Title:

Understanding the Role of TLRs and Associated Signalling Pathways in Triple Negative Breast Cancer

Introduction:

Globally, breast cancer is one of the most commonly diagnosed malignancies in women (1). Similarly, the burden of breast cancer is high in India ranking as one of the top most malignancies in Indian women (2). Breast cancer can be classified into four distinct molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 gene (HER2) in breast cancer (3). This includes luminal A, Luminal B, (estrogen and progesterone receptor positive), HER2 positive and triple negative breast cancer. Triple negative breast cancer (TNBC) is a subtype of breast cancer that lacks the expression of ER, PR, and HER2 receptors and is frequently associated with disease aggressiveness and the worst prognosis compared to other subtypes of breast cancer (4).

TNBC accounts for about 15–20 percent of all breast cancer cases globally, with higher prevalence rates in the Indian population (25-31%) (5,6). This subtype of breast cancer is unresponsive to conventional therapies known to be effective for treating other subtypes of breast cancer such as endocrine therapy (for luminal A or B subtypes) or Her-2 targeted therapy (for Her 2 enriched breast cancer) (3). Thus surgery, radiotherapy, and chemotherapy remain the only approved treatment modalities for TNBC patients (7). However, the recurrence and metastasis rate remain very high in this subtype of breast cancer (3).

TNBC is considered to be a highly immunogenic subtype of breast cancer, as these tumors show high immune cell infiltration, PD L1 expression, genomic instability, mutation rate making it a good candidate for treatment with immunotherapeutic approaches (8). Distinct immunotherapeutic strategies have been researched for the treatment of TNBC patients which includes checkpoint inhibitors, antibody-drug conjugates (ADC), chimeric antigen receptor (CAR) T-cell therapy (8). However, most of these strategies are adaptive immune response centric which showed promising response to a small fraction of TNBC patients. Thus, there is a need to evaluate

alternative strategies that can benefit a larger fraction of TNBC patients. The efficacy of immunotherapeutic strategies is based on the infiltrating immune cells and its interaction with the tumor cells. Since one of the larger proportion of cells within the tumor microenvironment (TME) is innate immune cells, there is a need to explore these cells as an alternate immunotherapeutic option. (9)

Innate immune system is first to generate an immune response and prime the adaptive immune cells through an interplay that takes place via the pattern recognition receptors (PRRs)in the body. These PRRs are expressed by the innate immune cells that recognize tumor associated antigens (TAAs) released by the tumor cells during the development and progression of breast cancer or post chemotherapy treatments thereby triggering the innate immune response. This further leads to the recruitment and expansion of adaptive immune cells that helps in the generation of antitumor response against the tumor cells (10).

Toll-like receptors (TLRs) are one among the PRRs that have been most extensively studied for their role in the pathogenesis of different cancers including TNBC (11). TLRs are known to be expressed on all the immune cells and non-immune cells like Dendritic cells, fibroblast and epithelial cells. These are type 1 transmembrane receptors with extracellular leucine rich repeats for recognition, a transmembrane domain, and an intracellular toll interleukin receptor domain (TIR) for downstream signaling. Upon activation of TLRs by their respective ligands, different adaptor proteins (MYD88, TIRAP, TRIF and TRAM) are recruited and associate with the TIR domain of TLR. Upon recruitment of MYD88 and TIRAP adaptor proteins to the cytosolic domain of the TLR receptors, activation of the downstream signaling network leads to activation and release of proinflammatory cytokines like IL6, and TNF alpha. while recruitment and association of TRIF and TRAM adaptor proteins to the cytosolic domain of the TLR receptors, leads to activation of the downstream signaling thereby releasing of anti-inflammatory cytokines such as type 1 interferon like IFN alpha IFN beta (12).

A number of clinical trials (NCT03435640; NCT01421017; NCT00899574; NCT02643303) are presently evaluating the TLR agonists in combination with chemotherapy/ immunotherapy for their impact on reactivating anti-tumor immune response in solid tumors including TNBC which achieved only limited success. One reason could be the heterogeneity in TLR expression within tumors of TNBC patients

(11,13). Multiple studies have indicated both pro- and anti-tumorigenic role of TLRs in regulating the development and progression of TNBC. Furthermore, some studies have also suggested that expression of distinct TLRs in different cells within the TME varies and it was correlated with different clinical outcomes in TNBC patients. (14,15). The molecular cues that drive the pro- and anti-tumorigenic role of TLRs are still unexplored. Thus, the present study will be focusing on evaluating the role of these TLRs and its associated signaling pathways in tumor epithelial cells and tumor infiltrating immune cells in progressive stages of TNBC.

Study Rationale:

Accumulating studies indicate the pro and anti-tumorigenic role TLRs and TLR regulated pathways in pathogenesis of TNBC (13- 15, 23- 24). However the relevance of targeting TLR signalling pathways either alone or in combination with immunotherapy or chemotherapy drugs in immune cell enriched (immunologically hot) and immune cells low (immunologically cold) TNBC tumors is incompletely understood. Also the potential of developing a targeted cell based therapy based on TLR expression in individual cell type has not been much explored as a strategy to improve the clinical outcomes of TNBC patients.

Objectives:

1. To study the cell type-specific expression of TLRs and TLR-associated signalling genes in TNBC tumors
2. To determine the effects of perturbing candidate TLR(s) /TLR pathway associated gene/s on transcriptomes of TNBC tumor epithelial cells and infiltrating immune cells
3. To evaluate the TLR perturbation regulated molecular signatures in human TNBC tumors

Methodology:

Objective1: To study the cell type-specific expression of TLRs and TLR-associated signalling genes in TNBC Tumors

The expression status of TLRs and associated signalling pathway genes (MYD88, TIRAP, TRIF, and TRAM) will be screened in individual Tumor and immune cells infiltrating TNBC tumors in progressive stages of human TNBC tumors and murine TNBC model.

A. Analysis of Human TNBC tumor samples:

The N=25 human TNBC tumors and tumor adjacent normal samples were collected from TNBC patients visiting Tata Memorial Hospital, Mumbai for treatment. The informed consent of all the patients and clearance from the Institutional Ethics Board were obtained (NIRRCH, IEC project # 355/2019). These tumors will be used to catalogue the expression status of TLRs and associated sigaling pathway genes (MYD88, TIRAP, TRIF, and TRAM) in tumor cells and infiltrating immune cells using qRT PCR, immunofluorescence or Immunohistochemistry and FACS. In addition to this the TLR-pathway regulated cytokines such as IL6, IL1β, TNF-∝, IFN∝, IFNß and IFNγ will be screened by Single or Multiplex ELISA.

1. Real Time Polymerase Chain Reaction:

The mRNA expression of TLR genes was analysed in N=12 human TNBC tumor samples along with patient-matched tumor-adjacent normal breast tissues. The total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, USA). The qRT PCR analysis was carried out using the primers. Real-time quantification of the TLR genes was performed using iTaq Universal SYBR Green Supermix kit (Biorad) according to manufacturer's instruction on the Bio-Rad™ CFX96™ Real Time Thermal Cycler PCR machine (Biorad) with 18S as control. Experimental samples were run in duplicate with the same concentration of cDNA (synthesised from 1µg RNA) per reaction. To check the amplicon contamination, each run contained no template controls in duplicate for each probe used. Cycle threshold (CT) values were recorded. To determine the relative level of gene expression, the comparative threshold cycle (ddCt) method was employed.

2. Immunofluorescence (IF) and Immunohistochemistry (IHC):

Immunofluorescence (IF) and Immunohistochemistry (IHC) based imaging is one of the routinely used techniques in our lab. It will be utilized in the current study for screening the TLRs and associated gene expression levels in the cells present in the human TNBC tumor-TME.

The multiplex immunofluorescence staining done in N=10 human TNBC tumor samples and tumor adjacent normal tissues using primary antibody for e cadherin (epithelial cell marker) (Santacruz, USA, 14472S, 1:50 dilution) and TLR4 (CST, sc-293072; 1:100 dilution) was done. The secondary antibody (1:200 dilution) was then tagged with a fluorescent dye (CF568) and the signals were further amplified by using tyramide amplification kit (Biotium, Inc. CA, USA). Further, the sections were mounted with Prolong gold antifade reagent with DAPI (CST, USA #8961) and images were captured using a fluorescence microscope equipped with an sCMOS camera (Leica Microsystems DMi8, Mannheim, Germany). The Fluorescence intensity was quantified using ImageJ (Fiji version) software.

3. Fluorescence-activated cell sorting (FACS) analysis:

The FACS analysis will be done on tumor cell suspension prepared from enzymatic digestion of early and late stage human TNBC tumors to compare the cell type-specific expression of TLRs and associated pathway genes in distinct immune cell types and tumor cells present in TME. For this, the tumor cell suspension will be prepared. The viability of the cells would be accessed by trypan blue staining and a cell count of viable cells will be measured. Thereafter the single cell suspension of TNBC tumor will be analysed for expression of TLRs and associated pathway genes by flow cytometry.

4.Enzyme-linked immunosorbent assay (ELISA)

Cytokine Analysis: The TLR-pathway regulated cytokines such as IL6, IL1β, TNF-∝, IFN∝, IFNß and IFNγ will also be quantified in the human TNBC tumor cell lysate using Single or Multiplex ELISA kit that are commercially available following the manufacturer's instructions for analysis.

In N=10 human TNBC tumor samples we have estimated the expression levels of TLR4 (Sigma, #RB1088) and IFNγ (Invitrogen, #A35576) using commercially available kits as per the manufacturer's protocol.

B. Development of a 4T1 cells induced murine syngeneic TNBC model:

Development of a syngeneic murine TNBC model was done as per the protocol previously mentioned (25) and screening the expression pattern of TLRs in distinct cell types present with TNBC TME. The animal protocols are approved by ICMR NIRRCH-IAEC, project no 06/23. Briefly, 4T1 luc2 murine TNBC cell line was purchased and cultured *in vitro* according to the manufacturer's instruction. This murine 4T1 luc2 cell line is injected in the 4th inguinal mammary gland of 12-16 weeks old primiparous female BALB/c mice. Primiparous mice were taken for ease of its 4th inguinal nipple detection and precise cell delivery into the ducts via nipple. Post inoculation of 4T1 luc2 cells, the tumor development was followed for a period of 4-5 weeks and animals were sacrificed at 3-time points, week 1(Normal like-early Breast Cancer, Tumor cut off- 20 mm^3), week 2 (Ductal Carcinoma *In situ*- DCIS or early stage, cut off - 50 mm^3), and week 4-5 (Metastatic stage, cut off -800 mm^3) post-inoculation of 4T1 cells for further studies as shown in figure 1.

As 4T1 MIND tumors became palpable after 1 week post-inoculation, the tumors were measured bi-weekly using digital callipers to calculate the tumor volume [(length×width^2)/2]. D-Luciferase was injected intraperitoneally and the tumors were monitored weekly by bioluminescence imaging using the IVIS Lumina system (PerkinElmer, Waltham, MA, USA) as described elsewhere (26). The images were captured within 10-15 min following D-Luciferase injection. For quantifying the photons emitted by the tumor cells we used Live imaging software (PerkinElmer). The tumors collected from the 4th inguinal mammary gland will be confirmed by H&E staining for presence of early stage and late stage histologically. They will then be used for screening TLRs and TLR associated signalling genes in tumor cells and infiltrating immune cells present within TNBC TME by FACS. In addition to this the TLR-pathway regulated cytokines such as IL6, IL1β, TNF-∝, IFN∝, IFNß and IFNγ will be screened by Single or Multiplex ELISA.

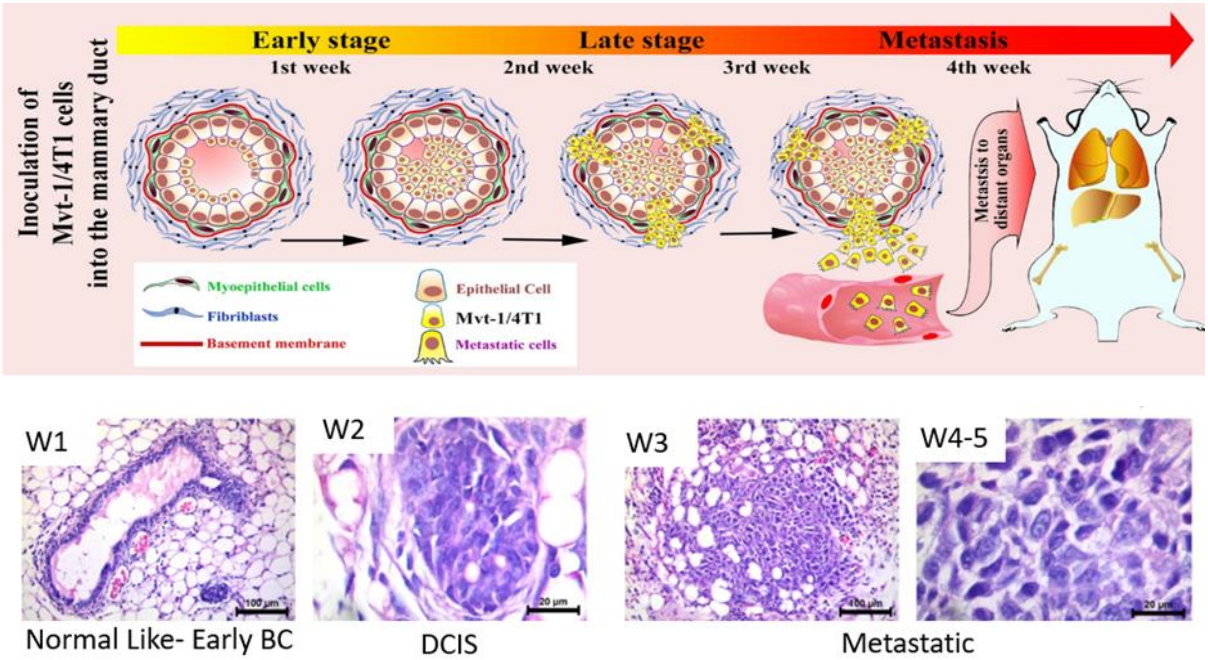

Figure 1: Progressive stages murine TNBC tumor model to be used in the study. Figure adapted from (25)

1. Immunofluorescence (IF) and Immunohistochemistry (IHC):

Immunofluorescence (IF) and Immunohistochemistry (IHC) based imaging will be utilised in the current study for screening the TLRs and associated gene expression levels in tumor epithelial cells and tumor-infiltrating immune cells present in the murine TNBC tumor-TME.

2. FACS analysis:

FACS analysis will be done on tumor cell suspension prepared from enzymatic digestion of murine early stage and late stage TNBC tumors collected will be examined for the cell type-specific expression of TLRs and associated pathway genes in distinct immune cell types and tumor cells present TME. Further, the same protocol will be utilised which was used previously for human TNBC samples.

3. ELISA:

Cytokine analysis: The TLR-pathway regulated cytokines such as IL6, IL1β, TNF-∝, IFN∝, IFNß and IFNγ will also be quantified in murine TNBC tumor cell lysate using Single or Multiplex ELISA kits following the manufacturer's instructions for analysis.

Objective 2. To determine the effects of perturbing candidate TLR(s) /TLR pathway associated gene/s in TNBC tumor epithelial cells and infiltrating immune cells transcriptome.

Based on the outcomes of objective 1 the TLR(s) or associated signalling molecules that showed a considerable response in the regulation of tumorigenesis in a murine tumor will be chosen for further analysis. For this the same 4T1-induced syngeneic TNBC mice model will be treated by TLR agonist or antagonist (in clinical trial and commercially available) delivered via liposomal nanoparticles targeted to specific cells in TNBC TME and their tumor progression would be studied.

1. Cell sorting: The tumors obtained from control and treated sets of animals (early stage and late stage) will then be subjected to cell sorting for segregating the tumor and stromal cells from murine TNBC tumors. The tumor epithelial and stromal cell suspension obtained will be used for transcriptome analysis.
2. Transcriptome analysis: Screening of both miRNA and mRNA profiles of early and late stage murine TNBC tumor derived epithelial and immune cells will be carried out by miRNA and mRNA sequencing.

Objective 3. To evaluate the TLR perturbation regulated molecular signatures in human TNBC tumours

The molecular signatures found to be perturbed in the murine model will then be confirmed in early-stage/late-stage human TNBC (high and low immune cell) tumor samples using RT2 profiler qPCR array. The molecular signatures identified common in human and mouse TNBC model capable of modulating tumor immune crosstalk will be further targeted with their specific inhibitors or agonists using *in vitro* coculture model.

Results:

1. Therapeutic relevance of tumor associated macrophages in TNBC tumors

Multiple studies suggest that distinct signalling cues driven by tumor associated macrophages and tumor cell cross talk play an important role in regulating pro and anti-tumorigenic effects in TNBC TME. These signalling cues could be targeted for developing different therapeutic strategies for improving the clinical outcomes of TNBC patients as presented in a review (27).

2. Heterogeneity of TLR expression of TNBC tumors.

Previous study from our lab suggests TLR 3, 4, 6 and 9 to be the most differentially expressed TLRs in TNBC TCGA analysis (Roychowdary et.al). Thus we confirmed the transcript expression pattern of these TLRs (TLR 3, 4, 6 and 9) in human TNBC tumor samples by qRT PCR. Wherein we observed these TLRs to be heterogeneously expressed. Multiple studies have reported the expression of TLR4 in distinct solid tumors with increased tumor progression, metastasis and poor clinical outcomes (15,28). So, we screened the localisation of TLR4 in E-cadherin positive tumor compartment and E-Cadherin negative stromal compartment of TNBC tumors and tumor adjacent normal tissues by immunofluorescence staining. In this analysis we found TLR4 to be downregulated in TNBC tumors as compared to tumor adjacent normal tissues. The TNBC tumor tissues were then segregated based on the number of tumor infiltrating lymphocytes (TIL) present in them into high TIL TNBC tumors (>30% TIL) and low TIL TNBC tumors (<30% TIL) and the expression pattern of TLR4 was screened in E-cadherin positive compartment and E-Cadherin negative compartment of TNBC tumors. Which revealed that TLR4 is highly expressed in E-Cadherin negative stromal compartment as compared to E-cadherin positive tumor compartment of high TIL TNBC tumors. While it was equally present in both the compartments of low TIL TNBC tumors.This altogether indicates that TLR4 is differently localised in distinct cell types of TNBC tumors based on the immune cell infiltration.

3. Immunomodulatory potential of TLR4 in TNBC

A trend of correlation (r= 0.15 p=0.33 N=10) was observed between TLR4 protein and a potential antitumor cytokine, IFNγ (28) in TNBC tumors. Additionally we also got a strong correlation (r= 0.62, p= 0.11 N=5) between TLR4 and IFNγ in high TIL TNBC samples. This indicates the immunomodulatory potential of TLR4 in TNBC. Currently we are extending this analysis in more samples.

This analysis is a part of a manuscript that has been submitted for publication in cancer letters.

4. Establishment of mammary intraductal model murine syngeneic TNBC model

The 4T1 (murine mammary carcinoma cell line) was injected in the 4th inguinal mammary glands of primiparous female Balb/C syngeneic mice model of TNBC. The progressive increase in tumor volume was obtained up to week 4 (figure 2). Studies are currently underway to characterise the tumors developed in terms of increase in tumor volume, tumor histology, extracellular matrix density, immune cell repertoire and TLR expression across different cell types in tumor TME. The model thus developed will then be used to test the therapeutic efficacy of TLR agonist/ antagonist by cell based therapy.

Figure 2: Invivo Image of Balb/c mice with 4T1-Luc2 generated TNBC tumors.

Statistical Analysis:

The statistical analysis was performed using the Graph Pad Prism, or SPSS software. All data was expressed as the mean ± SD. Statistically significant differences between groups was determined by using the unpaired Student's t-test. Associations between categorical variables were examined using Pearson's correlation test. P-values <0.05 were considered to be statistically significant.

Discussion:

In the present study we are evaluating the potential role of TLR and TLR associated signalling pathways in TNBC. Evaluation of mRNA levels of TLRs (TLR3, 4,6 and 9) present within human TNBC tumors revealed their heterogeneous expression pattern across the TNBC patients. Similarly, a group of investigators have reported differential expression patterns of TLRs in breast tumors as compared to the tumor adjacent normal tissues (11). A number of studies have reported TLR4 expression to be associated with poor prognosis in breast cancer patients (11, 28, 30). Also, it has been found that downregulation of TLR4 expression in immune cells leads to enhanced tumor progression and metastasis and upon treatment with TLR4 agonists like OM-174 can significantly inhibit tumor progression in mouse TNBC tumors. Thus we further screened the localisation of TLR4 within the tumor and stromal compartment of TNBC patients. Interestingly, it revealed a variable expression pattern of TLR4 protein based on the immune cell infiltration in the TNBC TME. Further assessment of immunomodulatory potential of TLR4 showed it to be correlated with downstream antitumorigenic cytokine IFNγ. Similar observations were reported by a group of investigators wherein they found TLR4 and IFNγ receptor (INFR2) expression by murine mammary tumor cells are involved in tumor growth inhibition (31). However, further studies will be done to understand the role of TLR4 expression in TNBC tumors will high and low immune cell infiltration. Altogether this part of the study aids in understanding the immunomodulatory role of TLR4 signalling in TNBC.

Impact of the research in the advancement of knowledge or benefit to mankind:

1. The study will add in knowledge by understanding the cell-type specific expression pattern of TLRs in TNBC tumors.

2. The study outcome might eventually help in development of cell based therapies targeting TLR4 expressing cells.

3. It will help identify TLR-driven genes signatures that have antitumor potential in human TNBC tumors.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209–49.

2. Mehrotra R, Yadav K. Breast cancer in India: Present scenario and the challenges ahead. World J Clin Oncol. 2022 Mar 24;13(3):209–18.

3. Harbeck N, Penault-Llorca F, Cortes J, et al. Breast cancer. Nat Rev Dis Primers. 2019 Dec 1;5(1).

4. Lehmann BD, Jovanović B, Chen X, et al. Refinement of triple-negative breast cancer molecular subtypes: Implications for neoadjuvant chemotherapy selection. PLoS One. 2016 Jun 1;11(6).

5. Suvobrata S, Murtaza A. Triple Negative Breast Cancer Prevalence in Indian Patients over a Decade: A Systematic Review. Int J Clin Biostat Biom. 2022 Jan 12;8(1).

6. Sandhu GS, Erqou S, Patterson H, et.al Prevalence of Triple-Negative Breast Cancer in India: Systematic Review and Meta-Analysis. J Glob Oncol. 2016 Jun 29;2(6).

7. Wahba HA, El-Hadaad HA. Current approaches in treatment of triple-negative breast cancer. Cancer Biol Med. 2015 Jun;12(2)

8. Valencia GA, Rioja P, Morante Z, et al. Immunotherapy in triple-negative breast cancer: A literature review and new advances. World J Clin Oncol. 2022 Mar;13(3).

9. Li Y, Zhang H, Merkher Y, et.al. Recent advances in therapeutic strategies for triple-negative breast cancer. J Hematol Oncol. 2022 Aug 29;15(1).

10. Rameshbabu S, Labadie BW, Argulian A, et.al. Targeting Innate Immunity in Cancer Therapy. *Vaccines*. 2021 Feb; 9(2).

11. Shi S, Xu C, Fang X, et al. Expression profile of Toll-like receptors in human breast cancer. Mol Med Rep. 2020 Feb;21(2).

12. El-Zayat SR, Sibaii H, Mannaa FA. Toll-like receptors activation, signaling, and targeting: an overview. Bull Natl Res Cent. 2019 Dec;43(1).

13. Roychowdhury A, Jondhale M, Saldanha E, et al. Landscape of toll-like receptors expression in tumor microenvironment of triple negative breast cancer (TNBC): Distinct roles of TLR4 and TLR8. Gene. 2021 Aug 5;792.

14. Singh A, Bandyopadhyay A, Mukherjee N, et al. Toll-Like Receptor 9 Expression Levels in Breast Carcinoma Correlate with Improved Overall Survival in Patients Treated with Neoadjuvant Chemotherapy and Could Serve as a Prognostic Marker. Genet Test Mol Biomarkers. 2021 Jan 1;25(1).

15. Papadaki MA, Monastirioti A, Apostolopoulou CA, Aggouraki D, Papadaki C, Michaelidou K, Vassilakopoulou M, Alexakou K, Mavroudis D, Agelaki S. TLR4 and pSTAT3 Expression on Circulating Tumor Cells (CTCs) and Immune Cells in the Peripheral Blood of Breast Cancer Patients: Prognostic Implications. Cancers. 2022; 14(4)

16. Fan, L., Sui, XY., Jin, X. et al. High expression of TLR3 in triple-negative breast cancer predicts better prognosis—data from the Fudan University Shanghai Cancer Center cohort and tissue microarrays. BMC Cancer 23, 298 2023.

17. Tomela J, Sandholm J, Karihtala P, Ilvesaro J, Vuopala KS, Kauppila JH, et al. Low TLR9 expression defines an aggressive subtype of triple negative breast cancer. Breast Cancer Res Treat. 2012 Sep;135(2):481–93.

18. Mehmeti, M., Allaoui, R., Bergenfelz, C. et al. Expression of functional toll like receptor 4 in estrogen receptor/progesterone receptor-negative breast cancer. Breast Cancer Res 17, 2015 Sep.

19. Zanker DJ, Spurling AJ, Brockwell NK, et al. Intratumoral administration of the Toll-like receptor 7/8 agonist 3M-052 enhances interferon-driven tumor immunogenicity and suppresses metastatic spread in preclinical triple-negative breast cancer. Clin Transl Immunology. 2020 Sep 28;9(9)

20. Shi, D., Zhao, S., Jiang, W. et al. TLR5: A prognostic and monitoring indicator for triple-negative breast cancer. Cell Death Dis, 2019 Dec.

21. Ma FJ, Liu Z bin, Hu X, et al. Prognostic value of myeloid differentiation primary response 88 and toll-like receptor 4 in breast cancer patients. PLoS One. 2014 Oct 31;9(10).

22. Khor Poh Yen, Johnson Stanslas, Tianshu Zhang, et al. Synthesis of small molecules targeting paclitaxel-induced MyD88 expression in triple-negative breast cancer cell lines, Bioorganic & Medicinal Chemistry, Volume 49, 2021 Nov.

23. Rajput S, Volk-Draper LD, Ran S. TLR4 is a novel determinant of the response to paclitaxel in breast cancer. Mol Cancer Ther. 2013 Aug;12(8).

24. Ahmed A, Huai Wang J, Redmond HP. Silencing of TLR4 increases tumor progression and lung metastasis in a murine model of breast cancer Ann Surg Oncol. 2013;20

25. Ghosh A, Sarkar S, Banerjee S, et al. MIND model for triple-negative breast cancer in syngeneic mice for quick and sequential progression analysis of lung metastasis. PLoS One. 2018 May 1;13(5).

26. Evans MS, Chaurette JP, Adams ST Jr, et al. A synthetic luciferin improves bioluminescence imaging in live mice. Nat Methods. 2014 Apr;11(4):393-5.

27. Shettigar A, Salunke R, Modi D, Mukherjee N. Targeting molecular cross-talk between tumor cells and tumor associated macrophage as therapeutic strategy in triple negative breast cancer. Int Immunopharmacol. 2023 Jun;119:110250.

28. Saponaro C, Scarpi E, Sonnessa M, et al. Prognostic Value of NLRP3 Inflammasome and TLR4 Expression in Breast Cancer Patients. *Front. Oncol.* 2021 Sept, 11:705331.

29. Xu, B., Sun, H., Liu, S. *et al.* IFI35 limits antitumor immunity in triple-negative breast cancer via CCL2 secretion. *Oncogene* 43, 2024 Jan.

30. Koliaraki V, Chalkidi N, Henriques A, et al. Innate Sensing through Mesenchymal TLR4/MyD88 Signals Promotes Spontaneous Intestinal Tumorigenesis. *Cell Rep.* 2019 Jan ;26(3).

31. Lamrani M, Sassi N, Paul C, Yousfi N, Boucher JL, Gauthier N, Labbé J, Seignez C, Racoeur C, Athias A, Guerreiro R, Vergely C, Rochette L, Bettaieb A, Jeannin JF. TLR4/IFNγ pathways induce tumor regression via NOS II-dependent NO and ROS production in murine breast cancer models. Oncoimmunology. 2015 Dec 29;5(5):e1123369.