
<td>TLR2</td>
<td>Cell surface</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lipoproteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycoproteins</td>
</tr>
<tr>
<td>TLR3</td>
<td>Cell surface</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td></td>
<td>Cell Compartment</td>
<td>Poly I:C</td>
</tr>
<tr>
<td>TLR4</td>
<td>Cell surface</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>TLR5</td>
<td>Cell surface</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR6</td>
<td>Cell surface</td>
<td>Diacyl Lipopeptides</td>
</tr>
<tr>
<td>TLR7</td>
<td>Cell compartment</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bropirimine (experimental drug with anti-cancer properties) [60]</td>
</tr>
<tr>
<td>TLR8</td>
<td>Cell compartment</td>
<td>Single-stranded RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synthetic compounds</td>
</tr>
<tr>
<td>TLR9</td>
<td>Cell compartment</td>
<td>Unmethylated CpG of DNA</td>
</tr>
<tr>
<td>TLR10</td>
<td>Cell surface</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

TLRs have not only been recognized as activators of innate immunity but also as important bridges for the activation of adaptive immunity [70]. Upon the recognition of a ligand, they often activate the production of inflammatory cytokines such as the interleukins as well as co-stimulatory molecules, the latter of which facilitate acquired immunity via T helper cells. Also, they have a special function in providing antiviral activity through the induction of type I interferon response [70]. All TLRs except for TLR3 mediate their downstream signaling through the adaptor molecule called Myeloid...
Differentiation primary response gene 88 (MyD88), closely related to an inflammatory response through IRAK signaling which eventually activates the NF-κB (nuclear factor kappa light chain enhancer of activated B cells) pathway [65]. Other adapter proteins that are involved in TLR signaling are TIR-domain containing adapter protein (TIRAP), TLR-related adapter molecule (TRAM) and TIR domain-containing adapter-inducing interferon β (TRIF) [65].

TLR3 operates through a MyD88-independent pathway, involving the adapter molecule TRIF which eventually leads to both the interferon regulatory factor (IRF) signaling as well as NF-κB signaling. TLR4 can also act through this same pathway in addition to its ability to utilize MyD88. Also, TLR7, TLR8 and TLR9 activate the IRF pathway, although through MyD88 [61, 65, 66]. The activation of the IRF pathway is instrumental in stimulating antiviral protection, which, as discussed earlier, is mediated mostly by the endosomal TLRs. Additionally, it is well established that NF-κB is a key factor in regulating both adaptive and innate immune responses [71]. Moreover, a derailed NF-κB pathway has wide implications in cancer and some autoimmune diseases [72, 73]. The following figure indicates the functioning of NF-κB as mediated by the TLR pathways, the dysfunction of which will affect transcription, in turn impacting several cellular functions including immune regulation.
Figure 1: TLR pathway diagram. Figure shows the downstream signaling of different TLRs resulting in the control and activation of various cellular and immune functions [65].
1.3.2. Toll-Like Receptors and Diseases

As seen in the previous paragraphs, TLRs have a wide variety of immunological functions, ranging from the activation of innate immunity through cytokines up to triggering the production of costimulatory molecules that assist in adaptive immunity. Therefore it is not surprising that a deregulation in the downstream signaling of TLRs can result in a wide range of diseases. There is also something to be said about the improper stimulation of TLRs. While less stimulation can lead to reduced immunity to infections, over stimulation can also result in inflammation-related conditions. In this light, TLRs have recently been implicated in different infectious as well as non-infectious diseases. For instance, non-immune cells such as pancreatic beta cells have been shown to express TLR3 in individuals with new-onset type I diabetes mellitus [74]. Polymorphisms in TLRs, resulting in aberrant downstream signaling has also been shown to aggravate inflammatory bowel disease [75]. Furthermore, abundant expression and activation of TLRs has been implicated in other chronic conditions such as rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis and a number of other autoimmune diseases [76]. For this reason, TLR antagonists have been attempted to be used for treatment purposes in certain disease conditions. The expression of TLRs in non-immune cells was at first surprising as well as confusing, considering their close affiliation with immunological functions. It has been suggested that their involvement in diseases could be due to their unique abilities to activate multiple inflammatory pathways not only through expression in immune cells, but also other cell types, resulting in aggravated
autoimmune conditions in particular, and all inflammation-dependant conditions in general.

Additionally there are a number of publications, indicating the involvement of TLR signaling in different cancer types. For instance, TLR4 expression in ovarian cancer cells has been shown to promote chemotherapeutic resistance [37], while in another studies, sufficient activation of TLR5 with its ligand flagellin actually showed some antitumor activity in mice [77]. Therefore, the roles of TLRs in cancer are still being explored and there is clearly a lot more to be understood in terms of defining their contributions to the development or the suppression of tumors. A better understanding of the consequences of activated TLRs in different tumors can potentially lead to the development of targeted drugs that can manipulate their downstream signaling and hence the disease condition.

1.3.3. Toll-Like Receptor 3

As noted earlier, TLR3 recognizes double-stranded RNA (dsRNA) molecules and generally provides immunity to viral infections. This is understandable, considering that there are a wide range of RNA viruses with dsRNA as their genetic material, such as the rotaviruses. TLR3 is also unique in that, it follows a MyD88-independent signaling pathway upon recognizing dsRNA about 40-50 base-pairs in length, in contrast to the rest of the TLR family [78]. Besides dsRNA, TLR3 can be activated in vitro by polyinosinic:polycytidylic acid (poly I:C), a synthetic compound analogous in structure to that of a dsRNA [79].
1.3.4. TLR3 and Diseases

Recently, some endogenous ligands have been identified as potential activators of TLRs in mammals. Particularly, TLR3 has been observed to recognize endogenous RNA, generated from necrotic cells, leading to the stimulation of dendritic cells [64]. Such activation of TLRs by endogenous ligands is suggested to be a major contributing factor to the chronic inflammation associated with the growth and spreading of different kinds of cancer [80]. It is believed that the endogenous activation of TLRs sets off a slew of interlinked inflammatory pathways that result in the massive recruitment of leukocytes to the tumor microenvironment. For instance, studies on both malignant melanoma cell lines and human melanoma tumors have indicated that TLR3 signaling induces the expression of inflammatory factors that assist in chronic inflammation [81]. Wnt5a is a part of the Wnt family of proteins known to be involved in embryonic development and the deregulation of the Wnt signaling pathway is notorious for oncogenesis. Active expression of TLR3 along with Wnt5a in papillary thyroid cancer has been found to be correlated with prolonged tumor cell survival, enhanced proliferation and migration [82, 83].

Taking into account the adequate indications in the literature that TLR3 signaling could lead to the production of different cytokines that are suggestive of tumor progression, it is important to examine the downstream signaling triggered in response to TLR3 activation in cancers.
2. **Hypothesis and Objectives**

The introductory chapter elucidates the relationship between breast cancer and the immune system, the role of TLR3 in providing innate immunity as well as its association with several diseases including breast cancer.

With this valuable information, my research project has explored the association between leukocyte infiltration in breast cancer and the innate immune response associated with the downstream signaling of TLR3. In other words, my study hypothesizes that the stimulation of TLR3 in breast cancer cells causes the secretion of chemokines that attract leukocytes responsible for chronic inflammation in the tumor microenvironment.

This was done by examining the effects of *in vitro* activation of TLR3 on the expression levels of different chemokines and cytokines that have been implicated in murine and human breast cancer. Migration studies to determine the role of activated TLR3 in promoting leukocyte attraction have also been performed, followed by inhibition studies, involving the *in vitro* knock down of TLR3 expression in the cells. The following sections describe experimental details, the results obtained and a summary section, describing the significance and future direction of my studies.
3. Materials and Methods

3.1. Cell Culture

4T1 mouse breast cancer cells: 4T1 mouse breast cancer cells, originally derived from BALB/c mouse mammary tumor model, were cultured in RPMI medium containing 10% FBS and 1% antibiotic for use in experiments. Cultures were prepared as monolayers and sub-cultured into 12-well plates after washing with PBS and trypsinization [84].

Human breast cancer cells: MCF-7 human breast cancer cells, cultured in ATCC media were primarily used for experiments. Additional human breast carcinoma cell lines namely BT20, MDA-MB231, MDA-MB468, ZR75-1 and T47D cells (ATCC, Manassas, VA) were also used for the study of TLR3 expression [85].

3.2. Treatment with Poly (I:C)

Poly (I:C) (Invivogen, San Diego, CA), a synthetic compound that is structurally analogous to double-stranded RNA, was used to treat 4T1 cells in order to stimulate TLR3 signaling. 4T1 cells were exposed to different concentrations of poly (I:C), ranging from 0 µg/ml (used as control) up to 10 µg/ml for different lengths of time, in low serum-containing OptiMEM medium (Invitrogen, Carlsbad, CA). The supernatant from each well was subsequently collected and tested for the presence of several chemokines by ELISA. Treated cells were also harvested for RNA extraction, followed by quantitative PCR (qPCR) analysis. Poly (I:C) treatment was facilitated by the use of the lipofectamine™ transfecting reagent (Invitrogen) for more efficient delivery of poly(I:C).
into the cells [86]. Later, a similar synthetic analog poly (A:U) was also used to stimulate TLR3 by lipofectamine™-mediated transfection.

3.3. ELISA

Supernatant samples collected upon treatment of 4T1 cells with poly (I:C) were tested for the expression of various chemokines by a sandwich ELISA [87]. The chemokines to be tested were selected based on their ability to attract various immune cells such as dendritic cells, lymphocytes, macrophages and neutrophils. An HRP-based detection system was used for analysis and the OD was measured at 405 nm to obtain the protein concentration using a standard curve. All samples were tested for the expression of RANTES, SDF-1α, MIP-3α and MIP-3β, each sample being tested in triplicate. Each of the experiments was also performed thrice to ensure repeatability. Results obtained in a protein array experiment performed later were also confirmed by ELISA in the same manner, repeating each experiment thrice for reproducibility. Also, each sample was tested in triplicate in a single experiment to minimize colorimetric and spectrophotometric errors.

3.4. RNA Extraction and Reverse Transcription Reaction

Total RNA was extracted from cells treated with different concentrations of poly (I:C) using TRizol® and the quality of the RNA was evaluated by measuring its absorbance at 260 nm. The RNA samples thus obtained were subjected to DNase treatment, followed by Reverse Transcription PCR (RT-PCR), using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). One part of the complementary DNA (cDNA) sample thus obtained was run in an agarose gel (AGE) and
observed under UV radiation to ensure that the reverse transcription reaction was successful [87].

3.5. Reverse Transcription PCR and Quantitative PCR (qPCR)

Complementary DNA samples obtained from different breast cancer cell lines and tissues by the above method were subjected to a regular PCR analysis for the expression of TLR3, using the respective primers. This was repeated thrice to ensure consistency of the results. Similarly, the cDNA obtained from tumor-associated dendritic cells were checked for the expression of several angiogenic factors by PCR. The PCR kit from Invitrogen, Inc. containing Taq DNA polymerase, MgCl₂, dNTPs and PCR buffer was used to run the PCR. The PCR reactions for each angiogenic factor were performed twice to check for reproducibility.

Following this, a quantitative PCR was performed for poly I:C-treated cDNA samples from 4T1 cells using SYBR® green (Applied Biosystems, Foster City, CA) and the expression of TLR3, RANTES, SDF-1α, MIP-3α and MIP-3β were analyzed by the absolute quantification method in the Bio-Rad iCycler thermocycler, using the Bio-Rad iQ5 software (Bio-Rad, Hercules, CA) for analysis. This was done to additionally evaluate the expression of these chemokines at the mRNA level. The real-time PCR was run twice for each target molecule to ascertain reproducibility of the data obtained [87].

3.6. Antibody Array

Supernatant samples of cells subjected to lipofectamine™-mediated poly I:C treatment, collected after 6 hours, were subjected to a protein array experiment. Commercially available RayBio® Mouse Cytokine Antibody Arrays (RayBiotech,
Norcross, GA) were used for the experiment following the manufacturer’s instructions. Only samples treated with a poly I:C concentration of 1 μg/ml were used for the arrays, along with controls (no treatment). The protein array consisted of antibodies for several mouse cytokines, embedded in a membrane provided with the product. Samples were incubated over the membrane and a secondary biotinylated antibody cocktail was added for detection purposes, mimicking the principle of a sandwich ELISA. Following addition of streptavidin-HRP, the array was exposed to Kodak X-Omat AR film and the signals were detected using a film developer. Exposure times ranged between 20 seconds up to 2 minutes. Subsequently, a similar protein array experiment was performed for MCF-7 human breast cancer cells treated with poly I:C, using the RayBio® Human Cytokine Antibody Arrays (RayBiotech, Norcross, GA), to do a semi-qualitative analysis of cytokine expression in the human breast cancer cells following manufacturer’s instructions. The protein arrays themselves were run only once each for murine cell samples and human cell samples, but consisted of pool supernatants recovered from different experiments. However, follow-up ELISA studies were performed to confirm quantitatively, the significant changes observed in the cytokine expression levels.

3.7. PCR Array

The RT$^2$ Profiler™ PCR Array (SA Biosciences, a part of Qiagen, Frederick, MD) for mouse cytokines and receptors was used to analyze the gene expression profile of about 84 genes encoding different chemokines and their receptors in cDNA samples from poly I:C-treated 4T1 cells (with samples from untreated 4T1 cells as control). For this purpose, the RNA isolation and RT-PCR was carried out using the RT$^2$ qPCR-grade
RNA Isolation Kit and RT² First Strand Kit respectively, also provided by SA Biosciences, Inc. The protocol provided along with the product was followed and the qPCR results were analyzed using the iQ5 software (Bio-Rad, Hercules, CA). This experiment was performed once as a cross-checking tool to validate the expression levels of some molecules such as RANTES which had been analyzed already at the protein level, as well as to identify some potential new cytokines that could have been upregulated at the gene level.

3.8. TLR3 Inhibition

Lentivirus that possess the ability to stably knockdown mouse TLR3, obtained commercially (MISSION® shRNA Lentiviral Transduction Particles, Sigma) were used for the knock-down of TLR3 in 4T1 cells. The cells were exposed to lentiviral vector preparations (10 MOI) for 24 hours in the presence of polybrene (SIGMA Chemicals Co., St. Louis, MO) [88]. Lentivirus particles with scrambled sequences served as controls. Cells were then cultured in RPMI media with 10% FBS and 2.5 µg/ml of Puromycin (added for selection of transduced cells). In order to evaluate the efficacy of the knockdown process, 4T1 cells were stained with an antibody to TLR3 and analyzed by immunofluorescence. Those cells transduced with a scrambled vector were stained for comparison. The knock-down of TLR3 expression was also analyzed at the mRNA level by RT-qPCR. The knock-down experiments were performed thrice and the knock-down efficiency was evaluated each time by the above methods (qPCR and IF).
3.9. Flow Cytometry

Cells obtained from 4T1 mouse breast tumors were stained for the surface markers CD11c, CD45, CD80, CD86 and CD40 and analyzed on a FACsort™ flow cytometer using CellQuest 3.2.1f1 (BD biosciences, San Jose, CA), to observe the population of dendritic cells and other leukocytes in the sample [87]. Further, in another three-color staining experiment, the mouse breast tumor-derived cells were stained for CD45, TLR3, CD11c, CD3, CD14 and VE-Cadherin to identify populations of leukocytes, endothelial cells and cells expressing TLR3 by flow cytometric analysis. Antibodies conjugated with Fluorescein isothiocyanate (FITC), phycoerythrin (PE) or Peridinin chlorophyll protein (PerCP) were used for each staining. Each experiment was performed thrice to ensure consistency.

3.10. Immunomagnetic Purification

Tumor-associated dendritic cells were recovered from single cell suspension of 4T1 solid tumors excised from mice by immunomagnetic purification. Upon blocking Fc receptors in the cells, CD11c positive cells were labeled with anti-mouse CD11c magnetic beads (MACS® Miltenyi Biotec) and isolated by passing through a column under a strong magnetic field [87]. CD11c is a surface marker found abundantly in dendritic cells and some other immune cells such as neutrophils, macrophages, mast cells, etc. However, these other cells also express Fc receptor, which the dendritic cells lack. Hence a blocking step of the Fc receptor was carried out to selectively isolate dendritic cells.
Following this, cDNA samples were prepared by reverse transcription following total RNA isolation from these cells. The expression of angiogenic factors MMP-2, MMP-7, MMP-8, MMP-9, VEGF-A, bFGF, heparanase and angiogenin were being measured by RT-PCR. As mentioned earlier, the samples were run twice with each primer to ensure repeatability. The primers for these molecules were designed such that the amplicon was about 100 base pairs long, using the web-based Primer 3 program [87].

### 3.11. Immunofluorescence

Immunofluorescence (IF) was used to study the presence of TLR3 expressing cells in mouse mammary tumor sections, which were first snap-frozen and embedded in OCT media [89]. Cryosections, each of 7μm thickness were fixed in acetone and then blocked in 5% milk to prevent non-specific binding. Subsequently, the cells were stained with a primary antibody against TLR3 (Invivogen, San Diego, CA) and a secondary AlexaFlour® 488 conjugate, using an IgG isotype control simultaneously, on the same slide. The slides were then counterstained with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) in order to visualize the nuclei of the cells, and inspected for TLR3-positive cells under the fluorescence microscope in the FITC channel. Eight slides were analyzed in total in this manner, each slide containing a section stained with the antibody and another section with the isotype control.

A similar staining was done in both cultured 4T1 cells and TLR3-knock down 4T1 cells to confirm the expression of TLR3 and to determine the efficiency of knock-down respectively. Four slides were prepared in total and the knock-down experiment itself was performed thrice as mentioned. Besides, different human cell lines were also
analyzed by IF for the expression of TLR3. Four slides were prepared for each cell line: MCF7, BT20, MDA-MB231, MDA-MB468, ZR75-1 and T47D.

3.12. Migration Assay And Cell Counting

A migration assay was performed using the ChemoTx® 96-well microplates (Neuro Probe, Gaithersburg, MD), following manufacturer’s instructions, in order to study the ability of poly(I:C)-treated samples to cause the migration of mouse macrophages and DC. The RAW 264.7 mouse macrophage cell line and the JAWS I mouse dendritic cell line were used respectively. The bottom portion of the transwell microplates was filled with untreated and treated samples as well as PBS (blanks), covered by a semi-permeable membrane, over which 60µL of the respective cells in suspension was added. The following figure represents the plate design used for the experiment. The cells were washed, re-suspended and the cell count was determined beforehand. The entire set-up was incubated at 37ºC for 4 hours.

![Figure 2: Plate design for migration assay](image)
Following this, the remaining cells on top of the membrane were washed away and the microplate was centrifuged, so as to also collect cells that had migrated but remained loosely attached to the lower surface of the membrane. Subsequently, the number of cells in each well was counted using the FACSort™ flow cytometer. This was done by adding a known volume of CountBright™ absolute counting beads (Molecular Probes™, Invitrogen, Carlsbad, CA), a calibrated suspension of microspheres that have the ability to fluoresce across a broad range of wavelengths. Therefore, 10 µL of the beads, containing 10,000 beads in total, was added to the cells that had been made up to 300 µL by volume using PBS. The samples were then run on the flow cytometer and a total of 2000 to 10,000 events were counted. A dot plot between the forward scatter and the linear side scatter was generated, in which the beads were observed as a distinctly separate population and hence gated. The cell concentration, represented in cells/µL, was calculated using the following formula:

\[
\frac{\text{Number of cell events}}{\text{Number of bead events}} \times \frac{\text{Assigned bead count of the lot (approx 10,000)}}{\text{Volume of sample (µL)}}
\]

Additionally, the percentage of cells migrated was calculated using the total cell count and plotted for both untreated and poly (I:C) treated cells for comparison. The migration assay was performed four times and the data collected were compared to ascertain reproducibility.
3.13. Statistical Analysis

All the quantitative data obtained in the above experiments, which include the results of ELISA, RT-qPCR and migration assay were put through statistical analysis. All the data obtained from repeated experiments were compared and the variance as well as the standard deviation among the different sets of data was calculated. A student ‘t’ test assuming unequal variances was performed for each set of data to determine the p value and to evaluate the statistical significance of the data. P value ≤ 0.05 was considered significant. P value ≤ 0.01 was considered very significant and P value ≤ 0.001 was considered extremely significant. All statistical analyses were done on Microsoft Excel 2007 using the ‘Data Analysis Toolpak’. In the results, the statistically significant data are represented using asterisk (*).
4. Results and Discussion

4.1. A population of leukocytes is found in 4T1 mouse mammary tumor

Cells were prepared as a suspension upon mechanical disaggregation of 4T1 mouse mammary tumors induced in BALB/c mice and stained with an antibody to CD45 (BD, San Jose, CA). They were then analyzed by flow cytometry and a distinct population of CD45 expressing cells was identified as shown in figure 3. CD45 is a pan leukocyte marker and has been extensively used to identify leukocyte populations by immunohistochemistry, immunofluorescence or flow cytometry in tissue samples. This result suggests that leukocytes can be recruited to the tumor microenvironment, indicating interactions of the immune system with the tumor microenvironment.

This result is not surprising, as several studies have in the past shown the presence of leukocytes in advanced tumor conditions, irrespective of whether or not the mechanism of underlying this process was studied [47, 90]. As shown in figure 4, a great proportion of these cells express CD11c, a typical murine DC marker.
Figure 3: Leukocyte population in 4T1 tumors by flow cytometry. Dot plot obtained from the flow cytometric analysis of cells derived from 4T1 mouse mammary tumor. Cells were stained with CD45 antibody. The CD45 positive cells shown in 3(ii) represent the population of leukocytes. The experiment was repeated 3 times. An isotype control was used for each repetition.
Figure 4: Dendritic cell population in 4T1 tumors by flow cytometry. Dot plot obtained from gating CD45 positive to show a population of cells dually positive for both CD45 and CD11c. This is the population of dendritic cells found within the cells obtained from 4T1 mouse mammary tumor. The experiment was repeated 3 times for consistency. An isotype control was used for each repetition.
4.2. 4T1 mouse mammary tumor-associated dendritic cells express pro-angiogenic factors

As mentioned in the methods section, tumor-associated DC were selectively isolated from 4T1 mouse mammary tumor using MACS® anti-CD11c beads. The total RNA was then isolated from the cells and RT-PCR was performed to obtain complementary DNA samples. Then primers designed by the web-based Primer 3 program were custom ordered through Invitrogen and were used to perform PCR and amplify selected regions encoding pro-angiogenic factors. The following figures show the electrophoresis gel images, indicating the expression of different angiogenic factors.

In the top panel, the image on the left shows the expression of the matrix metalloproteinases (MMP)-2 and 9. In all experiments, the house-keeping gene GAPDH was used as a loading control. The top right image shows the expression of MMP8 and MMP9. The bottom panel images indicate the expression of angiogenin, arginase I and heparanase. As mentioned in the materials and methods section, an array of pro-angiogenic factors were chosen and tested in this experiment: MMP2, MMP7, MMP8, MMP9, heparanase, VEGF-A, bFGF and angiogenin. The panel of images below only represents those that were found to be expressed in the experiments.
Figure 5: Expression of pro-angiogenic factors in tumor-associated DCs by RT-PCR. GAPDH was used along with each experiment set as a house-keeping gene, acting as control. MM stands for molecular marker (DNA ladder). Since each primer was custom-designed to be around 100 bp, all bands fall in the same range. The arrow in Figure 5D shows the 100 bp mark. Bands below this are primer dimers. The experiments were repeated three times for consistency.

The matrix metalloproteinases were chosen due to their notorious links to metastasis in different cancers in the literature. These are a family of proteases that function to degrade the various protein components of the ECM. Therefore in cancers, their excessive activity tends to eliminate the boundaries delimited in a primary tumor, providing for metastasis. They function in conjunction with heparanase, an enzyme that executes the degradation of polymeric heparin sulfate on the cell surface as well as the ECM. Vascular endothelial growth factor (VEGF-A) and angiogenin, as the names
suggest, helps in vasculature and new blood formation. Their over-expression in tumors can lead to angiogenesis, thereby resulting in the nourishment of the tumor via added blood and nutrient supplements.

The results of the RT-PCR experiments showed that tumor-associated dendritic cells express pro-angiogenic factors implicated in tumor progression. Therefore, it would be beneficial to find the cause for and then reduce or eliminate the influx of innate immune cells to the tumor microenvironment.

4.3. TLR3 is expressed in mouse and human breast cancer cell lines and tissues

As discussed in the introduction section, the signaling through toll-like receptors is found to be linked with different types of cancer. Therefore, the expression of TLR3 was first tested in various human and mouse breast cancer cell lines as well as tissues. A combination of both RT-PCR and immunofluorescence was used to confirm that the results were consistent. The first figure below represents the expression of TLR3 in both 4T1 mouse breast tumor cells as well as MCF7 human breast cancer cells by RT-PCR. The figures that follow subsequently confirm the expression of TLR3 in both 4T1 breast mammary tumor tissues as well as MCF7 cells by immunofluorescence. For the immunofluorescence experiments, the secondary antibody used was conjugated to Alexa Fluor® 488, a dye that fluoresces in the green region and which can be observed using a FITC (fluorescein isothiocyanate) filter due to its coinciding absorption and emission ranges.
Figure 6: TLR3 expression in 4T1 cells and MCF7 cells by RT-PCR. MM represents molecular marker (DNA ladder). GAPDH was used as a control in each experiment. Each primer was custom-designed to be around 100 bp in size and the arrow denotes this 100 bp mark. Bands below this are primer dimers. The experiments were repeated three times for consistency.
Figure 7: TLR3 expression in 4T1 mouse tumor tissues by IF. DAPI was used for nuclear staining (top panel) and TLR3 antibody was used followed by an AlexaFluor® 488 conjugated secondary antibody (bottom panel). A total of 8 such slides were prepared, each containing 2 fixed tissue sections. One was used as a control (left panel) and the other was stained with TLR3 antibody (right panel). The image above shows tissues viewed at 20X in a fluorescence microscope in the FITC channel. All IF images were taken at the same exposure time and brightness settings.
Figure 8: TLR3 expression in MCF7 cells by IF. DAPI was used for nuclear staining (top panel) and TLR3 antibody was used followed by an AlexaFluor® 488 conjugated secondary antibody (bottom panel). A total of 4 such slides were prepared, each containing 2 sets of fixed cells. One was used as a control (left panel) and the other was stained with TLR3 antibody (right panel). The image above shows 40X in a fluorescence microscope in the FITC channel. All IF images were taken at the same exposure time and brightness settings.

It can be seen from the above images that TLR3 is expressed in both human and mouse breast cancer cells. In order to demonstrate the relevance of studying TLR3 signaling in cancer further, the expression of TLR3 was examined by PCR in some other human breast cancer cell lines as well. In a similar experiment in which cultured breast cancer cell lines of MDA-MB468, MDA-MB231, ZR75-1, BT20 and T47D were used to obtain cDNA sequences amplified by PCR, TLR3 expression was determined using primers around the TLR3 region. The figure below shows the agarose gel images obtained upon electrophoresis, indicating expression of TLR3 by RT-PCR in different human cell lines. The top part of the gel shows GAPDH expression, as a control and the
bottom half shows TLR3 expression. It can be seen from the bands observed that TLR3 is expressed in MDA-MB231, ZR75-1, BT20 as well as T47D cells.

Figure 9: TLR3 expression in different human tumor cell lines by RT-PCR. GAPDH was used as a control in the experiment. The last column represents a molecular marker (MM), also called a DNA ladder.
Subsequently, since all studies were primarily designed in the mouse system, it made sense to study the expression of TLR3 in normal disease-free mouse tissue sections. Frozen tissue sections of unaffected mouse liver, heart, kidney, lungs, mammary glands, spleen, testes and skin were tested, by RT-PCR, as seen in the figure below.

Figure 10: TLR3 expression in normal mouse tissues by RT-PCR. GAPDH was used as a control in the experiment. The last column represents a molecular marker (MM) or a DNA ladder.
From the figure, it can be seen that there is a basal expression of TLR3 in all of the tissue types tested, except for mouse testes. This shows that the molecule is broadly distributed in several tissues and is not a marker of either leukocytes or tumor cells.

4.4. RANTES levels in 4T1 mouse cells increase with poly(I:C) treatment

The synthetic oligonucleotide resembling the structure of a double stranded RNA, called polyinosinic:polycytidylic acid (poly I:C) was used at varying concentrations in 4T1 cell culture and incubated for varying number of hours to allow enough time for TLR3 activation and downstream response. Initially, the incubation time was for 24 hours as well as 48 hours and poly (I:C) was directly incubated without the use of a carrier at 2 different concentrations: 0.1 µg/ml and 1 µg/ml, with ‘no treatment’ or 0 µg/ml as control. ELISA was used to analyze the supernatants collected for the expression of RANTES, SDF-1α, MIP-3α and MIP-3β. These chemokines were specifically chosen due to their roles in chemotactic attraction of various leukocytes when produced. Of the 4 different chemokines studied at 2 different time points, RANTES was found to be significantly increased in expression in 4T1 cells treated for 24 hours with poly I:C. As seen in the following figure, treatment with poly(I:C) at both 0.1 µg/ml and 1 µg/ml showed a statistically significant increase in RANTES expression in the supernatants. This finding was noteworthy due the role of RANTES in causing leukocyte infiltration into diseased or inflammatory sites. As explained earlier, this leukocyte infiltration may cause the production of more cytokines that interact with the existing tumor cells and stimulate the production of angiogenic factors. The concentration of RANTES is
expressed in nanograms per milliliter of supernatant. As can be seen, the most significant increase occurs at a concentration of 1 mg/ml of poly(I:C) treatment.

Figure 11: RANTES expression in poly(I:C) treated 4T1 cells by ELISA. Figure shows increased RANTES expression upon in vitro stimulation of TLR3 with poly (I:C) incubation at varying concentrations. ‘No treatment’, represented by ‘0 ug/ml’ in the figure was used as a control to measure endogenous expression in comparison to poly (I:C) treatment.

* denotes P value ≤ 0.05, statistically significant.
Due to the expression of TLR3 primarily in endosomal compartments, it was decided that the most effective way to stimulate TLR3 would be by the use of a transfection agent that could penetrate the cellular surface and deliver poly(I:C) into the cells. Therefore, a lipofectamine™ was used along with poly(I:C) in the experiments that followed to see if the results obtained initially were reproducible. This transfecting agent, commonly used for nucleic acid delivery, was apt for the delivery of poly(I:C) owing to its structural similarity. The lipofectamine™ is basically a liposome, whose membrane is made up of a phospholipid bilayer, the same as that of a cell membrane. However, because of its aggregation with poly(I:C), the net charge becomes positive, which allows for its fusion with the negatively charged lipid bilayer of the cell membrane. This process of delivery of nucleic acid material is called lipofection.

The following figure shows the analysis of RANTES expression by ELISA in 4T1 cells treated with 1 μg/ml and 3 μg/ml of poly(I:C) using lipofectamine™ for transfection.

It is clear from the figure that there was a significantly elevated expression of RANTES upon transfection with 1 μg/ml of poly(I:C). This led to determining a concentration of 1μg/ml of poly(I:C) and an exposure time of 24 hours as ideal conditions for TLR3 stimulation in future experiments. It further confirmed the elevated RANTES expression levels in TLR3 stimulated 4T1 cells.
Figure 12: RANTES expression in poly(I:C) transfected 4T1 cells by ELISA. Figure shows increased RANTES expression upon in vitro stimulation of TLR3 with poly (I:C) transfection by lipofectamine™ at varying concentrations. ‘No treatment’, represented by ‘0 ug/ml’ in the figure was used as a control to measure endogenous expression in comparison to poly (I:C) treatment.

** denotes P value $\leq 0.01$, statistically very significant.
Finally, in order to study the mRNA expression levels of RANTES, a qPCR was performed with results in agreement with former experiments, as shown in the figure.

![qPCR chart](image)

**Figure 13:** RANTES expression in poly(I:C) transfected 4T1 cells by qPCR. Figure shows relative increase in RANTES expression upon in vitro TLR3 stimulation with poly (I:C) at 1 ug/ml. ‘No treatment’, represented by ‘0 ug/ml’ in the figure was used as a control to measure endogenous expression in comparison to poly(I:C) treatment. *** denotes P value ≤ 0.001, statistically extremely significant. GAPDH expression was used to normalize values and obtain relative expression.

4T1 cells transfected with 1 μg/ml of poly(I:C) for 24 hours were also harvested and the total RNA was extracted. Following this, a reverse transcription qPCR was also carried out using primers designed specifically for RANTES to study the relative expression of RANTES in controls versus treated cells.
It was once again revealed that RANTES expression was significantly elevated in Poly(I:C) treated cells, as seen in the figure. GAPDH, as a housekeeping gene, was run alongside and the values obtained were used to normalize and calculate the relative expression levels of RANTES in the control and treated samples. Since RANTES is a fairly prominent breast cancer marker [56], these results were very promising for the ongoing studies.

4.5. IL-6 and MIP-2 are also elevated in poly(I:G) treated 4T1 cells

Upon identifying RANTES as a component elevated upon TLR3 stimulation, it was decided to examine the expression levels of other chemokines and cytokines playing important roles in stimulating immunological responses. For this purpose, a protein microarray membrane from RayBio®, containing capture antibodies to selected mouse cytokines embedded in it was chosen for analysis. The membrane contained the cytokines indicated in the following figure in the pattern shown. There were 6 positive controls and 4 negative controls in total to ensure accuracy.
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<tr>
<th>Positive</th>
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<th>6Ckine</th>
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<td>MIP-2</td>
<td>MIP-3β</td>
<td>RANTES</td>
<td>SCF</td>
<td>sTNFR1</td>
<td>TARC</td>
<td>TIMP-1</td>
<td>TNF-α</td>
<td>Thrombopoietin</td>
<td>VEGF</td>
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*Figure 14: Layout of cytokine capture antibodies in the mouse antibody membrane array.*
The supernatant from 4T1 cells treated with 1 μg/ml of poly (I:C) was used for the experiment, along with ‘no treatment’ samples used as control. Signals were subsequently detected on X-Ray film upon adding the detection solution provided with the kit.

As seen in figure 15, IL-6 and MIP-2 were found to be increased in expression. Predictably, RANTES also showed elevated expression in the treated sample. In addition, TIMP-1 was observed to be reduced in expression in the treated sample, as compared to the control. IL-6 is generally implicated in mediating fever as a response to infection, inflammation, etc., but its expression level has also been shown in the past to be related to the stage of breast cancer [31]. Similarly, MIP-2 is another chemokine generally playing a role in recruiting hematopoietic stem cells and polymorphonuclear leukocytes and assisting in local inflammatory processes. TIMP-1 on the other hand is a member of the family of glycoprotein called tissue inhibitor of metalloproteinases. As the name suggests, these inhibit the functioning of MMPs (matrix metalloproteinases) – enzymes that facilitate the degradation of the extracellular matrix (ECM). Since the activity of MMP in the degradation of the ECM has been popularly associated with the ability of tumor cells to metastasize, the functioning of TIMP-1 would logically be concluded as beneficial. However, a reduction in TIMP-1 expression in treated samples of 4T1 cells, could indicate that there is insufficient inhibition of the MMPs, leading to their uncontrolled activity in ECM degradation. Hence, the reduced expression of TIMP-1 proved to be an interesting observation.
Figure 15: Mouse Cytokines Antibody Array for poly(I:C) treated and control 4T1 samples. Figure shows over-expression of RANTES, IL-6 and MIP-2 in 4T1 cells with TLR3 activation using poly(I:C). TIMP-1 shows reduced expression in the treated sample.
Although the antibody array allowed for a quick qualitative study of the expression of several cytokines all at once in the treated versus untreated samples, it was not sufficient to quantitatively visualize the change in expression levels. Therefore, the expression of IL-6 and MIP-2 was further analyzed in poly(I:C) transfected 4T1 cells by ELISA. The results were in agreement with that of the antibody array. Both IL-6 and MIP-2 were found elevated in the 4T1 cell samples treated with poly(I:C), as represented in figure 16 and figure 17.

![ELISA for IL-6 expression in poly(I:C) transfected 4T1 cells](image)

**Figure 16: IL-6 expression in poly(I:C) transfected 4T1 cells by ELISA.** Figure shows increased IL-6 expression upon in vitro stimulation of TLR3 with poly (I:C) at varying concentrations.

* denotes \( P \text{ value} \leq 0.05 \), statistically significant.

** denotes \( P \text{ value} \leq 0.01 \), statistically very significant.
Figure 17: MIP-2 expression in poly(I:C) transfected 4T1 cells by ELISA. Figure shows increased MIP-2 expression upon in vitro stimulation of TLR3 using 1 µg/ml of poly(I:C). *** denotes P value \( \leq 0.001 \), statistically extremely significant.

### 4.6. Cytokines including RANTES, IL-6, IL-8 are elevated in treated MCF7 cells

Following the antibody array experiment with 4T1 cells, the next step was to treat MCF7 cells with 1 µg/ml for 24 hours for TLR3 activation and then perform a similar antibody array experiment consisting of a membrane with capture antibodies to a set of popular cytokines known to be involved in angiogenesis. The procedure used for the mouse antibody array experiment was replicated in this instance. The layout of cytokines in the commercially available membrane array from RayBio® was as per the following figure.
### Table

<table>
<thead>
<tr>
<th>Positive</th>
<th>Positive</th>
<th>Negative</th>
<th>Negative</th>
<th>ENA78</th>
<th>GCSF</th>
<th>GMCSF</th>
<th>GRO</th>
<th>GRO-α</th>
<th>I-309</th>
<th>IL-1α</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>ENA78</td>
<td>GCSF</td>
<td>GMCSF</td>
<td>GRO</td>
<td>GRO-α</td>
<td>I-309</td>
<td>IL-1α</td>
<td>IL-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>IL-3</td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-6</td>
<td>IL-7</td>
<td>IL-8</td>
<td>IL-10</td>
<td>IL-12 p40p70</td>
<td>IL-13</td>
<td>IL-15</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>IL-2</td>
<td>IL-3</td>
<td>IL-4</td>
<td>IL-5</td>
<td>IL-6</td>
<td>IL-7</td>
<td>IL-8</td>
<td>IL-10</td>
<td>IL-12 p40p70</td>
<td>IL-13</td>
<td>IL-15</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>MCP-1</td>
<td>MCP-2</td>
<td>MCP-3</td>
<td>MCSF</td>
<td>MDC</td>
<td>MIG</td>
<td>MIP-1δ</td>
<td>RANTES</td>
<td>SCF</td>
<td>SDF-1</td>
<td>TARC</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>MCP-2</td>
<td>MCP-3</td>
<td>MCSF</td>
<td>MDC</td>
<td>MIG</td>
<td>MIP-1δ</td>
<td>RANTES</td>
<td>SCF</td>
<td>SDF-1</td>
<td>TARC</td>
<td>TGF-β1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNF-β</td>
<td>EGF</td>
<td>IGF</td>
<td>Angio genin</td>
<td>Onco -statin M</td>
<td>Thrombo poietin</td>
<td>VEGF</td>
<td>PDGF BB</td>
<td>Leptin</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TNF-β</td>
<td>EGF</td>
<td>IGF</td>
<td>Angio genin</td>
<td>Onco -statin M</td>
<td>Thrombo poietin</td>
<td>VEGF</td>
<td>PDGF BB</td>
<td>Leptin</td>
<td>Negative</td>
<td>Positive</td>
</tr>
</tbody>
</table>

**Figure 18:** Layout of cytokine capture antibodies in the human antibody membrane array.  
GRO detects CXCL1, CXCL2, CXCL3; GRO-α detects only CXCL1  
IL-12 p40p70 detects both IL-12 p40 and IL-12 p70  
VEGF detects VEGF-165 and VEGF-121
Several cytokines were found to be upregulated in the treated samples of MCF7, as seen in figure 19. Notable among them were RANTES (CCL5), IL-6, IL-8 and GRO. IL-8 is a ligand that binds to CXCR2, a receptor that is also able to bind MIP-2 in mice. Therefore IL-8 in humans has a high sequence homology to MIP-2 in mouse. The GRO antibody in the blot recognizes CXCL1, CXCL2 and CXCL3. Therefore, the observable prominent blot with GRO could indicate an increased expression of either one or more of the mentioned chemokines. It is highly significant that the results obtained from treated MCF7 cells were in very close agreement with those obtained from treated 4T1 cells.

Other cytokines found to be upregulated in the poly(I:C) treated samples of MCF7 cells were IL-10, MCSF, SDF-1, IGF-1, PDGF and angiogenin, as seen in the figure. While all of these cytokines are active participants in inflammatory responses, SDF-1 and angiogenin are particularly important due to their implications in breast cancer and neoangiogenesis specifically [58]. Also, angiogenin has been shown in studies to be conducive to adhesion of cancer cells and promote metastasis [91]. Additionally, PDGF is a well-known molecular marker in human breast cancer and has been shown to be correlated with decreased survival rates in human clinical samples [92, 93]. These results emphasized the importance of the studies and there was enough reason to indicate that TLR3 signaling in human breast cancer could be relevant to the progression of the disease.
Figure 19: Human cytokines antibody array for poly(I:C) treated and control MCF7 samples. Figure shows over-expression of RANTES, IL-6, IL-8, IL-10, MCSF, GRO, SDF-1, IGF-1, PDGF and angiogenin in poly(I:C) treated samples.
4.7. TLR3 activation induces inflammatory cytokines at the mRNA level

Upon obtaining promising results from the antibody array experiments, a PCR array was used to profile the expression of a large number of cytokines at the mRNA level. The RT² Profiler™ PCR Array (SA Biosciences, Frederick, MD) for mouse cytokines and receptors was used and the samples, as with other experiments, were from 4T1 cells treated with 1 µg/ml of poly(I:C), compared with untreated control samples. After performing a RT-qPCR, the relative expression of different cytokines in the treated versus control samples was obtained.

The figure below represents the expression of each cytokine or receptor in the treated samples, relative to the control. Black boxes represent increased expression and the gray boxes represent decreased expression. As expected, RANTES (marked CCL5 in the figure) showed increased expression in treated samples, along with several other cytokines and receptors. Noteworthy among the receptors is CCR5, which binds RANTES to initiate downstream signaling. Fold changes in expression were analyzed and only those that indicated over 4-fold change were taken into account. The tables below list the cytokines that showed increased expression relative to the control samples and decreased expression respectively. This further emphasized that several inflammatory cytokines are upregulated at the mRNA level upon stimulation of TLR3 with poly(I:C) in mouse 4T1 cells.
Figure 20: PCR array results showing relative expression of poly(I:C) treated versus control 4T1 samples.
Table 2:  
Cytokines showing greater than 4-fold increase in poly(I:C) treated 4T1 samples relative to control in a PCR array experiment.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone morphogenic protein 10 (Bmp10)</td>
<td>4.9</td>
</tr>
<tr>
<td>Macrophage Inflammatory Protein 1β (Ccl4)</td>
<td>417.7</td>
</tr>
<tr>
<td>RANTES (Ccl5)</td>
<td>7.8</td>
</tr>
<tr>
<td>Macrophage Inflammatory Protein 1γ (Ccl9)</td>
<td>7.7</td>
</tr>
<tr>
<td>CD195 (Ccr5)</td>
<td>4.5</td>
</tr>
<tr>
<td>Interferon γ induced protein 10 (Cxcl10)</td>
<td>82.5</td>
</tr>
<tr>
<td>Interferon γ inducible protein 9 (Cxcl11)</td>
<td>96.8</td>
</tr>
<tr>
<td>Macrophage Inflammatory Protein 2α (Cxcl2)</td>
<td>7.8</td>
</tr>
<tr>
<td>Monokine induced by γ interferon (Cxcl9)</td>
<td>66.6</td>
</tr>
<tr>
<td>Inhibin, beta B (Inhbb)</td>
<td>11.3</td>
</tr>
<tr>
<td>Tumor Necrosis Factor (Tnf)</td>
<td>61.2</td>
</tr>
<tr>
<td>Lymphotactin (Xcl11)</td>
<td>6.4</td>
</tr>
</tbody>
</table>
Table 3:
Cytokines showing greater than 4-fold decrease in poly(I:C) treated 4T1 samples relative to control in PCR array experiment.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein C10 (Ccl6)</td>
<td>-7.6</td>
</tr>
<tr>
<td>Neurotactin (Cx3cl1)</td>
<td>-4.9</td>
</tr>
<tr>
<td>Epithelial-derived neutrophil-activating peptide 78 (Cxcl5)</td>
<td>-10.4</td>
</tr>
<tr>
<td>Pro-platelet basic protein (Pppbp)</td>
<td>-8.7</td>
</tr>
<tr>
<td>G-protein coupled receptor 9 (Cxcr3)</td>
<td>-6.6</td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1 α (Hif1a)</td>
<td>-10.2</td>
</tr>
<tr>
<td>Regulator of G protein signaling 3 (Rgs3)</td>
<td>-7.4</td>
</tr>
<tr>
<td>Toll-like receptor 4 (Tlr4)</td>
<td>-4.6</td>
</tr>
<tr>
<td>Beta glucuronidase (Gusb)</td>
<td>-18.0</td>
</tr>
<tr>
<td>Hypoxanthine-guanine phosphoribosyltransferase (Hprt1)</td>
<td>-8.8</td>
</tr>
<tr>
<td>Heat shock protein 90β (Hsp90ab1)</td>
<td>-13.7</td>
</tr>
<tr>
<td>Beta-actin (Actb)</td>
<td>-9.7</td>
</tr>
</tbody>
</table>
4.8. Murine macrophages RAW267.4 and dendritic cells JAWS I show increased migration with poly(I:C) treated 4T1 samples

The next step was to see if the TLR3 activated samples were indeed significant in causing leukocyte infiltration in murine mammary tumor. For this purpose, a transwell migration assay was performed on murine macrophage cell line RAW267.4 and murine dendritic cell line JAWS I. Poly(I:C) treated 4T1 cell samples were used in the 96-well microplates, with untreated samples used as controls. Over the transwell membrane, the RAW267.4 cells and JAWS I cells were added, in separate experiments. After 4 hours, the samples were collected from the 96-well plate and the cells were counted. It was found in both experiments, as seen in the following figures that there was a significant increase in the percentage of migrated cells when in contact with treated samples, compared to the controls. This indicated that the increased total expression of inflammatory cytokines in the poly(I:C) treated samples helped in recruiting dendritic cells and macrophages, while the controls lacking a high level of cytokines did not attract as many cells. This in turn indicated that activating TLR3 using poly(I:C) in 4T1 cells resulted in increased attraction of dendritic cells and macrophages by the production of chemokines and cytokines.
Figure 21: Percentage migration of RAW267.4 cells in contact with poly(I:C) treated 4T1 samples versus control. Each set of graphs (1-4) represents the cumulative data obtained from each experiment. The experiment was performed 4 times.

Figure 22: Percentage migration of JAWS I cells in contact with poly(I:C) treated 4T1 samples versus control. Each set of graphs (1-4) represents the cumulative data obtained from each experiment. The experiment was performed 4 times.
4.9. Poly(A:U) as an alternative TLR3 stimulant yields comparable results

In order to ensure that TLR3 was indeed being activated by the use of poly(I:C), the results obtained were compared with an alternative TLR3 activating ligand, polyadenylic: polyuridylic acid or poly(A:U). 4T1 cells were similarly transfected with 1 µg/ml of poly(A:U) with Lipofectamine™ for 24 hours. The supernatant was collected and tested for RANTES, IL-6 as well as MIP-2 by ELISA. The figure below shows the percentage increase in these protein with poly(A:U) induced stimulation compared alongside poly(I:C) induced stimulation.

![Figure 23: Percentage increase in expression of RANTES, IL-6 and MIP-2 upon TLR3 stimulation. ELISA results showing percentage change in RANTES, IL-6 and MIP-2 expression upon in vitro stimulation of TLR3 with 1 µg/ml poly(A:U) compared to 1µg/ml poly(I:C). Treated sample was compared against untreated sample used as control.](chart.png)
From the comparison chart above, it can be seen that the percentage change in the expression levels of IL-6 and MIP-2 are both comparable for both methods of TLR3 stimulation. However, it can be seen that poly(I:C) treatment caused a drastic increase in RANTES expression as compared to poly(A:U). This as well as the slight increase in the percentage induced expression of IL-6 and MIP-2 by poly(I:C) compared to poly(A:U) can be attributed to the fact that poly(I:C) has been shown to also act as a synthetic ligand to RIG-I and MDA5, proteins belonging to the family of RIG-I-like receptors (RLRs) [94]. RLRs are a family of receptors involved closely with antiviral responses and have been observed to interact with TLRs to provide a complete antiviral response [95].

4.10. **TLR3 can be knocked down in 4T1 cell cultures by siRNA**

The subsequent step was to see if TLR3 expression could be knocked down by siRNA using the RNA interference principle. Stable knock down cell lines were produced in both 4T1 cells and the efficiency of knock down was studied by both immunofluorescence as well as RT-qPCR. As seen in the images below, the knock down efficiency was significant using siRNA.
Figure 24: TLR3 knock down in 4T1 cells observed by immunofluorescence. shRNA was used to knock down TLR3 in 4T1 mouse mammary cells. Cells were observed under 40X in the FITC channel of a fluorescence microscope. Each knock down sample (right panel) was compared against a scrambled vector (left panel) used as control.

Figure 25: Quantitative RT-PCR showing shRNA mediated knock down of TLR3 expression. Control sample was obtained from 4T1 cell lysates treated with a scrambled vector. TLR3 expression in the control sample is considered 100%. The knock down sample shows 94.3% inhibition in TLR3 expression.
The purpose of this experiment was to determine if there was a possibility to use shRNA as a tool to reduce leukocyte migration and infiltration by knocking down the expression of TLR3. Correlating to previously obtained results, it would follow that a significant knock down of TLR3 expression could result in a reduction in the release of pro-inflammatory cytokines and chemokines, which would in turn reduce the migration of dendritic cells, macrophages and other leukocytes. Further *in vivo* studies would help in a better understanding of the dynamics and effect of TLR3 expression in leukocyte infiltration in a mammary tumor animal model. Also, it may be suggested that as an extrapolation of these results, pre-clinical studies could help understand the effectiveness and feasibility of the targeted silencing of TLR3 as an adjuvant therapy for breast cancer treatment.
5. Summary and Future Direction

There are several factors that govern the progression of breast cancer. One of the reasons that have been indicated in the progression of the disease is the infiltration of leukocytes into the tumor microenvironment, which is said to aggravate the disease state and increase the tendency to metastasize [42]. Toll like receptors are a group of receptors that have been identified to play an important role in innate immunity [61, 65, 66, 70]. They have however, also been implicated in several diseases recently [37, 67, 75, 80-83]. The goal of the project was to examine the possibility of a relationship between TLR3 activation and the release of chemokines and cytokines known to cause leukocyte infiltration. TLR3 was found to be expressed in both mouse mammary tumor cell line 4T1 as well as human breast cancer cell lines. Activation of TLR3 using 1 μg/ml of poly(I:C) or poly(A:U) in 4T1 cells led to the increase in expression of RANTES, IL-6 and MIP-2, all implicated in breast cancer progression. Similarly, activation of TLR3 using 1 μg/ml of poly(I:C) in MCF7 human breast cancer cells led to an increase in the expression of RANTES, IL-6 as well as GRO and IL-8 – both of which are part of the CXCL2 family, to which MIP-2 belongs. This correlation in the results between the mouse and human tumor cell lines was very encouraging and indicated that the mouse cell line chosen was ideal and relatable to the human system. Subsequently, studying the effect of TLR3 activated 4T1 cells on the migration of mouse dendritic cells and macrophages (JAWS I and RAW267.4 cells) showed that there was a significantly increased percentage of migration when treated with poly(I:C). Further knock down studies using shRNA to silence the expression of TLR3 in 4T1 cells were successful.
These results are significant in that they bring us one step closer to understanding the contributors to leukocyte infiltration in breast cancer, a crucial step leading to metastasis. The hypothesis stated earlier has been proven in the 4T1 mouse mammary tumor cell line at the \textit{in vitro} level. This can be summarized by the figure below.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure26.png}
\caption{Summary of hypothesis.}
\end{figure}

In summary, this thesis has elucidated the significance of TLR3 signaling in murine and human breast cancer cells \textit{in vitro}. Stimulation of the TLR3 downstream signaling using poly(I:C) in lab conditions results in the production of several chemokines including RANTES, IL6 and MIP2 in 4T1 cells and MCF7 cells. This leads to a massive increase in the migration of macrophages and dendritic cells, as tested with 4T1 cells. PCR results have shown the production of angiogenic factors by tumor associated dendritic cells. The figure depicts this sequence of events in a concise way.
Additional studies have to be performed on *in vivo* models of breast cancer as well as clinical samples in order to demonstrate the relevance of TLR3 as a molecular target. Upon showing the proof of concept in animal models, the feasibility and effectiveness of TLR3 silencing *in vivo* needs to be evaluated. Another useful study would be to determine the best method to target TLR3 expression specifically in the tumor microenvironment and not normal cells expressing TLR3. These studies would help in evaluating the usefulness of TLR3 knock down in acting as a less invasive adjuvant therapy to already existing methods.
References

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82. McCall, K.D., et al., *High basal levels of functional toll-like receptor 3 (TLR3) and noncanonical Wnt5a are expressed in papillary thyroid cancer and are coordinately decreased by phenylmethimazole together with cell proliferation and migration*. Endocrinology, 2007. 148(9): p. 4226-37.


