# Role of Toll-like Receptors in Cancer Immunotherapy– A Review

By

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the
requirements for the degree of
Bachelor of Pharmacy (Hons.)

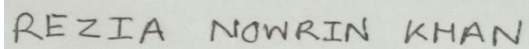The Department of Pharmacy
Brac University
August, 2021

# Declaration

It is hereby declared that

1.  The thesis submitted is my own original work while completing degree at Brac University.

2.  The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3.  The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4.  I have acknowledged all main sources of help.

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# Approval

The project titled "Role of Toll-like Receptors in Cancer Immunotherapy– A Review" submitted by Rezia Nowrin Khan (17346039) of Spring, 2017 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons.) on August, 2021.

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# Ethics Statement

This study does not involve any human or animal trial.

# Abstract

Cancer is recognized as the major concern in today's world. It is a condition or a group of diseases in which cells are abnormally divided. In the past century, the understanding and treatment of cancer has developed a lot and treatment such as chemotherapy, radiation therapy, surgery etc. have emerged. However, these are not enough to meet the desirable expectations and there is still need of new approaches to tame the immune system in the fight against cancer to make it a curable disease in the future. A series of current reports described that TLRs play a pivotal role in activating immune response against a number of pathogens, several disease conditions including pathogenesis of cancer. Besides, activation of TLRs interplay a crucial role in the initiation, progression and treatment of cancer. This review has discussed the basic features of TLR- ligands, their mechanism of action and how a limiting factor can contribute towards carcinogenesis. Moreover, it also involves the research and efforts of the scientists towards the evaluation of anti-cancer therapeutics that target TLR-signaling pathways.

**Keywords:** Toll-like receptors (TLRs); cancer progression; TLR-ligands; anti-cancer therapeutics; signaling.

# Dedication

*Dedicated to my parents*

# Acknowledgement

I would like to begin by thanking the Almighty Allah, our creator, the source of our life, strength, knowledge, wisdom, blessings and mercy. All praises to the Almighty Allah for blessing me with immense patience and strength to complete this project. This project would not have been completed without the support of the people who are recognized here.

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# List of Acronyms

TLR             Toll- like receptor

CART-cells      Chimeric Antigen Receptor T cells


MHC             Major histocompatibility complex

MoAb            Monoclonal antibody

mRNA            Messenger ribonucleic acid

MyD88           Myeloid differentiation primary response

                88

NF-kappa B      Nuclear Factor-κB

NK cells        Natural Killer cells

INF             Interferon

IL              Interleukin

TNF             Tumor necrosis factor

TRIF            TIR-domain-containing adapter-inducing interferon-β

Poly (I:C)      Polyinosinic-polycytidylic acid

PAMPs           Pathogen-associated molecular patterns

DAMPs           Damage-associated molecular patterns

LRR             Leucine-rich repeats

DC              Dendritic cell

PRRs            Pattern recognition receptors

LNCap           Lymph Node Carcinoma of the Prostate

EGF             Epidermal growth factor

APC             Antigen-presenting cell

ADCC            Antibody-dependent cellular cytotoxicity

SAA            Serum Amyloid A

HMGB1          High mobility group box 1 protein

BCG            Bacille Calmette Guerin

MAPK           Mitogen-activated protein kinase

IRAK           Interleukin-1 receptor associated kinase

CAFs           Cancer-associated fibroblast

TGA            Therapeutic Goods Administration

TGGA           The Cancer Genome Atlas

VEGF           Vascular endothelial growth factor

FGF            Fibroblast growth factor

HER2           Human epidermal growth factor receptor 2

TRAMP          Transgenic Adenocarcinoma of the Mouse Prostate

# Chapter 1 Introduction

## 1.1 What is Cancer?

Cancer is defined as a group of related diseases sharing six main hallmarks:

- self- sufficiency in growth signals

- insensitivity to growth- inhibitory signals

- evasion of programmed cell death

- limitless replication potential

- sustained angiogenesis and

- tissue invasion and metastasis (Hanahan and Weinberg, 2000).

So, Cancer basically refers to abnormal cell growth with an uncontrollable ability to infiltrate and damage normal body tissues. It is one of the second leading cause of death in the United States. According to CDC's National Center for Health Statistics, in 2018, there were 599,274 cancer deaths; 283,721were among females and 315,553 among males. And there were 9.6 million deaths from cancer worldwide in 2018. Lung (1.76 million deaths) liver (782,000 deaths), stomach (783,000 deaths) and Breast (627,000 deaths) are the most common causes of cancer death worldwide. (World Health Organization, 2018). Cancer progression arises as a result of the interaction between a person's genetic factors and 3 categories of external agents, including:

- Physical carcinogens, such as ultraviolet and ionizing radiation;
- Chemical carcinogens, such as components of tobacco smoke, arsenic (water contaminant) and biological carcinogens, such as infections from certain viruses, bacteria or parasites.

There are many types of tumors, which can be characterized based on their origin or their ability to spread cancer to different parts of the body. The most common classification is based on invasivity and the ability to metastasis:

1. benign tumors are considered less dangerous due to their incapability to invade and spread into different parts of the body. 2)

2. Malignant tumors are more fatal because they can enter the bloodstream or lymphatic system and develop secondary tumors in several body locations. (Chambers et al, 2002).

Different types of cancer have different mutation signatures. Certain signatures are related with age of a patient, known defect in DNA, or exposure to mutagens. However, the origin of many mutation signatures is unspecified. (Alexandrov et al.., 2013).
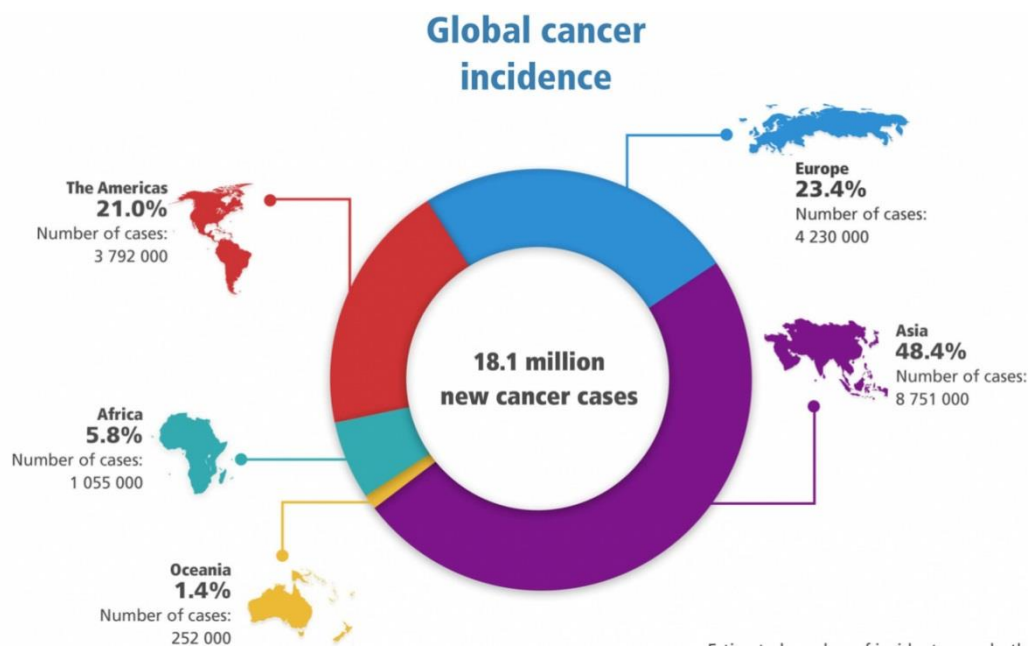


*Figure 1: Global cancer incidence (New Global Cancer Data, GLOBOCAN 2018).*

Usually, in men over the age of 65 prostate cancer is one of the most commonly found followed by lung cancer. Similarly, women with older age making up 48% of all malignant cancer which are- breast, lung, colon and stomach cancer. But cancer might show up at any age and it is not gender specific. Even children can develop malignant tumors like Leukemia, Brain and spinal

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cord tumors, Neuroblastoma, Lymphoma (including both Hodgkin and non- Hodgkin). While childhood cancers are often associated with leukemia, U.S Centers for Disease Control and Prevention (CDC) have found that brain cancer is now the life-threatening pediatric cancer.

## 1.2 The Need of New Approaches in Cancer Treatment

There are many types of cancer treatment which basically depends on the type of cancer and how advanced the progression of tumor is. Some people may have only one type of cancer treatment but most people have a combination of treatments such as surgery with chemotherapy or immunotherapy.

However, there are three standard treatment options. 1) Surgery: if the tumor is in solid form and location is optimal, then surgery is the first treatment option. It is a procedure where surgeon removes the tumor and nearby tissues during an operation. 2) Chemotherapy: during chemotherapy, drugs inhibiting cellular division and growth are used. This therapy are often very effective in some kinds of tumors, but the cytotoxic effect on healthy cells remains a serious concern (DeVita and Chu, 2008). (3) Radiotherapy: during radiotherapy, high doses of radiation are used to control or kill the malignant cells. But, this also does some serious damage to healthy tissue (Sebag-Montefiore et al., 2009).

In addition, several novel approaches in cancer treatment are emerging. For example, hormone therapy, targeted therapy, angiogenesis inhibitors, and immunotherapy. Immunotherapy is one of the most extensively studied cancer therapies, where the immune cells of the patient are used for tumor cells detection and elimination (Rosenberg et al., 2004).

Although the advancement in the understanding of cell proliferation and dissemination are satisfactory but this expectancy does not meet by the drugs that are currently available in the market (Agency & Evaluation, 2003). So, it can be said that, despite an enormous

advancement in cancer treatment, there is still need for improvement for the early diagnosis and better treatment toward this life-threatening disease.

Early detection of cancer and several treatment options make it convenient for cancer management. But nevertheless, most of the currently available treatments are still not effective enough against the wide variety of cancers. Also, the toxic effects remaining in healthy cells are still a matter of concern. Hence, new therapies need to discover for a better approach towards cancer management.

## 1.3 Immunotherapy of Cancer

One of the newly emerging therapy is introduced which is immunotherapy and it basically acts by using a patient's own immune system to fight cancers. Immunotherapy enhances the immune system's ability to acknowledge, target and eliminate cancer cells, wherever they're within the body which is making it a possible universal answer to cancer. It has been approved within the U.S and elsewhere as a first line treatment option for several types of cancers and works as an effective procedure for patients who are resistant to prior treatment. As of December 2019, immunotherapies have been approved as treatment for nearly 20 types of cancers including breast cancer, brain cancer, cervical cancer, colorectal cancer, kidney, liver and lung cancer, Leukemia, lymphoma, melanoma, childhood cancer as well as cancers with a particular genetic mutation.

There are several types of immunotherapies based on cellular immunity, antibody-based immunity, TLR agonists-based immunity. Cellular based immunotherapies can be classified as active or passive. Active cellular based immunotherapies include cell- based vaccines and passive cellular based immunotherapies include adoptive transfer of NK cells or T-lymphocytes. (Borghaei et al., 2009). Cell based vaccines require for their function expression of tumor specific antigens or tumor associated antigens on the surface of tumor cells.

**Cellular based immunotherapies:**

**Protein/peptide subunit vaccines**: It is made of amino acid sequences mimicking tumor antigens which is generally boosted by vaccine adjuvants stimulants monocytes and macrophages. The peptide proteins are presented by antigen presenting cells to CD8+ T-lymphocytes. The greater advantage of it is their low cost and easy manipulation as well as effective function in some types of tumors. On the other hand, low affinity, non-specific binding and rapid degradation are some of their disadvantages.

**DNA vaccines**: These vaccines are made up of a bacterial plasmid which after modifying are delivered by injection of encoding DNA allowing access to multiple antigen-presenting pathway. Immune cells then recognize these artificially expressed tumor antigens and activates their immune responses. The flexible design of DNA vectors, innate immunity and CD4+/CD8+ cells activation makes it a convenient approach. However, the efficiency of DNA vaccines is still not widely used because of their low immunogenicity of tumors.

**Whole cell vaccines**: It represents one active form of cancer immunotherapy which can express proteins in patient's cancer lesions and provide multiple tumor antigens for immune recognition. Tumor cells for whole cell vaccination can be autologous or allogenic. Autologous vaccines are separated during removal of tumor and prepared for immunization whereas allogenic vaccines are prepared from specific tumor cell lines which are largely available. However, variances of tumor cell antigen and patient's tumor antigen cells may result in less efficiency in comparison with autologous tumor cell vaccines.

**Dendritic cell vaccines**: Dendritic cells are antigen presenting cells which play a dual role in induction of both innate and adaptive immunity including T cell tolerance. This also can be used as preventive vaccines as well as therapeutic vaccines against cancer. Preventive vaccines induce pathogen-specific T- cells to keep immune memory and prevent diseases, while

therapeutic vaccines raise a particular immune response against existing tumor cells. It has become a considerable therapy due to its T-cell activation. DC cells are isolated from a patient's monocytes and loaded by tumor antigens. These antigens are combined with other stimulating molecules which cause DC maturation and activation.

**Adoptive transfer**: Adoptive cell transfer is basically the transfer of cells which may have been originated from the immune system of the patient or other individual for improving immune functionality and characteristics. There are two types of adoptive transfers which are – Nk cell adoptive transfer and T- lymphocyte adoptive transfer. NK cells are innate immune cells which have a power to distinct virus infected cells with unusual expression of MHC molecules. Besides, the production of IFN$\gamma$ can polarize T-lymphocytes to Th1 phenotype and initiate DC maturation. NK cells can be derived from either autologous (cells isolated directly from patients) or allogenic (cells donated from healthy relatives). The isolation of NK cells is then activated by cytokines (using cytokine, IL-2, IL-12, IL-15, IL-18); (Iliopoulou et al., 2010). In fact, some newly identified memory like NK cells is also taken as a potential source of adoptive transfer.

On the other side, T- lymphocytes are adaptive immune cells and unlike the innate immune system, it relies on B lymphocytes and T lymphocytes which are derived from specific types of stem cells. It either produces a direct cytotoxic effect on targeted cells or activate other immune responses. When isolated tumor infiltrating leukocytes are expanded ex vivo and administer backwardly into patients' body, then T- lymphocytes are used as adoptive transfer (Besser et al., 2010). Biological manipulation of T- lymphocytes results in the expression of high affinity antigen receptors on the surface of T- lymphocytes, successful recognition of tumor antigens and elimination of tumor cells from the patient's body. Also, several viral vectors like retrovirus, lentivirus are used for T-lymphocytes transfection which causes expression of chimeric antigen receptors on T-lymphocytes surface (Grupp er al., 2013). This

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therapy is known as CAR T-cell which may show difficulties in interaction with normal cells expressing the same antigens as tumor cells with autoimmune reactions.

**Antibody-based immunotherapy**: Antibody based immunotherapy includes monoclonal antibodies which are widely utilized in targeting specific proteins (antigens) expressed on the surface of tumor cells. There are several types of MAbs such as:

- Naked monoclonal antibodies which are most common and mostly based on antibody-dependent cellular cytotoxicity (ADCC).

- Conjugated monoclonal antibodies which is conjugated with chemotherapeutic or radiotherapeutic particles protecting healthy cells against serious side effects of chemotherapy. Simultaneously, these conjugated antibodies are excellent examples of chemotherapy and radiotherapy with immunotherapy (Witzig et al.,2002).

- Bispecific monoclonal antibody designs two different antibodies together and are able to attach two different antigens. This enhances the therapeutic effect (Shen and Zhu, 2008).

**TLRs-agonist based immunotherapy:**

Toll-like receptors plays a vital role in activating both innate and adoptive immune responses by engaging on various T- cell subsets to augment their responses and thus show possibilities to enhance the efficacy of cancer immunotherapies. When TLRs recognize their specific ligands, two different pathways can be activated and results in different immune system action. The first pathway is MyD88 dependent pathway which results in generation of inflammatory cytokines and the second pathway is MyD88 independent pathway which eventually results in stimulation of interferon-beta production and DC maturation (Kawasaki and Kawai, 2014; Zhu and Mohan, 2010). At present, several TLR agonists are being used in cancer treatment. For instance, Attenuated *Mycobacterium bovis* is used for TLR 2, TLR 4, TLR 9 activation. This therapy is also known as Bacilus Calmete Guerin (BCG) vaccine which has a great therapeutic

approach for superficial bladder carcinoma treatment. Another example **is Imiquimod** which is a synthetic TLR agonist activating TLR 7. It is mostly administered as a 5% cream for skin malignancies and skin premalignant conditions. Besides, it is also used as a vaccine adjuvant.

## 1.4 Immune System and Cancer

Immune system plays a vital role as body's defense system that consists of cells, tissues and organs, which mainly protect body against pathogens. There are two main parts of immune system: innate immunity and adaptive immunity. The major function of immune cells is to recognize the pathogens invading human body and their elimination from organism (Akira et al., 2006; Medzhitov and Janeway, 1998). Usually, the immune system has also the power to acknowledge and reject tumor cells.

Although it has the power to detect and eliminate tumor cells, the human body is not entirely resistant to cancer. The tumor cells regulation is controlled by a process named immunoediting.

### 1.4.1 Immunoediting of Cancer

Immunoediting is a very vigorous process consisting of three phases (Figure -2): elimination, equilibrium and escape (Dunn et al.,2002). The primary phase (=elimination), innate and adaptive immune cells are ready to recognize and shrink tumor cells. This phase is initiated by inflammation within the location of originating tumor followed by recruiting immune cells and synthesis of cytokines and chemokines (Dunn et al., 2002; Dunn et al.,200 4b).
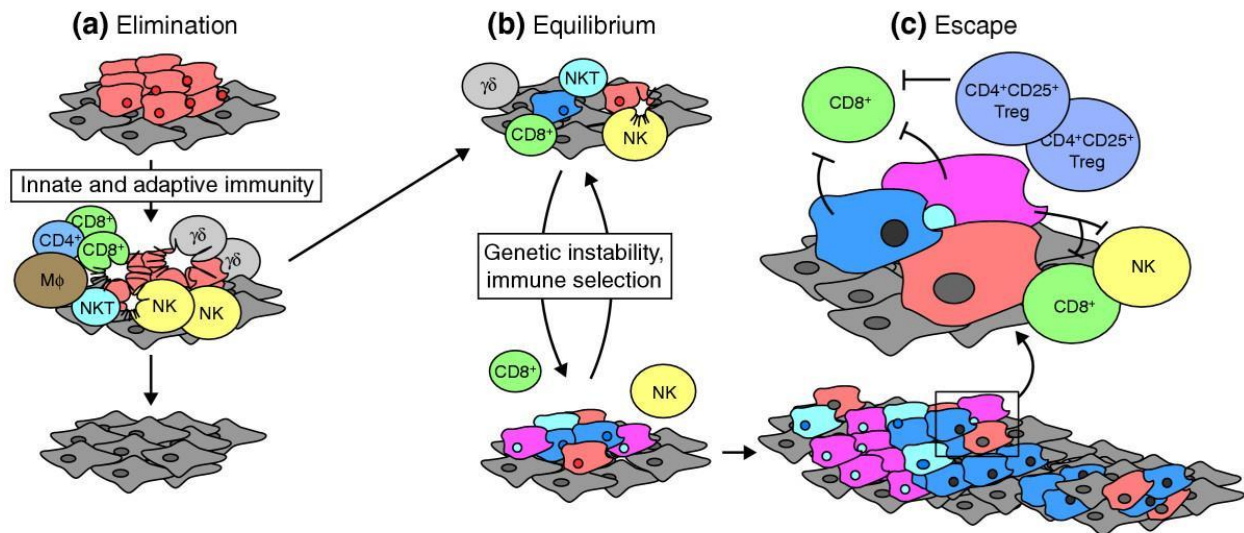
*Figure 2: Three phases of cancer immunoediting*

Then, the second phase (=equilibrium), the surviving cells from the elimination phase which generate clones with cumulative numbers of mutations. These mutations result into greater resistance to immune system and permit tumor cells to flee from immunosurveillance (Dunn et., al 2004b). The third phase (=escape), the immune cells will lose control under the tumor cells. Subsequently, tumor cells can expand and generate primary tumor and metastases (Dunn et al., 2006; Dunn et al., 2004b; Kim et al., 2007).

During the elimination phase, the tumor cells are controlled by immune system whereas during the phase equilibrium it acts as a dormant stage which can lead to the emergence of tumor variants with decreased immunogenicity providing the immune system unable to detect the altered tumor. As a result, tumor cells become genetically instable and become more resistant to immune cells. This resistance induces tumor cells escape and tumor growth (Dunn et al., 2004a).

There are various mechanisms of how tumor cells escape from immune control. For instance, downregulation of MHC class I antigen expression, immunosuppressive tumor microenvironment or upregulation of non-classical MHC I antigens. Generally, cancer

immunoediting occurs during tumor progression but also for patients receiving anticancer immunotherapies. Innate and acquired resistance acts as important barriers for the effectiveness of immunotherapy treatment. Moreover, the availability of large amounts of new information on the genomic and transcriptomic profile of different human malignancies provide an additional depth to stratify the microenvironment of tumor.

### 1.4.2 Innate Immunity and Cancer

Innate immunity serves as a first line defense against infectious agents and germ line-encoded pattern receptors help to recognize stressed and infected cells which extort potent effector activities that attain efficient microbe containment. Besides, it is required to the onset and maintenance of adaptive immunity and completely integrates the cancer-immunity cycle. The most significant role of pattern recognition receptors (PRRs) is to detect pathogen associated molecular patterns (PAMPs) during the inflammatory reaction. This recognition results into mobilization of immune cells and pathogen elimination (Akira et al., 2006; Medzhitov and Janeway, 1998).

Another major function of innate immunity is the complement activation. Complement is a cascade of plasma proteins and its activation can cause inflammatory cell attraction, pathogen opsonization or perforation of pathogen plasmatic membrane. So, the main characteristic of complement system is to aid in the extinction of pathogens by piercing their outer membrane (cell lysis) or by making them more prone to phagocytic cells such as macrophages (a process known as opsonization). There are three main pathways associated with complement activation which are: The classical pathway – triggered by directly or indirectly by pathogen and antibody respectively, the MB – lectin pathway and, the alternative pathway, which also give an amplification loop for the other two pathways.

Anatomical barriers, such as skin, gastrointestinal tract or respiratory tract are also important parts of innate immunity. These anatomical barriers play a role as a first mechanical blockage for invading pathogens (Hornef et al., 2002).

Interestingly, natural killer (NK) cells are primarily liable for killing cancer cells lacking expression of major histocompatibility complex (MHC) molecules. Besides, expression of interferon gamma (IFNγ), perforins and inflammatory cytokines are activated by stimulatory receptors of NK cell surfaces. These molecules initiate apoptosis of tumor cells (Waldhauer and Steinle, 2008; Zamai et al.,2007). On the other hand, macrophages are other innate immune cells which interacts with tumor cells and some tumors express "eat me "molecules (e.g., phosphatidylserine and low-density lipoproteins) on their surfaces. These expression of molecules results in activation of macrophage phagocytosis (Mantovani and Sica, 2010). Another crucial innate immune cell which also interacts with tumor cells via integrins and other receptors by causing phagocytosis of apoptotic cancer cells are dendritic cells (DC).

### 1.4.3 Adaptive Immunity and Cancer

Adaptive immunity acts as second line of immune defense. T- lymphocytes and B-lymphocytes are the key adaptive immune cells which have high specificity and immunological memory to certain pathogens. Generally, adaptive immunity is initiated when a pathogen evades the innate immune system for a longer period of time to produce a threshold level of antigen. It functions on evolutionary principles of selection because the B cells and T cells have genetic variety to activate and execute humoral and cellular immune responses as 'effector' cells. T- lymphocytes are mainly divided in two main groups which are- Th–lymphocytes and Tc-lymphocytes. Several kinds of cytokines are produced by activated Th-lymphocytes which is also known as CD4+ lymphocytes. These cytokines are important activators for other immune cells.  Tc-lymphocytes, also called CD8+ lymphocytes, shows an immediate cytotoxic effect on viral infected cells or other abnormal cells. B-lymphocytes are

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the second crucial adaptive immune cells which have the power to generate antibodies as professional antigen presenting cells. Antibodies have various functions including neutralization of bacterial cells, agglutination of foreign cells, precipitation of serum antigens or complement activation. Besides, production of specific types of B-lymphocytes, memory B-lymphocytes is crucial for intense immune reaction during invasion of secondary pathogen. (Bonilla and Oettgen, 2010; Chaplin, 2010). Apart from that, adaptive immune cells also make interaction with tumor cells via tumor antigens. Tumor antigens can be classified into two main groups:

- Tumor specific antigens which are merely expressed on the tumor cell surface.
- Tumor associated antigens which are expressed predominantly on tumor cells but their expression is also detected in some normal cells. Recognition of tumor antigens by adaptive immune cells, following by antigen presentation, can cause tumor cell destruction.

### 1.4.4 Tumor Immune Escape Mechanism

Tumor cells can escape from immune recognition via development of tumor immune escape mechanisms. This plays an important role in tumor growth it includes:

Modulation of tumor antigens: It is basically loss of antigenicity or a difference in the markers by which tumor cells can avoid immunologic elimination. Antigenic modulation has been denoted with murine leukemia cells expressing thymic lymphocyte (TL) antigens. Tumor cells can translocate their antigens from surface to cytoplasm and avoid recognition. Some of the (S. Shi et al., 2020) tumors can also make immunologically invisible antigens (Khanna, 1998).

- Masking of tumor antigens: antigens can me masked by production of mucoproteins and sialomucin (Becker et al., 1993).

- Induction of tolerance: expression of PD-L1 molecules on tumor cell surface can signify immune tolerance. For instance, PD-L1 protein can bind to PD1 receptors on the surface of T-lymphocytes and prohibit their activation

- (Blank et al., 2005).

- Production of blocking antibodies: blocking of complement activation and production of C3a C3b can happen by blocking antibodies. Some blocking antibodies can also conceal tumor cells and provide protection against immune recognition (Sjogren et al., 1972).

- Production or expression of immunosuppressants: tumor cells are able to produce or express immunosuppressants, such as – IL10, TGF beta, or VEGE. These immunosupressants help to initiate escape of tumor cells from immune surveillance and create typical tumor environment (Itakura et al., 2011; Yang et al., 2010)

## 1.5 Rationale of the study

Cancer is such a deadly disease which has a high mortality rate and existing treatments are still not effective enough to provide full protection from this disease. So, it is very important to find a successful treatment option in order to reduce the mortality rate of cancer. Therefore, targeting toll-like receptor agonists and phagocytosis stimulating ligands for their intatumoral activity can be a potential option for treating cancer. This study is to understand:

1. Amplification of the therapeutic mixture
   - Find interaction of effective and nontoxic agonists.
   - Physical constraints and forces involved in TLR stimulating ligands.
   - Maximization of the functions of TLRs ligands in the recognition between self and non-self-antigens.

2. Justification of the therapeutic efficiency.

3. Investigation of the underlying mechanisms:

   - Adaptive immunity participation

   - Innate immunity response

   - Cytotoxic effects of immune cells in vitro.

## 1.6 Aims and Objectives of the Study

The aim and objective of this review is to highlight the importance of therapeutic combination of phagocytosis stimulating ligands and TLRs agonists, which has been reported to be successful in patients suffering from breast cancer. The other objectives are to aid in research and to develop interest so that the scientists concentrate more to understand the mechanism of TLRs receptor agonists in various forms of cancer.

# Chapter 2 Research Methodology

Thorough literature review was done to attain all the information used in this review paper. The information was collected from various credible sources, which includes peer reviewed journals, online scholarly database. Following are the list of some of the many databases that were search extensively for the present study.

- Journal Database

- Library Catalogue

- Newspaper Database

- Professional website

# Chapter 3 Toll-like Receptors in Breast Cancer

## 3.1 Regulation of Toll-like Receptors for Cancer

TLRs are assigned to different subcellular compartments based on the chemical composition of their subsequent ligands. TLRs adapt proteins including Mal, MyD88, TRAM, and TRIF after ligation with their respective ligands. TLR signaling is mediated by at least two distinct signaling pathways: MyD88-dependent and TRIF-dependent signaling pathways.
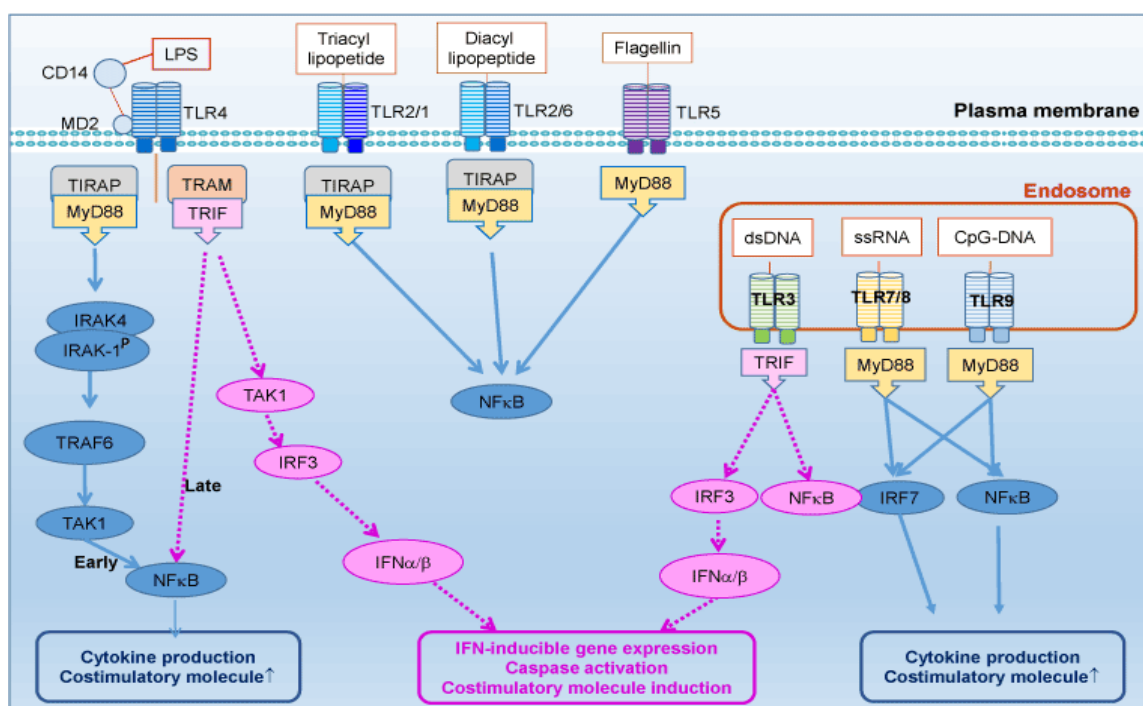


*Figure 3: TLRS signaling and their pathways. (Sun et al., 2016)*

TLR is essential for the activation of innate immunity against invading pathogens, the generation of cytokines, and the development of adaptive immune responses. TLR signaling, on the other hand, can increase the growth of tumorigenic effects in the tumor microenvironment as well as boost antitumor immunological tolerance (antitumor effects) in tumor cells, immune cells, and pattern recognition molecules, such as PAMPs and DAMPs. As a result, TLR activation in tumor cells acts as a double-edged sword (Basith et al., 2012).

Table 1:  *Expression of TLRs in various tumor cells/cell lines*

| TLRs expressed | Tumor cells/cell lines | References |
|---|---|---|
| TLR 1,7 and 9 | Multiple myeloma cells | (Jego et al., 2006) |
| TLR 2 | Oral squamous cell carcinoma | (Ng et al., 2011) |
| TLR 2,3 and 4 | Laryngeal carcinoma | (Szczepanski et al., 2007) |
| TLR 2,4 and 5 | Intestinal adenocarcinoma | (Pimentel-Nunes et al., 2011) |
| TLR 2,3,4 and 5 | Ovarian carcinoma | (Zhou et al., 2009) |
| TLR 3 | Human neuroblastoma (NB) cells, breast adenocarcinoma, cervical, hepatocellular, papillary thyroid, murine colon carcinoma | (Salaun et al., 2006, 2011; McCall et al., 2007; Jiang et al., 2008; Yoneda et al., 2008; Zhang et al., 2009a; Chuang et al., 2011; Guo et al., 2012) |
| TLR 3,4 and 9 | Breast and prostate carcinomas | (Gonzalez-Reyes et al., 2010; Gonzalez-Reyes et al., 2011) |
| TLR 3, 4, 7 and 9 | Esophageal squamous cell carcinoma | (Sheyhidin et al., 2011) |
| TLR 4 | Colon carcinoma, human head and neck squamous cell carcinoma, melanoma cell lines, NB-1 neuroblastoma. | (Hassan et al., 2006; Molteni et al., 2006; Doan et al., 2009; Qian et al., 2009; Szajnik et al., 2009; Szczepanski et al., 2009; |

| | | Kanczkowski et al., 2010; Tang et al., 2010; Zhang et al., 2010; Xu et al., 2011) |
|---|---|---|
| TLR 4 and 9 | Lung carcinoma | (Zhang et al., 2009b) |
| TLR 4,5 and 9 | Gastric carcinoma | (Schmausser et al., 2005) |
| TLR 5 | Cervical tumor cells, breast cancer cells, colon carcinoma, gastric cancer cells | (Kim et al., 2008; Rhee et al., 2008; Cai et al., 2011; Song et al., 2011 |
| TLR 7 and 8 | Lung carcinoma, colorectal carcinoma | (Cherfils-Vicini et al., 2010; Grimm et al., 2010) |
| Multiple TLRs | Human breast cancer cells, MDA-MB-231, murine tumor cell lines (MC 26, 4T1, RM1 B16, LTC1) | (Huang et al., 2005; Yang et al., 2010) |

TOLL-like receptor activation has been proven to be beneficial against many types of cancer. TLR receptors, however, appear to be a viable approach for breast and prostate cancer therapy via apoptosis, proliferation, as well as interferon (IFN), cytokine, and chemokine production (active role by TLR-3, TLR-4 and TLR-9).

## 3.2 TLR Family and Breast cancer

Breast cancer is the leading cause of mortality among women in the industrialized countries. Because Toll-like receptors are widely expressed on tumor cells and play key roles in tumor initiation and progression, they may serve as important targets and have a positive impact on breast cancer treatment. TLRs clearly play a significant role in both breast cancer cells and the

microenvironment. Although TLRs are abundant in macrophages, dendritic cells, and other innate immune cells, there is evidence that particular TLRs receptors are highly expressed in breast cancer cells.

TLR activation in cancer cells can result in tumor growth or inhibition. TLR 2,4, and 7/8 stimulation can promote tumor development by the generation of immunosuppressive cytokines, enhanced cell proliferation, and resistance to apoptosis. TLR 2, 3, 4, 5, 7/8 activation, on the other hand, is frequently coupled with chemo or immunotherapy, which might promote tumor inhibition via various pathways. Furthermore, TLR activation on NK cells and APCs (DC and macrophages) can affect CTLs to promote tumor development (Urban-Wojciuk et al., 2019)
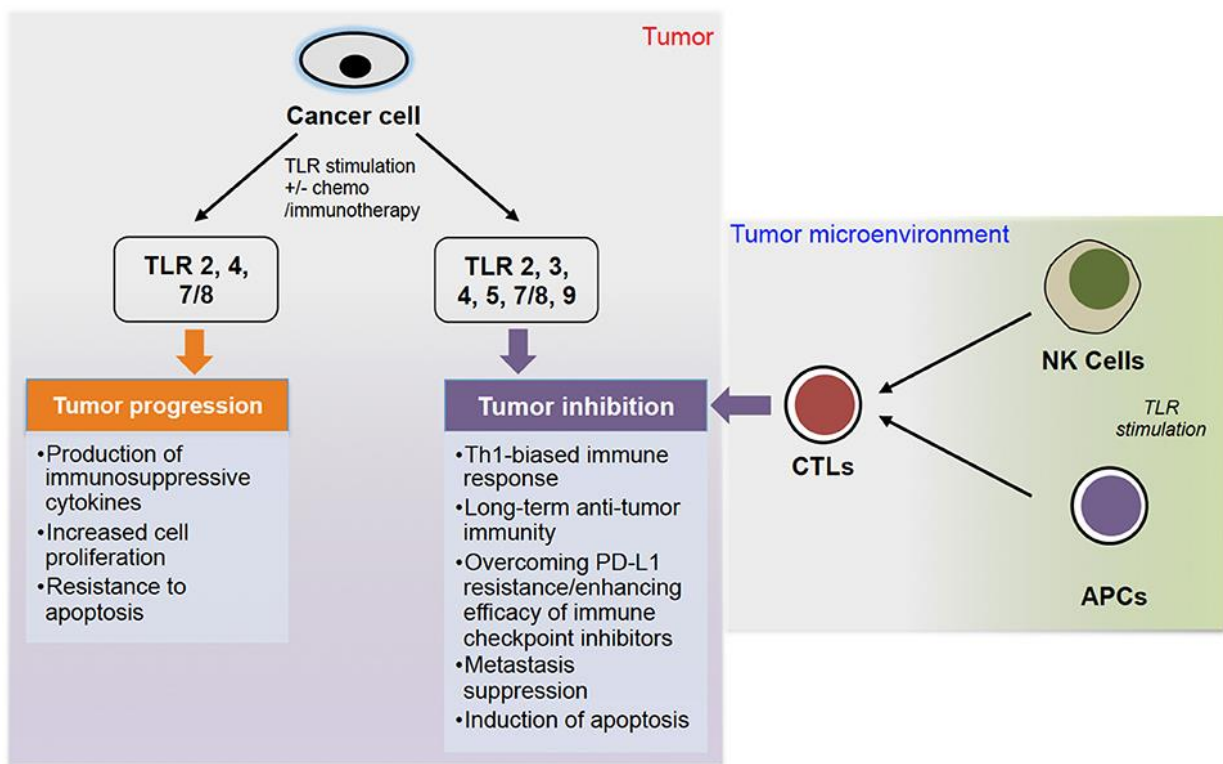


*Figure 4: The role of TLR stimulation in cancer progression  (Urban-Wojciuk et al., 2019)*

TlR3 primarily functions as a tumor suppressor and may limit tumor development via effects on cell proliferation and survival. Furthermore, activation of TLR3 by its agonist dsRNA induces apoptosis in human cancer cells. As a result, it has been discovered that tumor cells with high TLR3 expression have a significantly higher risk of metastasis (Li et al., 2014).

TLR4 promotes cancer cell invasiveness and the activation of pro-inflammatory and chemokine genes during cancer cell migration. Several clinical investigations have found a strong link between increased TLR4 expression and lymph gland metastasis and local cancer growth (Yang, Huan et al., 2014). Furthermore, TLR4 activation assists in controlling the expression of integrin v3, which improves the v3-mediated adhesion and invasiveness of metastatic breast malignant cells. Cancer cell proliferation is hampered by TLR4 knockdown, which eventually leads to a decrease in IL-6 and IL-8 levels. HMGB1, on the other hand, functions as an endogenous TLR4 ligand to induce carcinogenesis (Chen, Yan et al., 2015). Current breast cancer research has discovered that TLR5 is primarily expressed in breast carcinomas by its ligand flagellin, which has significant antitumor action and inhibits breast cancer cell growth (Cai, Zhenyu et al., 2011). Cancer cell growth is suppressed by the release of soluble substances that stimulate flagellin in an autocrine way. TLR-5 deleted breast cancer tumor cells were implanted, causing tumor development to be exaggerated. Flagellin, on the other hand, a TLR5 agonist, may slow tumor development by boosting necrobiosis and decreasing tumor cell proliferation.

TLR9 expression in epithelial malignant cells is linked to cancer development. Sandholm J et al. discovered that women with cancer have higher circulating levels of TLR9 than healthy controls (Sandholm, Jouko et al., 2012). TLR9 expression and invasiveness in cancer cells may be regulated by sex steroid hormones and estrogen receptors. Overexpression of involuntary ERa in ER-negative MDA-MB-231 cells reduce TLR9 expression and modulates their invasive capability in response to TLR9 ligands. (Qiu, J et al., 2011).

Each TLR has its own set of ligands, such as bacterial lipoprotein (TLR2), viral double-stranded RNA (TLR3), CpG-DNA (TLR9), and so on. TLRs, in essence, identify the molecular pattern of (DAMP), which functions as a hallmark in cancer development by triggering inflammation. The link between inflammation and breast cancer is gradually becoming clear. TLR-mediated NF-k and IRF activation has a multifaceted function in linking inflammation to cancer and autoimmune regulation.
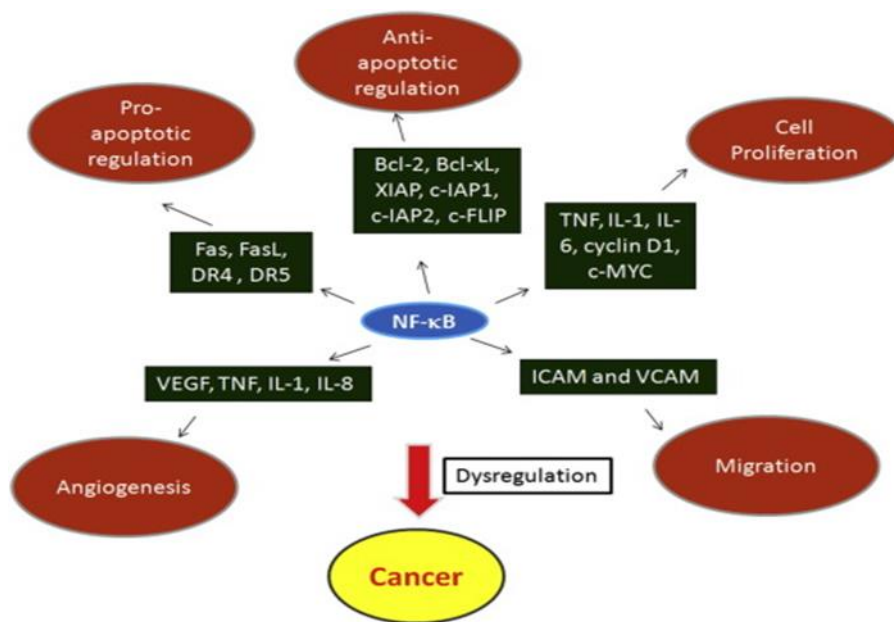


*Figure 5: Role of TLRs in linking inflammation and breast cancer (Bhatelia, K., Singh, K., & Singh, R. 2014).*

The etiology of breast cancer is not utterly clear, and so the early stage of breast cancer does not have typical symptoms and signs, therefore making the diagnosis and treatment complicated. But recent studies have reported that multiple SNPs which have been found in TLRs are acting as innate immunity receptors for detecting invading pathogens and are related with altered susceptibility to many diseases, including cancer. The emerging evidences denote that activation of inflammatory pathways of TLRs cause elevated level of pro- inflammatory cytokines and chemokines mounting inflammation which can lead to cancerous condition. Consequently, inflammation aids in tumor progression and metastasis. Expression of TLR4 has been described in various human tumors. One study has reported that 63% of breast cancer patients were stated to express TLR4 on tumor cells and the level of expression inversely

correlated with the survival. Dysregulation of TLR4 owing to single nucleotide polymorphisms (SNPs) have the ability to convulse the normal cellular immune response that may alter ligand binding and the balance between pro- and anti-inflammatory cytokines, hence rising the chance of chronic inflammation and cancer. So, TLRs have a distinctive role in breast cancer pathogenesis and recurrence.

## 3.3 Molecular Mechanism of TLRs in Breast Tumor Microenvironment

TLRs may recognize molecular patterns of PAMPs and DAMPs in order to create innate immunity and help in the control of adaptive immunity. In reality, the establishment of a tumor microenvironment is caused by the production of cytokines that recruit friendly immune cells, ultimately leading to cancer growth (Lu, Qi et al., 2013). Furthermore, TLR activation in innate immune cells and/or breast cancer cells promotes inflammation and cell survival in the tumor microenvironment. (Bhattacharya, D., & Yusuf, N. 2012). TLR activation can specifically attract various immune cells and Cancer-associated fibroblasts (CAFs) to the areas of inflammation, therefore initiating epithelial proliferation. As a result of an imbalance of pro-tumorigenic and anti-tumorigenic factors, it generates a microenvironment that inhibits apoptosis. Several signals with various effectors, such as the traditional anti-inflammatory cytokines such as IL-10 and TGF-, might impart to the tumor microenvironment. These effectors are produced by both tumor and stromal cells, and as a result, they have an influence on the secretion of pro-inflammatory molecules of immune cells (Heckel, Mark C et al., 2011).

In fact, regulatory T-cells (Tregs) are the most common kind of T-cell identified in human breast cancer, and Tregs have a proclivity to release anti-inflammatory cytokines. Tregs have the ability to destroy innate immune cells by releasing granzymes and perforin at the same time. In addition, an increase in spontaneous cell death may also result in an inhibiting immune environment (Mantovani, A., & Sica, A. 2010). Tumor-associated macrophages (TAM) may

produce a wide variety of growth factors, including fibroblast growth factor (FGF)-2 (also known as basic FGF), which promotes tumor cell proliferation. TAMs frequently produce and accumulate vascular endothelial growth factor (VEGF), prostaglandin E2, FGF-2, and IL-8 in hypoxic tumor areas (Mantovani, A., & Sica, A. 2010). Furthermore, TAMs promote and enhance tumor cell invasion and metastasis by the release of matrix metalloproteinases (MMPs), which have the capacity to destroy ECM components. In addition, EGF production promotes the formation of protrusions and an invasive phenotype in breast cancer cells. TLR4 is widely expressed in the tumor microenvironment by both cancer cells and immune cells. TLR4 activation selectively upregulates pro-inflammatory cytokines and chemokines such as-

(CCL, CXCL, CCR, CXCR, IL-6, IL-8,) tumor necrosis fiber (TNF)-, as well as immunosuppressive cytokines such as transforming growth factor- (TGF)-1, IL-10, and others specifically in tumor microenvironment. TLR4 in Foxp3+ CD4+, CD25+ cells mediate IL-10 and TGF- secretion inside the tumor microenvironment, limiting the antitumor impact (Ahmed, Abubakr et al., 2013). Inflammation is addressed through feedback mechanisms under normal settings. When these feedback systems are disrupted, as in cancer, chronic inflammation occurs, which is frequently referred to as "the wound that does not heal". As previously stated, TLRS may mediate inflammation and predict cancer survival, implying that they may also be involved in these feedback processes.

Furthermore, more severe disease phenotypes promote persistent inflammation in the tumor microenvironment, which can happen in tumor, immune, and stromal cells via TLR expression. These processes appear to take advantage of a critical cross-talk link between TLR and the production of nitric oxide synthase/COX2 (NOS2/COX2), both of which are key mediators of inflammation. Although NOS2 is an essential immune-surveillance regulator, a substantial increase in NOS2 expression was seen in apoptotic infiltrating mononuclear cells, which is

linked to increased Bax and caspase-3 expression in these cells and is thought to contribute to immunosuppression.
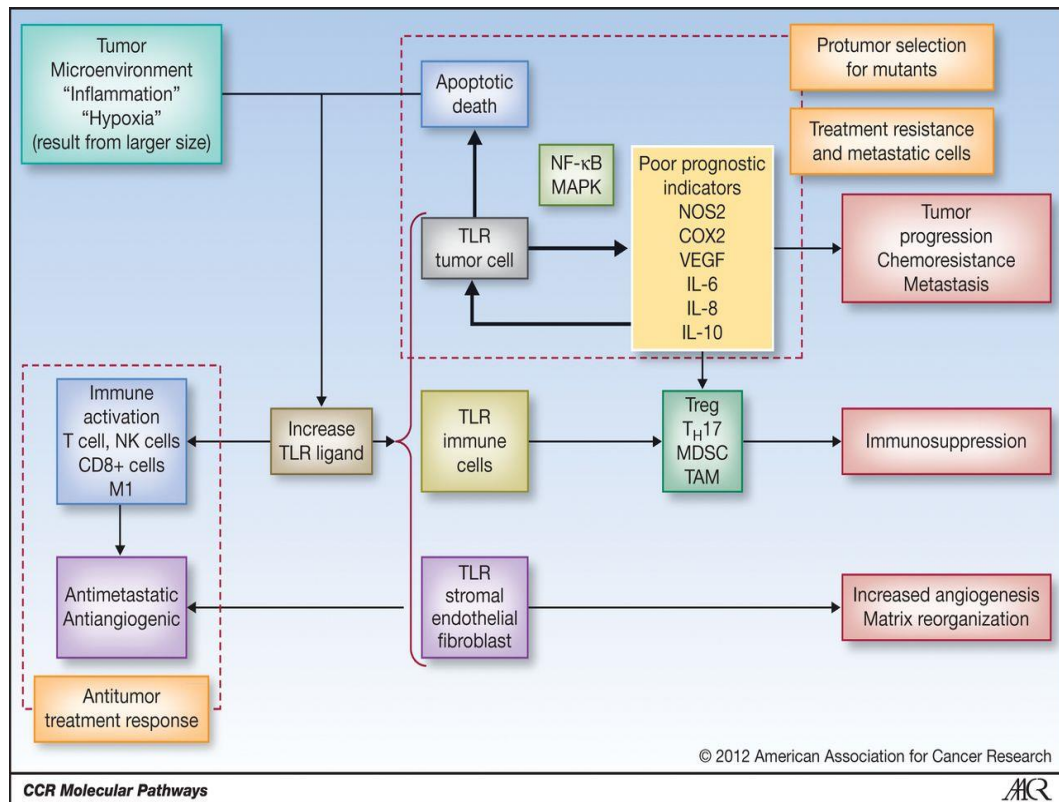


*Figure 6: Influence of TLR signaling on patient with cancer therapeutic outcome tumor cells in a chronically inflamed tumor microenvironment (Ridnour et al., 2013).*

Similarly, COX2 expressing colon cancer cells have been found to trigger T-cell cytotoxicity, suggesting that tumor immune surveillance may be involved. TLR activation increases the likelihood of the production of both pro-inflammatory enzymes, NOS2 and COX2, which are linked to tumor growth (Glynn et al., 2010). TLR activation, which results in increased NOS2/COX2 and the recruitment of immunosuppressive cell types, aids in the reduction of host tumor surveillance and the reduction of treatment response. Alternatively, TLR activation expressed on CD8+ T cells mediates an M1 antitumor response (Ridnour et al., 2013).

## 3.4 Statistical Analysis
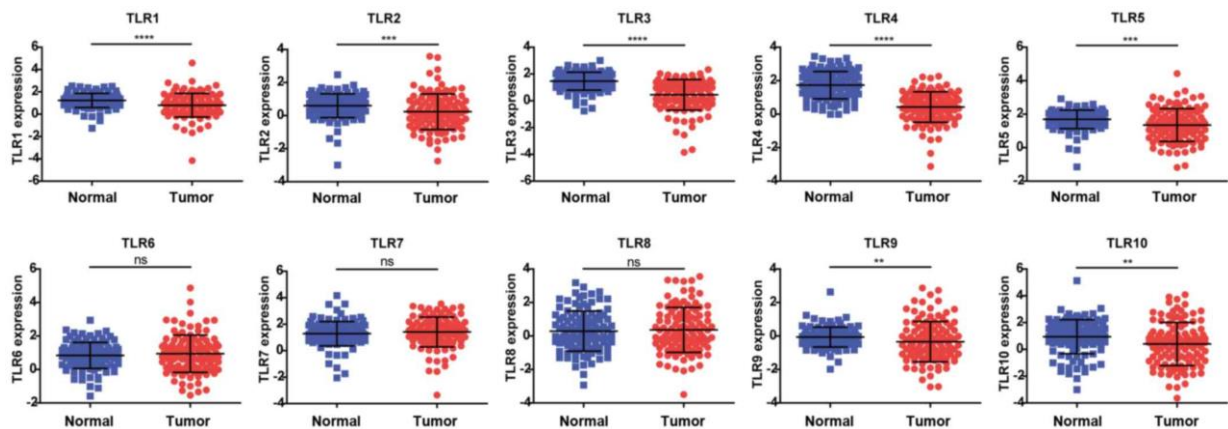


*Figure 7: TLR expression levels of breast cancer cases included in TGA database (S. Shi et al., 2020).*

The raw expression data showed nearly 113 tumor samples (red) and matched normal tissue (blue). The publicly accessible TCGA database had 2.5 petabytes of data representing tumor tissues and matched normal tissues. At the beginning, TLR 1- TLR 10 was evaluated by TCGA breast carcinoma database in order to get the clarity of understanding between normal and tumor tissues in comparison with the traditional control tissues, the expression levels of TLR1, TLR2, TLR3, TLR4, TLR5, TLR9 and TLR10 were notably decreased in breast cancer tissues. In contrast, the expression levels of TLR6, TLR 7 and TLR 8 were slightly increased in breast cancer tissues, although statistical significance was failed to achieve (Figure- 7). Here, data were expressed as the mean ± standard deviation. Moreover, paired student's T-test was representing statistical significance of differential mRNA expression levels of candidate genes between normal and tumor groups. **p≤0.01, *** p ≤ 0.001 and **** p≤ 0.0001 . (S. Shi et al., 2020).
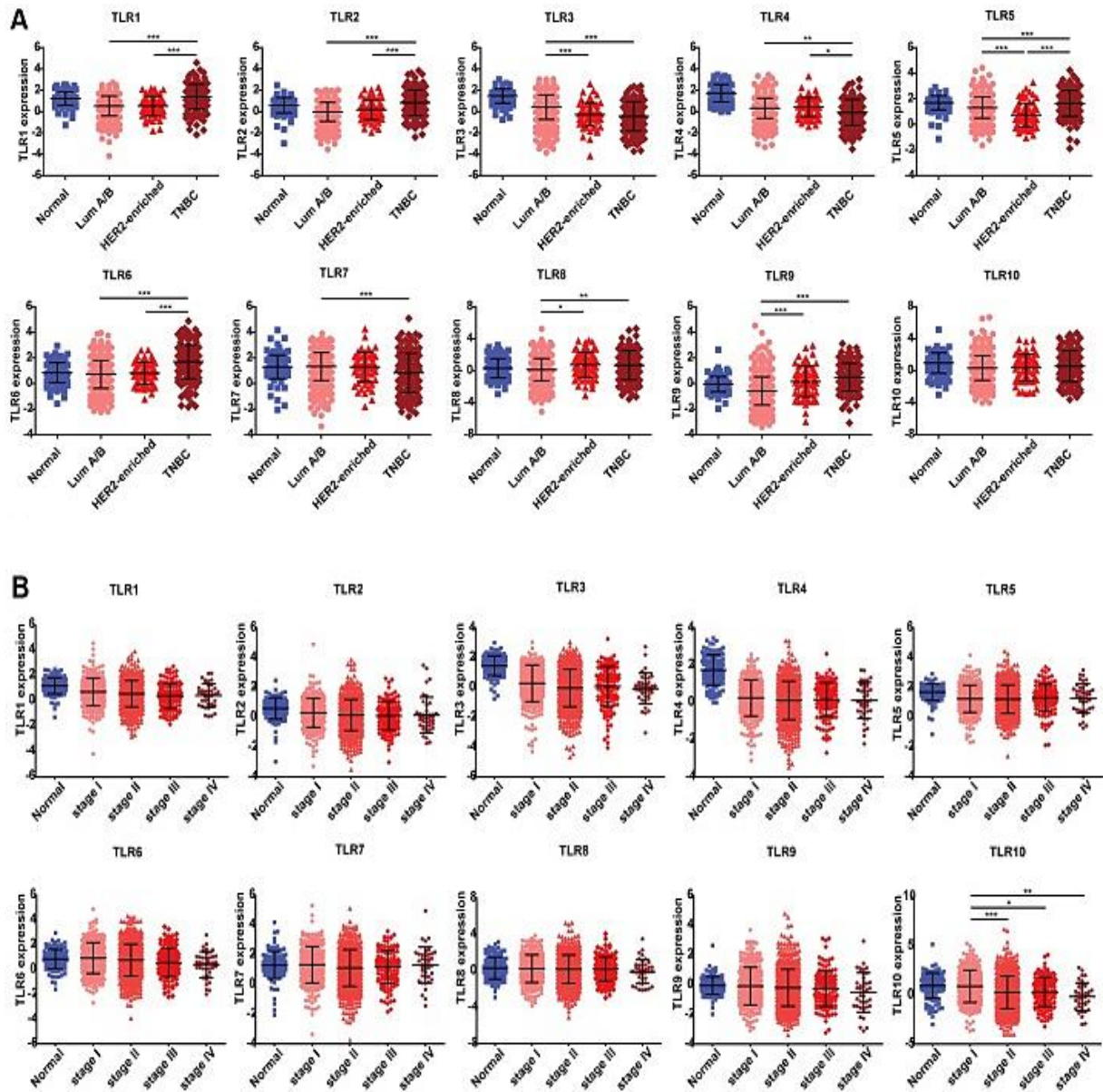
*Figure 8: Expression of TLRs among various subtypes and stages of breast cancer.*

Subtype information includes 113, 628, 67 and 142 samples from normal tissues (A), Luminal A/B, HER2- enriched and triple- negative subtypes, respectively (B). TNM stage information was dispersed from TCGA breast cancer database, including 113, 279, 622, 131and 40 samples from normal tissues, stage I, II, III, IV, respectively. As a result, data was evaluated by one-way analysis of variance which exhibited as the mean ± standard deviation to succeed in the differential mRNA expression levels of candidate genes among groups. In Figure 8, TLR 1, TLR 2 and TLR 6 exhibited vast level of expression in the triple- negative breast cancer subtype

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as compared to those in Lum A/B and HER2- enriched subtypes. Although the expression of TLR3 had a gradient fall among normal tissues and all the three subtypes. On the other hand, the variation between the HER-2 enriched and triple-negative subtypes was not analytically crucial. Interestingly, TLR5 was the sole gene that showed a notable difference among all the three subtypes, with the least expression observed in the HER2- enriched subtype, followed by the Lum A/B and triple negative subtypes. TLR-9 expression was increased to a great extent among all the three subtypes. As per observing TLR-3, there was no crucial differentiation between the HER2-enriched and triple- negative subtypes. On the contrary, expression of TLR-10 did not vary much among three subtypes of breast cancer (Fig 2A ). (S. Shi et al., 2020).

To sum up, these results give a clear view regarding the role of TLR-3 and TLR-9 as successful biomarkers of ER-/PR- negative breast cancer (HER-2 enriched and triple-negative subtypes), whereas TLR-5 may synchronize with HER-2 enriched breast cancer. (Chew & Abastado, 2013)


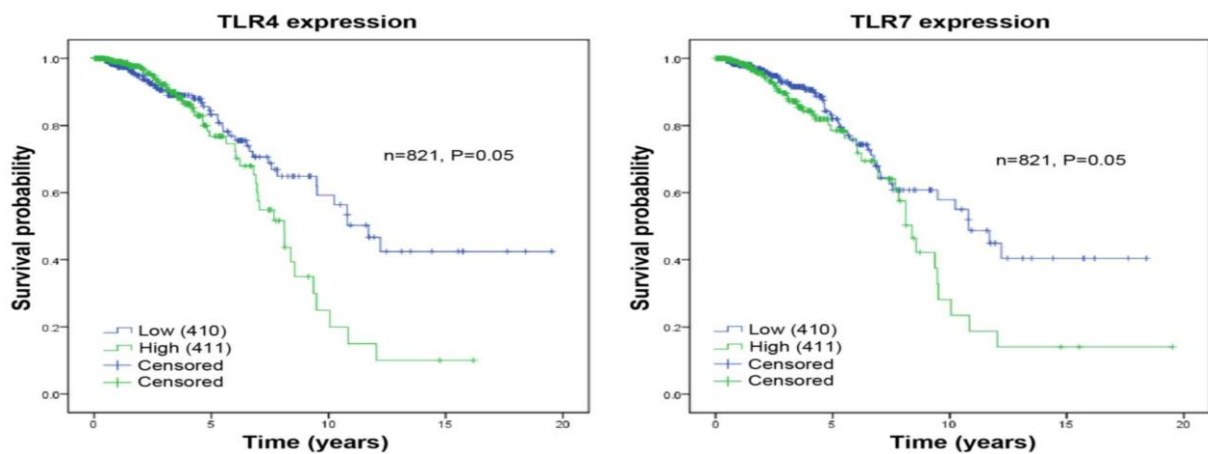
*Figure 9: Kaplan-Meier curves for overall survival stratified by TLR4 and TLR7 mRNA expression (S. Shi et al., 2020).*

The final outcome was dispersed from The Cancer Genome Atlas breast cancer database which involved 410 and 411 samples with low (blue lines) and high (green lines) TLR expression, respectively. Cases representing Cancer-associated mortality were only involved in this survey.

The symbol + stands for censored observation and the log-rank test was for comparison with the total survival curves between groups.

## 3.5 TLR Gene Polymorphism in Breast Cancer

Polymorphisms in TLR genes transfer balance between pro and anti-inflammatory cytokines, regulating the danger of infection, chronic inflammation and cancer. The list for TLR polymorphisms perspective based on oncogenomic investigation include rs10008492, rs4833103, rs5743815, rs11466657, rs7696175 (TLR1-TLR6-TLR-10 gene cluster); rs3804100, rs4696480, -196- - 174 del (Delta22). GT- microsatellite polymorphism (TLR2); 829 A/C (TLR3); rs5743836, rs352140 (TLR9). The extended list can additionally include rs4833095, rs5743551, rs5743618 (TLR1); rs5743704, rs62323857, rs1219178642 (TLR2); rs5743305, rs3775291, rs121434431, rs5743316 (TLR3); rs5744168 (TLR5); rs179008 (TLR7); rs3764880, rs2407992 (TLR8); rs352139, rs187084, rs41308230, rs5743844 (TLR9); rs4129009 (TLR10). (Source: American Society for Histocompa, 2011).

Immunopolymorphism play a crucial role behind the resistance or susceptibility of the host to an infectious disease. Over the years, researchers have found many genetic factors working as immune surveillance against certain tumor cells. Few single-nucleotide polymorphisms (SNPs) within the TLR genes are related to tumorigenesis. A deletion from -174 to -196 of TLR2 gene and Asp299Gly of TLR 4 gene polymorphisms might expand the susceptibility to cancer cell growth (Ruano et al., 2016). TLR9 SNP in rs352140 has been reported to be related with cancer risk. Moreover, TLR4 SNP at rs4986790 is responsible for significant increasing chance of cancer whereas SNP (rs1927911) is related to decreased cancer risk. Additionally, the prevalence of TLR4 +3725GC and CC genotypes notably increased in cancer patients in comparison with healthy controls. Cancer patients with TLR4 +3725g/c polymorphism, have significantly shorter survival time. Comprehensively, polymorphism in TLR4 gene seems to

be related to expanding susceptibility to cancer and TLR4 +3725G/C polymorphism might act as a prognostic and risk factor for the cancer (Yang, C-X et al., 2013).

Generally, decreased expression of TLR2 leads to reduced immune response favoring tumor development. On the contrary, Xie et al demonstrated that TLR2 activation by infectious bacterial PGN plays a significant role in breast cancer cell invasiveness. Additionally, stimulation of TLR2 expressed in breast cancer cells is related to increased tumor's invasiveness through NF-κB while blockage of TLR-2 may result into tumor suppression.

However, regarding the Asp299Gly and Thr399Ile polymorphisms in TLR4 encoding gene, there is a notable association between the presence of Gly allele and the risk for breast cancer development (Coughlin & Piper, 1999).
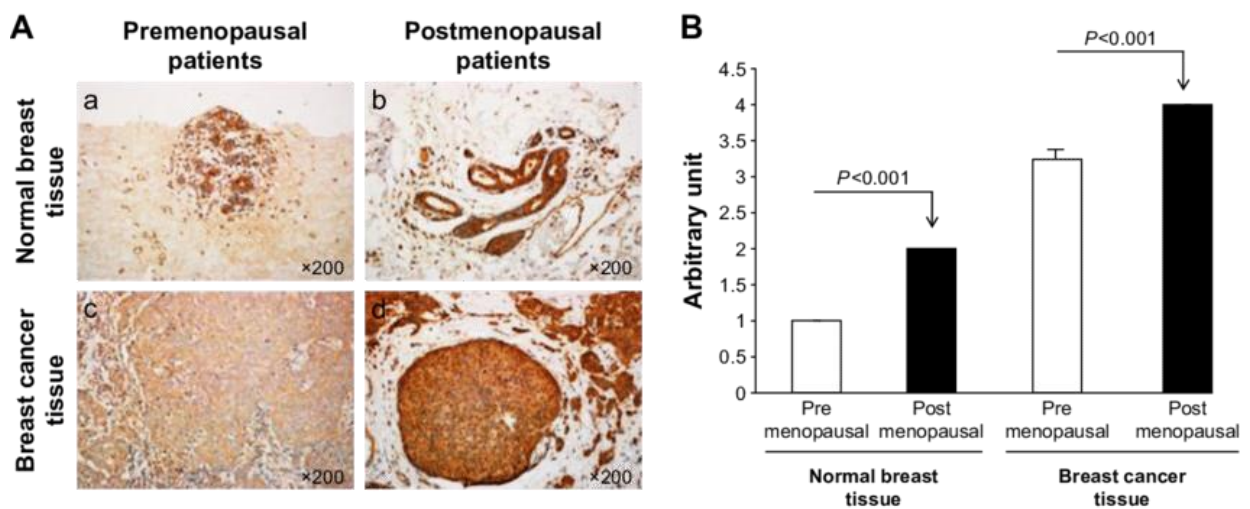


*Figure 10: Immunohistochemical determination of TLR-4 protein expression in normal and breast cancer tissues (A) and (B).*

Representative sections (×200) of positive immunostaining TLR-4 protein expression in normal breast tissues (a and b) and ER-breast cancer tissues (c and d).

The staining samples from ER-premenopausal patients is illustrated in (a and c) and from ER-postmenopausal patients in (b and d). Tissues were immunostained using particular TLR -4 antibodies.(B)   TLR-4   positive staining was evaluated   as follows : 0 point, no positive

staining, 1 point, ≤ 20% positive staining; 2 points, 21%-50% positive staining; 3 points, 51%-75% positive staining; and 4 points, ≥ 75% positive staining (Semlali et al., 2017).

## 3.6 Breast Cancer Treatment with TLRs

Conventional treatments for breast cancer include chemotherapy, radiation and surgery. Hormonal therapy which can decrease the elevated level of estrogen and progesterone, may also be used to restrain from recurrence of disease in women with hormone receptor- positive tumors. But Treatment with immunomodulators like - TLRs, take advantage of a person's own immune system to help in fighting against cancer cells. Many pharmaceutical companies and biotechnologies have different scheme of plan to formulate new drugs that act as either. (Table 2)

*Table 2: Compounds that affect toll-like receptor (TLR-2 and TLR-4) activity in breast cancer*

| Ligand | Role in normal cell | Effect in breast cancer | References |
|---|---|---|---|
| Serum amyloid A | Chemo-attractant | Depending on stage, it enhances in breast cancer patients | Zhang G et al., 2012 |
| HMGB1 | Regulate apoptosis | More express in tumor cell than normal cell | Tang D et al., 2010 |
| Peptidoglycan | Forms cell wall of bacteria | Cause of invasive breast cancer. | Xie W et al., 2010 |

1. Agonists of TLRs to increase immune responses against tumors and infectious agents or to improve allergic responses or;

2. Antagonists designed to alleviate inflammation due to infection or autoimmune disease. Immunomodulators manipulate the 'brakes' and "gas pedals" of the immune system. There are various options for the targeting TLRs, because the key role is to induce cytokines. It is well authorized in cancer and effectively being targeted in the clinic. Several compounds that are capable to stimulate TLRs 3,4,7,8 and 9 have now been tested in clinical trials (M. Shi et al., 2016). Therefore, activation of Toll-like receptors can help to stimulate vaccine like response against tumors which have opened up a productive area of treatment by detecting in the early stages of tumors.

**Serum amyloid A (SAA)**: It is an acute-phase protein which can function as an endogenous DAMP by binding to pattern recognition receptors like TLRs on both breast cancer cells and cancer associated fibroblasts (CAFs). In the tumor microenvironment (TME), binding of serum amyloid A to toll-like receptors trigger inflammasome which results in the notable increase in interleukin-1β production. So, once interleukin is increased into the TME, it originates a favorable environment to promote breast tumor growth. It is therefore can be acclaimed that in the TME, SAA binds to various TLRs on cell types like- immune cells, cancer cells  (Malle et al., 2009).  As a result, once it is bound, secretion of interleukin-1β, initiates tumor growth and eventually metastasis. Therefore, by inhibiting the inflammasome or by neutralizing antibodies which target SAA, potential therapeutic approaches could be taken into account for breast cancer patients.

Clinically, SAA increases gradually with tumor progression and the SAA value represents a direct correlation with the stage of tumor. In conventional renal cell carcinomas, expression of SAA1 protein in tumor cells associated with low course-specific survival. Also, SAA1 aids in tumor invasion and MMP expression. With regard to breast cancer, the proteomic mass

spectrometry shows that the blood level of SAA is remarkably higher in stage IV than stage I. High fat diet in F2 mouse, which was originated from a cross between the M16i polygenic obese and MMTV-PyMT mammary cancer models, is able to alter the expression of SAA gene. So apparently, these mice show a decline in mammary cancer latency and growth in pulmonary metastases (La Merrill et al., 2010). SAA concentrations varies significantly in stage I, II, III and IV patients with a higher median compared to those of healthy, benign breast diseases and stage I groups (p<0.05). So basically, patients with lymph node metastasis or distant metastasis have greater SAA concentrations than those without metastases which make it a suitable candidate marker for the staging and prognosis of breast cancer.

**HMGB1:** High mobility group box 1 is a number of the DAMPs which can be noticeable to various compartments of cell and serves in maintenance of genomic stability, autophagy, immune regulation, and tumor growth, accordingly. Some finding reveals that HMGB1 could initiate development of breast cancer cells in-vitro. Using many orthotopic, syngeneic mouse models of basal-like breast (4T1, 67NR and EpRas) or non-small cell lung (tc-1) cancer, the activity of several hbgb1 inhibitors alone and in combination with immune checkpoint blockade antibodies (anti-PD-1/anti-PD-L1) was evaluated later. Clearly, without affecting the global number of (CD45+) immune cells, substantial reduction of monocytic/granulocytic myeloid-derived suppressor cells (MDSC) and regulatory T lymphocytes, a higher M1/M2 ratio of macrophages as well as intensifying activation of both DC and pDC were simultaneously noticed following HMBG1 inhibition. Besides, blocking HMGB1 facilitated the efficacy of anti-PD-1 cancer monoimmunotherapy. The mammary carcinoma for DAMP expression indicates that increased HMGB1 compared to normal mammary epithelium. Besides, Myd88 inhibitory peptide analysis swapped the HMGB1 expression level in cancerous cells which was moderated in a Myd88 dependent manner. So, the inhibition of Myd88 function lowered the HMGB1 gene expression. Increasing expression of ∞-Smooth

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Muscle Actin (ASMA), a cancer-related fibroblast and reduced expression of cytoplasmic HMGB1 conjointly illustrate poor prognosis of breast cancer (Sohun & Shen, 2016). However, the HMGB1 level in cancerous tissue could be interrelated with lymphatic metastasis but were not connected with the age of patient and tumor growth. Recent reports suggests that invasion and migration of breast cancer cells can be inhibited by miR-200c which was modulated via HMGB1.Therefore, the miR-200c and HMGB1 may considered to be beneficial biomarkers to detect the progression of breast cancer and could be a progressive option of immunotherapy technique for cure (Chang, Bao-Ping et al., 2014).

**Peptidoglycan:** Breast cancer cells with large metastasis (MDA-MB-231) have high level of expression on Toll-like receptor 2 (TLR2) in comparison with inadequate metastatic breast cancer cells and homogenous untransformed breast cells. Consequently, TLR-2 is predominantly triggered by peptidoglycan (PGN) from infectious bacterium Staphylococcus aureus (PGN-SA) which therefore, results into promoted invasiveness and adhesiveness of the cancer cells in vitro. Phosphorylation of TAK1 and IkB in the TLR2-NF-kB is induced by PGN-SA, resulting in secretion of stimulated IL-6 and TGF-β in MDA-MB-231 cell and all these effects are revoked by blockage of TLR-2. Also, further studies showed that the functions of NF-kB, STAT3 and Smad3 were increased sequentially in MDA-MB-231 cells after PGN-SA stimulation. So, there was an increasing phosphorylation of NF-kBp65 and then followed by phosphorylation of STAT3 and Smad3 in next 4 or 6 hours. Apparently, there is an attenuated STAT3 and Smad3 by inhibition of NF-kB wherein supernatants of PGN-SA stimulated cell countermand these inhibitory cells. To sum up, it can be said that activation of TLR-2 by infectious bacterial PGN had an active contribution in breast cancer cell invasiveness and demonstrated a new connection between infectious bacteria and cancer cells, which potentially recommending the association of antibiotic therapy to treat cancer with bacterial infection. In a new study, it has been seen that activation of TLR5 on breast cancer cells by its

agonist flagellin, results into suppression of cell proliferation and anchorage-based cell maturation which was again confirmed in vivo using mouse xenograft models of human breast cancer cells (Cai, Zhenyu et al., 2011)

There is a growing aspect towards prevention and treatment of breast cancer through the signaling of Toll-like receptors. Currently available preclinical and clinical data strongly indicates that successful anticancer immunotherapy is needed for combinatorial approaches in a wide fraction of patients. In this seeing, TLR agonists have potential functions to accelerate the immune response in patients with an effort to contribute for better clinical outcomes.

# Chapter 4 Toll-like Receptors in Prostate Cancer

## 4.1 TLR Family and Prostate Cancer

Prostate cancer is one of the leading reasons of death in worldwide for men. The fundamental mechanism of immune recognition receptors is still under inspection to understand its immunogenic role in cancer treatment. Some common TLR-agonists like- HMGB1 can activate TLR2 and TLR4, (Apetoh et al., 2007) Versican acts as a TLR 2 agonist and peroxiredoxin1 (prx1) has an agnostic contribution for TLR4 in prostrate adenocarcinoma (Kim et al., 2010) . Perhaps, there may exist more endogenous TLR ligands that are undergoing further identification and verification.

There are few TLR ligands which can restrain the tumor growth of prostrate adenocarcinoma (Figure 11). In fact, it's found that TLR3 mRNA is discovered in three prostrate adenocarcinoma cell lines including LNCaP, PC3 and DU-145 (Harashima, N., Inao, T., Imamura, R. et al, 2012). Besides, poly (I.C) helps to increase the mRNA level of TLR3. Additionally, TLR 3 protein was also expressed at similar levels in LNCaP and DU-145 cells, with a relatively lower expression in PC3 cells. Also, stimulation with poly (I:C) have a suppressing nature in prostrate tumor growth in vivo, perhaps due to the occurrence of infiltration of T- lymphocytes and NK cells in type I IFN-dependent manner. Furthermore, greater expression level of TLR-3 was substantially interlinked with high probability of the recurrence of prostate cancer (González-Reyes et al., 2011).  TLR 3 could synchronize the procedure of angiogenesis and apoptosis in prostate cancer cells through hypoxia-inducible factor 1α (HIF-1α) and PKC-dependent mechanism (Paone et al., 2010).  On the other hand, TLR 5 is expressed in LNCap and DU-145 by which stimulation activates the generation of chemokines which recruit immune cells, along with NK cells and also cytotoxic CD8 cells , which eventually likely participate to tumor inhibition (Galli et al., 2010)

The activation of other TLRs might have a different role in the development phase of tumor (Figure -11). Several animal models were demonstrated to understand the expression of TLR4 as it might act as a potential preventive strategy in the treatment of prostate cancer. Studies showed a fundamental expression of TLR 4 in the epithelial cells of rat ventral prostrate along with rat adenocarcinoma cell line and in prostrate primary culture cells. Moreover, TLR 4 is also demonstrated in DU-145, PC3, and normal prostate gland in both stroma and epithelium. Furthermore, expression of TLR 4 has also been noticed in clinical samples of prostate cancer. A significant clinical study also revealed that TLR 9 is expressed in prostate cancer specimens. Joanna et al. found that TLR 9 is expressed in human prostate cancer cell lines LnCaP, C4-2B, Du-145, PC3 and also in clinical samples of prostate cancer via immunohistochemistry and western blotting. Also, it was statistically expanded in prostate cancer epithelium and stroma, in comparison with the indistinguishable compartments in benign hyperplasia, significantly in the most poorly differentiated forms (Väisänen, Marja-Riitta et al., 2010). Therefore, the functional and biological significance of TLRs in prostate cancer appears as a complex mechanism.

The therapeutic approach of specific TLR agonists alone or in combination with standard chemo- or radio-therapy has been considered as a justifiable anti-cancer strategy in various in vitro or in vivo cancer models and different types of molecules have been evaluated in clinical trials ( www.clinicaltrials.gov ). (Galluzzi et al., 2012; O'Neill et al., 2010).

William Coley's investigations demonstrating gram positive / negative – inactivated toxins with an appropriate effect in cancer treatment were the first to validate the anti-cancer method of pathogen-derived compounds (Coley, 1991). Coley's toxin components (bacterial proteins, lipids, and DNA) were then considered, and a large number of pathogen-derived medications or synthetic chemicals were produced which are capable of selective stimulation by TLRs (O'Neill et al., 2010). So, the activity of TLR-agonists has been evaluated alone, with

combination treatment, with other cytotoxic medicines, or as vaccine adjuvants to acknowledge the role of these molecules on the suppression of tumor growth.
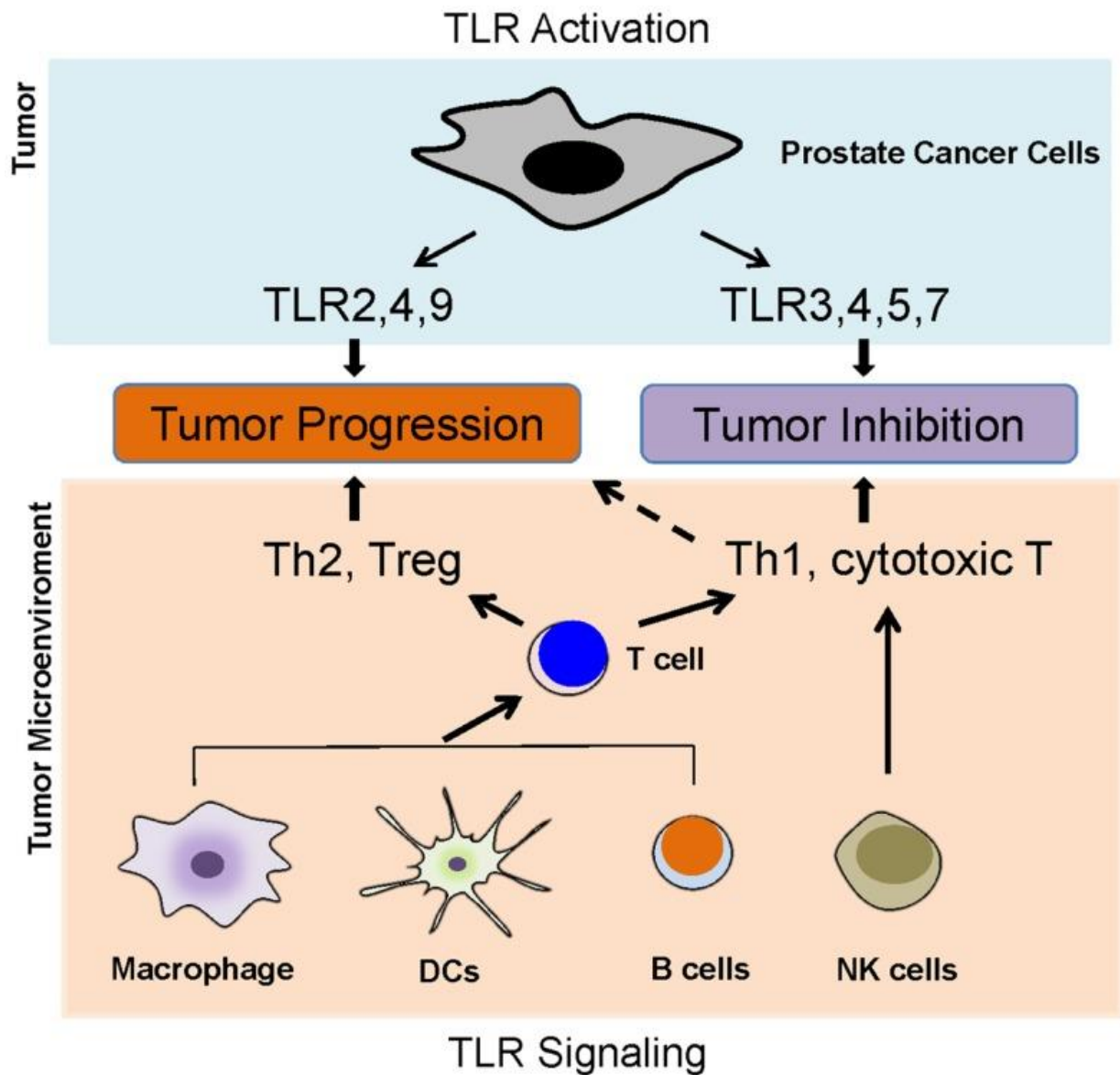


*Figure 11 : Toll-like receptors and prostate cancer (Zhao et al., 2014)*

TLR activation in antigen-presenting cells including DCs, macrophages, and B cells can cause either Th1 or T cytotoxic responses or Th2 or Treg responses. TLR2, 4 and 9 activations in prostate cancer cells appears to enhance tumor growth, but TLR 3, 4, 5 and 7 activation helps to inhibit prostate cancer.

## 4.2 MicroRNA Regulation and Signaling of TLRs in Prostate Cancer

MicroRNAs (miRNAs) are a kind of tiny non-coding RNAs (~22 nt in length) that control gene expression in a contradictory manner after transcription (He & Hannon, 2004). MiRNAs persuade gene silence by slowing translation or causing mRNA degradation due to their binding nature to target regions inside the 3′ UTR of mRNA.

Furthermore, dysregulation of miRNA is thought to be a major factor in carcinogenesis and many miRNAs have been identified as oncogenes or tumor suppressors (Ventura & Jacks, 2009; Zhang et al., 2007).

In the control of TLR signaling, microRNAs serve as a primitive mechanism (Nahid et al., 2011). In prostate cancer, researchers have discovered a connection between the two miRNAs and TLRs. In fact, in the instance of metastatic prostate cancer, MiR-29a served as a possible tumor suppressor via regulating TRAF-4 expression (Ahmed et al., 2013). Furthermore, upregulation of miRNAs such as miR-29b, -29c, -148b, and -152, which primarily target DNA methyltransferases, is aided by TLR 3 activation by poly (I:C), resulting in the re-expression of oncosuppressor RAR in prostate cancer cells (Galli et al., 2013). So, either it facilitates or inhibits the process.

TLR ligation may recruit one or more adaptor proteins with TLR domain contacts, such as MYD88, TRIF, TRAM, and MAL. TLR 3 is primarily activated by the action of IL-1 receptor associated kinase via a MyD88-dependent signaling pathway (IRAK). MAPK and NF-kB signaling may be activated as a result of this. TRIF is activated in response to stimulation, resulting in the production of NF-K and type I IFN signaling. TLR 3 can be activated in prostate cancer cells, although the molecular signaling pathway has not yet been fully explored. TLR 3 appears to aid in the apoptosis and growth arrest of LNCaP cells by downregulating the PI3K/Akt pathway, according to several recently conducted research. In poly (I:C) - treated

LNCaP cells, CyclinD1, c-Myc, p53, and NOXA are thought to have a role (Harashima, N.,

Inao, T., Imamura, R., Okano, S., Suda, T., & Harada, M. (2012)).
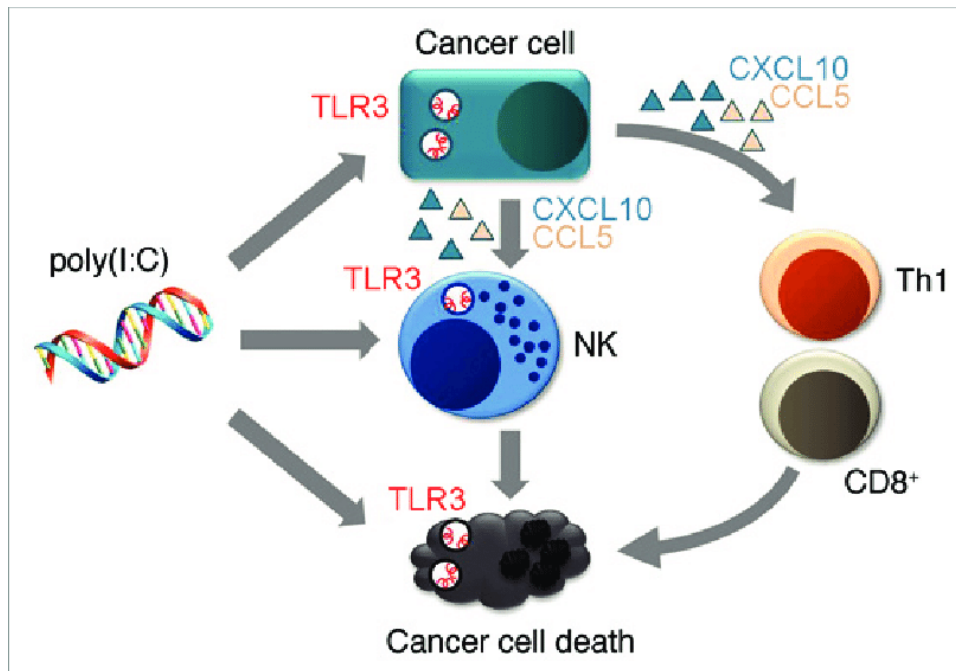


*Figure 12: proposed mode of action of TLR-3 ligands (Chew & Abastado, 2013)*

HIF-1 promotes apoptosis in poly (I:C)-treated prostate cancer cells through a PKC-dependent

mechanism, according to earlier research. TLR 3 also activates JNK and p38 through PKC-

and causes apoptosis in a caspase-8-dependent way (Paone et al., 2008, 2010). Poly (I:C)

treatment causes a cascade of chemokines to be released in LNCap cells, including CCL3,

CCL4, CCL5, CCL8, CXCL9, and CXCL10, which might lead to large NK cell and CD8 T

cell chemotaxis. Recent research on antibacterial and anti-inflammatory treatments for prostate

cancer has shown some interesting results. In reality, TLR-4 gene silencing in combination

with small interfering RNA (siRNA) has been shown to be effective to decrease tumor cell

motility and invasion, to reduce cell viability and cause cell death. Downsizing the expression

of (MyD88) or phosphorylation (TRIF, IRF-1) of TLR-4 signaling pathways achieves these

results (Hua, Dong et al.,2009).

Furthermore, TLR-4 siRNA inhibits tumor growth and survival in a mouse prostate cancer model. As a result, these findings clearly show that TLR-4 plays a role in prostate cancer oncogenesis and development. Also, selenium-mediated suppression of the LPS-mediated TLR-4 signaling pathway in human PCs cells may result in reduced pro-inflammatory and, most likely, anticancer activity. Another truth is that TLR-4 mutations have been found to be sensitive to Gram-negative bacteria (Poltorak et al., 1998). TLR-4 recognition on antigen-presenting cells has been shown to boost antigen-specific antitumor immunity. Hence, TLR-4 agonists have been proposed as an anticancer treatment (Nie et al., 2018). Moreover, long-term low doses of microbial or endogenous TLR-4 ligand activation in local microenvironments (e.g., prostate) can begin cancer development, whereas one or two doses of TLR-4 ligands (e.g., vaccination adjuvant) can enhance antigen-specific antitumor responses (Ou et al., 2018).

In addition, poly (I:C) contributes in the development of inflammatory molecules, including IL-6 and IL-12, most of which are connected with NF-Kb signaling pathways. In the TRAMP tumor model, poly (I:C) therapy attracts NK cells and T lymphocytes through a type 1 IFN agonist-dependent pathway, which leads to tumor growth suppression.
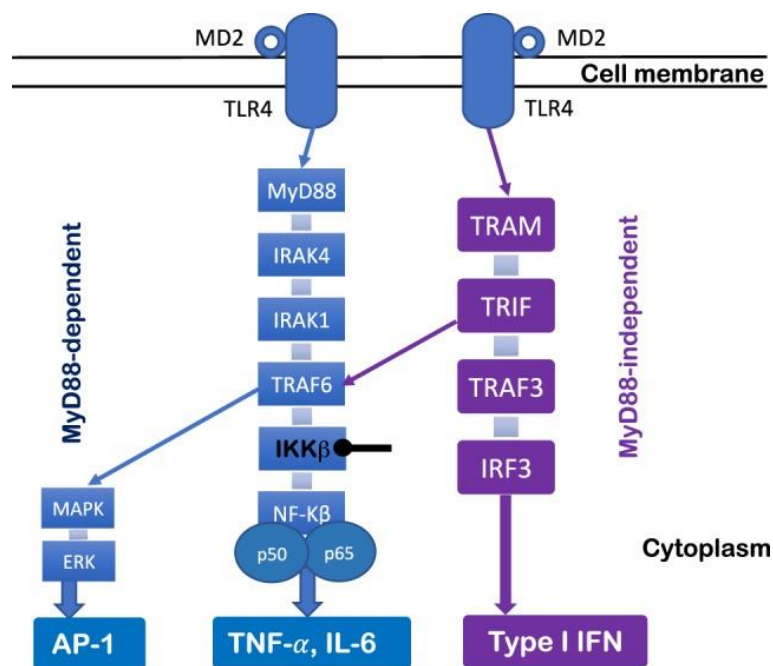
*Figure 13 : signaling pathway of TLR-4 (Ou et al., 2018)*

LPS activation of TLR 4 in DU 145 stimulates the NF-k signaling pathway, which leads in the production of pro-inflammatory cytokines such as IL-6 and IL-1 via the Myd88 dependent pathway. TLR 4 activation can also increase the production of VEGF and TGF-1 in PC3 cells, both of which promote tumor growth. Through TRAF6, the MyD88 pathways also produce MAKP and other extracellular signal-regulated kinases (ERK), which results in AP-1 activation. Furthermore, when the TRIF-related adaptor molecule (TRAM) is activated, TRIF, TRAFs are recruited, resulting in the activation of the interferon regulatory factor 3 (IRF 3) and the generation of type 1 IFN. Flagellin, on the other hand, is a TLR-5 agonist that activates NF-Kb signaling in LNCaP and DU145 cells and begins the production of pro-inflammatory chemicals. The stimulation of TLR-9 by CpG-ODN is critical for prostate cancer invasion. This result is mediated by NF-kB activation and COX-2 overexpression. Later, after some study, it was shown that TLR-9 expression contributes to increase invasiveness in vitro by inducing MMP-13. As a consequence, CpG-ODN stimulation had no effect on cell proliferation in either study, indicating that TLR-9 signaling plays a role in cancer development and metastasis.

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Hossain et al. discovered that myeloid–derived suppressor cells express TLR-9 in the blood of individuals with metastatic prostate cancer, inhibiting the function of CD8+ T lymphocytes (Hossain et al., 2015). Another study found that effective engraftment of TLR-9 expressing prostate cells into mice occurs as a result of the cross-talk between TLR-9 and leukemia inhibitory factor, which promotes immunosuppressive activity with the help of STATs and PMN-MDSCs (Won et al., 2017)

Furthermore, TLR-9 plays an important role in the propagation and self-renewal of prostate cancer by activating the NF-B/RELA and STAT3 pathways, and TLR-9 positive tumors have a unique gene profile that is linked to inflammation and stem cells (Hossain et al., 2015). In patients with prostate cancer cells, about 66.7 percent had strongly positive cytoplasmic immune staining and 31.7 percent had mildly positive cytoplasmic immune staining (Väisänen et al., 2013). Despite the fact that TLR-9 is linked with prostate cancer-specific progression-free survival in individuals who have undergone radical prostatectomy, it is not consistent with most disease etiology and progression indicators  (e.g., pT-class, Gleason score, and preoperative PSA level) (Väisänen et al., 2013). As a result of these findings, TLR-9 plays a pathologic function in prostate cancer progression, and targeting it with a curative goal may provide viable therapeutic methods to battle prostate cancer. To summarize, it is difficult to explain why activation of some TLRs, such as – TLR3, limits tumor development while activation of other TLRs, such as – TLR-2, promotes tumor growth (Figure-10). To detect the particular effectors in TLR activations leading to opposing outcomes, some observable TLR signaling pathways must exist.

## 4.3 TLR Gene Polymorphism and Prostate Cancer Risk

TLR gene polymorphisms make people more vulnerable to a wide range of infections and inflammatory diseases. Some compelling data suggests that persistent intra-prostatic

inflammation accelerates the growth of prostate cancer. It was also shown that TLR gene polymorphism may alter TLR signaling, therefore influencing inflammation and prostate cancer risk. Certain research has been done to determine whether there is a relationship between TLR gene polymorphism and prostate cancer risk, and the findings have been mixed (Stevens et al., 2008; J. Sun et al., 2005). In previous research, single nucleotide polymorphisms (SNPs) in TLR 4 were thought to be associated with prostate cancer progression.

Furthermore, sequence variations in the TLR gene cluster (TLR6-TLR1-TLR10) were thought to be linked to prostate cancer risk. Nonetheless, some contentious results were gathered. Shui and colleagues examined 10 TLR-4 SNPs and discovered no significant link between TLR-4 genetic variation and the risk of developing prostate cancer (Shui et al., 2012). According to Chen et al., sequence variations of the gene cluster TLR6-TLR1-TLR10 were not associated with the likelihood of developing prostate cancer. Furthermore, Lindstrom et al. found no significant link between TLR gene variation and prostate cancer risk in a meta-analysis.

The disparity in these results might be attributed to a variety of factors such as detection technique, population race, and sample size. This problem must be resolved since it will establish a result regarding the feature of TLR polymorphism as a diagnosis/prognosis marker, as well as if we can build a novel approach to treat prostate cancer by targeting TLRs and their signaling system. Polymorphisms in genes encoding TLRs and TLR pathway proteins do not have a role in the etiology and pathophysiology of prostate cancer. More in-depth studies should be conducted in order to understand the relationship between TLR single nucleotide polymorphisms and prostate cancer risk.

# Chapter 5 Future Perspectives

## 5.1 Potential Approach of TLR Agonists as Anti-Cancer Therapy

The potential use of TLRs to control prostate cancer growth has heightened interest in the development of immunotherapy against prostate cancer using TLR agonists or antagonists. The FDA has authorized three TLR-targeting medicines for use in cancer patients: Bacillus calmette-Guerin (BCG), Monophosphoryl lipid A (MPL), and Imiquimod (Galluzzi et al., 2012). BCG is typically made from an attenuated strain of Mycobacterium bovis and triggers TLR2/4. It is mostly used as a TB vaccine, but it is also used to treat in situ bladder cancer. Monophosporyl lipid A, on the other hand, is a dynamic component of Cervarix, which is used against cancer-causing human papillomavirus (HPV) (Mata-Haro, Van et al., 2007; Schiffman, M., & Wacholder, S., 2012).

Imiquimod (TLR-7 agonist) is another effective TLR-targeting medication that is derived from synthetic imidazoquinoline and is more commonly utilized in the treatment of skin cancers such as basal cell carcinoma and Bowen's disease. Furthermore, imiquimod increases proinflammatory cytokines such as IFN, IL-6, and TNF- (O'Neill et al., 2010). Furthermore, TLR-7/8 activation triggers Th 1 responses and anti-tumor activity, both of which rely on IFN. In terms of prostate cancer, Han et al. demonstrated that Imiquimod can inhibit both human and animal prostate cancer growth by inducing apoptosis (Han, Ju-Hee et al., 2013)

*Table 3: Targeted TLR-ligands in clinical trials.*

| Molecule | TLR target | Cancer | Phase | Reference no. |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| VTX-2337 | TLR – 8 | Squamous cell cancer of head and neck | Phase I | NCT01334177 |
| Resiquimoid (R848) | TLR-7, 8 | Melanoma | Phase II | NCT00960752 |
| Imiquimoid (IMQ) | TLR- 7 | Breast cancer | Phase I/II | NCT01421017 |
| CBLB502 | TLR- 5 | Solid tumor | Phase I | NCT01527136 |
| VTX-2337 | TLR- 8 | Fallopian tube cancer | Phase I | NCT01294293 |
| VTX-2337 | TLR- 8 | Low grade B-cell lymphoma | Phase I/II | NCT01396018 |
| 852A | TLR-7 | Breast cancer, ovarian cancer, cervical cancer | Phase II | NCT00319748 |
| Ampligen | TLR- 3 | Ovarian cancer | Phase I/II | NCT01312389 |
| Poly- ICLC | TLR-3 | Melanoma | Phase I/II | NCT01079741 |
| CpG 7909 | TLR- 9 | Lymphoma, non- Hodgkin | Phase I/II | NCT00185965 |
| IMO - 2055 | TLR-9 | Non- small cell lung cancer | Phase I | NCT00633529 |

| | | Colorectal cancer | Phase I | |
|---|---|---|---|---|
| IMO - 2055 | TLR-9 | Colorectal cancer | Phase I | NCT00719199 |
| BCG | TLR 2/ 4 | Bladder cancer | n.s | (Mackern-Oberti et al., 2006) |
| Poly A: U | TLR- 3 | Breast cancer | n.s | (Gatti et al., 2006) |
| Imiquimod | TLR- 7 | Basal cell carcinoma | Phase III | (Rakoff-Nahoum & Medzhitov, 2009) |
| Imiquimod | TLR- 7 | Breast cancer, skin metastasis | Phase III | (König, Jens Ekkehard et al., 2004) |
| 852A | TLR- 7 | Melanoma | Phase II | (Zheng et al., 2004) |

Many preclinical and clinical investigations are being conducted to assess the immunotherapeutic efficacy of TLR agonists against cancer. Surprisingly, TLR-3 activation directly induces death in prostate cancer cells. As a result, TLR-3 agonists appear to be a promising clinical strategy for the development of anti-tumor medicinal medicines. In fact, Ampligen, which is comprised of poly (I:C) (a TLR-3 agonist), has reduced tumor development in the early phases of clinical studies (Galluzzi et al., 2012)

Cadi-05, a poly-TLR agonist, has been found to inhibit the development of murine myeloma and thymoma in mice (Rakshit et al., 2012). This chemical has also been utilized in clinical

studies for the treatment of prostate and bladder tumors (NCT00525408 and NCT00694915: the recruitment status of this experiment is unknown due to the lack of unambiguous verification of the information). Hiltonol (TLR-3 agonist), a specific formulation of poly (I:C), is now being tested in phase I/II clinical trials to determine its therapeutic safety and effectiveness (Galluzzi et al., 2012). In the meantime, a phase 2 clinical trial (NCT00514072) using the BCG vaccination to treat prostate cancer is still ongoing. In prostate cancer patients, a multi-peptide, dual-adjuvant telomerase vaccine (GX301) including Imiquimod demonstrated reduced toxicity and immunogenicity. It appears that adequate monitoring is also required to evaluate its therapeutic efficacy (Fenoglio et al., 2013). Furthermore, LPS activation of TLR-4 is thought to cause chemoresistance to docetaxel in prostate cancer cells. On the other hand, Lipid-A, the active component of LPS, and other lipid-A –derived synthetic compounds, such as OM-174, are TLR4 agonists that can regulate tumor growth in the murine B16 melanoma experimental model by activating natural killers (NK) and cytotoxic T lymphocyte (CTL) mediated anti-tumoral response (D'Agostino, Cartesio et al., 2005). Conversely, a TLR5 synthetic agonist, CBLB502, demonstrated radioprotective benefits exclusively in non-transformed mouse and primate experimental models, offering up a new avenue for the use of TLR5 agonists as radiotherapy adjuvants (Burdelya et al., 2008). A phase I clinical trial is enrolling patients with locally advanced or metastatic solid tumors to investigate the efficacy and tolerability of the TLR5 agonist CBLB502, and the second goal of the study is to comprehend the preliminary evidence of efficacy of this drug.

As a result, the superior immunostimulatory characteristics of TLR agonists are frequently coupled with traditional cancer treatments such as radiation or chemotherapy to enhance oncogenesis treatment. Antitumor efficacy has been demonstrated in many types of cancer, and TLR-agonists are currently undergoing extensive clinical research to better understand the response in both mice and human cells, which may differ from one another.

## 5.2 Clinical Prospectus

Using TLR agonists as adjuvants to mature DCs either ex vivo or in situ, is a useful method for initiating or boosting antitumor responses persuaded by cancer vaccines. A multi-TLR agonist approach resulted into resistance against tumor cells, apoptosis and inhibition of tumor progression with less side effects, made it a promising topic of future research (Zhao et al., 2014).

A fundamental concern of which TLR ligand to include in a vaccine formulation for receiving most effective antitumor responses still exists to puzzle the field of immunotherapy. Choosing the right combination of antigen(s) and adjuvant(s) may require recommendations from precise immunomonitoring. Most beneficial would be to develop tests directly evaluating the potential of the different TLR agonists to activate innate immunity, but presently, such adjuvanticity tests are lacking. Another problem that hinders the field from moving forward stems from the fact that vaccine development is a very pragmatic process and tumor surveillance needs different types of immune responses (Baxevanis et al., 2013)

Since cancers are heterogeneous in nature, so new immunotherapies may play integral role of multi-treatment regimens or may be beneficial in some cases as monotherapy. Moreover, there is ample clinical evidence to suggest that TLR ligands may act as a successful immunotherapy adjuvant to intensify treatment efficacy and give positive patient outcomes to treat not only breast and prostate cancer, but also highly potential in – Melanoma, ovarian cancer, peritoneal fallopian tube, head and neck cancer, gastric cancer, lung cancer and bladder cancer.

## 5.3 Future Studies

Despite the positive results of the TLRs in clinical trials of mouse models, further studies need to prove the safety and efficiency for human use. The future research will be focused on:

First, to apply combinatorial treatments with the association of metronomic doses of chemotherapeutics, targeted therapy, irradiation, or blockade of immune checkpoint inhibitors in order to develop Th- 1 type antitumor immunity which may help to reduce the level of tumor-induced immunosuppression (Pradere et al., 2014). In fact, combination of immunotherapy and chemotherapy act as a beneficial approach for the immune system to find and destroy cancer cells. Hence, drastically modify the effect of therapeutic cancer vaccines/ TLR ligand formulations.

Second, to choose for the suitable patient population. There are references which exhibits that patient with less amount of immunosuppression, having small tumor burden (being in the adjuvant or in the metastatic setting, but with inactive form of disease), have potential chances to get advantages from therapeutic vaccination (Baxevanis, Constantin N et al., 2013). Besides, TLR agonists should also update to improve their functions by identifying recent stimulatory elements as illustrated, for instance, in the present evaluation of P-class of CpG ODNs, which incorporates appropriate properties of the familiar CpG ODN classes. Consequently, this P-class provokes superior cytokine production upon in vivo application (Samulowitz et al., 2010). Such initiatives will help to facilitate our understanding of how TLR agonists regulate distinct elements of the immune system.

# Chapter 6 Conclusion

Therefore, to sum up we can say that, from this review, it is clear that Toll-like receptor agonists /antagonists can be targeted as therapeutics against various types of cancer. The activation of various cells such as – DCs, T- cell subsets, and even tumor cells along with their downstream signaling pathways may lead to tumor immunity. Successful studies have involved a TLR7 agonist, imiquimod, and a nonspecific agonist of TLR2/TLR4, BCG. There is an emerging area of identifying and developing new immunotherapeutic agents, combinatorial therapies and indications which will facilitate in cancer treatment and some of them are being studied in clinical trials. An attractive possible tumor therapy can be developed with the association of TLR- specific agonists into cancer vaccination based on DCs. By keeping this in mind, the development of new TLR-agnostic drugs should be done in such a way that it does not exert pro-tumor response instead of anti-tumor immune response by discovering their special characteristics. As a result, TLRs will be able to induce local expression of IFN types I and II that are known to instigate cell death, and activate cell immunity in response to the recognition of pattern receptors (PAMPs and DAMPs) which can be considered as a potential hallmark of cancer treatment. Further in-depth studies will help us to gain better understanding of TLRs role in tumorigenesis, tumor immunity and tumor metastasis which in turn may provide more distinct, structurally more effective and safer drugs to treat the cancer patients. So, Toll-like receptors in cancer immunology should undergone further mechanistic investigations as it has vast opportunities.

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